12-10-2015

Effects of Resistance Training and Creatine Monohydrate on Doxorubicin-Induced Muscle Dysfunction

Eric Christopher Bredahl

UNIVERSITY OF NORTHERN COLORADO

Greeley, Colorado
The Graduate School

EFFECTS OF RESISTANCE TRAINING AND CREATINE MONOHYDRATE ON DOXORUBICIN-INDUCED MUSCLE DYSFUNCTION

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

Eric Christopher Bredahl

College of Natural and Health Sciences
School of Sport and Exercise Science
Exercise Science

December 2015

This Dissertation by: Eric Christopher Bredahl

Entitled: *Effects of Resistance Training and Creatine Monohydrate on Doxorubicin-Induced Muscle Dysfunction*

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

Accepted by the Doctoral Committee

_____
David Hydock, Ph.D., Research Advisor

_____
Reid Hayward, Ph.D., Committee Member

_____
Gary Heise, Ph.D., Committee Member

_____
Patrick Burns, Ph.D., Faculty Representative

Date of Dissertation Defense _____

Accepted by the Graduate School

_____
Linda L. Black, Ed.D.
Associate Provost and Dean
Graduate School and International Admissions

ABSTRACT

Bredahl, Eric C. *Effects of Resistance Training and Creatine Monohydrate on Doxorubicin-Induced Muscle Dysfunction.* Published Doctor of Philosophy dissertation, University of Northern Colorado, 2015.

Doxorubicin (DOX) is a powerful chemotherapy agent that is associated with a number of deleterious side effects, including skeletal muscle dysfunction. The exact mechanisms behind the observed skeletal muscle dysfunction have yet to be fully understood. Nonetheless, the observed myotoxicity is believed to be the result of an increased oxidative stress. Resistance training (RT) has been shown to preserve skeletal muscle function in DOX treated muscles. Conversely, creatine (Cr) has been shown to improve skeletal muscle function. Yet, there has been no investigation into the effect of combined RT and Cr on DOX-induced muscle dysfunction. Thus, the possibility exists that in combination, Cr and RT could attenuate the myotoxic effects of DOX beyond that of when the interventions are administered separately. PURPOSE: To investigate the effects of prior RT and Cr treatment on DOX-induced skeletal muscle dysfunction. METHODS: Male Sprague-Dawley rats were randomly assigned to a RT or sedentary group. Resistance training was simulated by using an elevated food model. After six weeks of training, the soleus (SOL) and extensor digitorum longus (EDL) were excised and placed in an *ex vivo* tissue bath containing a Krebs buffer (K) where initial force was measured. Muscles were then incubated with either K or a K containing Cr (25 mM) for 30 minutes. The buffers were refreshed with either new K or K containing DOX (24 μM) and incubated for 30 minutes. Muscles were then supplied with new K and twitch force

data were collected. The muscles were again supplied with fresh K and subjected to a 100 sec fatigue protocol where force production was recorded every 10 seconds to analyze fatigue. After functional data were collected, tissues were analyzed for total Cr content. RESULTS: This study failed to demonstrate DOX-induced muscle dysfunction on maximal twitch characteristics but DOX-induced dysfunction did become apparent under prolonged stimulation. Furthermore, this investigation demonstrated that, in combination, RT and Cr could significantly attenuate fatigue in the DOX treated muscle. This study also failed to show any significant change total intracellular Cr after incubation with Cr or prior RT. CONCLUSION: Evidence from this study provides insight into the effectiveness of a combined treatment with RT and Cr to minimize fatigue and offset the myotoxic effects of DOX. Such evidence provides more support to the effectiveness and capacity of exercise interventions to improve functioning of cancer patients during their treatment process.

DEDICATED TO DAVID L. CROSSON, M. D.

In grateful acknowledgement of the continued support, love, guidance, and council which aided me in realization of my dreams.
Thank you.

ACKNOWLEDGMENTS

Theodore Roosevelt once said, "No man needs sympathy because he has to work, because he has a burden to carry. Far and away the best prize that life offers is the chance to work hard at work worth doing." This dissertation would not have been possible without the continued guidance and counsel of my committee, family, and friends. You all have taught what it means to work hard on something worth doing.

To my committee, words cannot express my gratitude for your time, patience, and invaluable expertise. You have each provided me with a unique perspective and opinions that have turned this project into something of which I am very proud.

First and foremost, I must extend my deepest appreciation and respect to my friend and mentor, Dr. David Hydock, for his unconditional kindness, counsel, expertise, and his seemingly endless supply of patience throughout my graduate work. You are an incredible person and demonstrate all of the qualities of what a researcher and teacher should be. Furthermore, you have helped me overcome some of the greatest challenges that I have ever known, and you have also helped me through them in the most positive way possible. I feel honored and blessed to have had the chance to work with you. Your philosophies on life, academia, and science have helped me grow as a person and helped to change my outlook on life. Thank you, my friend.

Dr. Reid Hayward, you have shown the greatest level of tenacity in the face of adversity and have helped me become a better scientist. Your expertise, suggestions, and

perspectives have made me a better academic. You have helped to turn a fledgling graduate student into a proud scientist, and for that, I thank you.

Dr. Patrick Burns, your encouragement and humor have helped me progress through my graduate studies. You have always been a source of inspiration, and your work ethic is second to none. I must also thank you for your technical expertise and continual guidance on all aspects of science and academia. Your approach to teaching and sincerity towards students serve as a continual source of inspiration. You are the epitome of a great teacher!

I also must extend a special thanks to Dr. Keith Phanesteil, Dr. Colin Quinn, Ms. Raquel Bourgeis, Ms. Leanne Hochberg, Mr. Pete Graham, and all other graduate and undergraduate students who have helped me with various projects and other aspects of my life. I also extend a personal thanks to Mr. James Morgante. Thank you for your service to our country and for being an excellent friend to me these last couple years. I feel truly blessed to have had such fantastic friends.

Finally, I must thank my unbelievably supportive family. Your love and unconditional support has left me awestruck. You have seen me through the tough times and the good times, and you have been my staunchest supporters. Without you, this would have never been possible. This achievement is as much yours as it is mine. Thank you for always believing in me, even when I didn't believe in myself.

Thank you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

The use of exercise as a means of minimizing cancer related fatigue and improving quality of life during and after chemotherapy treatment is becoming more commonplace. With this growing trend toward improving quality of life and reducing patient fatigue during and after chemotherapy, there is an immense need to develop safe and effective guidelines based on quality research.

Fatigue is one of the most common side effects of cancer treatment. Up to 70% of patients experience fatigue during chemotherapy and radiation therapy (Smets, Garssen, Schuster-Uitterhoeve, & de Haes, 1993). Additionally, many cancer patients suffer severe limitations in their ability to perform activities of daily living (ADLs). These activities include feeding, bathing, dressing, grooming, work, and homemaking. There is also evidence that fatigue can adversely affect the efficacy of chemotherapy; fatigue as a side effect can be a reason to slow or stop treatment altogether. In recent years, there has been a large amount of information encouraging cancer patients to exercise prior to and during chemotherapy treatment. Existing research has found that exercise is particularly effective during treatment with the chemotherapeutic drug doxorubicin (DOX), which is classified as an anthracycline.

Anthracyclines are arguably the most effective and commonly prescribed antineoplastic chemotherapy drugs (Weiss, 1992). They are used to treat numerous types

of solid tumors (Green et al., 2001), leukemias (Lipshultz et al., 2004), and lymphomas (Abraham, Basser, & Green, 1996). Anthracyclines are named based on their chemical structures, which consist of an anthraquinone-chromophore and the polycyclic ring system (Weiss, 1992). To date, there are more than 2,000 analogs of anthracyclines; however, only four analogs of anthracyclines are used clinically. Of these four, DOX stands alone in its effectiveness and commonality.

Doxorubicin can be traced back to 1958 when Farmitalia Research Laboratories of Milan, Italy, discovered an antibiotic (daunomycin) derived from a new species of *Streptomyces peucetius* (Di Marco et al., 1964). A few years later, the laboratory of Rhone-Poulenc in France also publicized a new antibiotic (rubidomyocin) from a different species of *Streptomyces peucetius* (Dubost et al., 1964). Both laboratories had uncovered the exact same substance, and the antibiotic was subsequently named DNR (Weiss, 1992). In 1964, DNR was approved to treat leukemia and lymphoma (Weiss, 1992). Farmitalia laboratory researchers then created DOX from a 14-hydroxy analog of DNR derived from a mutant form of *Streptomyces peucetius* (Arcamone et al., 1969), although it was not until 1974 that cancer patients in the United States started receiving DOX as a chemotherapy treatment (Weiss, 1992). Although the structures of DOX and DNR are very similar, the antitumor activity of DOX is more potent than that of DNR (Di Marco, Gaetani, & Scarpinato, 1969), and the spectrum of antineoplastic activities of DOX are much wider than those of DNR (Weiss, 1992).

Despite the positive anticancer effects of DOX, it was not until years later that scientists learned about its damaging side effects. The first report of DOX-induced cardiotoxicity was reported in 1973. It was characterized by deterioration of the

myocardium, assessed through echocardiograms and postmortem examinations (Lefrak, Pitha, Rosenheim, & Gottlieb, 1973). Later on it was discovered that DOX given at high doses (550mg/m^2) was associated with an increased incidence of heart failure (Swain, Whaley, & Ewer, 2003). As DOX use became more prolific, so did the evidence suggesting that it had a negative effect on more systems beyond that of the cardiovascular system (Goorin et al., 1990; Haq et al., 1985; Von Hoff et al., 1979).

As DOX use increased, so did clinicians' reports that its use was associated with a higher than average incidence of fatigue (Gilliam & St Clair, 2011). These reports of excessive fatigue were generally attributed to the cardiotoxic effects of DOX. As researchers became more aware of DOX's toxic effects on various physiological systems, they began to understand that fatigue could not be attributed to reductions in the cardiovascular system alone. Later, multiple investigators discovered that DOX does, in fact, exert a myotoxic effect that can interfere with normal skeletal muscle function (Hydock, Lien, Jensen, Schneider, & Hayward, 2011).

As researchers gained a better understanding of DOX, they searched to identify a means of overcoming its cardiotoxic side effects. To date, one of the most effective treatments capable of attenuating DOX-induced cardiotoxicity is with aerobic exercise (Chicco, Schneider, & Hayward, 2005; Hydock, Lien, Schneider, & Hayward, 2008; Wonders, Hydock, Schneider, & Hayward, 2008).

The ability to reduce the myotoxic effects of DOX could make a difference in a patient's ability to perform ADLs and maintain a degree of normalcy in his or her life. If true, this could greatly alter the patient's outlook on life and could mean the difference

between living independently or becoming dependent on others, which has a profound impact on a patient's quality of life.

Clinically, DOX administration results in a number of harmful effects to skeletal muscle, including weakness (Harada et al., 2004), reduced functional capacity (Gilliam & St Clair, 2011), reduced fiber size (Bonifati et al., 2000), increased rates of atrophy (Pfeiffer et al., 1997), and reduced body weight (Tozer et al., 2008). If these myotoxic effects can be mitigated, it might be possible to minimize cancer related fatigue, improve the cancer patient's quality of life, and increase his or her capacity to perform activities of daily living.

Perhaps no treatment is better suited to minimize these effects than with a form of resistance training (RT). Resistance training has been shown to increase fiber size (Mero et al., 2013), increase maximal strength in cancer patients (De Backer, Schep, Backx, Vreugdenhil, & Kuipers, 2009), minimize cancer related fatigue (Segal et al., 2003), and improve bone mineral density (Layne & Nelson, 1999). All of these adaptations stand in stark contrast to the myotoxic effects of DOX. Thus, it might be possible to mitigate the DOX-induced muscle dysfunction with a form of RT.

Previous research has shown that cardiac tissue is particularly sensitive to treatment with DOX (Hayward & Hydock, 2007; Ito et al., 1990). Subsequently, research has also demonstrated that DOX-induced cardiotoxicity can be attenuated by various forms of aerobic exercise (Chicco, Hydock, Schneider, & Hayward, 2006; Chicco et al., 2005; Hydock et al., 2008; Wonders et al., 2008). There are reports that DOX will also affect skeletal muscle. One such report demonstrated that DOX treatment increased calcium (Ca^{2+}) release from the sarcoplasmic reticulum (SR; Abramson et al., 1988).

Other reports have claimed that along with a rise in free intracellular Ca^{2+}, there is a decrease in the antioxidant capabilities of the cell, leading to an increase in free radical production (Doroshow, Tallent, & Schechter, 1985; Kanter, Hamlin, Unverferth, Davis, & Merola, 1985; Smuder, Kavazis, Min, & Powers, 2011; Wallace, 2003). Taken together, this rise in free intracellular Ca^{2+} is likely the result of oxidative damage to the SR, a reduced energy supply for SR Ca^{2+} uptake via compromised mitochondria, and alterations in the contractile properties of skeletal muscle. Thus, any treatment that could act as a short-term energy buffer could minimize the energy deficit caused by DOX.

For instance, creatine monohydrate (Cr) could be used to help minimize DOX-induced muscle dysfunction. The benefits of Cr as an ergogenic aid have been well documented (Clark, 1997; Kreider, 2003; Persky & Brazeau, 2001). It has the ability to improve muscular performance (Volek et al., 1997), enhance the phosphocreatine energy system (Persky & Brazeau, 2001), increase total Cr (Bemben & Lamont, 2005), and act as a mild antioxidant (Lawler, Barnes, Wu, Song, & Demaree, 2002). Because of these properties, Cr could be used as a possible approach to overcome DOX-induced muscle dysfunction. One of the key points of this study was to examine the role of Cr treatment on DOX-induced muscle dysfunction. If effective, it could be used as the first step on the road for its use as a possible nutraceutical to help alleviate the harmful effects of DOX.

Statement of the Purpose

The purpose of this study was fourfold: (a) to examine the effects of RT on DOX-induced muscle dysfunction and; (b) to investigate the effects of Cr on DOX-induced muscle dysfunction; (c) to investigate the combined effects of Cr and RT on DOX-

induced muscle dysfunction; and (d) to investigate the effects of RT and Cr on total Cr concentration.

Research Hypotheses

H1 Resistance training will protect against doxorubicin-induced myotoxicity by preserving maximal twitch force and attenuating fatigue.

H2 Creatine treatment will protect against doxorubicin-induced myotoxicity by preserving maximal twitch force and reducing fatigue.

H3 Combined resistance training and creatine treatment will provide additional protection against doxorubicin-induced dysfunction when compared to resistance training and creatine administered alone.

H4 Creatine treatment will increase in the total creatine concentration.

Need for the Study

Treatment with doxorubicin is associated with a number of debilitating side effects, such as its dose dependent cardiotoxicity and more recently discovered myotoxicity. The cardiotoxicity is commonly manifested in the form of dilated cardiomyopathy and congestive heart failure (Chatterjee, Zhang, Honbo, & Karliner, 2010). The mechanisms behind these negative effects are the result of an increase in oxidative stress, increase in intracellular iron, inhibition of NRG1/Erb signaling cascade, impairment of progenitor cell renewal and cardiac repair, and decreased vasculogenesis (Menna, Salvatorelli, & Minotti, 2008; Shi, Moon, Dawood, McManus, & Liu, 2011). To date, there is not an effective pharmacological agent that has the ability to prevent these side effects. However, aerobic exercise has been used effectively as a means for attenuating the decline in cardiac function (Chicco et al., 2005; Hydock et al., 2008; Wonders et al., 2008).

There is growing evidence that DOX also negatively affects skeletal muscle function (Ascensao et al., 2011; Hydock et al., 2011; van Norren et al., 2009). Although there is consensus that DOX has a negative impact on skeletal muscle function, the mechanisms behind the dysfunction remain elusive (Gilliam & St Clair, 2011). A number of mechanisms have been proposed to explain DOX-induced dysfunction. It has been suggested that the dysfunction could be a result of variations in tumor necrosis factor receptors (Gilliam & St Clair, 2011), changes in Ca^{2+} handling (van Norren et al., 2009), and disruptions in the SR (Abramson, Buck, Salama, Casida, & Pessah, 1988). Another possibility is that DOX could be interfering with key Ca^{2+} handling proteins such as the sarcoendoplasmic reticulum calcium ATPase (SERCA), which is instrumental in Ca^{2+} uptake during skeletal muscle relaxation (Periasamy & Kalyanasundaram, 2007). In cardiac tissue treated with DOX, there is a decrease in the antioxidant capabilities of the cardiac myocytes (Wallace, 2003). In diaphragm muscle treated with DOX, there is a similar reduction in antioxidant capabilities (Gilliam, Moylan, Callahan, Sumandea, & Reid, 2011). This idea is further evidenced by the activation of caspase-3, a key regulatory component of apoptosis triggered by high levels of oxidative stress (Smuder, et al., 2011). The increase in free radical production could lead to an altered functional capacity of the SR, possibly through lipid peroxidation. Furthermore, this altered Ca^{2+} handling via damaged SR could be a factor in the increased muscular fatigue commonly seen during DOX treatment.

In regard to the proposed mechanisms of DOX-induced muscle dysfunction, Cr can serve as a possible means of attenuating the DOX-induced muscle dysfunction. Creatine is a very popular ergogenic aid and its benefits on the phosphagen system have

been well studied (Clark, 1997; Kraemer & Volek, 1999; Persky & Brazeau, 2001). Creatine has been shown to increase muscular performance variables in combination with short-term, high intensity exercise (Persky & Brazeau, 2001). Other research has demonstrated that when a skeletal muscle is incubated in an *ex vivo* tissue bath with Cr, there is an increased force production and improved rates of time to fatigue (Head, Greenaway, & Chan, 2011). It has also been shown that supplemental Cr can improve the rates of muscle fatigue in mice with mitochondrial dysfunction (Eisner, Lenaers, & Hajnoczky, 2014).

Part of this study examined the effects of incubating muscles *ex vivo* with Cr prior to treatment with DOX. This treatment, in combination with RT, had the possibility of attenuating DOX-induced myotoxicity. This study is the first step in establishing guidelines for improving the quality of life for those with DOX-induced muscle dysfunction. To date, there is a gap in the literature as to the effects that RT has on DOX treated skeletal muscle with and without Cr supplementation. It is important for there to be a better scientific understanding of these factors since RT and Cr supplementation may be an effective treatment strategy in the palliative care of those undergoing chemotherapy with DOX. Table 1.1 provides abbreviations pertinent to this study.

Table 1.1

Abbreviations

Acronym	Definition
ADLs	Activities of daily living
ADP	Adenosine diphosphate
AGAT	Arginine glycine aminotransferase
AKT	Protein kinase b
AMP	Adenosine monophosphate
ARE	Antioxidant response element
ATP	Adenosine triphosphate
Ca^{2+}	Calcium
CAT	Catalase
CK	Creatine kinase
Cr	Creatine monohydrate
CreaT	Creatine transporter
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
EDL	Extensor digitorum longus
FOKO	Fox-head box O
GMAT	Guanidinoacetate methyltransferase
GPX	Glutathione peroxidase
IGF	1- insulin like growth factor 1
K	Krebs-Henseleit buffer
KEAP1	Kelch-like ECH-associated protein 1
MAPK	Mitogen activated protein kinases
mTOR	Mammalian target of rapmaycin
NADH	Nicotinamide adenine dinucleotide
Nrf2	Nuclear like factor 2
PCr	Phosphocreatine
P70 S6K	Ribosomal protein S6K
ROS	Reactive oxygen species
SERCA	Sarco/endoplasmic reticulum calcium-ATPase
SOD	Super oxide dismutase
SOL	Soleus
SR	Sarcoplasmic reticulum

Delimitations of the Study

Samples in this study were collected form 10 week-old male Sprague Dawley rats. Resistance training was simulated by using an elevated food and water model for six weeks. Skeletal muscle function was assessed using an *ex vivo* muscle function apparatus. Muscle dysfunction was induced by incubating excised skeletal muscle tissue in a 25μM DOX+Krebs-Henseleit (K) solution. Creatine was added to the incubating buffers at a concentration of 25 mM prior to DOX treatment.

Definition of Terms

Apoptosis. Programed cell death.

Creatine monohydrate. Synthetic form of creatine.

Doxorubicin. Chemotherapy agent approved by the Food and Drug Administration for the treatment of a wide variety of cancers.

Fatigue rate. Recording over time of the ability of a muscle to produce a less than anticipated response for a given stimulus.

Lipid peroxidation. Oxidative degradation of lipids.

Maximal twitch force. Highest recorded contractile response.

Protein carbonyl. Modification of amino acids side chains in proteins to carbonyl derivatives.

Rate of force decline. Rate at which a muscle relaxes from a contraction following stimulation.

Rate of force development. Rate at which a muscle develops contractile strength.

Reactive oxygen species. Chemically reactive molecules containing oxygen.

CHAPTER II

REVIEW OF LITERATURE

Cancer is an ever-growing threat to human life and longevity. As humans continue to surpass their life expectancy, the risk of cancer becomes ever more prevalent. Males have a 43.2% chance of developing cancer in their lifetime, whereas females have a 38% chance of developing cancer (Howlader et al., 2013). This cancer prevalence requires treatments that are versatile and effective.

One such treatment is the chemotherapy agent known as doxorubicin (DOX). Doxorubicin is an anthracycline antibiotic commonly used in the treatment of a wide range of cancers including leukemia, lymphoma, many types of carcinoma, and soft tissue sarcomas (Tacar, Sriamornsak, & Dass, 2013). Despite the therapeutic benefits of DOX, it also comes with a number of negative side effects that, if left unchecked, could severely alter the ability of the patient to perform activities of daily living (ADLs), and in some cases, even become life threatening (Martin, Vogel, Crown, & Mackey, 2005). These adverse side effects can become apparent within 2-3 days of administration (Chatterjee et al., 2010).

The most common side effects of DOX treatment include nausea, vomiting, neutropenia, alopecia, arrhythmias, heart failure, and skeletal muscle dysfunction (Greene, Nail, Fieler, Dudgeon, & Jones, 1994; Hydock et al., 2011; Shi, Moon, Dawood, Mcmanus, & Liu, 2011). Skeletal muscle dysfunction is of particular concern because of

its direct involvement ADLs and is relatively common in those patients undergoing treatment with DOX (Jennings-Sanders & Anderson, 2003; Schwartz, 2000; Smuder et al., 2011).

All ADLs involve varying degrees of skeletal muscle activity. Although these tasks are typically comprised of short bursts of activity, they are often taken for granted in healthy populations. However, in the patient undergoing chemotherapy, being unable to perform ADLs can have a profound effect on quality of life (Fukuse, Satoda, Hijiya, & Fujinaga, 2005; Jacobsen et al., 1999).

Doxorubicin

Doxorubicin exerts its antitumor activity through two main methods. First, DOX inhibits DNA Topoisomerase II, which prevents DNA replication (Tewey, Rowe, Yang, Halligan, & Liu, 1984). Secondly, DOX generates reactive oxygen species (ROS) leading to cellular, organelle and DNA damage. In addition, there is evidence that generation of ROS can lead to apoptotic cell death through activation of p53-DNA binding and activation of the intrinsic cascade (Janeesh & Abraham, 2014).

Reactive oxygen species are chemically reactive molecules that are generated from a variety of sources (Kohen & Nyska, 2002). While ROS are implicated as signaling molecules that help to maintain physiological homeostasis, excessive production of these ROS may overwhelm endogenous antioxidant defense mechanisms, leading to oxidative modification of key cellular macromolecules (Deavall, Martin, Horner, & Roberts, 2012) and possibly membrane damage through lipid peroxidation (Halliwell & Chirico, 1993). In the cancer cell, this is beneficial because it can eventually lead to apoptosis. But in healthy tissue, this is a cause of concern, especially

in cardiac and skeletal muscle cells. Doxorubicin has also been shown to increase the freely available amount of intracellular iron (Xu, Presson, & Richardson, 2005). This is important because as intracellular iron increases, it begins to form a redox cycle with the appropriate electron donors in the mitochondria, which can lead to the formation of superoxide at complex I in the electron transport chain (Berthiaume & Wallace, 2007). This disruption of mitochondrial function could eventually reduce the availability of adenosine triphosphate (ATP) and possibly interfere with energy-dependent actions of the cell.

Doxorubicin is unique because its structure allows for the formation of a semiquinone through loss of an electron (Zucchi & Danesi, 2003). This reduction is the result of exogenous nicotinamide adenine dinucleotidedehydrogenase (NADH) in the mitochondria. The formation of the semiquinone can then donate an electron to oxygen, which forms a superoxide anion. The production of superoxide molecules could be a possible mechanism for lipid peroxidation. Lipid peroxidation has been found to be increased by as much as four times in the DOX treated cell (Mimnaugh, Trush, Bhatnagar, & Gram, 1985). This suggests DOX can damage lipid bound organelles, further disrupting normal cellular function.

Lipid peroxidation is an important process in many disease states in both adults and infants (Mylonas & Kouretas, 1999). The process of lipid peroxidation does take place in small amounts in the human body, but in excess, it can lead to severe organelle dysfunction. With lipid peroxidation, ROS readily attack the polyunsaturated fatty acids of any fatty acid membrane, which leads to a domino effect of continuous oxidation

(Mylonas & Kouretas, 1999). The destruction of membrane lipids and the end products of such lipid peroxidation reactions are especially dangerous for the viability of cells.

Natural antioxidants such as superoxide dismutase (SOD) and catalase (CAT) can quickly be overwhelmed, causing a continual process of lipid peroxidation to take place (Mukhopadhyay, Ghosh, & Chatterjee, 1995). Since lipid peroxidation is a chain reaction, the initial oxidation of only a few lipid molecules can result in significant cell damage or cell death (Deviatkina, Tarasenko, & Voskresenskii, 1984; Mylonas & Kouretas, 1999).

Although ROS are important in maintaining normal physiological function, in excess they can lead to severe damage of cellular components (Valko et al., 2007). Normally, the generation of ROS is balanced by antioxidant defense systems regulated by a web of pathways to ensure the correct response. Nuclear like factor 2 (Nrf2) is the primary regulator of cellular resistance to ROS (de Grey, 2000; Itoh et al., 1999). Normally, Nrf2 binds to the antioxidant response element (ARE) in the promoter region of genes that code for proteins that are ROS defenses, such as glutathione transferase, NADPH dehydrogenase, and glucuronsyltransferase (Vomhof-Dekrey & Picklo, 2012).

Understanding how Nrf2 typically works is essential in order to better understand how its lack of activation would affect the DOX treated cell. Nuclear like factor 2 regularly remains bound by a reducer known as kelch-like ECH-associated protein (KEAP1). The KEAP1 keeps Nrf2 in the inactive state unless activated by ROS sensitive cysteine residues. Once these cysteine residues are activated, it causes a conformational change in KEAP1, resulting in the release of Nrf2. Once free, Nrf2 is free to bind to

ARE and lead to the transcription of anti-oxidant enzymes (Nordgren & Wallace, 2014). These systems are often compromised with DOX treatment (Kohen & Nyska, 2002).

Redox cycling plays an important role in normal cellular function, but when this redox cycling is disturbed, it can cause the activation of a number of proteolytic pathways, including activation of calpains and caspase 3 (Smuder et al., 2011). Caspase 3 is important because it plays a key role in muscle protein degradation (Du et al., 2004). It is important to note that caspase 3 can be activated by both intrinsic and extrinsic pathways, eventually leading to apoptosis (Earnshaw, Martins, & Kaufmann, 1999). The possibility exists that the rise in mitochondrial oxidative stress and its eventual dysfunction could be a trigger for the activation of caspase 3 and eventual apoptosis. Further complicating this issue could be the activation of calpains. Calpains are a class of proteins that play an important role in proteolysis and cell cycle progression (Kawasaki & Kawashima, 1996). Activation of calpains is often Ca^{2+}-dependent and they play an important role in skeletal muscle atrophy (Goll, Thompson, Li, Wei, & Cong, 2003). It should be noted that when there is a rise in ROS, there is often a rise in Ca^{2+} (Goll et al., 2003). The rise in ROS could potentially lead to direct proteolysis via direct oxidative modification.

Doxorubicin's toxic effects have usually been attributed to its ROS formation, but there is some evidence to support the idea that DOX directly interacts with apoptotic cell signaling (Eom et al., 2005; Zhang, Shi, Li, & Wei, 2009). As mentioned before, DOX will lead to mitochondrial DNA damage, mitochondrial membrane disruption, mitochondrial dysfunction, and ATP depletion, which can lead to eventual necrosis and cell death (Lebrecht & Walker, 2007).

Previous research has shown that DOX will lead to apoptosis via activation of p53 (X. Liu, Chua, & Gao, 2004), down regulation of GATA-4 (Kim, Ma, & Kitta, 2003), and degradation of p300 (Poizat, Puri, Bai, & Kedes, 2005). It is important to note that GATA-4 is an upstream activator of BCL-X. This is a key survival factor for postnatal, differentiated cardiomyocytes. P300 is an important component of the cell cycle, differentiation, tumor genesis, and apoptosis (Polich, 1998). In the DOX treated cell, the activation of p38 kinases alpha and beta actively degrade p300, which can lead to apoptosis. Evidence also supports the idea DOX can lead to activation of apoptosis via the antigen 1 (FAS) receptor and its ultimate activation of caspase-8 in the S-type neuroblastoma (Fluda, Sieverts, Friesen, Herr, & Debatin, 1997). Interestingly, there is evidence to suggest that DOX may still lead to apoptosis even when caspase-8 has been silenced, as well as in cells that have been silenced for FAS mediated apoptosis (Shain, Landowski, Buyuksal, Cantor, & Dalton, 2000). Taken together, it is clear to see that DOX can be effective at triggering apoptosis even when key signaling elements have been removed. It has also been proposed that DOX may also exert its effects through the up-regulation of the E3 upiquitin-ligase atrogin 1/MAFbx, suggesting that atrophy could be occurring through the proteasome pathway (Yamamoto et al., 2008).

Cardiac Dysfunction

The first report of DOX-induced cardiotoxicity was in 1973. It was characterized by deterioration of the myocardium assessed through echocardiograms and postmortem examinations (Lefrak, Pitha, Rosenheim, & Gottlieb, 1973). Later on it was discovered DOX given at high doses (550mg/m^2) was associated with an increased incidence of heart failure (Swain, Whaley, & Ewer, 2003).

In human studies, DOX administration has been associated with a decreased ejection fraction and altered diastolic function (Haq et al., 1985; Swain et al., 2003). Similarly, animals treated with DOX have reduced left ventricle pressure, reduced ejection fraction, altered fractional shortening, and increased diastolic pressure (Hayward & Hydock, 2007; Hydock et al., 2008; Kanter et al., 1985; Singal, Li, Kumar, Danelisen, & Iliskovic, 2000; Wonders et al., 2008). With higher levels of DOX administration, there is a corresponding reduction of myocardium around the left ventricle (Chatterjee et al., 2010; Hayward & Hydock, 2007). The reduction of the myocardium is a major factor in the development of heart failure and skeletal muscle dysfunction (Hydock et al., 2011).

Several studies have shown the changes resulting from heart failure will eventually result in reduced blood flow and become a contributing factor in the development of skeletal muscle dysfunction (Dalla Libera, Vescovo, & Volterrani, 2008; Harrington et al., 1997; Hydock et al., 2011). Furthermore, the altered cardiovascular dynamics could also lead to physiological changes within the musculature (Hydock et al., 2011). Changes in the musculature could include fiber type shifts towards a more anaerobic type. Yet, there are also other external factors to consider such as the proclivity of cancer patients to engage in sedentary activities (Inui, 2002) and inadequate energy consumption (Adamsen et al., 2009), which could further alter skeletal muscle characteristics. All of these would eventually become compounding variables that would only serve to enhance fatigue and muscle dysfunction. Together, this demonstrates the need for understanding DOX-induced muscle dysfunction outside of these compounding effects.

Muscle Dysfunction

Physician reports have noted lower extremity muscle weakness in patients undergoing chemotherapy with DOX (Harada et al., 2004). Functionally, patients receiving DOX have reduced 12-minute walk distances and increases in reported fatigue (Gilliam & St Clair, 2011). In animal models, it has been shown that DOX administration results in a loss of muscle mass, altered myofilament structure, and depressed force production (McLoon, Luo, & Wirtschafter, 1993). Doxorubicin-induced muscle dysfunction most commonly manifests itself in the form of reduced maximal twitch force, reduced rate of force decline and rate of force development, and increased fatigability (De Beer, Finkle, Voest, Van Heijst, & Schiereck, 1992; Hydock et al., 2011).

Clinical studies have shown that patients receiving DOX often report lower limb weakness (Jhamb et al., 2007) and a reduced exercise tolerance (Schwartz, 2000). Furthermore, leukemia patients who received treatment with DOX have exercise intolerance for one to five years following chemotherapy (Elbl et al., 2006). Moreover, limb strength is often compromised in patients undergoing treatment with DOX, providing further evidence of the effects of DOX on skeletal muscle (Galvao, Taaffe, Spry, Joseph, & Newton, 2010; Galvao et al., 2009). Additionally, patients receiving isolated limb perfusions of DOX have shown a reduction of size in both type 1 and type 2 fibers (Bonifati et al., 2000). Similarly, patients undergoing chemotherapy with DOX often display decreased body weight and muscle mass (Tozer et al., 2008). It has also been shown that the degree of limb atrophy correlates with the DOX concentration within the quadriceps (Pfeiffer et al., 1997).

Further complicating the issue is that DOX also exerts a negative effect on the respiratory muscles. Up to 70% of patients receiving DOX show a depressed maximal oxygen consumption and premature fatigue during exercise testing (Elbl et al., 2006). There is further evidence that those receiving DOX also have reduced maximal inspiratory pressures (Travers et al., 2008). This suggests a compromised diaphragm and possible dyspnea. Dyspnea is often associated with fatigue during chemotherapy (Ripamonti, 1999).

Other studies have noted DOX's myotoxic effects from a therapeutic aspect. Local DOX injections have been used clinically to treat blepharospasm, hemifacial spasm, and other related disorders permanently and non-surgically (McLoon, Ekern, & Wirtschafter, 1992). In a study by Goding and Pernell (2000), clinical use of DOX for management of spasmodic dysphonia was investigated as a possible long-term treatment. The authors attempted to use DOX to weaken the canine vocal cord closure without a notable disruption in the mucosal wave. They found that 3 mg of DOX was capable of reducing tension by 74% compared to an average side to side difference of 12% in the control group (Goding & Pernell, 2000).

Another study investigated the use of DOX on rabbit sternocleidomastoid muscle and found similar results. Rabbits injected with 2 mg/kg of DOX were found to have a reduced cross-sectional area of 71% versus control animals. Total muscle strength was 50% of controls two months post injection. It is also interesting to note that the DOX treated sternocleidomastoid muscle had lower force development at all frequencies, although the lower frequency stimulation failed to produce a significant response (Falkenberg, Iaizzo, &McLoon, 2001).

In one of the first studies to examine the direct role of DOX-induced muscle dysfunction, it was observed that DOX produced an increase in tension that was dependent on the time and Ca^{2+} concentrations used (De Beer et al., 1992). The authors of this study believed the increased tension generated was likely a result of DOX-myosin ATPase interaction (De Beer et al., 1992). Doxorubicin injected directly into the eyelids of primates showed myofibrillar dissolution within the orbicularis oculi muscle at five minutes post injections (McLoon, Luo, &Wirtschafter, 1993). Similar results were found by McLoon and colleagues (1992) who injected DOX into the eyelids of monkeys with a combination of 1 mg of DOX and 0.25 mg Verapamil, which led to significant loss in muscle mass (McLoon et al., 1992). Loss of myofibrillar organization and interstitial edema has been found following a single injection of DOX (Doroshow et al., 1985). Further studies have noted that DOX treatment will also result in nucleolar segregation and altered perinucleolar chromatin distribution (Merski, Daskal, & Busch, 1978; Van Vleet & Ferrans, 1980). It is clear that DOX has a powerful effect on skeletal muscle, but the mechanism by which DOX exerts this effect is less clear.

Doxorubicin has been known to interfere with the respiratory chain and inhibit oxidative phosphorylation (Smuder et al., 2011; van Norren et al., 2009). This distorted mitochondrial function is believed to increase the formation of ROS and leads to altered energy availability and altered cellular function. Further complicating the issue is that when energy stores start to deteriorate, there is often a rise in free intracellular Ca^{2+} (Honda, Kondo, Zhao, Feril, & Kitagawa, 2004). In a normal cell, the rise in free intracellular Ca^{2+} is the result of Ca^{2+} flowing down its concentration gradients and then is actively pumped out of the cytosol. In the DOX treated cell, the picture becomes less

clear because DOX's possible effect on the SR could be helping to contribute to a prolonged rise in free intracellular Ca^{2+} via damage from the generation of ROS or the direct interference in Ca^{2+} handling proteins. This idea was best evidenced by an early study done by De Beer and colleagues (1992) found that there is increased Ca^{2+} sensitivity in single skinned muscle fibers treated with DOX during low frequency stimulation, but there was not an increased force production during high frequency stimulation. Direct incubation with 100-175 μm of DOX for one hour resulted in a time and contraction dependent decrease in maximal force (van Norren et al., 2009). Furthermore, in the presence of DOX, maximal force increased during the contraction phase at lower frequencies (40 Hz) and tended to disappear at higher frequencies (van Norren et al., 2009). This suggests there is an effect from DOX on the SR, or at least some effect on the Ca^{2+} handling that results in a transient Ca^{2+} increase in the cytosol. The authors of the previous study suggested the rise in Ca^{2+} was not a result of DOX acting directly on the SR itself, but action of DOX affecting Ca^{2+} reuptake by the SR, which leads to increased Ca^{2+} responses and prolonged relaxation times.

Doxorubicin at a low dose of 1.15 mg/kg leads to a reduction in force production in both type 1 and type 2 muscles (Ertunc, Sara, Korkusuz, & Onur, 2009). The authors of this study believed the loss of force was the result of a reduction in the muscle-specific isoform of the sarco/endoplasmic reticulum ATPase (SERCA). This loss of the muscle-specific isoform of SERCA could also help to explain the rise in free intracellular Ca^{2+}.

There is consensus that DOX has the ability to interfere with the contractile properties of skeletal muscle, but the exact mechanism by which this occurs is still elusive. The most plausible explanation behind this dysfunction is that it is the result of

two distinct properties. First, the SR dysfunction is the result of oxidative damage to the lipid membranes and the oxidation of Ca^{2+} handling proteins. Second, the damage that occurs to the mitochondria via the excessive generation of free radicals leads to a reduced adenosine triphosphate (ATP) supply and reduced energy availability for cellular functions. If these two possible modes of dysfunction are correct, it could be possible to alter the toxic effects of DOX with interventions that have the ability to enhance ATP supply and preserve function.

Exercise and Doxorubicin

Doxorubicin is a powerful chemotherapy agent with potent anticancer properties. Because DOX's use is limited by the development of its side effects (Chatterjee et al., 2010), any treatment that mitigates the progression of these side effects should receive increased attention. If these side effects can be mitigated, the amount of DOX given during chemotherapy treatment could be increased, it could improve the patient's quality of life, and increase the survivability of treatment with DOX. One of the most successful methods to mitigate the side effects of DOX is with exercise.

Studies examining the role of aerobic exercise prior to DOX treatment found that trained animals typically have a higher end systolic pressure, left ventricular developed pressure, and maximal rate of left ventricular pressure development (Chicco et al., 2005; Hydock et al., 2008; Wonders et al., 2008). Low intensity exercise training prior to and during treatment was also shown to preserve cardiac function (Chicco et al., 2005, 2006). There is also evidence that a single bout of exercise at 25 m/min for 60 min prior to DOX treatment with 15 mg/kg, resulted in a cardio protective effect with preserved end systolic pressure and maximal developed left ventricle pressure (Wonders et al., 2008). Aerobic

exercise can also lead to the attenuation of the DOX-induced mitochondrial dysfunction at complexes I and V, decreased caspase 3 and 9 activities, and increased cardiac mitochondrial SOD activity (Ascensao et al., 2011). Furthermore, chronic exercise training has been beneficial in the protection of DOX-induced vascular dysfunction. Rats that engaged in voluntary wheel running for 14 weeks had significantly greater smooth muscle contractile force and improved endothelium-independent relaxation times 24 hours after a single DOX dose of 15 mg/kg (Gibson, Greufe, Hydock, & Hayward, 2013).

In order to maintain homeostasis, it is critical that the body be able to correctly manage its antioxidant status. This suggests that there is balance between the production of ROS and the ability of the cell to quench these through its antioxidant defenses. Exercise has been shown to increase superoxide dismutase 1 (SOD1), superoxide dismutase 2 (SOD2), and glutathione peroxidase (GPX) levels in skeletal muscle (Ji, 2008). As mentioned earlier, DOX is a powerful chemotherapy agent that can greatly increase the oxidative state of the cell. This rise in the ROS is troublesome to non-cancerous tissues and is likely a contributing mechanism to the cardiotoxicity, myotoxicity, and fatigue commonly seen with DOX treatment. Although there is little doubt that aerobic exercise has a potent effect on mitigating DOX-induced cardiotoxicity, there has been no investigation on the ability of exercise to offset DOX-induced muscle dysfunction.

There is growing evidence that exercise can be effective at minimizing cancer related fatigue and improving quality of life (Quist et al., 2006; Spence, Heesch, & Brown, 2010). Empirical evidence does overwhelmingly suggest that exercise, in general, is a safe and effective during the administration of adjuvant cancer therapies

(Cheema, Gaul, Lane, & Fiatarone Singh, 2008; Jones & Demark-Wahnefried, 2006; McNeely et al., 2006). However, the appropriate modalities and dosages required to produce these positive effects have yet to be fully elucidated. Even with the prevailing focus upon the effectiveness of aerobic exercise, there is a growing body of data for the benefits of other forms of exercise (Focht et al., 2013). For example, resistance training (RT) produces a number of beneficial adaptations that could be effective at minimizing the clinically relevant physiological (muscle mass, strength, bone density, and body composition) and quality of life outcomes in cancer patients and survivors.

In humans, RT has the capability to produce a wide range of adaptations such as improved muscular performance, increased rates of hypertrophy, improved body composition, increased appetite, and increased bone strength (Winett & Carpinelli, 2001). Furthermore, RT has been used as a means of palliative care and has been shown to reduce cancer-related fatigue (Adamsen et al., 2009; Meneses-Echavez, Gonzalez-Jimenez, & Ramirez-Velez, 2014), improve muscular strength in patients with heart failure (Gielen, Laughlin, O'Conner, & Duncker, 2014), preserve muscle mass in patients with heart failure (Gielen et al., 2014), promote increased blood cell counts in those going through chemotherapy (Karvinen, Esposito, Raedeke, Vick, & Walker, 2014), and improve breast cancer outcomes during chemotherapy (Courneya et al., 2014). These demonstrate the benefits from RT stand in sharp contrast to myotoxicity seen with DOX. Thus, it can be concluded that RT could be used to minimize DOX-induced muscle dysfunction.

At the cellular level, RT produces a number of favorable adaptations, which could be used to offset the myotoxic effects of DOX. Resistance training results in the

activation of protein kinase B (Akt), mammalian target of rapmacyacin (mTOR), and mitogen-activated protein kinase (MAPK) signaling pathways (Bodine et al., 2001). The activation of these cellular pathways can lead to the downstream activation of the ribosomal protein S6K (p70 S6K) (Terada et al., 1994). The activation of p70 S6K ultimately results in an increase in protein synthesis and an increase in muscle size (Spiering et al., 2008). In addition, AKT can also phosphorylate the fox-head box O (FOXO) family of transcription factors, which can prevent FOXO from stimulating the transcription of some proteolytic ubiquitin ligases (Sartorelli & Fulco, 2004). Furthermore, RT can also increase circulating concentrations of insulin like growth factor 1(IGF-1) and growth hormone (Spiering et al., 2008). Elevations in both of these hormones help to improve the likelihood for an increase in protein synthesis and overall muscle size. Furthermore, a greater muscle size could increase the capacity of cancer patients to perform ADLs and improve their overall quality of life.

Resistance training has been shown to be beneficial for minimizing fatigue and improving quality of life in cancer patients (Courneya & Friedenreich, 1999). However, there is substantially less information about the appropriate dosages for the application of RT. It is important that further studies be done to identify the appropriate application of RT, and in a manner that is clinical relevant to cancer patients

Current evidence suggests that a RT period from six weeks to six months is required to minimize the side effects of the cancer treatment process. The inclusion of RT produces meaningful improvements in a wide variety of physiological and quality of life outcomes during the cancer treatment process. However, there is a great deal of variability in the improvements seen with RT (Focht et al., 2013). In general, evidence

does suggest that RT will produce a statistically significant increase in muscular strength, muscular endurance, improvements in physical function, and some improvements in quality of life (Howlader et al., 2013; Speck, Courneya, Masse, Duval, & Schmitz, 2010). It is difficult to extrapolate the findings of clinical studies to general cancer rehabilitation recommendations because of the different cancer types, the kinds of treatment, and treatment stage at which the RT was implicated. As a result, it can be hard to generalize the findings of these studies to larger populations. Animal-based studies, on the other hand, can provide an alternative to the compounding variables commonly seen with clinical type studies. By utilizing these types of studies, scientists can develop treatment recommendations based on the treatment type and level.

Most studies involving small animals generally report that RT produces a similar set of adaptations to that of humans (Cholewa et al., 2014). In response to RT, both humans and small animals show an increase in hypertrophy, improvements in skeletal muscle strength, and an increase in protein synthesis (Cholewa et al., 2014). Furthermore, studies involving animals allow for the researchers to standardized the treatment, and reduce the overall variability of the study. In addition, it lets researchers study RT in a setting away from the secondary disease process (e.g., heart failure, cachexia, inadequate energy consumption) that take place in some cancer patients.

Most animal studies use a weighted vest, tail weights, weighted ladder climbing, or weighted swimming to simulate anaerobic exercise (Cholewa et al., 2014). However, there is little carryover from these investigations that can be translated into future clinical recommendations. A secondary concern from these types of investigations is the level of distress experienced by the animal. The experienced distress could become a

compounding variable in the interpretation of the results of these studies. Thus, it would be advisable to incorporate a model in which animal distress is minimized, is clinically relevant, and produces a meaningful and reproducible effect.

In comparison to aerobic training, there is considerably less evidence regarding the benefits of RT on minimizing the side effects of chemotherapy. However, the available data suggested RT represents an exercise intervention that may produce a wide range of health benefits during the cancer treatment process (Antonelli, Freedland, & Jones, 2009; Courneya, 2009; Courneya & Friedenreich, 2001; Focht et al., 2013). From this, we can conclude that exercise not only has the potential to minimize the myotoxic effects of DOX but could also help minimize its effects on other bodily systems. To date, there have been no investigations on the ability of prior RT to minimize DOX-induced skeletal muscle dysfunction.

Creatine

Creatine is a natural occurring substance that is typically supplied through the diet and is located throughout the body, with the vast majority of it being stored in skeletal muscle (Walker, 1979). The use of supplemental Cr can be traced back to the early 20th century when Harvard researchers first discovered that ingesting Cr produced an increase in intramuscular Cr stores (Folin & Denis, 1912). A few years later, scientists started to understand the ability of phosphocreatine (PCr) to donate a phosphate to adenosine diphosphate (ADP) and produce ATP (Fiske & Subbarow, 1927). The use of supplementary Cr as an ergogenic aid has become increasingly popular since the early 1990s (Demant & Rhodes, 1999). Supplementation with exogenous Cr can produce a wide range of benefits including preventing reductions in PCr, increased fiber size,

increases in total lean body mass, and increased rates of protein synthesis (Persky & Brazeau, 2001). It has also been shown that Cr can provide powerful performance-enhancing effects in the form of increased force production, reduced fatigue, and increased muscle mass (Persky & Brazeau, 2001). The benefits of Cr could therefore provide a means of overcoming DOX-induced muscle dysfunction.

Creatine is a naturally occurring compound obtained from endogenous production and dietary intake (Persky & Brazeau, 2001). The synthesis of Cr is the product of 3 key amino acids: arginine, methionine, and glycine. The primary reaction in the formation of Cr is the formation of guanidinoacetate. In this reaction, the compounds glycine and arginine are catalyzed by the kidney enzyme arginine glycine amino transferase (AGAT) (Wyss & Kaddurah-Daouk, 2000). This primary reaction is considered to be the rate-limiting step in the formation of Cr. The second reaction takes place in the liver and is the methylation of guanidinoacetate, which produces Cr. The process of methylation involves the donation of the methyl group from methionine via the enzyme guanidinoacetate N-methyltransferase (GAMT; Persky & Brazeau, 2001; Wyss & Kaddurah-Daouk, 2000). Once Cr reaches a particular level, it acts as a negative feedback mechanism, which is able to inhibit AGAT mRNA (Wyss & Kaddurah-Daouk, 2000).

Once produced, Cr is transported throughout the body by the blood stream where it is taken up by working tissues via a sodium- and chloride-dependent transporter (CreaT; Guerrero-Ontiveros & Wallimann, 1998). The CreaT transporter is expressed in a variety of tissues including kidney, heart, skeletal muscle, brain, testis, and colon, but

not in the liver, pancreas or intestine (Guimbal & Kilimann, 1993; Nash et al., 1994; Sora et al., 1994).

In the muscle cell, total Cr content is dependent on fiber types, catecholamines, IGF-1, insulin, and exercise (Persky & Brazeau, 2001); however, the most important factor regarding total Cr content is probably the fiber type itself. In the rat, type 1 fibers from the soleus show total concentrations of Cr at 23±1 μmol/gww, whereas type 2 fibers from the EDL show concentrations of 35±1 μmol/gww of Cr (Kushmerick, Moerland, & Wiseman, 1992; Willott et al., 1999). In human skeletal muscle, Cr levels can range from 110 to 160 mmol/kg (Bemben & Lamont, 2005; Harris, Hultman, & Nordesjo, 1974).

Upon entering the cell, Cr provides a number of protective and energy-sparing effects that are likely responsible for its improvements in muscle function. The primary benefits from Cr are its energy-preserving effects, its effects on protein synthesis, and its ability to facilitate membrane stabilization (Persky & Brazeau, 2001).

Adenosine triphosphate (ATP) is the energy currency for most biological systems in the human body (Lehninger, 1982). Excitable cells and tissues depend on large amounts of ATP that are used in a fluctuating manner, and maintaining this supply of energy is of critical importance. In addition, many key cellular functions are regulated by ATP, ADP and AMP, as well as the ATP/ADP ratio (Tornroth-Horsefield & Neutze, 2008). Given that most cells only contain a small amount of freely available ATP (2-5 mM) (Beis & Newsholme, 1975), which could only provide a few seconds of ATP, a system needs to be in place to rapidly provide the cell with the needed energy when the time arises. To facilitate this transient and localized energy demand, Cr and PCr are in

rapid exchange because of the process of trans-phosphorylation via creatine kinase (CK; Harris, Soderlund, & Hultman, 1992).

Creatine kinase works by transferring the phosphate group of ATP to Cr. This reaction results in the formation PCr. The stored inorganic phosphate can be utilized for the immediate ATP generation in periods of acute need. Phosphocreatine is unique because it protects the cell from a reduction in energy supply and acts as a pH buffer (Persky&Brazeau, 2001). The energy-sparing effects of PCr come from its ability to preserve ATP availability and prevent the excessive buildup of ADP, which could alter the rate of energy-dependent actions like cross bridge cycling. It should be mentioned that there are two key CK enzymes--one that functions within the cytosol and one that functions within the mitochondria (Clark, 1997).

The mitochondrial CK is found within the mitochondrial intermembrane space, where it works to produce PCr from mitochondrial generated ATP and imported Cr from the cytosol (Schlattner, Tokarska-Schlattner, & Wallimann, 2006). Together, both the cytosolic and mitochondrial CKs work together in an ATP dependent process known as PCr/Cr shuttle. This system involves the shuttling of mitochondrial produced PCr by mitochondrial CK to cytosolic CK. Once accepted, the PCr can be used to generate ATP. This suggests PCr is not only acting as an energy buffer, but also as a cellular energy transporter from areas of energy production to areas of energy utilization (Schlattner et al., 2006).

Exercise and Creatine

Well over 500 research studies have evaluated the effects of Cr supplementation on muscle physiology and/or exercise capacity in healthy, trained, and various diseased

populations (Kreider, 2003). A number of studies have analyzed the effects of Cr with RT or other short-term activities such as sprint performance; however, early data were not conclusive, with some reporting no measurable benefit and others reporting marked improvements with Cr. A review by Rawson and Volek (2003) found Cr supplementation with RT generally produced an 8% greater increase in muscular strength when compared to resistance training alone.

Another review found that nearly 70% of studies examining the effects of Cr supplementation found noticeable improvements in high-intensity performance with Cr supplementation while the other 30% showed no effect. It was reported that short-term supplementation with Cr was able to improve maximal strength by 5-15% (Kreider, 2003). Although most cancer patients do not engage in regular physical activity, they do need to perform ADLs. The nature of ADLs is quite similar to that of RT. Both RT and ADLs usually involve short bursts of activity and rely heavily on Cr/PCr system. If the same benefits can be attained in the DOX treated muscle, it is possible to offset DOX's myotoxic effects with the combination of RT and Cr supplementation. If true, this could provide the cancer patient with increased capacity to perform ADLs, improve muscular endurance, and minimize fatigue. Despite the fact that not all studies have found significant improvements in performance, the great preponderance of gathered evidence does suggest Cr is an effective nutritional ergogenic aid for a variety of exercises and possibly for clinical populations.

Exercise intolerance is a major limiting symptom in patients receiving chemotherapy (Jones et al., 2011). Part of this fatigue is attributable to chemotherapy-induced cardiotoxicity and the systemic effects of a reduced blood supply. Exercises that

improve hemodynamics and left ventricle function do not improve exercise capacity immediately when blood flow is increased (Wilson, Martin, & Ferraro, 1984; Wilson, Martin, Ferraro, & Weber, 1983). If short-term energy supplies can be increased, it might be possible to minimize this exercise intolerance until energy production from glycolytic and oxidative sources can catch up.

Studies that use phosphorus^{-31} nuclear magnetic resonance have shown significant reductions in PCr levels in patients with heart failure (Massie et al., 1988). In the DOX treated heart, there is a similar reduction of intracellular Cr and ATP (Pelikan et al., 1986). However, intracellular levels of Cr and ATP have not been examined in the DOX treated muscle. Given Cr's multifaceted role in energy maintenance, it could be plausible that changes in cellular metabolism resulting from alterations in the levels of Cr and PCr in skeletal muscle could be contributing to the development of exercise intolerance and excessive fatigue. If this reduction in intracellular Cr could be minimized, it might be possible to attenuate some of the negative changes in muscle metabolism during DOX treatment.

The use of RT and supplementary Cr could be a possible treatment to help minimize DOX-induced muscle dysfunction. Interestingly, heavy exercise has been shown to improve PCr resynthesis after exercise (Arnold, Matthews, & Radda, 1984). With the appropriate application of RT, it may be possible to facilitate Cr uptake in the DOX-treated muscle. If intracellular Cr can be increased, it could enhance short-term ATP supply, improve muscle function, and minimize fatigue.

Supplementation with Cr generally produces an increase in intracellular Cr during the initial loading phase, but as intracellular CR rises, it begins to reduce CreaT

expression and slow Cr uptake (Snow & Murphy, 2001). However, supplementary Cr will produce an overall increase in the rate of PCr resynthesis and total Cr (Greenhaff, Bodin, Soderlund, & Hultman, 1994). Interestingly, supplementation with Cr does enhance muscle PCr resynthesis during the second minute of recovery from intense muscular contraction (Greenhaff et al., 1994). Furthermore, elevated pre-exercise muscle PCr levels generated by Cr loading have been shown to be maintained during dynamic intermittent muscle contractions (Vandenberghe, Van Hecke, Van Leemputte, Vanstapel, & Hespel, 1999). Thus, it can be concluded that RT could enhance Cr uptake and help to preserve muscle function.

Taken together, the benefits of combined treatment with RT and Cr could improve muscle function and minimize fatigue, lessen the changes in cellular metabolism, increase intracellular Cr stores, and improve overall functional capacity. Each of these benefits could help alleviate some the deleterious side effects of treatment with DOX and provide cancer patients with a better quality of life and an increased capacity to perform ADLs.

Creatine and Doxorubicin

Doxorubicin is a common and well-studied chemotherapy agent that comes with a number of side effects that can lead to a reduction in skeletal muscle function via a variety of mechanisms, whereas Cr can improve skeletal muscle function and enhance the PCr system. Taken together, the possibility exists that pretreatment with Cr might mitigate some of the myotoxic effects of DOX. To date, few studies exist examining the role of supplementation with Cr on DOX-induced muscle dysfunction.

Deficits in energy metabolism are believed to be one of the mechanisms that lead to DOX-induced cardiotoxicity. Although the exact mechanisms behind DOX-induced alterations in cardiac energy metabolism have yet to be fully understood, it has been shown that DOX causes a decrease in high-energy phosphates and a reduction in the ratio of PCr to ATP (Darrabie et al., 2012; Maslov et al., 2010; Ohhara, Kanaide, & Nakamura, 1981; Seraydarian, Artaza, & Goodman, 1977). This suggests that as the cardiomyocytes begin to rely on more anaerobic sources of energy, they lose the ability to properly couple mitochondrially produced ATP to the recycling of PCr and ADP. Furthermore, it has also been noted that DOX can lower levels of Cr and CK in cardiac myocytes (Tokarska-Schlattner, Zaugg, Zuppinger, Wallimann, & Schlattner, 2006). Additionally, DOX has been shown to reduce Cr transport, decrease V_{max}, lower $K_{m,}$ and reduce the amount of CreaT present at the cell surface in cultured cardiomyocytes (Santacruz et al., 2014). Although this research was conducted on cardiomyocytes, it does provide some help in explaining the DOX-induced muscle dysfunction from an energy standpoint. The possibility of a reduction of ATP and a reduced PCr and ATP ratio could help explain the prolonged rise in Ca^{2+} in DOX treated muscle cells. Because of the reduced ATP availability, there is lack of energy to facilitate the movement of Ca^{2+} against its concentration gradient, thus leading to an altered cross bridge formation and compromised skeletal muscle function.

Despite Cr's notable benefit to the PCr system, there has been little investigation as to its ability to minimize DOX's cytotoxic effects. Cultured cardiomyocytes treated with a 5 nmol/L solution of Cr have shown reduced rates of apoptosis and lower levels of ROS production following DOX treatment when compared to non Cr treated controls

(Santacruz et al., 2014). Rats treated with 2 mg/kg of Cr per day for 30 days prior to a 15 mg/kg IP injection of DOX were found to have lower levels of serum lactate dehydrogenase and creatinine levels compared to DOX-treated controls (Santos, Batista, Caperuto, & Costa Rosa, 2007). These results demonstrate that there is some capacity for Cr to mitigate the toxic effects of DOX.

To date, no study has been conducted to assess the ability of Cr to mitigate myotoxic effects of DOX. A pilot study using 10-week-old male Sprague-Dawley rats was conducted on the feasibility of Cr to mitigate the myotoxic effects of DOX. In this study, excised SOL and EDL muscles were treated with either a Krebs-Henseleit buffer (K) or K containing 25 mM of Cr. Tissues were then treated with K or K containing 24 μM of DOX. The results for this pilot study indicated Cr pretreatment protected against DOX-induced muscle fatigue in the SOL (see Appendix A). Additionally, a trend did show some Cr-induced protection against DOX-dysfunction in the EDL. This pilot suggested Cr exerted a protective effect on skeletal muscle.

Summary

Doxorubicin-induced muscle dysfunction most commonly manifests itself in the form of reduced maximal twitch force, reduced rate of force decline and force development, and increased fatigue ability (De Beer et al., 1992; Hydock et al., 2008, 2011; van Norren et al., 2009). Creatine is unique in the fact that it is a well-studied ergogenic substance with documented effects on improving human performance (Persky & Brazeau, 2001). Typically, these benefits are the result of the enhancement of Cr/Pcr system and Cr-Pcr shuttle. The cardiotoxic side effects of DOX have been shown to be attenuated by aerobic exercise (Hydock et al., 2008; Kanter et al., 1985; Wonders et al.,

2008). Yet, there has been little investigation into the ability of RT to attenuate DOX-induced muscle dysfunction. Thus, the current study examined the combined effects of RT and Cr on DOX-induced myotoxicity.

CHAPTER III

METHODS

Doxorubicin (DOX) is well known for its anti-cancer effects, but it can cause severe disruptions in skeletal muscle function. The mechanisms behind this dysfunction have yet to be fully explained but likely result from a combination of excess levels of ROS and interference in energy availability. Given the proposed mechanisms behind DOX-induced muscle dysfunction, Cr and RT, individually, could each offer a reprieve from the harmful effects of DOX. In combination, they have the possibility of providing substantial protection against DOX-induced muscle dysfunction. This chapter describes the methodology used to examine the effects of Cr and RT on DOX-induced muscle dysfunction.

Animals and Animal Care

All procedures were approved by the University of Northern Colorado's Institutional Animal Care and Use Committee (IACUC; see Appendix B). Ten week-old male Sprague-Dawley rats ($N = 64$), weighing approximately 300 grams, were used in this study. Animals were housed two per cage in standard 20.32 cm high and 26.67 cm wide by 48.26 cm deep plastic rat cages and were put on a 12hr/12hr light/dark cycle in a temperature and humidity controlled environment. Animals had access to standard chow and distilled water *ad libitum* for the duration of the study.

Training Protocol

Animals were randomly assigned to either a RT group (n = 32) or sedentary (SED) group (n = 32) for six weeks. A six week RT training protocol was used because it represented the minimal amount of time required to reduce fatigue, improve muscular strength, and increase physical and functional activity in cancer patients undergoing chemotherapy (Adamsen et al., 2009). At the end the end of training period, animals were further divided into four groups (n = 16) based on the first treatment buffer (Cr or K). Animals were then further divided into eight groups (n = 10) based on the second treatment buffer (DOX or K). An illustration of the experimental design can be seen in Figure 3.1.

Resistance training was simulated by placing rats in cages where the food and water were progressively elevated over the course of two weeks until reaching a final height of 0.2 meters above normal (see Figure 3.2). Animals remained in the elevated food and water cages for four weeks. This elevated height caused the rats to rise to a bipedal stance or jump to reach their food and water, thus providing a representative model for chronic hind limb loading and RT. This model has been shown to increase hypertrophy of the hind limb muscles and increase hind limb bone density while minimizing animal distress (Yao, Jee, Chen, Li, & Frost, 2001). Animals were kept in the elevated food cages for a total of 6 weeks. Animals assigned to the SED group were kept in standard cages for an equivalent amount of time.

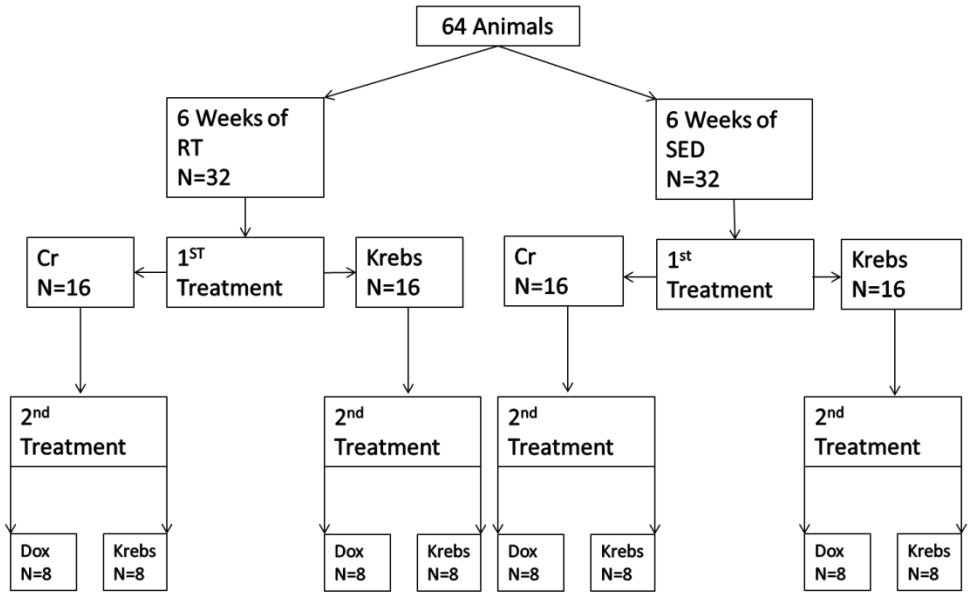


Figure 3.1. Illustration of experimental design. Abbreviations: RT- Resistance Training; SED- Sedentary; Cr- Creatine Monohydrate; Krebs- Krebs-Henseleit Buffer.

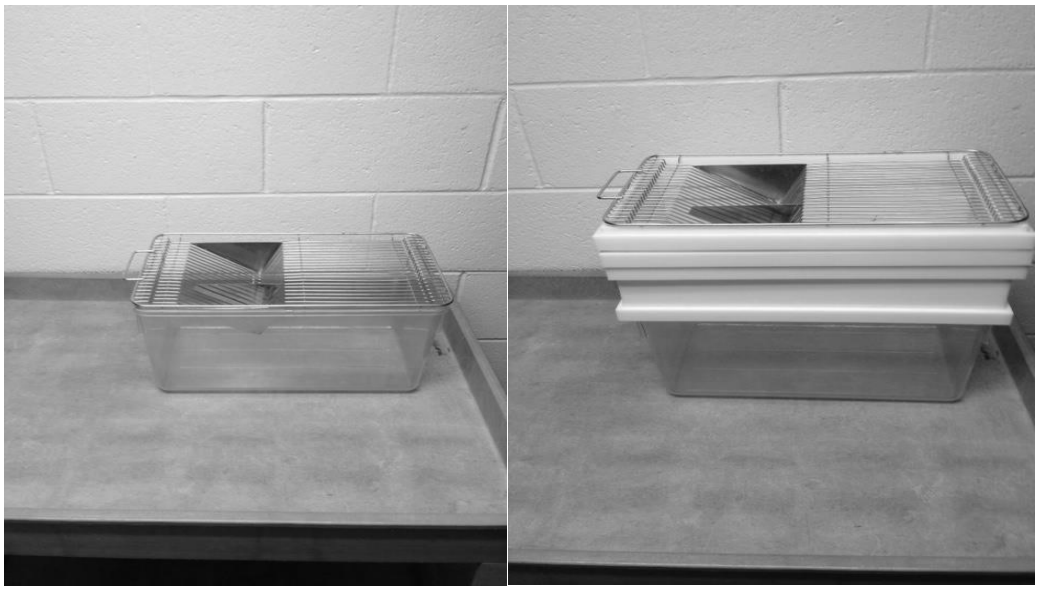


Figure 3.2. Photographs of standard rat housing (left) and an elevated food cage (right). The elevated housing causes the rat to rise to a bipedal stance, which provides a representative model for resistance training.

Skeletal Muscle Function

After six weeks of RT or sedentary conditions, each animal was anesthetized with an intraperitoneal injection of heparinized sodium pentobarbital (50 mg/kg). Supplemental sodium pentobarbital injections were given as needed. When the animal was anesthetized, and a tail pinch reflex was absent, the right and left SOL and EDL were quickly excised and placed in oxygenated (95% O_2 and 5%CO_2) Krebs Henseleit buffer (K) (120 mM NaCl, 5.9 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM MgCl, 25 mM $NaHCO_3$, 17 mM glucose, pH 7.4) heated to 36.8°C. Excised tissues were given two minutes to recover from surgery, and then the muscle was mounted in an *ex vivo* muscle function apparatus (see Figure 3.3). The proximal end was attached to a spring clip connected to a force transducer. The distal end was attached to a stationary glass hook. Stimulation of excised muscles was initiated with field stimulating electrodes. Maximal twitch characteristics (maximal twitch force, maximal rate of force development, and maximal rate of force decline) were recorded through ADInstruments Lab Chart software (Colorado Springs, CO).

Functional data were only collected from the SOL and EDL from the right hind limb. The muscles from left hind limb of non-DOX treated animals underwent the same treatment process as the right-sided muscle but without any functional testing.

Following the recovery period and prior to the first incubation, optimal maximal twitch characteristics were determined and recorded for the muscles of the right hind limb. A baseline tension of 0.5 grams was applied to the muscle and twitch force was measured. Tension was increased by 0.2 grams until an increase in twitch force was no longer present. The muscle was given a two minute recovery period after each

stimulation. Next, optimal voltage was determined using a similar process of increasing voltage from a baseline of 40 volts by 10 volts until an increase in contraction strength was no longer evident. The muscle was again given a two minute recovery period after each stimulation. When these settings were identified, the buffers were changed to the first treatment buffer for both sets of muscles.

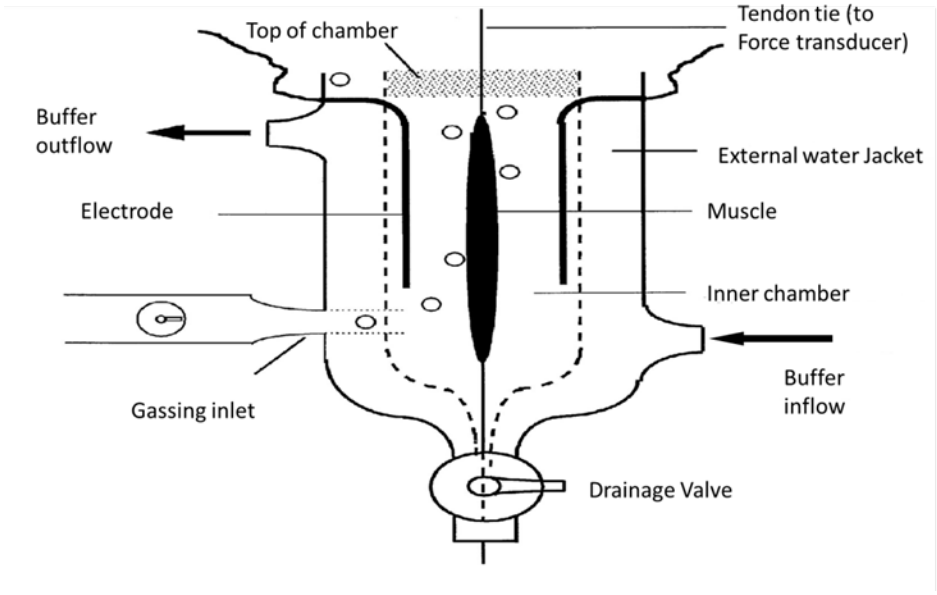


Figure 3.3. Schematic of *ex vivo* muscle function apparatus.

The first treatment buffer was either a K buffer or K buffer containing Cr (25 mM). During the incubation period, the muscle was stimulated at 100 Hz for 200 msec every five minutes using the same established twitch settings. This stimulation protocol has been previously shown to facilitate movement and activation of the Cr transporter (CreaT) (Head et al., 2011). Following the 30 minute incubation period, the buffer was changed to the second treatment buffer (K or K buffer with DOX [24 μM]), and both sets of muscles were allowed to incubate for another 30 minutes. A DOX concentration of 24

μM was used because it best represented the upper limits for serum concentrations immediately following infusion with DOX (Baurain, Deprez-De Campeneere, Zenebergh, & Trouet, 1982). The muscles were stimulated for 200 msec at 100 Hz once every five minutes for the duration of second incubation using the same voltage as before.

At the end of the final incubation, left hind limb muscles were removed from the tissue bath, weighed, frozen in liquid nitrogen, and stored in in -80°C for later biochemical analysis.

The buffers were replaced again with fresh K buffer and then muscle characteristics were reassessed using the initial maximal twitch settings. Following maximal twitch determination, the tissues were supplied with fresh K and analyzed for fatigue. Fatigue rate was determined using the same voltage settings from maximal twitch determination with a frequency of 83 Hz and pulse duration of 500 ms (square wave pulses). Muscles were stimulated to contract every second for 100 seconds and twitch forces through the course of the protocol were recorded. At the end of data collection, tissues from the right limb were weighed, frozen in liquid nitrogen, and stored in -80°C for biochemical analysis.

Creatine Content Analysis

A 1:4 weight/volume ratio of Cr assay buffer (Sigma-Aldrich, St. Louis, MO) and 5 μL of protease inhibitor (Sigma-Aldrich, St. Louis, MO) were added into an Eppendorf tube along with a portion of tissue and homogenized using a Dounce homogenizer. After five minutes of homogenization, a sonicator (Fisher Scientific model 100 sonic dismembrator; Waltham, MA) was used to further disrupt cell and organelle membranes

at 1-second pulses, repeated five times. After five minutes of homogenization, the homogenates were centrifuged through a 10 Kda micro spin filter (GE28-9322-47, Sigma-Aldrich, St. Louis, MO) at 10,000 *g* for 10 minutes at 4℃. After centrifugation, the supernatant was collected and transferred to a new Eppendorf tube. Total protein concentration of the sample was determined using the Bradford protein assay method (Bradford, 1976).

Analysis of total creatine content was done using the Sigma-Aldrich creatine assay kit (MAK079, Sigma-Aldrich, St. Louis, MO). Standards were prepared by mixing 10 µl of reconstituted creatine standard with 990 µl of assay buffer to generate a 1mM (nmole/µl) standard solution of working solution. Amounts of 0, 2, 4, 6, 8, and 10 µL of the 1 mM creatine standard solution were loaded onto a 96 well plate, generating 0 (blank), 2, 4, 6, 8, and 10 nmole/well standards. Next, the reaction mix was prepared by adding 44 µl of creatine assay buffer, 2 µl of creatinase, and 2 µl creatine enzyme mix, 2 µl of creatine probe. 50 µl of reaction mix was added to each sample and standard; each was allowed to incubate at 37°C for one hour. The standards were analyzed at 570 nm to develop a standard curve. Samples were then analyzed at 570 nm to determine total Cr content using the standard curve.

Statistical Analysis

All data were presented as mean ± standard deviation (mean ± *SD*). A three-factor analysis of variance (ANOVA) was used to determine main effects and interactions for the three treatments (RT, Cr, and DOX). Tukey's *post-hoc* analysis was used to determine significance between treatment combinations on maximal twitch characteristics. The dependent variables for maximal twitch characteristics were

maximal twitch force, maximal rate of force development, and maximal rate of force decline. A two-way ANOVA was used to determine the effects of RT and Cr on intracellular Cr content. Tukey's *post-hoc* analysis was used to determine significance between the groups. The dependent variable for Cr content was the calculated Cr concentration. Fatigue was analyzed over time using repeated measures ANOVA with a Dunnett's *post-hoc* analysis. A significance level of $\alpha = 0.05$ was used for all statistical analyses. Graph Pad Prism (San Diego, CA) and Statistical Analysis System (Cary, NC) were used for statistical analysis.

CHAPTER IV

RESULTS

Doxorubicin (DOX) is a powerful chemotherapy agent with potent anticancer properties, but it can cause severe disruptions in normal skeletal muscle function. The specific mechanisms behind this dysfunction have yet to be fully explained; however, they are likely the result of a combination of excess levels of ROS and interference in ATP availability. Thus, it might be possible to minimize the DOX-induced muscle dysfunction with either Cr or RT. Individually, each of these could offer a reprieve from the harmful effects of DOX. In combination, they have the possibility of providing substantial protection against DOX-induced muscle dysfunction.

General Observations

Prior to incubation, each animal was weighed and body mass recorded. An unpaired *t*-test was performed to determine if the body and tissue masses were significantly different. Resistance training (RT) animals had a significantly higher body mass of 393 ± 31 grams when compared to the body mass of 350 ± 29 grams in the SED, $p < 0.0001$. Furthermore, RT animals had a significantly higher SOL mass when then those in the SED group, $p < 0.0001$. No significant differences were found EDL mass. Muscle weights can be seen in Table 4.1.

Table 4.1

Muscle Masses

Group	L-SOL (g)	L-EDL (g)
Sedentary	0.2109±0.01	0.1975±0.03
Resistance Training	0.2496±0.07	0.2227±0.04

Maximal Twitch Characteristics

Following the incubation periods, maximal twitch characteristics (maximal twitch force, maximal rate of force development, and maximal rate of force decline) were assessed and recorded. The selected maximal twitch variables can be seen in Figures 4.1-4.6. A three-way ANOVA was performed to assess the main effects and interactions of activity (RT or SED), drug (DOX or K), and nutrition (Cr or K) on each maximal twitch characteristic in the SOL and EDL. As Figure 4.1 shows, no significant drug effect existed for SOL maximal twitch force, $F(1, 70) = 0.08$, $p = 0.7744$. Likewise, nutrition had no significant effect on SOL maximal twitch, $F(1, 70) = 0.37$, $p = 0.5437$; however, there was a significant activity effect, $F(1, 70) = 5.83$, $p = 0.0184$. In general, SED animals had a higher SOL max twitch than RT animals. Furthermore, a trend toward an interaction of drug and activity was found on SOL maximal twitch force, $F(1, 70) = 3.47$, $p = 0.0667$. No significant interactions existed between activity and nutrition, $F(1, 70) = 0.10$, $p = 0.7524$ or nutrition and drug, $F(1, 70) = 0.53$, $p = 0.698$. There was also no statistically significant three-way interaction among activity, drug, and nutrition, $F(1, 70) = 0.37$, $p = 0.05430$. To examine the significant effects further, Tukey's multiple

comparison tests were performed and no significant differences between the groups were observed (see Figure 4.2, $p > 0.05$).

When examining SOL maximal rate force development, no significant main effects from activity, $F(1, 69) = 0.10$, $p = 0.7482$; drug, $F(1, 69) = 0.15$, $p = 0.6966$; or nutrition, $F(1, 69) = 0.05$, $p = 0.8265$, were found (see Figure 5). In addition, no significant interactions were observed between activity and drug, $F(1, 69) = 0.03$, $p = 0.8536$; activity and nutrition, $F(1, 69) = 0.00$, $p = 0.9713$; or nutrition and drug, $F(1, 69) = 2.31$, $p = 0.1329$. Furthermore, no significant three-way interaction existed among activity, drug, and nutrition, $F(1, 69) = 0.67$, $p = 0.4163$.

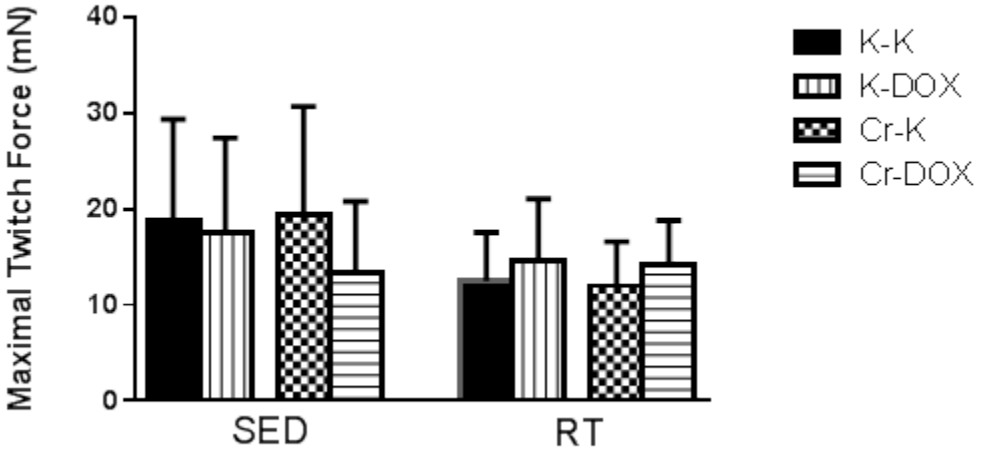


Figure 4.1. Soleus maximal twitch force. SED, sedentary; RT, resistance training; K, Krebs-Henseleit buffer; Cr, Creatine; DOX, doxorubicin. Significant activity effect, $F(1, 70) = 5.83$, $p = 0.0184$.

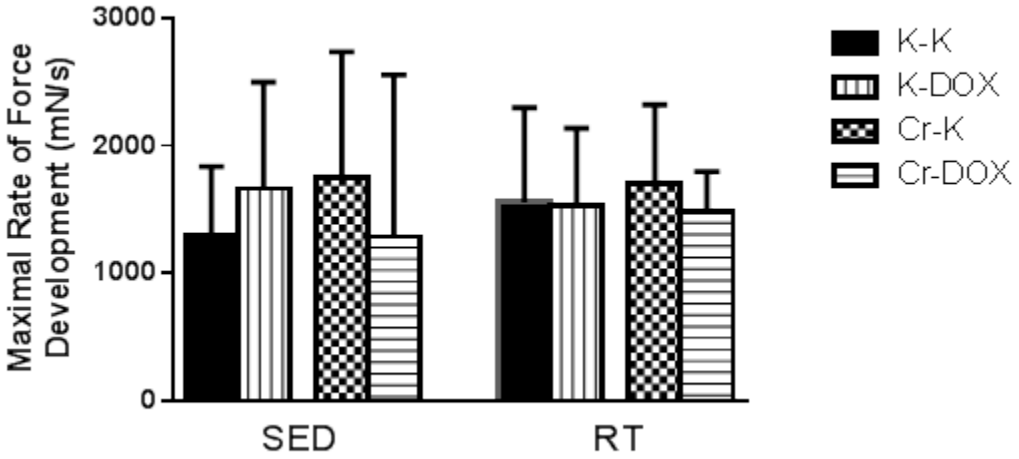


Figure 4.2. Soleus maximal rate of force development. SED, sedentary; RT, resistance training; K, Krebs-Henseleit buffer; Cr, Creatine; DOX, doxorubicin. No main effects or interactions.

Similarly, as illustrated in Figure 4.3, no significant main effects for activity, $F(1, 69) = 0.08$, $p = 0.7846$; drug, $F(1, 69) = 0.06$, $p = 0.7689$; or nutrition, $F(1, 69) = 0.00$, $p = 0.9559$, were observed for SOL maximal rate of force decline. Likewise, no significant interactions from either activity and drug, $F(1, 69) = 0.00$, $p = 0.9510$; activity and nutrition, $F(1, 69) = 0.03$, $p = 0.8542$; or nutrition and drug, $F(1, 69) = 1.54$, $p = 0.2182$, were observed on SOL maximal rate of force decline. Finally, no significant three-way interaction was observed among activity, drug, and nutrition, $F(1, 69) = 0.42$, $p = 0.5178$, on SOL maximal rate of force decline.

In the EDL, no significant main effects from the drug existed on EDL maximal twitch force, $F(1, 68) = 0.02$, $p = 0.8865$. Similarly, no significant main effects were detected with either the activity, $F(1, 68) = 0.0$, $p = 0.9609$; or drug, $F(1, 68) = 0.02$, $p = 0.8865$ on EDL maximal twitch force (see Figure 4.4). In addition, no interactions were observed between activity and drug, $F(1, 68) = 1.17$, $p = 0.2839$; activity and nutrition, $F(1, 68) = 0.28$, $p = 0.5999$, or drug and nutrition $F(1, 68) = 0.30$, $p = 0.5837$ on EDL maximal twitch force. No significant three way interaction among activity, drug, or nutrition was detected, $F(1, 68) = 0.74$, $p = 0.3937$.

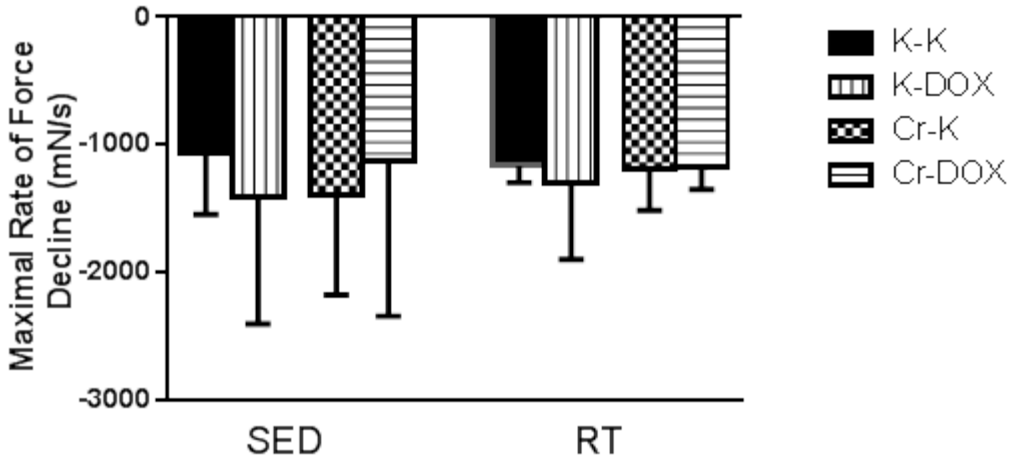


Figure 4.3. Soleus maximal rate of force decline. SED, sedentary; RT, resistance training; K, Krebs-Henseleit buffer; Cr, Creatine; DOX, doxorubicin. No main effects or interactions.

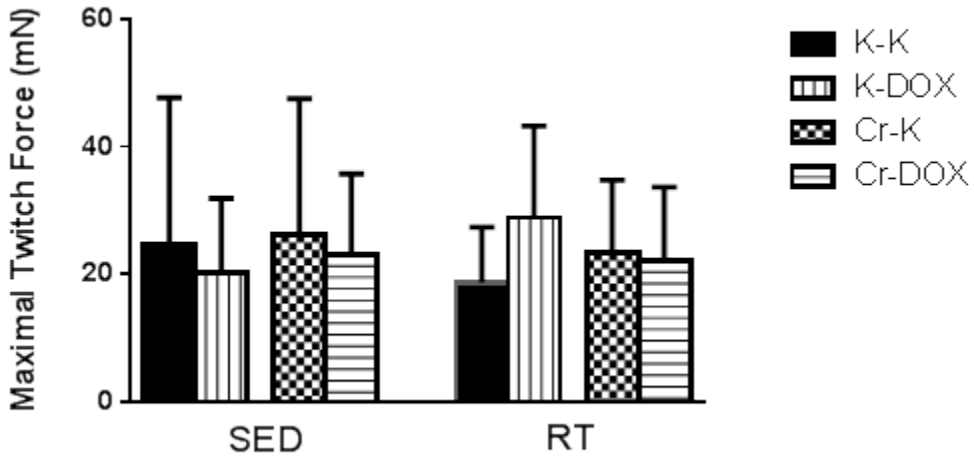


Figure 4.4. Extensor digitorum longus maximal twitch force. SED, sedentary; RT, resistance training; K, Krebs-Henseleit buffer; Cr, Creatine; DOX, doxorubicin. No main effects or interactions.

The analysis of EDL maximal rate of force development revealed no significant main effects from activity, $F(1, 66) = 0.52$, $p = 0.4739$; drug $F(1, 66) = 0.01$, $p = 0.9355$; or nutrition, $F(1, 66) = 0.69$, p = 0.4091 (see Figure 4.5). Moreover, the interaction of activity and drug was not significant, $F(1, 66) = 0.92$, $p = 0.3415$. In addition, activity and nutrition did not result in a significant interaction, $F(1, 66) = 0.17$, $p = 0.6858$ nor was the combination of drug and nutrition, $F(1, 66) = 0.18$, $p = 0.6750$. Also, no significant three-way interaction among activity, drug, and nutrition was observed, $F(1, 66) = 0.20$, $p = 0.6568$.

As Figure 4.6 depicts, no main effect from activity, $F(1, 65) = 0.91$, $p = 0.3524$; drug, $F(1, 65) = 0.87$, $p = 0.3543$; or nutrition, $F(1, 65) = 0.45$, $p = 0.5040$, was observed for EDL maximal rate of force decline. Additionally, no significant interactions between activity and drug, $F(1, 65) = 1.27$, $p = 0.2637$; activity and nutrition, $F(1, 65) = 1.60$, $p = 0.2098$; or drug and nutrition, $F(1, 65) = 1.27$, $p = 0.2640$, were noted. Similarly, the three way interaction among activity, drug, or nutrition was not significant, $F(1, 65) = 0.02$, $p = 0.8911$.

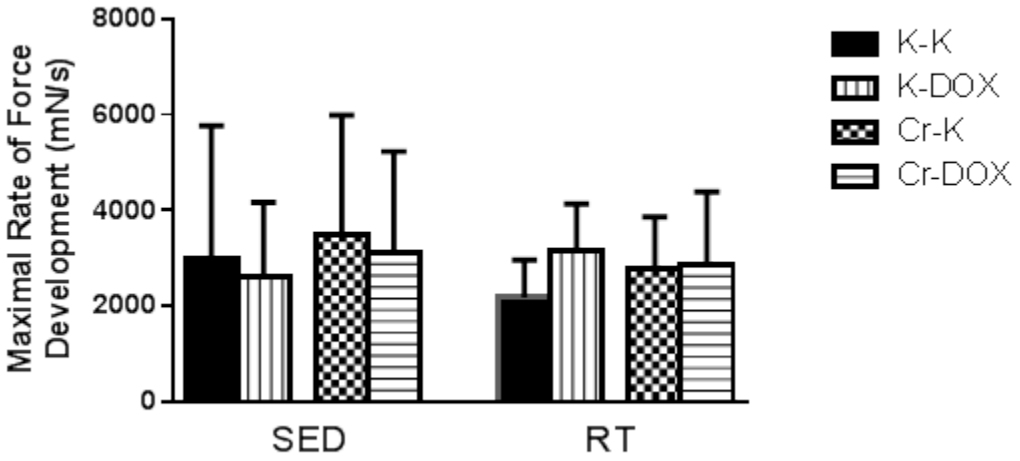


Figure 4.5. Extensor digitorum longus maximal rate of force development. SED, sedentary; RT, resistance training; K, Krebs-Henseleit buffer; Cr, Creatine; DOX, doxorubicin. No main effects or interactions.

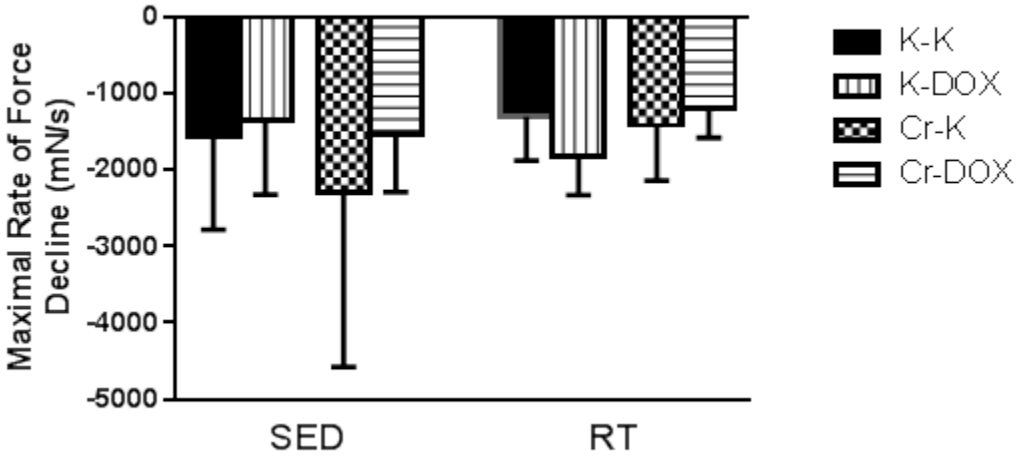


Figure 4.6. Extensor digitorum longus maximal rate of force decline. SED, sedentary; RT, resistance training; K, Krebs-Henseleit buffer; Cr, Creatine; DOX, doxorubicin. No main effects or interactions.

.

Fatigue

After determination of maximal twitch characteristics and the buffers refreshed with K, the muscles were stimulated once per second for 100 seconds. Forces were then recorded every 10 seconds. Significant reductions from baseline in the SOL were seen at 70, 50, and 50 seconds for SED-K-K, SED-Cr-DOX, and SED-K-DOX, respectively. Conversely, SED-Cr-K did not have a significant drop from baseline during the observed time period, which indicated that Cr did delay the onset of fatigue. The addition of RT to the SOL produced a profoundly different response. A significant drop from baseline was observed at 60 seconds for RT-K-K and 60 seconds for RT-K-DOX; however, the addition of RT delayed the onset of fatigue until 100 seconds for RT-Cr-DOX. No significant drop from baseline was found for RT-Cr-K group. Soleus fatigue data can be seen in Figure 4.7.

Sedentary-K-K fatigue for the EDL was significantly different from baseline at 60 seconds. With the addition of Cr, however, a significant reduction from baseline was not observed during the stimulation period. At the 50 second time point, SED-K-DOX was significantly lower than baseline. Sedentary-Cr-DOX was significantly lower than baseline at the 60 second time point. Significant reductions from baseline were found at 50 seconds for RT-K-DOX and 60 seconds for RT-K-K. The application of Cr delayed the onset of fatigue to 70 seconds in RT-Cr-DOX. This highlighted RT's unique response on fatigue rates in the EDL. Without DOX, the combination of RT and Cr did not result in a significant drop from baseline. Resistance training EDL fatigue data can be seen in Figure 4.8.

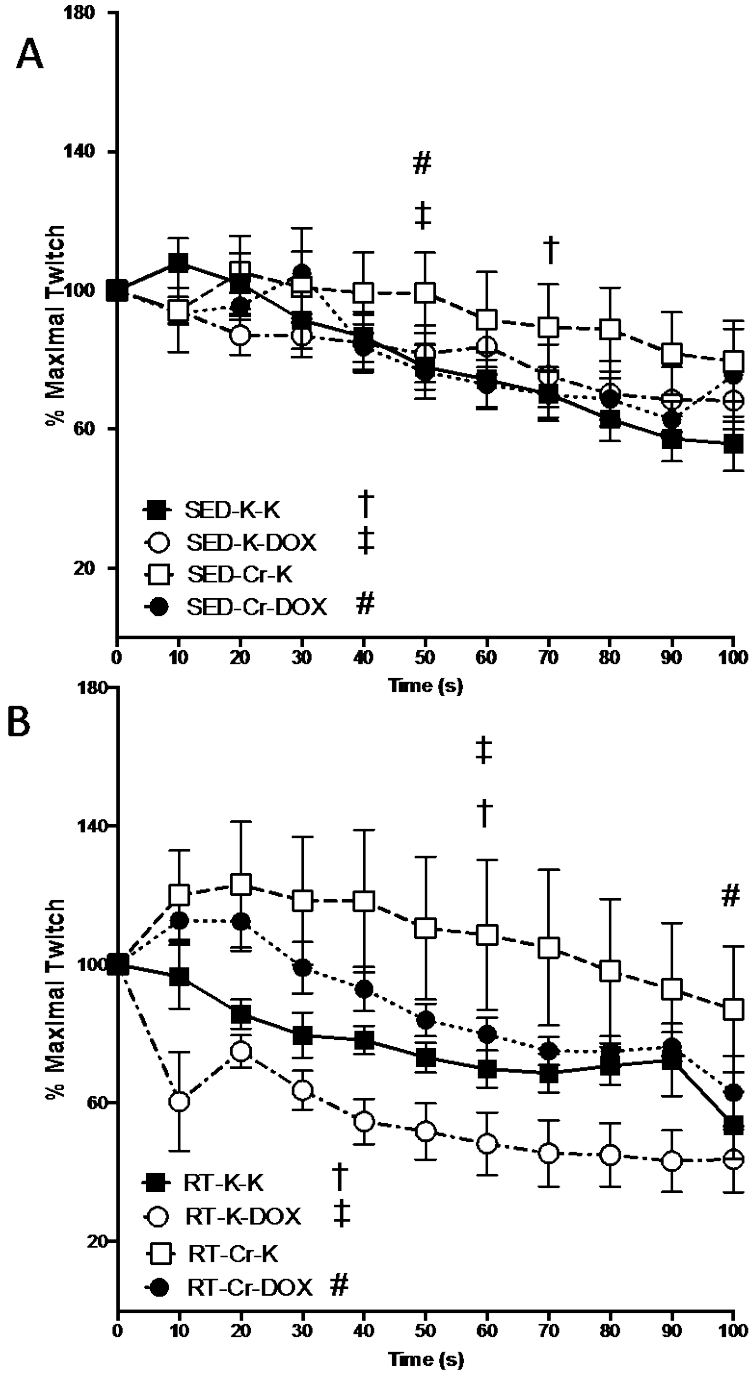


Figure 4.7. Soleus fatigue rates for a 100 second fatigue for the soleus of sedentary animals (A) and for the soleus of resistance trained animals (B). SED, sedentary; RT, resistance training; K, Krebs-Henseleit buffer; Cr, Creatine; DOX, doxorubicin. Symbols indicate significant reductions from baseline. † K+K, significantly lower than baseline ($p < 0.05$), ‡ K+DOX, significantly lower than baseline ($p < 0.05$), and # Cr+DOX, significantly lower than baseline ($p < 0.05$).

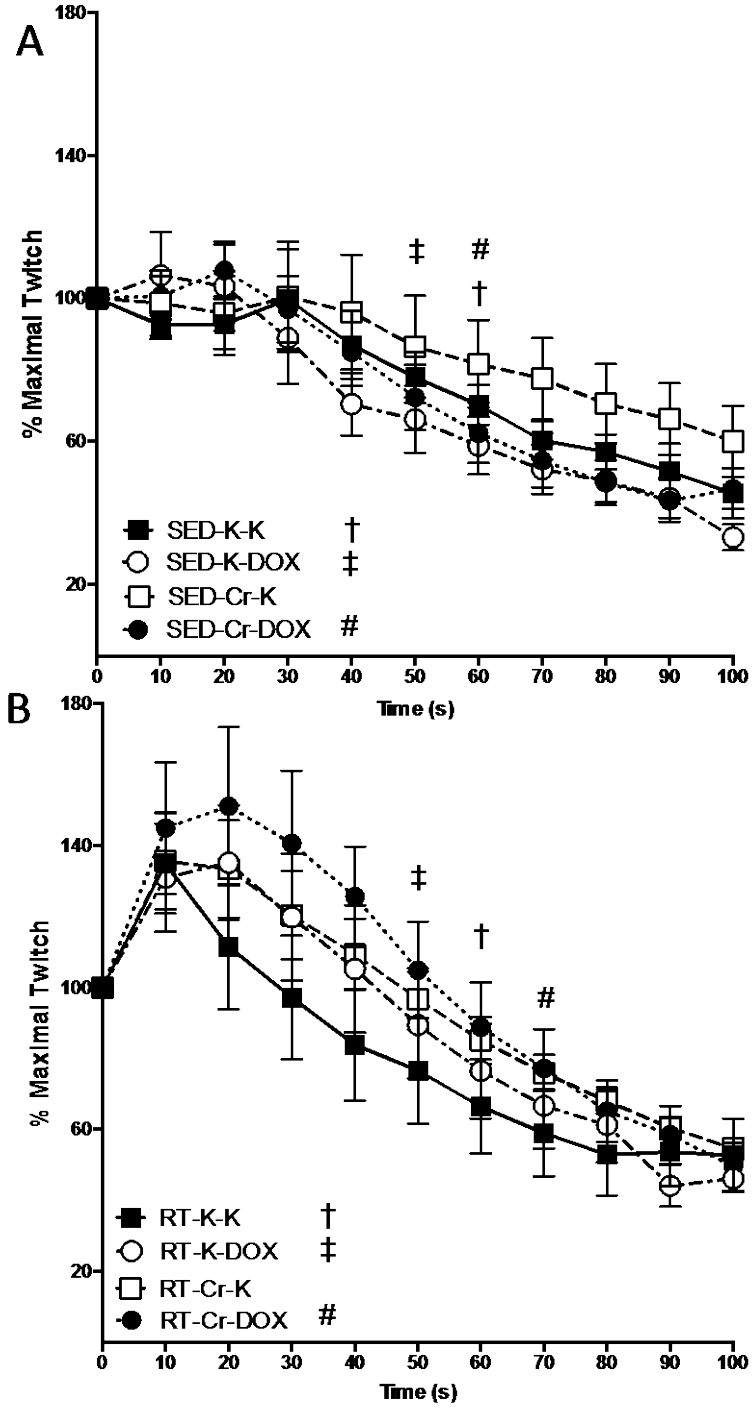


Figure 4.8. Fatigue rates for a 100 second fatigue for the extensor digitorum longus of sedentary animals (A) and for the extensor digitorum longus of resistance trained animals (B). SED, sedentary; RT, resistance training; K, Krebs-Henseleit buffer; Cr, Creatine; DOX, doxorubicin. Symbols indicate significant reductions from baseline. † K+K, significantly lower than baseline ($p < 0.05$), ‡ K+DOX, significantly lower than baseline ($p < 0.05$), and # Cr+DOX, significantly lower than baseline ($p < 0.05$).

Creatine Content

Tissue Cr concentration was analyzed using the left SOL and EDL from SED-K-K, Sed-Cr-K, RT-K-K, and RT-Cr-K animals. To test the effect of activity (RT and SED) and nutrition (Cr or K) on muscle Cr content, a two way ANOVA was performed. In the SOL, The results showed no significant main effect of activity, $F(1, 24) = 0.8469$, $p = 0.3666$, or nutrition, $F(1, 24) = 0.7668$, $p = 0.3899$; however, there was a significant interaction between activity and nutrition on SOL Cr content, $F(1, 24) = 4.760$, $p = 0.0392$. As can be seen in Figure 4.9, in the RT condition, SOL muscles incubated with Cr had higher Cr content than non-treated controls. To explore this effect further, Tukey's multiple comparison tests were performed and no significant differences in intracellular Cr were observed ($p > 0.05$).

In the EDL, there was a significant main effect, $F(1, 24) = 6.129$, $p = 0.0207$, of nutrition on total intracellular Cr concentration; however, no significant activity effect was observed, $F(1, 24) = 1.119$, $p = 0.3006$, and there were no significant interactions, $F(1, 24) = 0.5208$, $p = 0.4775$ (see Figure 13). Tukey's post hoc testing did not show any significant differences between the groups ($p > 0.05$; see Figure 4.10).

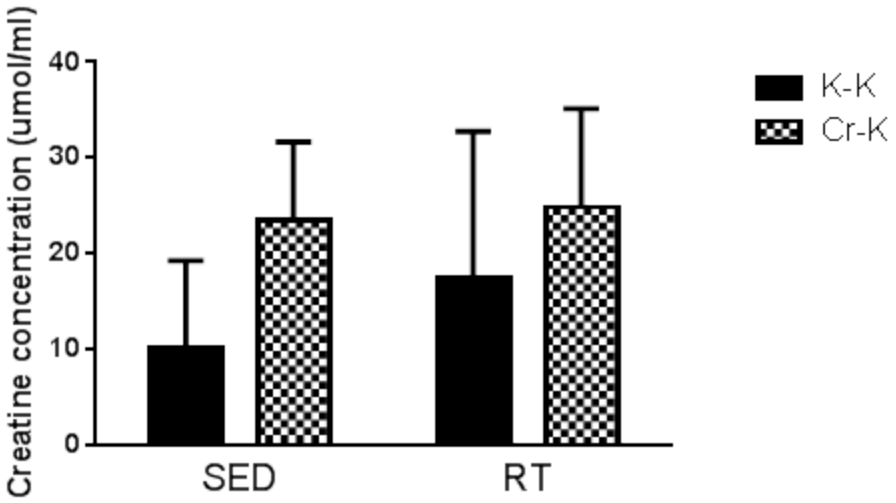


Figure 4.9. Soleus creatine concentration. SED, sedentary; RT, resistance training; K, Krebs-Henseleit buffer; Cr, Creatine. Significant interaction between activity (RT) and nutrition (Cr), $F(1, 24) = 4.760$, $p = 0.0392$.

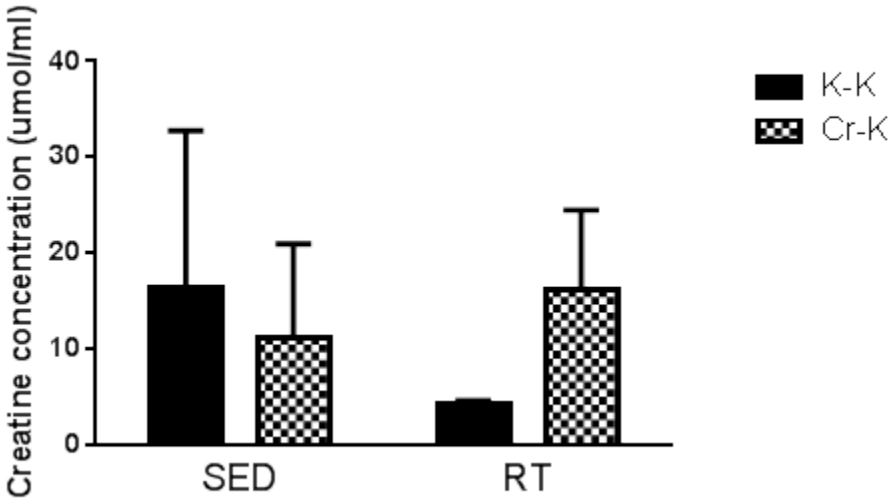


Figure 4.10. Extensor digitorum longus creatine concentration. SED, sedentary; RT, resistance training; K, Krebs-Henseleit buffer; Cr, Creatine. Significant main effect nutrition (Cr), $F(1, 24) = 6.129$, $p = 0.0207$.

Conclusions

With regard to the hypotheses, the following statistical conclusions were warranted.

1. With regard to the capacity of RT to protect against DOX-induced myotoxicity by preserving maximal twitch force, the statistical analysis rejected H1; however, the analysis of fatigue showed that RT delayed fatigue in the SOL but not in the EDL. Thus, statistically, H1 could not be completely rejected.
2. With respect to the ability of Cr to minimize DOX-induced myotoxicity by preserving maximal twitch force, the statistical findings rejected H2. Conversely, the statistical findings, with respect to capacity of Cr to minimize fatigue, suggested Cr could delay the onset of fatigue in the EDL but not in the SOL. Thus, H2 could not be completely rejected.
3. With respect to the ability of RT and Cr to provide a greater level of protection against DOX-induced myotoxicity than either treatment administered separately, the statistical results failed to reject H3. The results indicated the combination of RT and Cr could result in a substantial protection against DOX-induced fatigue.
4. In the determination of the ability of Cr to increase total Cr, the findings indicated Cr treatment did not increase total Cr. Thus, H4 was rejected.

CHAPTER V

MANUSCRIPT

Introduction

Use of the anthracycline chemotherapeutic drug doxorubicin (DOX) is associated with a number of debilitating side effects, such as its dose dependent cardiotoxicity and more recently discovered myotoxicity. The cardiotoxicity is commonly manifested in the form of dilated cardiomyopathy and congestive heart failure (Chatterjee et al., 2010). The mechanisms behind these negative effects are partly the result of an increase in oxidative stress (Gilliam & St Clair, 2011), an increase in intracellular iron (Ichikawa et al., 2014), inhibition of NRG1/Erb signaling cascade (Vasti et al., 2012), and a reduction in high-energy phosphates (Maslov et al., 2010). In addition, impairment of progenitor cell renewal, cardiac repair, and decreased vasculogenesis are common with DOX treatment (Menna et al., 2008; Y. Shi et al., 2011). To date, no pharmacological agent can prevent these side effects; however, aerobic exercise has been used effectively as a means for attenuating the decline in cardiac function (Chicco et al., 2005; Hydock et al., 2008; Wonders et al., 2008).

There is growing evidence that DOX not only has cardiotoxic effects but can also negatively affect skeletal muscle function (Ascensao et al., 2011; Hydock et al., 2011; van Norren et al., 2009). Although there is broad consensus that DOX has a negative impact on skeletal muscle function, the specific mechanisms behind this dysfunction

remain elusive despite several proposed explanations. For example, Gilliam and St Clair (2011) suggested the skeletal muscle dysfunction could be a result of the variation in tumor necrosis factor receptor. Similarly, van Noreen et al. (2009) identified the dysfunction as resulting from changes in calcium (Ca^{2+}) handling, while Abramson et al. (1988) attributed it to disruptions in the sarcoplasmic reticulum (SR). Another possibility is that DOX interferes with key Ca^{2+} skeletal muscle handling proteins such as the sarcoendoplasmic reticulum calcium ATPase (SERCA; Abramson et al., 1988). This protein is instrumental in Ca^{2+} uptake during skeletal muscle relaxation (Periasamy & Kalyanasundaram, 2007). These findings help explain DOX-induced dysfunction from a functional standpoint, but the effects of DOX are not limited to interference with Ca^{2+} alone.

Part of the myotoxic effects of DOX can be attributed to its generation of reactive oxygen species (ROS). It is plausible that the rise in ROS could be the culprit in the observed DOX-induced muscle dysfunction. Furthermore, cardiac myocytes treated with DOX display a decrease in their overall antioxidant capacity (Wallace, 2003). Additionally, in diaphragm muscle treated with DOX, there is a similar reduction in antioxidant capabilities (Gilliam et al., 2011). This antioxidant reduction is further evidenced by the activation of caspase-3, a key regulatory component of apoptosis triggered by high levels of oxidative stress (Smuder et al., 2011). This increase in free radical production could lead to an altered functional capacity of the SR, possibly through lipid peroxidation. Furthermore, altered Ca^{2+} handling via damaged SR could interfere with normal cross bridge cycling, which could account for some of the increased muscular fatigue commonly seen during DOX treatment.

There is mounting evidence that exercise can minimize cancer related fatigue and improve quality of life (Quist et al., 2006; Spence et al., 2010). Furthermore, evidence suggests that exercise, in general, is safe and effective during the administration of adjuvant cancer therapies (Cheema et al., 2008; Jones & Demark-Wahnefried, 2006; McNeely et al., 2006). However, the appropriate modalities and dosages required to produce these positive effects have yet to be fully elucidated. Even with the prevailing focus upon the effectiveness of aerobic exercise, there is a growing body of evidence supporting the benefits of other forms of exercise (Focht et al., 2013). For example, it is well known that resistance training (RT) yields a number of favorable adaptations that could be effective at reducing the physiological and quality of life outcomes in cancer patients and survivors.

Resistance training produces a wide range of adaptations such as improved muscular performance, hypertrophy, improved body composition, increased appetite, and increased bone strength (Winett & Carpinelli, 2001) . Moreover, RT has been used as a means of palliative care and has been shown to reduce cancer-related fatigue (Adamsen et al., 2009; Meneses-Echavez et al., 2014), improve muscular strength in patients with heart failure (Gielen et al., 2014), preserve muscle mass in patients with heart failure (Gielen et al., 2014), promote increased blood cell counts in those undergoing chemotherapy (Karvinen et al., 2014), and improve breast cancer outcomes during chemotherapy (Courneya et al., 2014). The benefits from resistance training stand in sharp contrast to myotoxicity seen with DOX. Thus, one purpose of this study was to examine the effects of RT on DOX-induced muscle dysfunction.

Creatine monohydrate (Cr) is a popular ergogenic aid and its benefits on the phosphagen system have been well studied (Clark, 1997; Kraemer & Volek, 1999; Persky & Brazeau, 2001). Previous research has demonstrated that when skeletal muscle is incubated in an *ex vivo* tissue bath with Cr, there is an increase in force production and improved rates of time to fatigue (Head et al., 2011). In addition, Eisner et al. (2014) demonstrated that supplemental Cr improves the rates of muscle fatigue in mice with mitochondrial dysfunction. Drawing on the model of DOX-induced muscle dysfunction, deficits in energy metabolism are believed to be one of the mechanisms that lead to DOX-induced muscle dysfunction. Doxorubicin has been shown to decrease high-energy phosphates and alter the ratio of phosphocreatine (PCr) to adenosine triphosphate (ATP; Darrabie et al., 2012; Maslov et al., 2010; Ohhara et al., 1981; Seraydarian et al., 1977). Furthermore, it has also been noted that DOX can lower levels of Cr and creatine kinase (CK) in cardiac myocytes (Tokarska-Schlattner et al., 2006). The possibility of a reduction of ATP and a reduced PCr and ATP ratio could help explain DOX-induced muscle dysfunction from an energy standpoint.

A major purpose of this study involved the examination of the effects of incubating muscles *ex vivo* with Cr prior to treatment with DOX in order to minimize muscle dysfunction. In addition, another purpose of this study was to examine the effects of a combined treatment of RT and Cr to minimize DOX-induced muscle dysfunction. Furthermore, this study hypothesized that Cr in combination with RT had the possibility of greatly attenuating DOX-induced myotoxicity. To date, there is a gap in the literature as to the effects RT has on DOX treated skeletal muscle with and without Cr supplementation. It is important to develop a better explanation of these factors because

they might be an effective treatment strategy in the palliative care of those undergoing chemotherapy with DOX.

Methods

All procedures were approved by the University of Northern Colorado's Institutional Animal Care and Use Committee (IACUC). Ten week-old male Sprague-Dawley rats (N=64) weighing approximately 300 grams were used in this study. Animals were housed two per cage in standard 20.32 cm high and 26.67 cm wide by 48.26 cm deep plastic rat cages and maintained on a 12hr/12hr light/dark cycle in a temperature and humidity controlled environment. Animals had access to standard chow and distilled water *ad libitum* for the duration of the study.

Training Protocol

Animals were randomly assigned to either an RT group (n = 32) or a sedentary (SED) group (n = 32) for six weeks. A six week RT training protocol was used because it has been reported to be the minimal amount of time necessary to reduce fatigue, improve muscular strength, and increase physical and functional activity in cancer patients undergoing chemotherapy (Adamsen et al., 2009). Resistance training was simulated by placing rats in cages where the food and water was progressively elevated over the course of two weeks until reaching a final height of 0.2 meters above normal. Animals remained in the elevated food and water cages for an additional four weeks (six weeks total). This elevated height caused the rats to rise to a bipedal stance or jump to reach their food and water, thus providing a representative model for chronic hind-limb loading and RT. This model has been shown to increase hypertrophy of the hind-limb muscles and increase hind-limb bone density while minimizing animal distress (Yao et

al., 2001). Animals assigned to the SED group were kept in standard cages for an equivalent amount of time. At the end of the training period, animals were further divided into four groups (n = 16) based on the first treatment buffer (Cr or K). Next, animals were further divided into eight groups based on the second treatment buffer (DOX or K). An illustration of the experimental design can be seen in Figure 5.1.

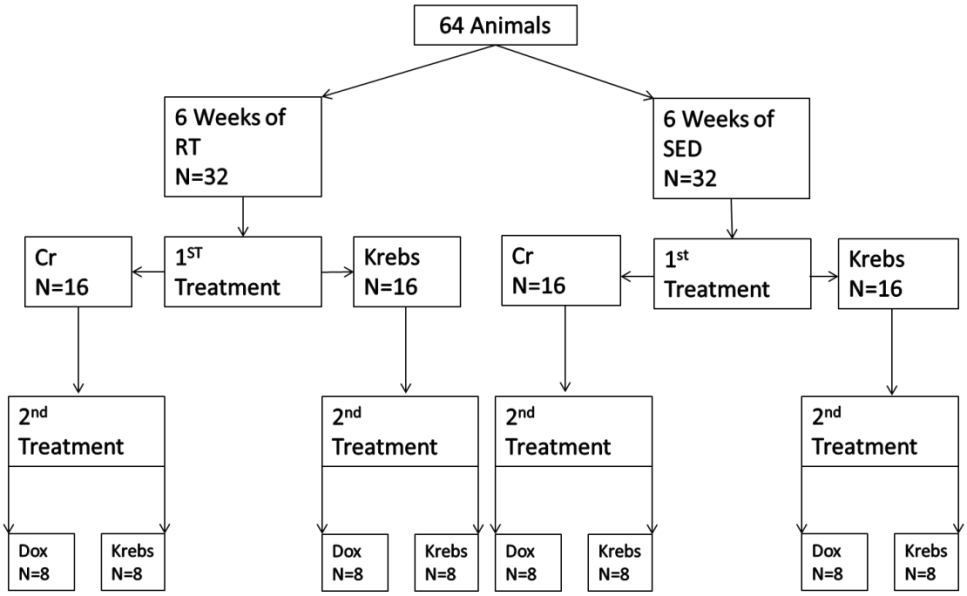


Figure 5.1. Illustration of experimental design. Abbreviations: RT- Resistance Training; SED- Sedentary; Cr- Creatine Monohydrate; Krebs- Krebs-Henseleit Buffer.

Muscle Function, Creatine, and Doxorubicin

After six weeks of RT or SED, each animal was anesthetized with an intraperitoneal injection of heparinized sodium pentobarbital (50 mg/kg). When the animal was anesthetized, and a tail pinch reflex was absent, the right and left soleus (SOL) and extensor digitorum longus (EDL) were excised and placed in oxygenated (95% O_2 and 5%CO_2) Krebs-Henseleit buffer (K) (120 mM NaCl, 5.9 mM KCl, 2.5 mM

CaCl2, 1.2 mM MgCl, 25 mM NaHCO3, 17 mM glucose, pH 7.4) heated to 37°C. Excised tissues were given two minutes to recover from surgery, and then the muscle was mounted in an *ex vivo* muscle function apparatus. The proximal end of the muscle was then attached to a spring clip connected to a force transducer. The distal end was attached to a stationary glass hook. Muscle characteristics (maximal twitch force, maximal rate of force development, and maximal rate of force decline) were recorded through ADInstruments Lab Chart software (Colorado Springs, CO). Functional data were only collected from the SOL and EDL from the right hind limb. The muscles from left hind limb of non-DOX treated animals underwent the same treatment process as the right sided muscle but without any functional testing.

Following the recovery period and prior to the first incubation, optimal maximal twitch characteristics were determined and recorded for the muscles of the right hind limb as described previously (Hydock et al., 2011). Following maximal twitch determination, the muscle was exposed to the first treatment buffer consisting of a K buffer or K buffer containing Cr (25 mM) for 30 minutes. During the incubation period, the muscle was stimulated at 100 Hz for 200 msec every five minutes using the established twitch settings. This stimulation protocol has been previously shown to facilitate movement and activation of the Cr transporter (CreaT; Head et al., 2011). Following the 30 minute incubation period, the buffer was changed to the second treatment buffer (K or K buffer with DOX [24 μM]), and both sets of muscles were allowed to incubate for another 30 minutes. A DOX concentration of 24 μM was used since it represented the upper limits for serum concentrations immediately following infusion with DOX (Baurain et al., 1982). The muscles were stimulated again for 200

msec at 100 Hz once every five minutes for the duration of second incubation using the same voltage as before. At the end of the last incubation period, the left hind limb muscles from all non-DOX treated animals were removed from the tissue bath, weighed, flash frozen in liquid nitrogen, and stored in a -80°C freezer for later biochemical analysis.

The buffers were replaced again with fresh K buffer, and muscle characteristics were reassessed using the initial maximal twitch settings. Following reassessment of maximal twitch, the tissues were supplied with fresh K and analyzed for fatigue. Fatigue rate was determined using the same voltage settings from maximal twitch determination with a frequency of 83 Hz and pulse duration of 500 ms (square wave pulses). Muscles were stimulated to contract every second for 100 seconds, and twitch forces through the course of the protocol were recorded.

Creatine Content

A portion of the left SOL or EDL was added to an Eppendorf tube along with a 1:4 weight/volume ratio of Creatine assay buffer (Sigma Aldrich, St. Louis, MO) and homogenized (ScilogexD160 homogenizer; Rocky hill, CT). After five minutes of homogenization, the homogenates were centrifuged through a 17 KDa micro spin filter at 10,000 *g* for 10 minutes at 4℃. After centrifugation, the supernatant was collected, and its total protein concentration was determined using the Bradford protein assay method (Bradford, 1976). Finally, 30µl of each sample was loaded on to a 96 well plate.

Analysis of creatine content was done using the Sigma Aldrich creatine assay kit (MAK079, Sigma Aldrich, St. Louis, MO). Samples and standards (0, 2, 4, 6, 8, and 10 nmole/well) were prepared and treated according to the manufacturers specifications.

Samples were analyzed at 570 nm using a 96 well plate reader (BioTek elx800, Winooski, VT) to determine total Cr content.

Statistical Analysis

All data were presented as mean ± standard deviation (mean ± *SD*). A three-factor analysis of variance (ANOVA) was used to determine main effects and interactions for the three treatments--RT, Cr, and DOX. Tukey's *post-hoc* analysis was used to determine significance between treatment combinations on maximal twitch characteristics and Cr content. The dependent variables for maximal twitch characteristics were maximal twitch force, maximal rate of force development, and maximal rate of force decline. A two-way ANOVA was used to determine the effects of RT and Cr on muscle Cr content. Tukey's *post-hoc* analysis was used to determine significance between the groups. The dependent variable for Cr content was the measured Cr concentration. Fatigue was analyzed over time using repeated measures ANOVA with a Dunnett's *post-hoc* analysis. A significance level of $\alpha = 0.05$ was used for all statistical analyses. Graph Pad Prism (San Diego, CA) was used for statistical analysis.

Results

Doxorubicin is a powerful chemotherapy agent with potent anticancer properties, but it can cause severe disruptions in normal skeletal muscle function. The specific mechanisms behind this dysfunction have yet to be fully explained; however, they are likely the result of a combination of excess levels of ROS and interference in ATP availability. Thus, it might be possible to minimize the DOX-induced muscle dysfunction with either Cr or RT. Individually, each of these could offer a reprieve from

the harmful effects of DOX. In combination, they have the possibility of providing substantial protection against DOX-induced muscle dysfunction.

General Observations

Prior to incubation, each animal was weighed and body mass recorded. An unpaired *t*-test was performed to determine if the body masses were significantly different. Resistance training animals had a significantly higher body mass of 393 ± 31 grams when compared to the body mass of 350 ± 29 grams in the SED, $p < 0.0001$. Furthermore, RT resulted in a significantly higher SOL mass than those in the SED group, $p < 0.0001$. No significant differences were found EDL mass. Muscle weights can be seen in Table 1.

Table 5.1

Muscle Masses

Group	L-SOL (g)	L-EDL (g)
Sedentary	0.2109±0.01	0.1975±0.03
Resistance Training	0.2496±0.07	0.2227±0.04

Maximal Twitch Characteristics

Following the incubation periods, maximal twitch characteristics (maximal twitch force, maximal rate of force development, and maximal rate of force decline) were assessed and recorded. The selected maximal twitch variables can be seen in Figures 5.2-5.7. A three-way ANOVA was performed to assess the main effects and interactions of activity (RT or SED), drug (DOX or K), and nutrition (Cr or K) on each maximal twitch

characteristics in the SOL and EDL. As Figure 5.2 shows, no significant drug effect existed for SOL maximal twitch force, $F(1, 70) = 0.08$, $p = 0.7744$. Likewise, nutrition had no significant effect on SOL maximal twitch, $F(1, 70) = 0.37$, $p = 0.5437$; however, there was a significant activity effect, $F(1, 70) = 5.83$, $p = 0.0184$. In general, SED animals had a higher SOL max twitch than RT animals. Furthermore, a trend toward an interaction of drug and activity was found on SOL maximal twitch force, $F(1, 70) = 3.47$, $p = 0.0667$. No significant interactions existed between activity and nutrition, $F(1, 70) = 0.10$, $p = 0.7524$, or nutrition and drug, $F(1, 70) = 0.53$, $p = 0.698$. There was also no statistically significant three-way interaction among activity, drug, and nutrition, $F(1, 70) = 0.37$, $p = 0.05430$. To examine the significant effects further, Tukey's multiple comparison tests were performed, and no significant differences between the groups were observed (see Figure 5.2, $p > 0.05$).

When examining SOL maximal rate force development, no significant main effects from activity, $F(1, 69) = 0.10$, $p = 0.7482$; drug, $F(1, 69) = 0.15$, $p = 0.6966$; or nutrition, $F(1, 69) = 0.05$, $p = 0.8265$, were found (see Figure 5.3). In addition, no significant interactions were observed aiming activity and drug, $F(1, 69) = 0.03$, $p = 0.8536$; activity and nutrition, $F(1, 69) = 0.00$, $p = 0.9713$; or nutrition and drug, $F(1, 69) = 2.31$, $p = 0.1329$. Furthermore, no significant three-way interaction existed among activity, drug, and nutrition, $F(1, 69) = 0.67$, $p = 0.4163$.

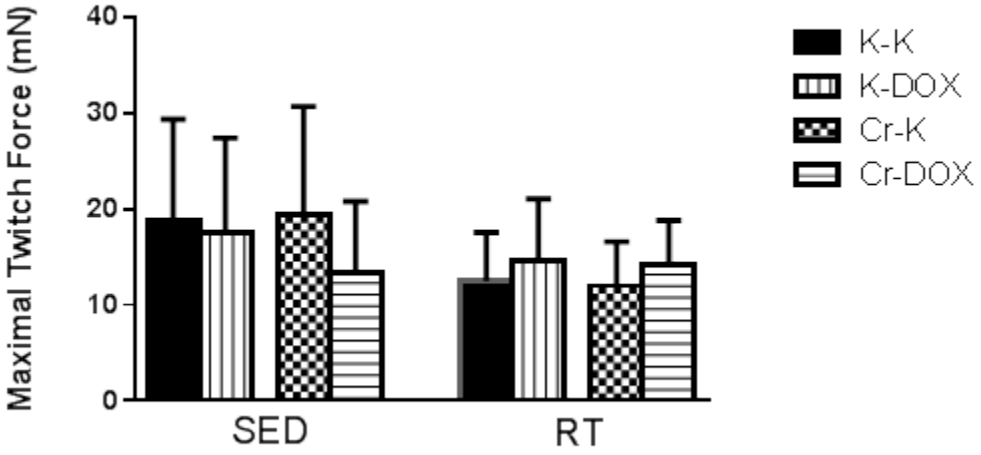


Figure 5.2. Soleus maximal twitch force. SED, sedentary; RT, resistance training; K, Krebs-Henseleit buffer; Cr, Creatine; DOX, doxorubicin. Significant activity effect, $F(1, 70) = 5.83$, $p = 0.0184$.

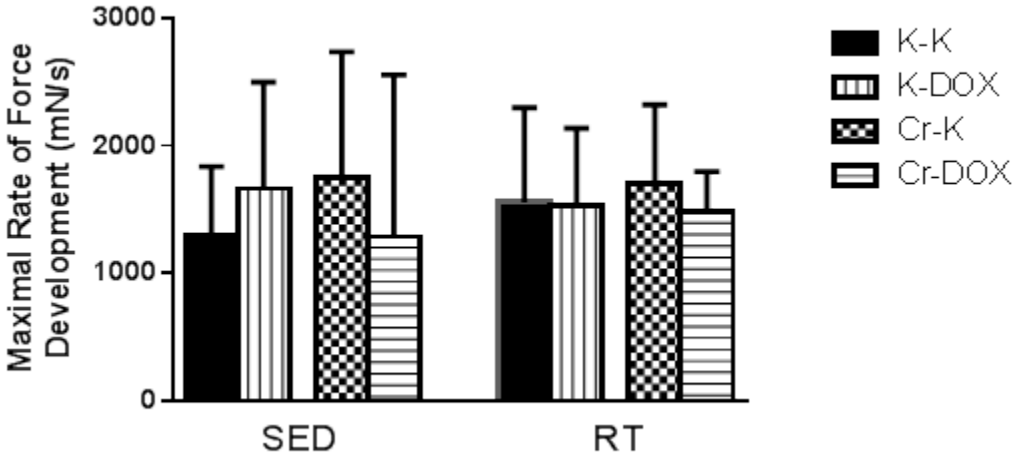


Figure 5.3. Soleus maximal rate of force development. SED, sedentary; RT, resistance training; K, Krebs-Henseleit buffer; Cr, Creatine; DOX, doxorubicin. No main effects or interactions.

Similarly, as illustrated in Figure 5.4, no significant main effects for activity, $F(1, 69) = 0.08$, $p = 0.7846$; drug, $F(1, 69) = 0.06$, $p = 0.7689$; or nutrition, $F(1, 69) = 0.00$, $p = 0.9559$, were observed for SOL maximal rate of force decline. Likewise, no significant interactions from either activity and drug, $F(1, 69) = 0.00$, $p = 0.9510$; activity and nutrition, $F(1, 69) = 0.03$, $p = 0.8542$; or nutrition and drug, $F(1, 69) = 1.54$, $p = 0.2182$, were observed on SOL maximal rate of force decline. Finally, no significant three-way interaction was observed among activity, drug, and nutrition, $F(1, 69) = 0.42$, $p = 0.5178$, on SOL maximal rate of force decline.

In the EDL, no significant main effects from the drug existed on EDL maximal twitch force, $F(1, 68) = 0.02$, $p = 0.8865$. Similarly, no significant main effects were detected with either the activity, $F(1, 68) = 0.0$, $p = 0.9609$; or drug, $F(1, 68) = 0.02$, $p = 0.8865$ on EDL maximal twitch force (see Figure 5.5). In addition, no interactions were observed between activity and drug, $F(1, 68) = 1.17$, $p = 0.2839$; activity and nutrition, $F(1, 68) = 0.28$, $p = 0.5999$, or drug and nutrition $F(1, 68) = 0.30$, $p = 0.5837$ on EDL maximal twitch force. No significant three way interaction among activity, drug, or nutrition was detected, $F(1, 68) = 0.74$, $p = 0.3937$.

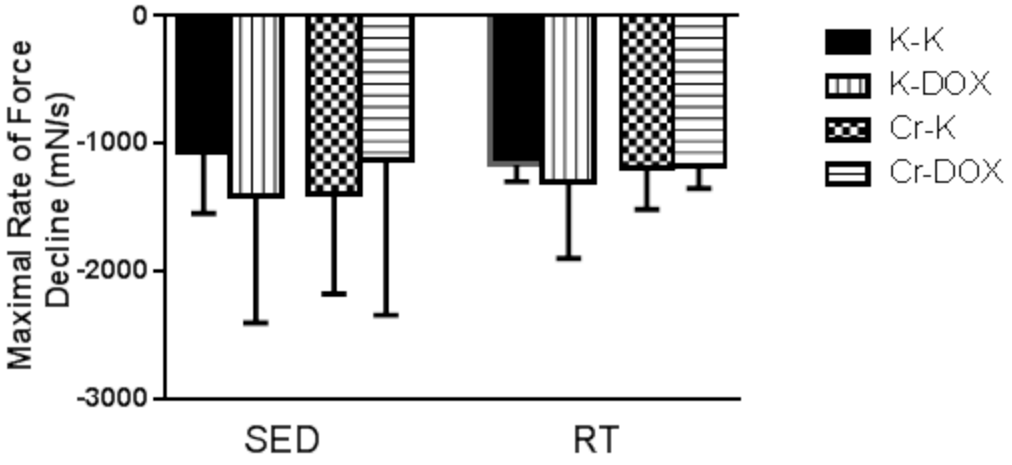


Figure 5.4. Soleus maximal rate of force decline. SED, sedentary; RT, resistance training; K, Krebs-Henseleit buffer; Cr, Creatine; DOX, doxorubicin. No main effects or interactions.

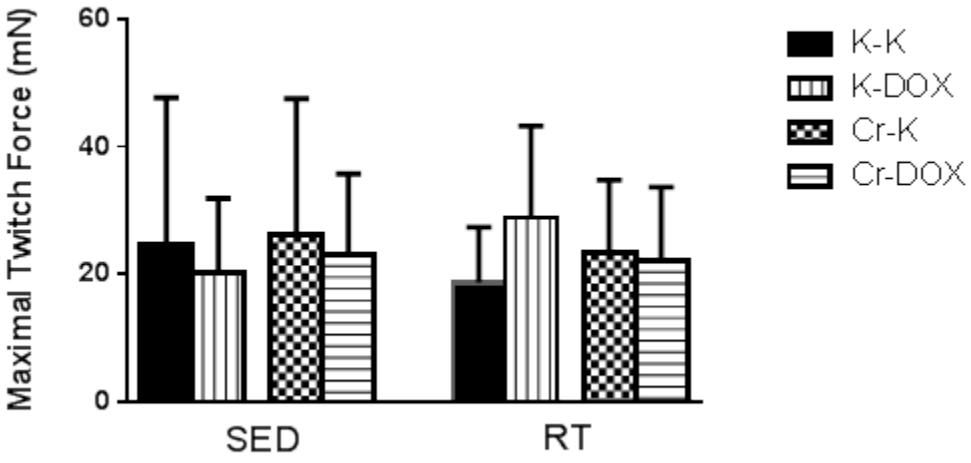


Figure 5.5. Extensor digitorum longus maximal twitch force. SED, sedentary; RT, resistance training; K, Krebs-Henseleit buffer; Cr, Creatine; DOX, doxorubicin. No main effects or interactions.

The analysis of EDL maximal rate of force development revealed no significant main effects from activity, $F(1, 66) = 0.52$, $p = 0.4739$; drug $F(1, 66) = 0.01$, $p = 0.9355$; or nutrition, $F(1, 66) = 0.69$, p = 0.4091 (see Figure 5.6). Moreover, the interaction of activity and drug was not significant, $F(1, 66) = 0.92$, $p = 0.3415$. In addition, activity and nutrition did not result in a significant interaction, $F(1, 66) = 0.17$, $p = 0.6858$ nor was the combination of drug and nutrition, $F(1, 66) = 0.18$, $p = 0.6750$. Also, no significant three-way interaction among activity, drug, and nutrition was observed, $F(1, 66) = 0.20$, $p = 0.6568$.

As Figure 5.7 depicts, no main effect from activity, $F(1, 65) = 0.91$, $p = 0.3524$; drug, $F(1, 65) = 0.87$, $p = 0.3543$; or nutrition, $F(1, 65) = 0.45$, $p = 0.5040$, was observed for EDL maximal rate of force decline. Additionally, no significant interactions between activity and drug, $F(1, 65) = 1.27$, $p = 0.2637$; activity and nutrition, $F(1, 65) = 1.60$, $p = 0.2098$; or drug and nutrition, $F(1, 65) = 1.27$, $p = 0.2640$, were noted. Similarly, the three way interaction among activity, drug, or nutrition was not significant, $F(1, 65) = 0.02$, $p = 0.8911$.

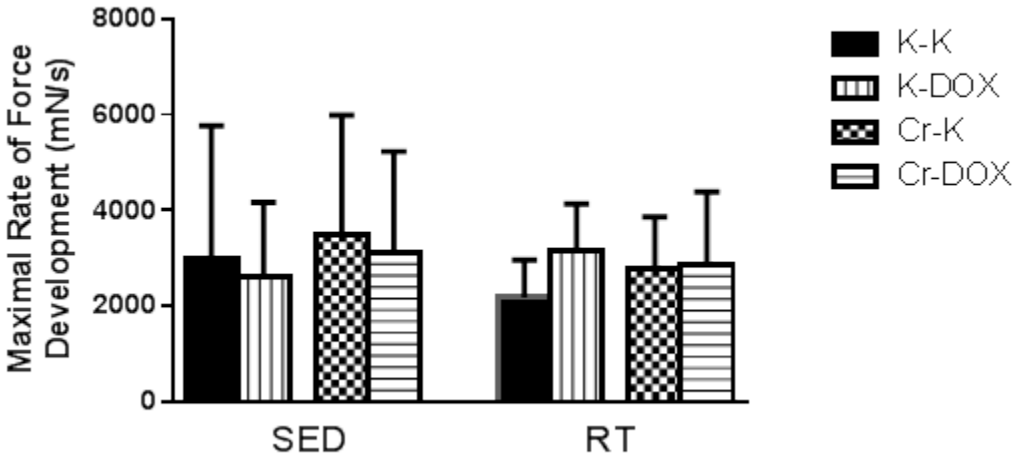


Figure 5.6. Extensor digitorum longus maximal rate of force development. SED, sedentary; RT, resistance training; K, Krebs-Henseleit buffer; Cr, Creatine; DOX, doxorubicin. No main effects or interactions.

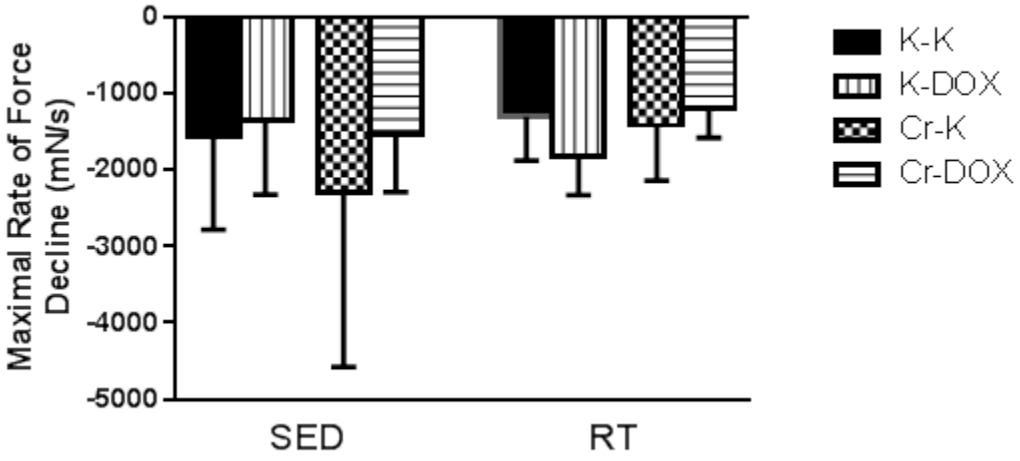


Figure 5.7. Extensor digitorum longus maximal rate of force decline. SED, sedentary; RT, resistance training; K, Krebs-Henseleit buffer; Cr, Creatine; DOX, doxorubicin. No main effects or interactions.

.

Fatigue

After determination of maximal twitch characteristics, and the buffers refreshed with K, the muscles were stimulated once per second for 100 seconds. Forces were then recorded every 10 seconds. Significant reductions from baseline in the SOL were seen at 70, 50, and 50 seconds for SED-K-K, SED-Cr-DOX, and SED-K-DOX, respectively. Conversely, SED-Cr-K did not have a significant drop from baseline during the observed time period, which indicated that Cr did delay the onset of fatigue. The addition of RT to the SOL produced a profoundly different response. A significant drop from baseline was observed at 60 seconds for RT-K-K, and 60 seconds for RT-K-DOX; however, the addition of RT delayed the onset of fatigue till 100 seconds for RT-Cr-DOX. No significant drop from baseline was found for RT-Cr-K group. Soleus fatigue data can be seen in Figure 5.8.

Sedentary-K-K fatigue for the EDL was significantly different from baseline at 60 seconds. With the addition of Cr, however, a significant reduction from baseline was not observed during the stimulation period. At the 50 second time point, SED-K-DOX was significantly lower than baseline. Sedentary-Cr-DOX was significantly lower than baseline at the 60 second time point. Significant reductions from baseline were found at 50 seconds for RT-K-DOX and 60 seconds for RT-K-K. The application of Cr delayed the onset of fatigue to 70 seconds in RT-Cr-DOX. This highlighted RT's unique response on fatigue rates in the EDL. Without DOX, the combination of RT and Cr did not result in significant drop from baseline. Resistance training EDL fatigue data can be seen in Figure 5.9.

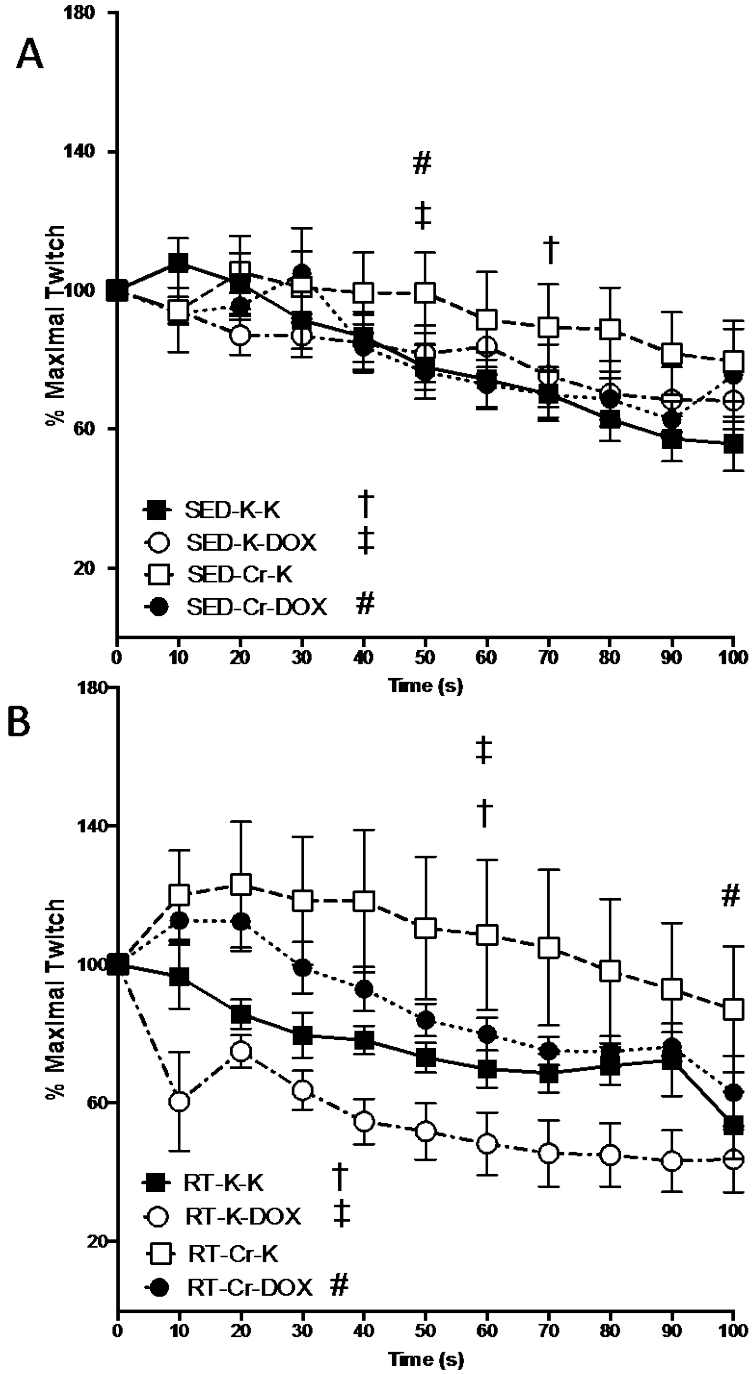


Figure 5.8. Soleus fatigue rates for a 100 second fatigue for the soleus of sedentary animals (A) and for the soleus of resistance trained animals (B). SED, sedentary; RT, resistance training; K, Krebs-Henseleit buffer; Cr, Creatine; DOX, doxorubicin. Symbols indicate significant reductions from baseline. † K+K, significantly lower than baseline ($p < 0.05$), ‡ K+DOX, significantly lower than baseline ($p < 0.05$), and # Cr+DOX, significantly lower than baseline ($p < 0.05$).

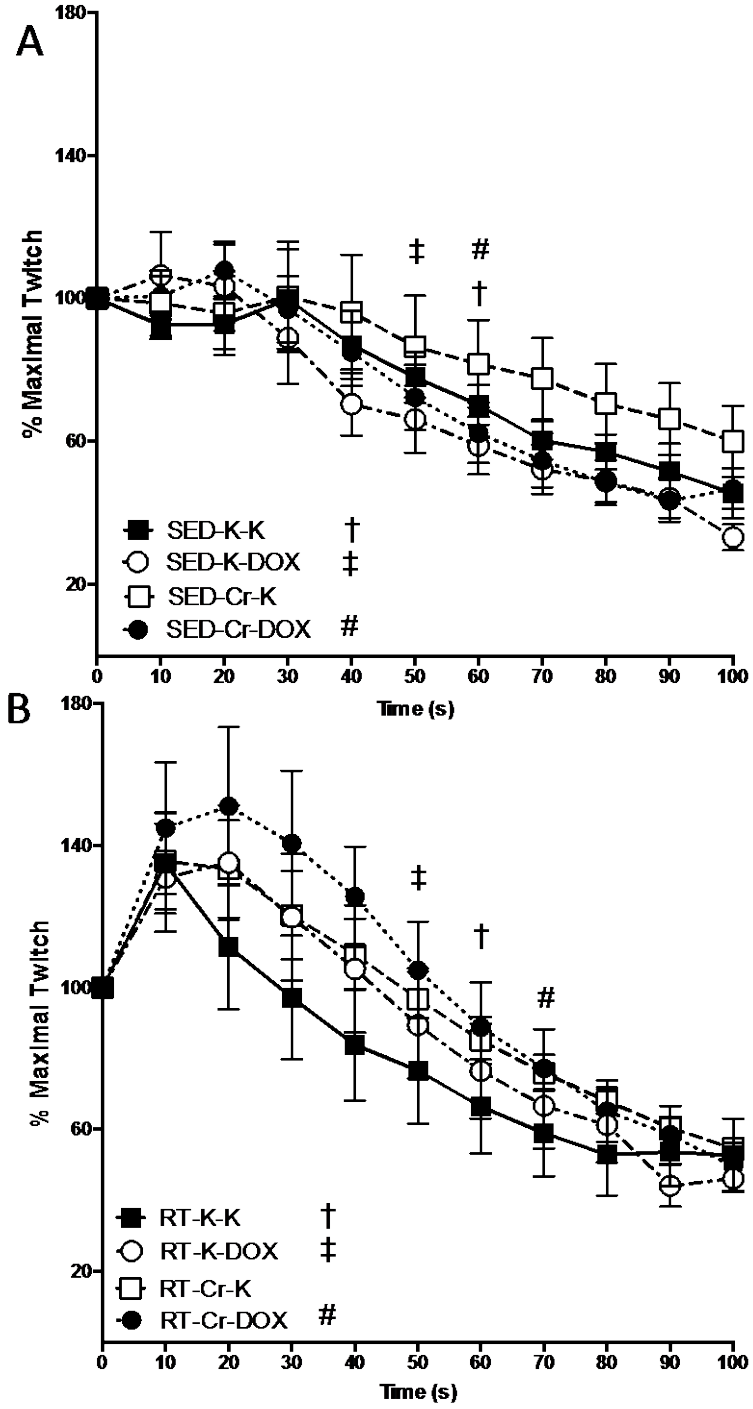


Figure 5.9. Extensor digitorum longus fatigue rates. Fatigue rates for a 100 second fatigue for the extensor digitorum longus of sedentary animals (A) and for the extensor digitorum longus of resistance trained animals (B). SED, sedentary; RT, resistance training; K, Krebs-Henseleit buffer; Cr, Creatine; DOX, doxorubicin. Symbols indicate significant reductions from baseline. † K+K, significantly lower than baseline ($p < 0.05$), ‡ K+DOX, significantly lower than baseline ($p < 0.05$), and # Cr+DOX, significantly lower than baseline ($p < 0.05$).

Creatine Content

Total Cr concentration was analyzed using the left SOL and EDL from SED-K-K, Sed-Cr-K, RT-K-K, and RT-Cr-K animals. To test the effect of activity (RT and SED) and nutrition (Cr or K) on muscle Cr content, a two way ANOVA was performed. In the SOL, results showed no significant main effect of activity, $F(1, 24) = 0.8469$, $p = 0.3666$, or nutrition, $F(1, 24) = 0.7668$, $p = 0.3899$; however, there was a significant interaction between activity and nutrition on SOL Cr content, $F(1, 24) = 4.760$, $p = 0.0392$. As can be seen in Figure 5.10, in the RT condition, SOL tissue incubated with Cr had higher Cr content than non-treated controls. To explore this effect further, Tukey's multiple comparison tests were performed and no significant differences in intracellular Cr were observed ($p > 0.05$).

In the EDL, there was a significant main effect, $F(1, 24) = 6.129$, $p = 0.0207$, of nutrition on total intracellular Cr concentration; however, no significant activity effect was observed, $F(1, 24) = 1.119$, $p = 0.3006$, and there was no significant interactions, $F(1, 24) = 0.5208$, $p = 0.4775$ (see Figure 5.10). Tukey's post hoc testing did not show any significant differences between the groups ($p > 0.05$; see Figure 5.11).

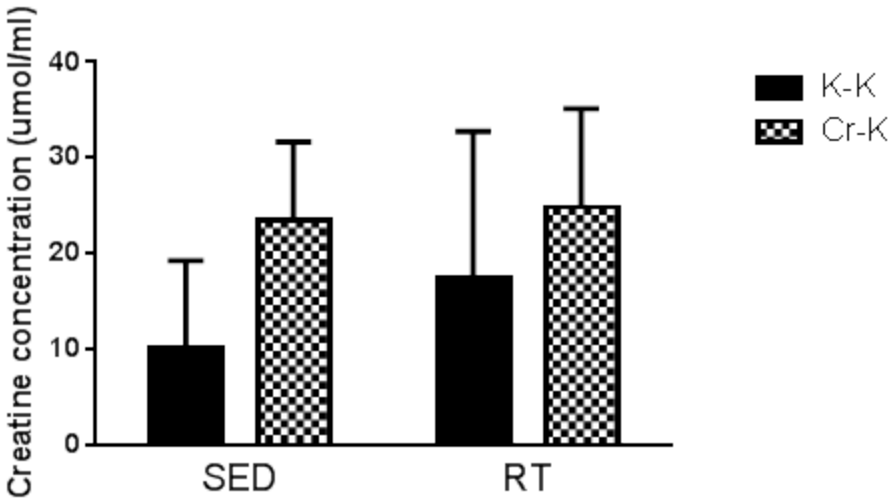


Figure 5.10. Soleus creatine concentration. SED, sedentary; RT, resistance training; K, Krebs-Henseleit buffer; Cr, Creatine. Significant interaction between activity (RT) and nutrition (Cr), $F(1, 24) = 4.760$, $p = 0.0392$.

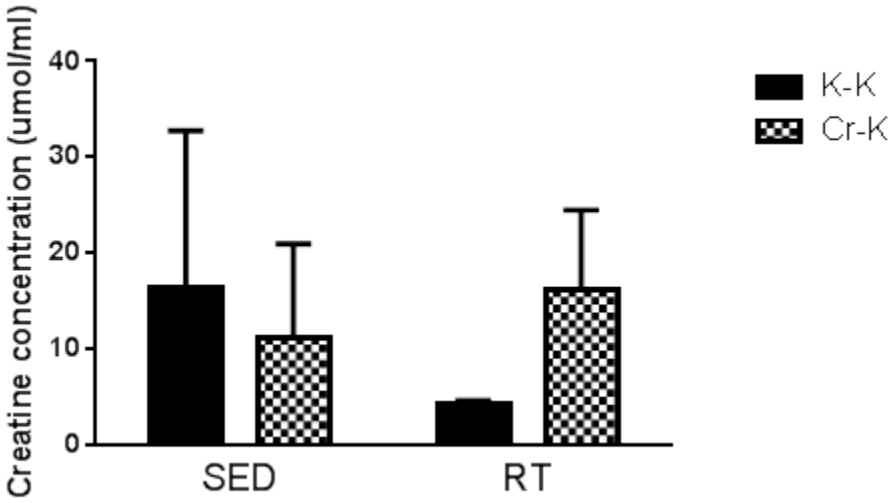


Figure 5.11. Extensor digitorum longus creatine concentration. SED, sedentary; RT, resistance training; K, Krebs-Henseleit buffer; Cr, Creatine. Significant main effect nutrition (Cr), $F(1, 24) = 6.129$, $p = 0.0207$.

Discussion

Resistance training (RT) represents a therapeutic intervention that could be utilized to improve the lives of cancer patients. In fact, RT has been used as a means of palliative care and has been shown to reduce cancer-related fatigue (Adamsen et al., 2009; Meneses-Echavez, Gonzalez-Jimenez, & Ramirez-Velez, 2014), improve muscular strength in patients with heart failure (Gielen, Laughlin, O'Conner, & Duncker, 2014), preserve muscle mass in patients with heart failure (Gielen et al., 2014), promote increased blood cell counts in those going through chemotherapy (Karvinen, Esposito, Raedeke, Vick, & Walker, 2014), and improve breast cancer outcomes during chemotherapy (Courneya et al., 2014). More importantly, RT has been shown to be beneficial for minimizing fatigue and improving quality of life in cancer patients (Courneya & Friedenreich, 1999). These benefits demonstrate the powerful benefits that can occur with RT. Thus, it can be concluded that RT can be used to minimize DOX-induced muscle dysfunction. However, there is substantially less information about the appropriate dosages for the application of RT. It is important that further studies be done to identify the appropriate application of RT and in a manner that is clinical relevant to cancer patients.

Current evidence suggests a RT period from six weeks to six months is required to minimize the side effects of the cancer treatment process. Given the meaningful improvements in a wide variety of physiological and quality of life outcomes during the cancer treatment process, it can be concluded that RT could and should be incorporated into the treatment plans of cancer patients. However, there is a great deal of variability in the improvements seen with RT (Focht et al., 2013). In general, evidence does suggest

that RT will produce a statistically significant increase in muscular strength, muscular endurance, improvements in physical function, and some improvements in quality of life (Howlader et al., 2013; Speck et al., 2010). Due to different cancer types, the kinds of treatment, and treatment stage at which the RT was implicated, it can be difficult to extrapolate these findings to general recommendations for cancer rehabilitation professionals. Animal-based studies, on the other hand, can provide an alternative to the compounding variables commonly seen with clinical type studies. With these types of studies, scientists can develop treatment recommendations based on the treatment type and level; it also allows the researcher a tremendous amount of control over the exercise intervention.

Aerobic exercise has been used to minimize DOX-induced cardiac dysfunction (Wonders et al., 2008; Chico et al., 2006), improve the antioxidants status (Ascensao et al., 2011), and improve vascular function (Gibson et al., 2013). However, to our knowledge, no study has been conducted on the ability of exercise to minimize DOX-induced muscle dysfunction. The usefulness of aerobic training to minimize DOX-induced muscle dysfunction still remains to be determined, but given the functional capacity of cancer patients, RT is perhaps the best choice for an exercise intervention to minimize DOX-induced muscle dysfunction. Resistance training by its nature can improve strength and muscular endurance, which could enhance the ability of cancer patients to perform ADL and minimize treatment-related fatigue.

The current study demonstrated the effects of DOX, Cr, and RT individually and in combination on DOX-induced muscle dysfunction. Given that DOX treatments commonly result in skeletal muscle dysfunction via changes in enzymatic (Smuder et al.,

2011), morphological (Stathopoulos et al., 1996), contractile (van Norren et al., 2009), and calcium handling (De Beer et al., 1992), cancer patients might require treatments that could augment multiple facets of skeletal muscle function. Despite the complex nature of these mechanisms, this study was able to show that resistance training in combination with Cr could mitigate DOX-induced muscular fatigue.

The skeletal muscle dysfunction seen with DOX treatment was unique in that it might stem from the cardiac dysfunction caused by DOX (Chicco et al., 2006; Hydock et al., 2008), and it has long been known that heart failure can lead to skeletal muscle wasting and changes in the contractile properties of skeletal muscle (Gosker, Wouters, van der Vusse, & Schols, 2000). However, identifying the exact cause of DOX-induced muscle dysfunction is complex because those who are undergoing DOX treatment are likely to have a number of secondary disease processes such as inadequate energy consumption, skeletal muscle disuse and atrophy, cachexia, or other syndromes that make it difficult to identify the true mechanism behind DOX-induced muscle dysfunction. These secondary disease processes might enhance the effects of heart failure (Liu et al., 2006), further contributing to the development of skeletal muscle dysfunction.

Although some mechanisms of DOX-induced muscle dysfunction have been identified, much of its effects on skeletal muscle are still unknown. In addition to secondary disease processes commonly accompanying the cancer treatments, we also know that DOX has direct effects on skeletal muscle function (van Norren et al., 2009). However, we show that Cr and RT could diminish DOX-induced muscle fatigue.

Muscle Dysfunction

To our knowledge, this is the first study to examine the effects of RT and Cr on DOX-induced muscle dysfunction. We show that incubation with DOX does not result in the skeletal muscle dysfunction commonly seen with *in vivo* treatments. Previous studies have shown that with lower frequency stimulation (40 Hz), there is an increase in maximal force following incubation with DOX (van Norren et al., 2009). Van Noreen et al. (2009) attributed the increase in maximal twitch force during low frequency stimulation to an increase in free intra-cellular Ca^{2+}. Work by Hydock et al. (2011) demonstrated a significant reduction in muscle force with DOX treatment; however, the dysfunction was more pronounced in the SOL than in the EDL. This is an important finding that highlights the effects of DOX on oxidative muscle like the SOL. Furthermore, evidence shows that treatment with DOX results in a significant decline in EDL function (Ertunc et al., 2009; Hydock et al., 2011; van Norren et al., 2009). In the current study, we were unable to find any significant reductions in maximal twitch characteristics with DOX treatment in either the SOL or EDL, but there was a significant main effect of RT on SOL maximal twitch. This finding suggested that, generally, RT produced a lower maximal twitch force in the SOL; however, post hoc testing revealed no significant differences between the groups. Contradictory results between this study and previous *in vivo* studies were likely due to differences in methodology in DOX administration. For example, work by van Noreen et al. (2009) showed that incubating mouse EDL in 100 or 175 μM DOX for one hour or more resulted in a time- and concentration-dependent decrease in maximal forces. In comparison, our investigation used 30 minute incubation and a DOX concentration of 24 μM, which might not have

been sufficient enough for DOX to exert its full myotoxic effects. A DOX concentration of 24 μM was used because it represented the upper limits for serum concentrations following infusion with DOX (Baurain et al., 1982). Thus, future investigations should utilize multiple incubation periods to gain a better understanding of the effects of DOX on skeletal muscle.

Fatigue

Doxorubicin use is associated with excessive patient fatigue and skeletal muscle dysfunction (Fairclough, Fetting, Cella, Wonson, & Moinpour, 1999; Liu et al., 2006), which can lead to significant reductions in the quality of life for cancer patients. Thus, it is imperative to develop approaches that can help minimize these negative effects. Fatigue represents a significant obstacle for cancer patients, and it can be reason to slow or stop treatment altogether. Although fatigue is relatively common in cancer patients going through the cancer treatment process, the exact mechanisms behind the observed chemotherapy-induced fatigue remains elusive. Even in the in the healthy individual, it can be extremely difficult to identify the exact cause of fatigue. Despite the complex nature of fatigue, there is a wealth of literature documenting DOX's effects on a variety of tissues. To that end, DOX has been shown to interfere with normal mitochondrial function, result in the excessive generation of ROS, and increase rates of atrophy (Giliam et al., 2011). The impaired mitochondrial function could alter the amount ATP available for normal cellular activities like cross bridge cycling and ion pumping. The increased rates of ROS generation can quickly overwhelm endogenous antioxidants and lead to an increase in protein and lipid oxidation, which can cause disruptions in normal organelle and protein function. Furthermore, DOX has been shown to lead to increased rates of

atrophy (Bonifati et al., 2000). This decrease in muscle mass as well as the compromised cellular function provides a multicomponent explanation to observed DOX-induced myotoxicity.

Although this study did not show significant reductions in maximal twitch characteristics, it did show that DOX treated tissue had a faster time to fatigue than untreated tissue. These results were in agreement with previous studies (Hydock et al., 2011; van Norren et al., 2009). For instance, *ex vivo* work by Hydock et al. (2011) demonstrated that DOX-induced fatigue in the SOL occurred at 20-30 seconds and at 70 seconds in untreated controls. Similarly, we showed that DOX treatment resulted in a faster time to fatigue than untreated controls. In addition, evidence from this study showed that RT prior to DOX treatment delayed the onset of fatigue by 10 seconds in the SOL when compared to SED-K-DOX. Moreover, the combination of RT and Cr prior to DOX was able to delay fatigue to the 100-second time point. This was an important finding that highlighted the unique nature of RT and Cr in minimizing DOX-induced SOL dysfunction and warrants further investigation.

This study demonstrated that treatment with DOX resulted in an earlier time to fatigue by 10 seconds when compared to SED-K-K. Unlike in the SOL, prior RT did not delay the onset of fatigue in the DOX-treated EDL. Conversely, Cr prior to DOX treatment delayed the onset of fatigue, while including a combination of RT and Cr before DOX helped delay the onset of fatigue by 20 seconds when compared to SED-DOX . Given that RT and Cr were able to delay the onset of fatigue beyond the effects of RT or Cr alone suggested the combined effects of these treatments could be used to offset

the myotoxic effects of DOX in cancer patients. Being able to mitigate the DOX-induced fatigue could do much to improve their lives.

These findings provided the first evidence that the combination of Cr and RT could be used to offset DOX-induced fatigue. Separately, Cr has been shown to improve intracellular Cr stores (Persky & Brazeau, 2001), increase PCr resynthesis (Volek et al., 2004), and act as antioxidant (Lawler et al., 2002). The ability to improve short-term energy supply and minimize oxidative damage could explain some aspects of the reduced fatigue rate. In addition, the ability to minimize DOX's capacity for ROS generation could help preserve mitochondrial integrity, which could help to preserve ATP production and help maintain normal muscle function. Yet, as the results showed, it was only in combination with RT when the DOX-induced fatigue was the least apparent. Thus, in combination with Cr, there was an increase in short term energy supply and a larger overall contractile apparatus capable utilizing the increase in ATP. In view of the current findings, the combination of Cr and RT could do much to help minimize DOX-induced fatigue and improve the lives of those going through the cancer treatment process.

Creatine Concentration

Creatine's effectiveness as an ergogenic aid has been well documented in healthy, trained, and diseased populations (Kreider, 2003). In the current investigation, a primary objective was to determine if incubating muscle tissue in Cr would result in an increase in total Cr levels. We also examined whether prior RT could have an effect on Cr content. Results from this investigation indicated a significant interaction between the combinations of RT and Cr toward a higher total Cr content; however, *post hoc* testing

did not reveal any significant differences across groups. In the EDL, there was a significant main effect from Cr on total Cr content. Although a significant main effect was found, no significance between group differences was observed with *post hoc* testing.

It should be noted that the Cr analysis used could not differentiate between Cr and phosphocreatine (PCr). Although these results were encouraging and provided evidence to warrant additional investigation, future studies should focus on the ratio of Cr to PCr. This is an important consideration given the significance of the PCr-shuttle and its role in maintaining ATP supply. Thus, it might be possible that the ratio of Cr to PCr could be altered by Cr or RT. Furthermore, evidence also suggested that mitochondrial PCr stores could change up to 100-fold when growth factors were removed (Vander Heiden et al., 2000). Thus, it is plausible that Cr might preserve mitochondrial function in the DOX treated cell by maintaining mitochondrial PCr stores. In addition to Cr effects on cellular energy, Cr also exerts an antioxidant effect (Lawler et al., 2002). Lawler et al. (2002) demonstrated that Cr acted as a direct antioxidant against radical ROS. Moreover, there is evidence that Cr can prevent oxidative damage to mitochondrial DNA (mtDNA; Guidi et al., 2008). Mitochondrial DNA is important because it can have profound effects on the energy state of the cell and has recently been implicated in a variety of oxidative stress disorders (Copeland, 2010). Taken together, these findings suggested that Cr could be effective at minimizing oxidative damage caused by DOX, maintaining energy supply, and preserving PCr stores.

Summary

The present study examined the effects of RT and Cr on DOX-induced muscle dysfunction within the SOL and EDL. Although this study did not show DOX-induced alterations in maximal twitch characteristics commonly seen with *in vivo* studies, it did show an earlier time to fatigue with DOX treatment in the SOL and EDL. Additionally, this investigation demonstrated that RT and Cr in combination could attenuate fatigue in the DOX-treated muscle. This finding provided the first evidence for the incorporation of RT and Cr into the treatment plan to help minimize DOX-induced muscle dysfunction. Adding RT and Cr to the treatment plan could provide cancer patients with an improved quality of life and increased capacity to perform ADL. Although more research should be done, these findings provided insight into the potential application of combined treatment with RT and Cr to preserve function and offset the myotoxic effects of DOX. Such evidence provided more support for the effectiveness and capacity of exercise and nutritional interventions to improve functional capacity for cancer patients during their treatment process.

REFERENCES

Abraham, R., Basser, R. L., & Green, M. D. (1996). A risk-benefit assessment of anthracycline antibiotics in antineoplastic therapy. *Drug Saf, 15*(6), 406-429. Retrieved from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd= Retrieve&db= PubMed&dopt=Citation&list_uids=8968695

Abramson, J. J., Buck, E., Salama, G., Casida, J. E., & Pessah, I. N. (1988). Mechanism of anthraquinone-induced calcium release from skeletal muscle sarcoplasmic reticulum. *J Biol Chem, 263*(35), 18750-18758. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3198599

Adamsen, L., Quist, M., Andersen, C., Moller, T., Herrstedt, J., Kronborg, D., . . . Rorth, M. (2009). Effect of a multimodal high intensity exercise intervention in cancer patients undergoing chemotherapy: randomised controlled trial. *BMJ, 339*, b3410. doi:10.1136/bmj.b3410

Antonelli, J., Freedland, S. J., & Jones, L. W. (2009). Exercise therapy across the prostate cancer continuum. *Prostate Cancer Prostatic Dis, 12*(2), 110-115. doi:10.1038/pcan.2009.4

Arcamone, F., Cassinelli, G., Fantini, G., Grein, A., Orezzi, P., Pol, C., & Spalla, C. (1969). Adriamycin, 14-hydroxydaunomycin, a new antitumor antibiotic from S. peucetius var. caesius. *Biotechnol Bioeng, 11*(6), 1101-1110. Retrieved from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=5365804

Arnold, D. L., Matthews, P. M., & Radda, G. K. (1984). Metabolic recovery after exercise and the assessment of mitochondrial function in vivo in human skeletal muscle by means of 31P NMR. *Magn Reson Med, 1*(3), 307-315. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/6571561

Ascensao, A., Lumini-Oliveira, J., Machado, N. G., Ferreira, R. M., Goncalves, I. O., Moreira, A. C., . . . Magalhaes, J. (2011). Acute exercise protects against calcium-induced cardiac mitochondrial permeability transition pore opening in doxorubicin-treated rats. *Clin Sci (Lond), 120*(1), 37-49. doi:10.1042/CS20100254

Baurain, R., Deprez-De Campeneere, D., Zenebergh, A., & Trouet, A. (1982). Plasma levels of doxorubicin after IV bolus injection and infusion of the doxorubicin-DNA complex in rabbits and man. Comparison with free doxorubicin. *Cancer Chemother Pharmacol, 9*(2), 93-96. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7172411

Beis, I., & Newsholme, E. A. (1975). The contents of adenine nucleotides, phosphagens and some glycolytic intermediates in resting muscles from vertebrates and invertebrates. *Biochem J, 152*(1), 23-32. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1212224

Bemben, M. G., & Lamont, H. S. (2005). Creatine supplementation and exercise performance: recent findings. *Sports Med, 35*(2), 107-125. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/15707376

Berthiaume, J. M., & Wallace, K. B. (2007). Adriamycin-induced oxidative mitochondrial cardiotoxicity. *Cell Biol Toxicol, 23*(1), 15-25. doi:10.1007/s10565-006-0140-y

Bodine, S. C., Stitt, T. N., Gonzalez, M., Kline, W. O., Stover, G. L., Bauerlein, R., . . . Yancopoulos, G. D. (2001). Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. *Nat Cell Biol, 3*(11), 1014-1019. doi:10.1038/ncb1101-1014

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 Chemother Pharmacol, 46*(6), 517-522. doi:10.1007/s002800000175

Chatterjee, K., Zhang, J., Honbo, N., & Karliner, J. S. (2010). Doxorubicin cardiomyopathy. *Cardiology, 115*(2), 155-162. doi:10.1159/000265166

Cheema, B., Gaul, C. A., Lane, K., & Fiatarone Singh, M. A. (2008). Progressive resistance training in breast cancer: a systematic review of clinical trials. *Breast Cancer Res Treat, 109*(1), 9-26. doi:10.1007/s10549-007-9638-0

Chicco, A., Hydock, D., Schneider, C., & Hayward, R. (2006). Low-intensity exercise training during doxorubicin treatment protects against cardiotoxicity. *Journal of appllied physiology*, 519-527.

Chicco, A. J., Schneider, C. M., & Hayward, R. (2005). Voluntary exercise protects against acute doxorubicin cardiotoxicity in the isolated perfused rat heart. *Am J Physiol Regul Integr Comp Physiol, 289*(2), R424-R431. doi:10.1152/ajpregu.00636.2004

Cholewa, J., Guimaraes-Ferreira, L., da Silva Teixeira, T., Naimo, M. A., Zhi, X., de Sa, R. B., . . . Zanchi, N. E. (2014). Basic models modeling resistance training: an update for basic scientists interested in study skeletal muscle hypertrophy. *J Cell Physiol, 229*(9), 1148-1156. doi:10.1002/jcp.24542

Clark, J. F. (1997). Creatine and phosphocreatine: a review of their use in exercise and sport. *J Athl Train, 32*(1), 45-51. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/16558432

Copeland, W. C. (2010). Understanding heterogeneous diseases in mtDNA maintenance. *Methods, 51*(4), 363. doi:10.1016/j.ymeth.2010.07.008

Courneya, K. S. (2009). Physical activity in cancer survivors: a field in motion. *Psychooncology, 18*(4), 337-342. doi:10.1002/pon.1546

Courneya, K. S., & Friedenreich, C. M. (1999). Physical exercise and quality of life following cancer diagnosis: a literature review. *Ann Behav Med, 21*(2), 171-179. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10499138

Courneya, K. S., & Friedenreich, C. M. (2001). Framework PEACE: an organizational model for examining physical exercise across the cancer experience. *Ann Behav Med, 23*(4), 263-272. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11761343

Courneya, K. S., Segal, R. J., McKenzie, D. C., Dong, H., Gelmon, K., Friedenreich, C. M., . . . Mackey, J. R. (2014). Effects of exercise during adjuvant chemotherapy on breast cancer outcomes. *Med Sci Sports Exerc, 46*(9), 1744-1751. doi:10.1249/MSS.0000000000000297

Dalla Libera, L., Vescovo, G., & Volterrani, M. (2008). Physiological basis for contractile dysfunction in heart failure. *Curr Pharm Des, 14*(25), 2572-2581. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/18991674

Darrabie, M. D., Arciniegas, A. J., Mantilla, J. G., Mishra, R., Vera, M. P., Santacruz, L., & Jacobs, D. O. (2012). Exposing cardiomyocytes to subclinical concentrations of doxorubicin rapidly reduces their creatine transport. *Am J Physiol Heart Circ Physiol, 303*(5), H539-548. doi:10.1152/ajpheart.00108.2012

De Backer, I. C., Schep, G., Backx, F. J., Vreugdenhil, G., & Kuipers, H. (2009). Resistance training in cancer survivors: a systematic review. *Int J Sports Med, 30*(10), 703-712. doi:10.1055/s-0029-1225330

De Beer, E. L., Finkle, H., Voest, E. E., Van Heijst, B. G., & Schiereck, P. (1992). Doxorubicin interacts directly with skinned single skeletal muscle fibres. *Eur J Pharmacol, 214*(1), 97-100. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1582454

De Grey, A. D. (2000). Antioxidants and redox signaling: Internet resources. *Antioxid Redox Signal, 2*(4), 937-940. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11213493

Deavall, D. G., Martin, E. A., Horner, J. M., & Roberts, R. (2012). Drug-induced oxidative stress and toxicity. *J. Toxicol*, 107-116.

Demant, T. W., & Rhodes, E. C. (1999). Effects of creatine supplementation on exercise performance. *Sports Med, 28*(1), 49-60. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10461712

Deviatkina, T. A., Tarasenko, L. M., & Voskresenskii, O. N. (1984). [Lipid peroxidation in stress-induced tissue damage in adrenal gland insufficiency]. *Probl Endokrinol (Mosk), 30*(6), 60-65. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/6542664

Di Marco, A., Gaetani, M., Orezzi, P., Scarpinato, B. M., Silvestrini, R., Soldati, M., . . . Valentini, L. (1964). 'Daunomycin', a New Antibiotic of the Rhodomycin Group. *Nature, 201*, 706-707. Retrieved from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=14142092

Di Marco, A., Gaetani, M., & Scarpinato, B. (1969). Adriamycin (NSC-123,127): a new antibiotic with antitumor activity. *Cancer Chemother Rep, 53*(1), 33-37. Retrieved from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=5772652

Doroshow, J. H., Tallent, C., & Schechter, J. E. (1985). Ultrastructural features of Adriamycin-induced skeletal and cardiac muscle toxicity. *Am J Pathol, 118*(2), 288-297. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3970141

Du, J., Wang, X., Miereles, C., Bailey, J. L., Debigare, R., Zheng, B., . . . Mitch, W. E. (2004). Activation of caspase-3 is an initial step triggering accelerated muscle proteolysis in catabolic conditions. *J Clin Invest, 113*(1), 115-123. doi:10.1172/JCI18330

Dubost, M., Ganter, P., Maral, R., Ninet, L., Pinnert, S., Preudhomme, J., & Werner, G. H. (1964). Rubidomycin: a New Antibiotic with Cytostatic Properties. *Cancer Chemother Rep, 41*, 35-36. Retrieved from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=14213139

Earnshaw, W. C., Martins, L. M., & Kaufmann, S. H. (1999). Mammalian caspases: structure, activation, substrates, and functions during apoptosis. *Annu Rev Biochem, 68*, 383-424. doi:10.1146/annurev.biochem.68.1.383

Eisner, V., Lenaers, G., & Hajnoczky, G. (2014). Mitochondrial fusion is frequent in skeletal muscle and supports excitation-contraction coupling. *J Cell Biol, 205*(2), 179-195. doi:10.1083/jcb.201312066

Elbl, L., Vasova, I., Tomaskova, I., Jedlicka, F., Kral, Z., Navratil, M., . . . Vorlicek, J. (2006). Cardiopulmonary exercise testing in the evaluation of functional capacity after treatment of lymphomas in adults. *Leuk Lymphoma, 47*(5), 843-851. doi:10.1080/10428190500402559

Eom, Y. W., Kim, M. A., Park, S. S., Goo, M. J., Kwon, H. J., Sohn, S., . . . Choi, K. S. (2005). Two distinct modes of cell death induced by doxorubicin: apoptosis and cell death through mitotic catastrophe accompanied by senescence-like phenotype. *Oncogene, 24*(30), 4765-4777. doi:10.1038/sj.onc.1208627

Ertunc, M., Sara, Y., Korkusuz, P., & Onur, R. (2009). Differential contractile impairment of fast- and slow-twitch skeletal muscles in a rat model of doxorubicin-induced congestive heart failure. *Pharmacology, 84*(4), 240-248. doi:10.1159/000241723

Fairclough, D. L., Fetting, J. H., Cella, D., Wonson, W., & Moinpour, C. M. (1999). Quality of life and quality adjusted survival for breast cancer patients receiving adjuvant therapy. Eastern Cooperative Oncology Group (ECOG). *Qual Life Res, 8*(8), 723-731. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10855346

Falkenberg, J. H., Iaizzo, P. A., &McLoon, L. K. (2001). Physiological assessment of muscle strength in vitro after direct injection of doxorubicin into rabbit sternocleidomastoid muscle. *Movement Disorders, 16,* 683–692. doi:10.1002/mds.1125

Fiske, C. H., & Subbarow, Y. (1927). The nature of the "inorganic phosphate" in voluntary muscle. *Science, 65*(1686), 401-403. doi:10.1126/science.65.1686.401

Fluda, S., Sieverts, H., Friesen, C., Herr, I., & Debatin, K. (1997). The cd95 (apo-1/fas) system mediates drug induced apoptosis in neuroblastoma cells. . *Cancer res.*, 57.

Focht, B. C., Clinton, S. K., Devor, S. T., Garver, M. J., Lucas, A. R., Thomas-Ahner, J. M., & Grainger, E. (2013). Resistance exercise interventions during and following cancer treatment: a systematic review. *J Support Oncol, 11*(2), 45-60. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/23967493

Folin, O., & Denis, W. (1912). Protein metabolism from the standpoint of blood and tissue analysis: Third paper. Further absorbtion experiments with especial reference to the behavior of creatine and creatinine and to the formation of UREA *Journal of Biological Chemistry, 12*, 141-162.

Fukuse, T., Satoda, N., Hijiya, K., & Fujinaga, T. (2005). Importance of a comprehensive geriatric assessment in prediction of complications following thoracic surgery in elderly patients. *Chest, 127*(3), 886-891. doi:10.1378/chest.127.3.886

Galvao, D. A., Taaffe, D. R., Spry, N., Joseph, D., & Newton, R. U. (2010). Combined resistance and aerobic exercise program reverses muscle loss in men undergoing androgen suppression therapy for prostate cancer without bone metastases: a randomized controlled trial. *J Clin Oncol, 28*(2), 340-347. doi:10.1200/JCO.2009.23.2488

Galvao, D. A., Taaffe, D. R., Spry, N., Joseph, D., Turner, D., & Newton, R. U. (2009). Reduced muscle strength and functional performance in men with prostate cancer undergoing androgen suppression: a comprehensive cross-sectional investigation. *Prostate Cancer Prostatic Dis, 12*(2), 198-203. doi:10.1038/pcan.2008.51

Gibson, N. M., Greufe, S. E., Hydock, D. S., & Hayward, R. (2013). Doxorubicin-induced vascular dysfunction and its attenuation by exercise preconditioning. *J Cardiovasc Pharmacol, 62*(4), 355-360. doi:10.1097/FJC.0b013e31829c9993

Gielen, S., Laughlin, M. H., O'Conner, C., & Duncker, D. J. (2014). Exercise Training in Patients with Heart Disease: Review of Beneficial Effects and Clinical Recommendations. *Prog Cardiovasc Dis*. doi:10.1016/j.pcad.2014.10.001

Gilliam, L. A., Moylan, J. S., Callahan, L. A., Sumandea, M. P., & Reid, M. B. (2011). Doxorubicin causes diaphragm weakness in murine models of cancer chemotherapy. *Muscle Nerve, 43*(1), 94-102. doi:10.1002/mus.21809

Gilliam, L. A., & St Clair, D. K. (2011). Chemotherapy-induced weakness and fatigue in skeletal muscle: the role of oxidative stress. *Antioxid Redox Signal, 15*(9), 2543-2563. doi:10.1089/ars.2011.3965

Goding, G. S., Jr., & Pernell, K. J. (2000). Doxorubicin chemomyectomy: effects on evoked vocal fold tension and mucosal wave. *Ann Otol Rhinol Laryngol, 109*(3), 294-300. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10737314

Goll, D. E., Thompson, V. F., Li, H., Wei, W., & Cong, J. (2003). The calpain system. *Physiol Rev, 83*(3), 731-801. doi:10.1152/physrev.00029.2002

Gosker, H. R., Wouters, E. F., van der Vusse, G. J., & Schols, A. M. (2000). Skeletal muscle dysfunction in chronic obstructive pulmonary disease and chronic heart failure: underlying mechanisms and therapy perspectives. *Am J Clin Nutr, 71*(5), 1033-1047. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10799364

Green, D. M., Grigoriev, Y. A., Nan, B., Takashima, J. R., Norkool, P. A., D'Angio, G. J., & Breslow, N. E. (2001). Congestive heart failure after treatment for Wilms' tumor: a report from the National Wilms' Tumor Study group. *J Clin Oncol, 19*(7), 1926-1934. Retrieved from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11283124

Greene, D., Nail, L. M., Fieler, V. K., Dudgeon, D., & Jones, L. S. (1994). A comparison of patient-reported side effects among three chemotherapy regimens for breast cancer. *Cancer Pract, 2*(1), 57-62. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8055007

Greenhaff, P. L., Bodin, K., Soderlund, K., & Hultman, E. (1994). Effect of oral creatine supplementation on skeletal muscle phosphocreatine resynthesis. *Am J Physiol, 266*(5 Pt 1), E725-730. Retrieved from http://www.ncbi.nlm.nih.gov /pubmed/8203511

Guerrero-Ontiveros, M. L., & Wallimann, T. (1998). Creatine supplementation in health and disease. Effects of chronic creatine ingestion in vivo: down-regulation of the expression of creatine transporter isoforms in skeletal muscle. *Mol Cell Biochem, 184*(1-2), 427-437. Retrieved from http://www.ncbi.nlm.nih. gov/pubmed/9746337

Guidi, C., Potenza, L., Sestili, P., Martinelli, C., Guescini, M., Stocchi, L., . . . Stocchi, V. (2008). Differential effect of creatine on oxidatively-injured mitochondrial and nuclear DNA. *Biochim Biophys Acta, 1780*(1), 16-26. doi:10.1016/j.bbagen.2007.09.018

Guimbal, C., & Kilimann, M. W. (1993). A Na(+)-dependent creatine transporter in rabbit brain, muscle, heart, and kidney. cDNA cloning and functional expression. *J Biol Chem, 268*(12), 8418-8421. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8473283

Halliwell, B., & Chirico, S. (1993). Lipid peroxidation: its mechanism, measurement, and significance. *Am J Clin Nutr, 57*(5 Suppl), 715S-724S; discussion 724S-725S. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8475889

Haq, M. M., Legha, S. S., Choksi, J., Hortobagyi, G. N., Benjamin, R. S., Ewer, M., & Ali, M. (1985). Doxorubicin-induced congestive heart failure in adults. *Cancer, 56*(6), 1361-1365. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/4027874

Harada, Y., Kato, S., Komiya, H., Shirota, T., Mukai, K., & Hayashi, T. (2004). Primary omental gamma/delta T-cell lymphoma involving the central nervous system. *Leuk Lymphoma, 45*(9), 1947-1950. doi:10.1080/10428190410001697368

Harrington, D., Anker, S. D., Chua, T. P., Webb-Peploe, K. M., Ponikowski, P. P., Poole-Wilson, P. A., & Coats, A. J. (1997). Skeletal muscle function and its relation to exercise tolerance in chronic heart failure. *J Am Coll Cardiol, 30*(7), 1758-1764. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9385904

Harris, R. C., Hultman, E., & Nordesjo, L. O. (1974). Glycogen, glycolytic intermediates and high-energy phosphates determined in biopsy samples of musculus quadriceps femoris of man at rest. Methods and variance of values. *Scand J Clin Lab Invest, 33*(2), 109-120. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/4852173

Harris, R. C., Soderlund, K., & Hultman, E. (1992). Elevation of creatine in resting and exercised muscle of normal subjects by creatine supplementation. *Clin Sci (Lond), 83*(3), 367-374. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1327657

Hayward, R., & Hydock, D. S. (2007). Doxorubicin cardiotoxicity in the rat: an in vivo characterization. *J Am Assoc Lab Anim Sci, 46*(4), 20-32. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/17645292

Head, S. I., Greenaway, B., & Chan, S. (2011). Incubating isolated mouse EDL muscles with creatine improves force production and twitch kinetics in fatigue due to reduction in ionic strength. *PLoS One, 6*(8), e22742. doi:10.1371/journal.pone.0022742

Honda, H., Kondo, T., Zhao, Q. L., Feril, L. B., Jr., & Kitagawa, H. (2004). Role of intracellular calcium ions and reactive oxygen species in apoptosis induced by ultrasound. *Ultrasound Med Biol, 30*(5), 683-692. doi:10.1016/j.ultrasmedbio.2004.02.008

Howlader, N., Noone, A., Krapcho, M., Garshell, J., Neyman, N., Altekruse, S., . . . Cronin, K. (Producer). (2013, november). National Cancer Institute. *National Cancer Institute*. Retrieved from http://seer.cancer.gov/csr/1975_2010/

Hydock, D., Lien, C.-Y., Jensen, B. T., Schneider, C. M., & Hayward, R. (2011). Characterization of the effect of in vivo doxorubicin treatment on skeletal muscle function in the rat. *Anticancer Res, 31*(6), 2023-2028. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/21737618

Hydock, D. S., Lien, C. Y., Schneider, C. M., & Hayward, R. (2008). Exercise preconditioning protects against doxorubicin-induced cardiac dysfunction. *Med Sci Sports Exerc, 40*(5), 808-817. doi:10.1249/MSS.0b013e318163744a

Ichikawa, Y., Ghanefar, M., Bayeva, M., Wu, R., Khechaduri, A., Naga Prasad, S. V., . . . Ardehali, H. (2014). Cardiotoxicity of doxorubicin is mediated through mitochondrial iron accumulation. *J Clin Invest, 124*(2), 617-630. doi:10.1172/JCI72931

Inui, A. (2002). Cancer anorexia-cachexia syndrome: current issues in research and management. *CA Cancer J Clin, 52*(2), 72-91. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11929007

Ito, H., Miller, S. C., Billingham, M. E., Akimoto, H., Torti, S. V., Wade, R., . . . Torti, F. M. (1990). Doxorubicin selectively inhibits muscle gene expression in cardiac muscle cells in vivo and in vitro. *Proc Natl Acad Sci U S A, 87*(11), 4275-4279. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2349236

Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., Igarashi, K., Engel, J. D., & Yamamoto, M. (1999). Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev, 13*(1), 76-86. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9887101

Jacobsen, P. B., Hann, D. M., Azzarello, L. M., Horton, J., Balducci, L., & Lyman, G. H. (1999). Fatigue in women receiving adjuvant chemotherapy for breast cancer: characteristics, course, and correlates. *J Pain Symptom Manage, 18*(4), 233-242. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10534963

Janeesh, P. A., & Abraham, A. (2014). Robinin modulates doxorubicin-induced cardiac apoptosis by TGF-beta1 signaling pathway in Sprague Dawley rats. *Biomed Pharmacother*. doi:10.1016/j.biopha.2014.09.010

Jennings-Sanders, A., & Anderson, E. T. (2003). Older women with breast cancer: perceptions of the effectiveness of nurse case managers. *Nurs Outlook, 51*(3), 108-114. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12830102

Jhamb, R., Gupta, N., Garg, S., Kumar, S., Gulati, S., Mishra, D., & Beniwal, P. (2007). Diffuse lymphomatous infiltration of kidney presenting as renal tubular acidosis and hypokalemic paralysis: case report. *Croat Med J, 48*(6), 860-863. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/18074421

Ji, L. L. (2008). Modulation of skeletal muscle antioxidant defense by exercise: Role of redox signaling. *Free Radic Biol Med, 44*(2), 142-152. doi:10.1016/j.freeradbiomed.2007.02.031

Jones, L. W., & Demark-Wahnefried, W. (2006). Diet, exercise, and complementary therapies after primary treatment for cancer. *Lancet Oncol, 7*(12), 1017-1026. doi:10.1016/S1470-2045(06)70976-7

Jones, L. W., Liang, Y., Pituskin, E. N., Battaglini, C. L., Scott, J. M., Hornsby, W. E., & Haykowsky, M. (2011). Effect of exercise training on peak oxygen consumption in patients with cancer: a meta-analysis. *Oncologist, 16*(1), 112-120. doi:10.1634/theoncologist.2010-0197

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. *J Appl Physiol (1985), 59*(4), 1298-1303. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/4055607

Karvinen, K. H., Esposito, D., Raedeke, T. D., Vick, J., & Walker, P. R. (2014). Effect of an exercise training intervention with resistance bands on blood cell counts during chemotherapy for lung cancer: a pilot randomized controlled trial. *Springerplus, 3*, 15. doi:10.1186/2193-1801-3-15

Kawasaki, H., & Kawashima, S. (1996). Regulation of the calpain-calpastatin system by membranes (review). *Mol Membr Biol, 13*(4), 217-224. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9116760

Kim, Y., Ma, A., & Kitta, K. (2003). Anthracycline-induced supression of GATA-4 transcription factor: implication in the regulation of cardiac myocycte apoptosis *Mol Pharmacol*, 368-377.

Kohen, R., & Nyska, A. (2002). Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. *Toxicol Pathol, 30*(6), 620-650. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12512863

Kraemer, W. J., & Volek, J. S. (1999). Creatine supplementation. Its role in human performance. *Clin Sports Med, 18*(3), 651-666, ix. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10410847

Kreider, R. B. (2003). Effects of creatine supplementation on performance and training adaptations. *Mol Cell Biochem, 244*(1-2), 89-94. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12701815

Kushmerick, M. J., Moerland, T. S., & Wiseman, R. W. (1992). Mammalian skeletal muscle fibers distinguished by contents of phosphocreatine, ATP, and Pi. *Proc Natl Acad Sci U S A, 89*(16), 7521-7525. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1502163

Lawler, J. M., Barnes, W. S., Wu, G., Song, W., & Demaree, S. (2002). Direct antioxidant properties of creatine. *Biochem Biophys Res Commun, 290*(1), 47-52. doi:10.1006/bbrc.2001.6164

Layne, J. E., & Nelson, M. E. (1999). The effects of progressive resistance training on bone density: a review. *Med Sci Sports Exerc, 31*(1), 25-30. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9927006

Lebrecht, D., & Walker, U. (2007). Role of mtDNAlesions in antracycline cardiotoxcity *Cardiovasc Toxicol*, 108-113.

Lefrak, E. A., Pitha, J., Rosenheim, S., & Gottlieb, J. A. (1973). A clinicopathologic analysis of adriamycin cardiotoxicity. *Cancer, 32*(2), 302-314. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/4353012

Lehninger, A. L. (1982). Proton and electric charge translocation in mitochondrial energy transduction. *Adv Exp Med Biol, 148*, 171-186. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7124514

Lipshultz, S. E., Rifai, N., Dalton, V. M., Levy, D. E., Silverman, L. B., Lipsitz, S. R., . . . Sallan, S. E. (2004). The effect of dexrazoxane on myocardial injury in doxorubicin-treated children with acute lymphoblastic leukemia. *N Engl J Med, 351*(2), 145-153. doi:10.1056/NEJMoa035153351/2/145 [pii]

Liu, J., Tu, D., Dancey, J., Reyno, L., Pritchard, K. I., Pater, J., & Seymour, L. K. (2006). Quality of life analyses in a clinical trial of DPPE (tesmilifene) plus doxorubicin versus doxorubicin in patients with advanced or metastatic breast cancer: NCIC CTG Trial MA.19. *Breast Cancer Res Treat, 100*(3), 263-271. doi:10.1007/s10549-006-9257-1

Liu, X., Chua, C., & Gao, J. (2004). Pifithrin-alpha protects aginst doxorubcin induced apoptosis and acute cardiotoxcity in mice. . *AM J Heart Circ Ohysiol*, h933-H939.

Martin, M., Vogel, C., Crown, J., & Mackey, J. (2005). Life-threatening complications from doxorubicin-docetaxel chemotherapy for breast cancer. *JAMA, 294*(17), 2166; author reply 2166-2167. doi:10.1001/jama.294.17.2166-a

Maslov, M. Y., Chacko, V. P., Hirsch, G. A., Akki, A., Leppo, M. K., Steenbergen, C., & Weiss, R. G. (2010). Reduced in vivo high-energy phosphates precede adriamycin-induced cardiac dysfunction. *Am J Physiol Heart Circ Physiol, 299*(2), H332-337. doi:10.1152/ajpheart.00727.2009

Massie, B. M., Conway, M., Rajagopalan, B., Yonge, R., Frostick, S., Ledingham, J., . . . Radda, G. (1988). Skeletal muscle metabolism during exercise under ischemic conditions in congestive heart failure. Evidence for abnormalities unrelated to blood flow. *Circulation, 78*(2), 320-326. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3396168

McLoon, L. K., Ekern, M., & Wirtschafter, J. (1992). Verapamil substantially increases the chemomyectomy effect of doxorubicin injected into rabbit or monkey eyelid. *Invest Ophthalmol Vis Sci, 33*(11), 3228-3234. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1399427

McLoon, L. K., Luo, X. X., & Wirtschafter, J. (1993). Acute morphologic changes in orbicularis oculi muscle after doxorubicin injection into the eyelid. *Muscle Nerve, 16*(7), 737-743. doi:10.1002/mus.880160708

McNeely, M. L., Campbell, K. L., Rowe, B. H., Klassen, T. P., Mackey, J. R., & Courneya, K. S. (2006). Effects of exercise on breast cancer patients and survivors: a systematic review and meta-analysis. *CMAJ, 175*(1), 34-41. doi:10.1503/cmaj.051073

Meneses-Echavez, J. F., Gonzalez-Jimenez, E., & Ramirez-Velez, R. (2014). Supervised exercise reduces cancer-related fatigue: a systematic review. *J Physiother*. doi:10.1016/j.jphys.2014.08.019

Menna, P., Salvatorelli, E., & Minotti, G. (2008). Cardiotoxicity of antitumor drugs. *Chem Res Toxicol, 21*(5), 978-989. doi:10.1021/tx800002r

Mero, A. A., Hulmi, J. J., Salmijarvi, H., Katajavuori, M., Haverinen, M., Holviala, J., . . . Selanne, H. (2013). Resistance training induced increase in muscle fiber size in young and older men. *Eur J Appl Physiol, 113*(3), 641-650. doi:10.1007/s00421-012-2466-x

Merski, J., Daskal, Y., & Busch, H. (1978). Comparison of adriamycin-induced nucleolar segregation in skeletal muscle, cardiac muscle, and liver cells. *Cancer Treat Rep, 62*(5), 771-778. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/657162

Mimnaugh, E. G., Trush, M. A., Bhatnagar, M., & Gram, T. E. (1985). Enhancement of reactive oxygen-dependent mitochondrial membrane lipid peroxidation by the anticancer drug adriamycin. *Biochem Pharmacol, 34*(6), 847-856. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3977958

Mukhopadhyay, C. K., Ghosh, M. K., & Chatterjee, I. B. (1995). Ascorbic acid prevents lipid peroxidation and oxidative damage of proteins in guinea pig extrahepatic tissue microsomes. *Mol Cell Biochem, 142*(1), 71-78. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7753044

Mylonas, C., & Kouretas, D. (1999). Lipid peroxidation and tissue damage. *In Vivo, 13*(3), 295-309. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10459507

Nash, S. R., Giros, B., Kingsmore, S. F., Rochelle, J. M., Suter, S. T., Gregor, P., . . . Caron, M. G. (1994). Cloning, pharmacological characterization, and genomic localization of the human creatine transporter. *Receptors Channels, 2*(2), 165-174. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7953292

Nordgren, K., & Wallce, K. (2014). Keap1 redox-dependent regulation of doxorubicin-induced oxidative stress response in cardiac myoblasts. *Toxicol Appl Pharmacol*, 107-116.

Ohhara, H., Kanaide, H., & Nakamura, M. (1981). A protective effect of coenzyme Q10 on the adriamycin-induced cardiotoxicity in the isolated perfused rat heart. *J Mol Cell Cardiol, 13*(8), 741-752. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7265263

Pelikan, P. C., Weisfeldt, M. L., Jacobus, W. E., Miceli, M. V., Bulkley, B. H., & Gerstenblith, G. (1986). Acute doxorubicin cardiotoxicity: functional, metabolic, and morphologic alterations in the isolated, perfused rat heart. *J Cardiovasc Pharmacol, 8*(5), 1058-1066. Retrieved from http://www.ncbi.nlm.nih.gov /pubmed/2429080

Periasamy, M., & Kalyanasundaram, A. (2007). SERCA pump isoforms: their role in calcium transport and disease. *Muscle Nerve, 35*(4), 430-442. doi:10.1002/mus.20745

Persky, A. M., & Brazeau, G. A. (2001). Clinical pharmacology of the dietary supplement creatine monohydrate. *Pharmacol Rev, 53*(2), 161-176. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11356982

Pfeiffer, T., Krause, U., Thome, U., Rajewski, A., Skorzek, M., & Scheulen, M. E. (1997). Tissue toxicity of doxorubicin in first and second hyperthermic isolated limb perfusion--an experimental study in dogs. *Eur J Surg Oncol, 23*(5), 439-444. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9393575

Poizat, C., Puri, P., Bai, Y., & Kedes, L. (2005). Phosphorylation dependent degradation of p300 by doxorubcin-activated p38 mitogen activted protien kinase in cardiac cells *Mol Cell Bio*, 2673-2687.

Polich, J. (1998). P300 clinical utility and control of variability. *J Clin Neurophysiol, 15*(1), 14-33. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9502510

Quist, M., Rorth, M., Zacho, M., Andersen, C., Moeller, T., Midtgaard, J., & Adamsen, L. (2006). High-intensity resistance and cardiovascular training improve physical capacity in cancer patients undergoing chemotherapy. *Scand J Med Sci Sports, 16*(5), 349-357. doi:10.1111/j.1600-0838.2005.00503.x

Ripamonti, C. (1999). Management of dyspnea in advanced cancer patients. *Support Care Cancer, 7*(4), 233-243. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10423049

Santacruz, L., Darrabie, M. D., Mantilla, J. G., Mishra, R., Feger, B. J., & Jacobs, D. O. (2014). Creatine Supplementation Reduces Doxorubicin-Induced Cardiomyocellular Injury. *Cardiovasc Toxicol*. doi:10.1007/s12012-014-9283-x

Santos, R. V., Batista, M. L., Jr., Caperuto, E. C., & Costa Rosa, L. F. (2007). Chronic supplementation of creatine and vitamins C and E increases survival and improves biochemical parameters after Doxorubicin treatment in rats. *Clin Exp Pharmacol Physiol, 34*(12), 1294-1299. doi:10.1111/j.1440-1681.2007.04717.x

Sartorelli, V., & Fulco, M. (2004). Molecular and cellular determinants of skeletal muscle atrophy and hypertrophy. *Sci STKE, 2004*(244), re11. doi:10.1126/stke.2442004re11

Schlattner, U., Tokarska-Schlattner, M., & Wallimann, T. (2006). Mitochondrial creatine kinase in human health and disease. *Biochim Biophys Acta, 1762*(2), 164-180. doi:10.1016/j.bbadis.2005.09.004

Schwartz, A. L. (2000). Daily fatigue patterns and effect of exercise in women with breast cancer. *Cancer Pract, 8*(1), 16-24. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10732535

Segal, R. J., Reid, R. D., Courneya, K. S., Malone, S. C., Parliament, M. B., Scott, C. G., . . . Wells, G. A. (2003). Resistance exercise in men receiving androgen deprivation therapy for prostate cancer. *J Clin Oncol, 21*(9), 1653-1659. doi:10.1200/JCO.2003.09.534

Seraydarian, M. W., Artaza, L., & Goodman, M. F. (1977). Adriamycin: effect on mammalian cardiac cells in culture. I. Cell population and energy metabolism. *J Mol Cell Cardiol, 9*(5), 375-382. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/559773

Shain, K., Landowski, T., Buyuksal, I., Cantor, A., & Dalton, W. (2000). Clonal variability in cd95 expression is the major determinant in fas mediated , but not chemotherapy-mediated apoptosis in the rpmi 8226 multiple myeloma cell line,. *Leukemia*, 830.

Shi, Y., Moon, M., Dawood, S., Mcmanus, B., & Liu, P. (2011). Mechanisms and management of doxorubicin cardiotoxicity. *Herz*, 296-305.

Singal, P. K., Li, T., Kumar, D., Danelisen, I., & Iliskovic, N. (2000). Adriamycin-induced heart failure: mechanism and modulation. *Mol Cell Biochem, 207*(1-2), 77-86. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10888230

Smets, E. M., Garssen, B., Schuster-Uitterhoeve, A. L., & de Haes, J. C. (1993). Fatigue in cancer patients. *Br J Cancer, 68*(2), 220-224. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8347475

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

Snow, R. J., & Murphy, R. M. (2001). Creatine and the creatine transporter: a review. *Mol Cell Biochem, 224*(1-2), 169-181. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11693194

Sora, I., Richman, J., Santoro, G., Wei, H., Wang, Y., Vanderah, T., . . . et al. (1994). The cloning and expression of a human creatine transporter. *Biochem Biophys Res Commun, 204*(1), 419-427. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7945388

Speck, R. M., Courneya, K. S., Masse, L. C., Duval, S., & Schmitz, K. H. (2010). An update of controlled physical activity trials in cancer survivors: a systematic review and meta-analysis. *J Cancer Surviv, 4*(2), 87-100. doi:10.1007/s11764-009-0110-5

Spence, R. R., Heesch, K. C., & Brown, W. J. (2010). Exercise and cancer rehabilitation: a systematic review. *Cancer Treat Rev, 36*(2), 185-194. doi:10.1016/j.ctrv.2009.11.003

Spiering, B. A., Kraemer, W. J., Anderson, J. M., Armstrong, L. E., Nindl, B. C., Volek, J. S., & Maresh, C. M. (2008). Resistance exercise biology: manipulation of resistance exercise programme variables determines the responses of cellular and molecular signalling pathways. *Sports Med, 38*(7), 527-540. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/18557656

Stathopoulos, G. P., Papadopoulos, N. G., Stephanopoulou, A., Dontas, I., Kotsarelis, D., & Karayannacos, P. E. (1996). An increase of serum lipids after cumulative doses of doxorubicin and epirubicin in experimental animals. *Anticancer Res, 16*(6B), 3429-3433. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9042202

Swain, S. M., Whaley, F. S., & Ewer, M. S. (2003). Congestive heart failure in patients treated with doxorubicin: a retrospective analysis of three trials. *Cancer, 97*(11), 2869-2879. doi:10.1002/cncr.11407

Tacar, O., Sriamornsak, P., & Dass, C. (2013). Doxorubicin: an update on anticancer molecular action, toxicity and novel drug delivery systems. *J Pharm Pharmacol*, 157–170.

Terada, N., Patel, H. R., Takase, K., Kohno, K., Nairn, A. C., & Gelfand, E. W. (1994). Rapamycin selectively inhibits translation of mRNAs encoding elongation factors and ribosomal proteins. *Proc Natl Acad Sci U S A, 91*(24), 11477-11481. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7972087

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. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/6093249

Tokarska-Schlattner, M., Zaugg, M., Zuppinger, C., Wallimann, T., & Schlattner, U. (2006). New insights into doxorubicin-induced cardiotoxicity: the critical role of cellular energetics. *J Mol Cell Cardiol, 41*(3), 389-405. doi:10.1016/j.yjmcc.2006.06.009

Tornroth-Horsefield, S., & Neutze, R. (2008). Opening and closing the metabolite gate. *Proc Natl Acad Sci U S A, 105*(50), 19565-19566. doi:10.1073/pnas.0810654106

Tozer, R. G., Tai, P., Falconer, W., Ducruet, T., Karabadjian, A., Bounous, G., . . . Droge, W. (2008). Cysteine-rich protein reverses weight loss in lung cancer patients receiving chemotherapy or radiotherapy. *Antioxid Redox Signal, 10*(2), 395-402. doi:10.1089/ars.2007.1919

Travers, J., Dudgeon, D. J., Amjadi, K., McBride, I., Dillon, K., Laveneziana, P., . . . O'Donnell, D. E. (2008). Mechanisms of exertional dyspnea in patients with cancer. *J Appl Physiol (1985), 104*(1), 57-66. doi:10.1152/japplphysiol.00653.2007

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. *Int J Biochem Cell Biol, 39*(1), 44-84. doi:10.1016/j.biocel.2006.07.001

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. *Br J Cancer, 100*(2), 311-314. doi:10.1038/sj.bjc.6604858

Van Vleet, J. F., & Ferrans, V. J. (1980). Clinical and pathologic features of chronic adriamycin toxicosis in rabbits. *Am J Vet Res, 41*(9), 1462-1469. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7447139

Vandenberghe, K., Van Hecke, P., Van Leemputte, M., Vanstapel, F., & Hespel, P. (1999). Phosphocreatine resynthesis is not affected by creatine loading. *Med Sci Sports Exerc, 31*(2), 236-242. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10063812

Vander Heiden, M. G., Chandel, N. S., Li, X. X., Schumacker, P. T., Colombini, M., & Thompson, C. B. (2000). Outer mitochondrial membrane permeability can regulate coupled respiration and cell survival. *Proc Natl Acad Sci U S A, 97*(9), 4666-4671. doi:10.1073/pnas.090082297

Vasti, C., Witt, H., Said, M., Sorroche, P., Garcia-Rivello, H., Ruiz-Noppinger, P., & Hertig, C. M. (2012). Doxorubicin and NRG-1/erbB4-Deficiency Affect Gene Expression Profile: Involving Protein Homeostasis in Mouse. *ISRN Cardiol, 2012*, 745185. doi:10.5402/2012/745185

Volek, J. S., Kraemer, W. J., Bush, J. A., Boetes, M., Incledon, T., Clark, K. L., & Lynch, J. M. (1997). Creatine supplementation enhances muscular performance during high-intensity resistance exercise. *J Am Diet Assoc, 97*(7), 765-770. doi:10.1016/S0002-8223(97)00189-2

Volek, J. S., Ratamess, N. A., Rubin, M. R., Gomez, A. L., French, D. N., McGuigan, M. M., . . . Kraemer, W. J. (2004). The effects of creatine supplementation on muscular performance and body composition responses to short-term resistance training overreaching. *Eur J Appl Physiol, 91*(5-6), 628-637. doi:10.1007/s00421-003-1031-z

Vomhof-Dekrey, E. E., & Picklo, M. J. (2012). The Nrf2-antioxidant response element pathway: a target for regulating energy metabolism. *J. Nutr. Biochem.*, 1201-1206.

Walker, J. B. (1979). Creatine: biosynthesis, regulation, and function. *Adv Enzymol Relat Areas Mol Biol, 50*, 177-242. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/386719

Wallace, K. B. (2003). Doxorubicin-induced cardiac mitochondrionopathy. *Pharmacol Toxicol, 93*(3), 105-115. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12969434

Weiss, R. B. (1992). The anthracyclines: will we ever find a better doxorubicin? *Semin Oncol, 19*(6), 670-686. Retrieved from http://www.ncbi.nlm.nih.gov/ entrez/ query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=1462166

Willott, C. A., Young, M. E., Leighton, B., Kemp, G. J., Boehm, E. A., Radda, G. K., & Clarke, K. (1999). Creatine uptake in isolated soleus muscle: kinetics and dependence on sodium, but not on insulin. *Acta Physiol Scand, 166*(2), 99-104. doi:10.1046/j.1365-201x.1999.00539.x

Wilson, J. R., Martin, J. L., & Ferraro, N. (1984). Impaired skeletal muscle nutritive flow during exercise in patients with congestive heart failure: role of cardiac pump dysfunction as determined by the effect of dobutamine. *Am J Cardiol, 53*(9), 1308-1315. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/6711433

Wilson, J. R., Martin, J. L., Ferraro, N., & Weber, K. T. (1983). Effect of hydralazine on perfusion and metabolism in the leg during upright bicycle exercise in patients with heart failure. *Circulation, 68*(2), 425-432. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/6861318

Winett, R. A., & Carpinelli, R. N. (2001). Potential health-related benefits of resistance training. *Prev Med, 33*(5), 503-513. doi:10.1006/pmed.2001.0909

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

Wyss, M., & Kaddurah-Daouk, R. (2000). Creatine and creatinine metabolism. *Physiol Rev, 80*(3), 1107-1213. Retrieved from http://www.ncbi.nlm.nih.gov/ pubmed/10893433

Xu, X., Presson, H., & Richardson, D. (2005). Molecular pharmacology of the interaction of anthracyclines with iorn. . *Mol harmacol*, 68:261-276.

Yamamoto, Y., Hoshino, Y., Ito, T., Nariai, T., Mohri, T., Obana, M., . . . Azuma, J. (2008). Atrogin-1 ubiquitin ligase is upregulated by doxorubicin via p38-MAP kinase in cardiac myocytes. *Cardiovasc Res, 79*(1), 89-96. doi:10.1093/cvr/cvn076

Yao, W., Jee, W. S., Chen, J. L., Li, C. Y., & Frost, H. M. (2001). A novel method to 'exercise' rats: making rats rise to erect bipedal stance for feeding - raised cage model. *J Musculoskelet Neuronal Interact, 1*(3), 241-247. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/15758498

Zhang, Y., Shi, J., Li, Y., & Wei, L. (2009). Cardiomyocyte Death in doxorubcin induced cardiotoxcity. *Arch Immunol*, 435-445.

Zucchi, R., & Danesi, R. (2003). Cardiac toxicity of antineoplastic anthracyclines. *Curr Med Chem Anticancer Agents, 3*(2), 151-171. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12678909

APPENDIX A

PILOT STUDY

A pilot study using 10-week-old male Sprague-Dawley rats was conducted to examine if Cr can mitigate the myotoxic effects of DOX. Animals were randomly assigned to one of four experimental groups: K +K (n =7), K+DOX (n=7), Cr+ K (n = 8), or Cr+DOX (n = 8). Three different buffers were made: K, Cr, and DOX. Each buffer started with a basic K buffer (in mM, 120 NaCl, 5.9 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 25 $NaHCO_3$, 17 glucose, and 0.5 EDTA, pH 7.4). The Cr buffer used a K buffer with 25 mM of Cr. For the DOX buffer, a K buffer was affixed with 24 μM of DOX. The SOL and EDL muscles from the right and left hind limb were excised and allowed to stabilize for 5 min in aerated (95 % O_2/5 % CO_2) K buffer. Following stabilization, sutures with micro-spring clips were attached to the distal and proximal tendons of each muscle, and muscles were submerged in an organ chamber filled with K buffer. Muscle function was analyzed using an *ex vivo* muscle function apparatus (Radnoti LLC, Monrovia, CA). The proximal end of the muscle was affixed to an isometric force transducer; the distal end was affixed to a stationary glass hook. Muscle function data were recorded via Lab Chart software (ADInstruments, Colorado Springs, Colorado) following the attachment of the muscles to the force transducer, maximal force determinations were made by adjusting tension by 0.2 grams from a baseline of 0.5 grams until an increase in force is no longer evident. Next, voltage was increased by 10 volts until maximal twitch force was achieved.

Next, the buffers were changed to the corresponding treatment buffer (K or Cr). During the incubation period, muscles were stimulated using platinum field stimulating electrodes for 200 msec at 100 Hz once every five minutes for 30 minutes at the same voltage achieved during the maximal force determinations. This stimulation helped

facilitate Cr uptake into the cell. Then, the second treatment buffer was added (K or DOX) and the muscles were again stimulated for 200 msec at 100 Hz once every five minutes for 30 minutes using the same voltage as before. Following the second buffer treatment, fresh K buffer was added and the muscles were again analyzed for maximal twitch force. The fresh K buffer was again added, and the muscles were subjected to a 100 second fatigue protocol, during which muscle force was recorded every 10 seconds for 100 seconds using the same voltage as before.

Examination of maximal twitch force (see Figures 1 and 2) showed an increase in maximal twitch force with Cr administration in both the SOL and EDL when compared to non-treated groups with the most pronounced differences occurring in the EDL. Furthermore, pretreatment with Cr did not protect against DOX-induced muscle fatigue in the SOL (see Figure 3). However, in the EDL, data did show some degree of Cr-induced protection for attenuating fatigue (see Figure 4). Furthermore, Cr might have had a more pronounced effect in type II (fast) muscle due to the higher levels of phosphocreatine (PCr) and a greater reliance on the PCr system when compared to more type I (slow) muscle. This suggested that Cr exerted a protective effect in skeletal muscle.

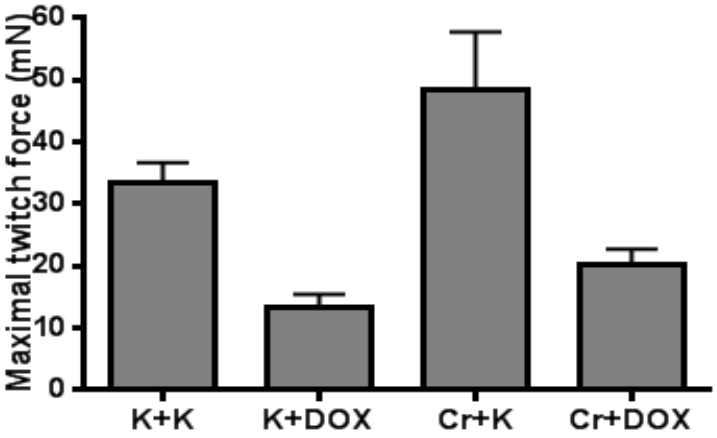


Figure 1. Maximal twitch force in the EDL. * vs. K+DOX & Cr+DOX($P < 0.05$).

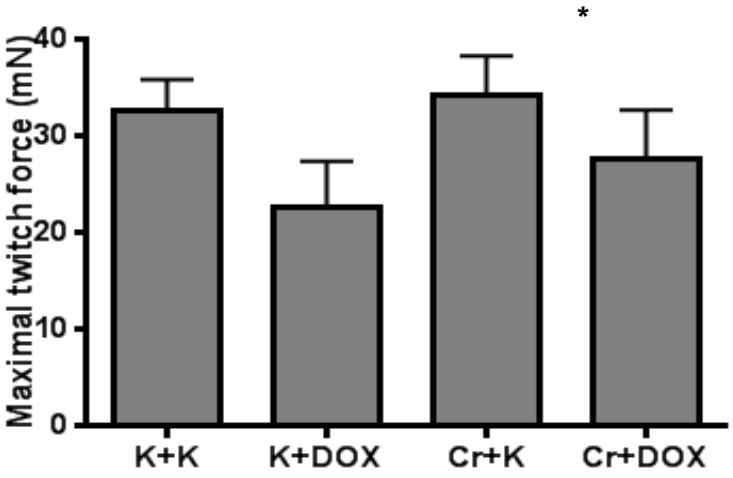


Figure 2. Maximal twitch force in the SOL. * vs. K+DOX ($P < 0.05$).

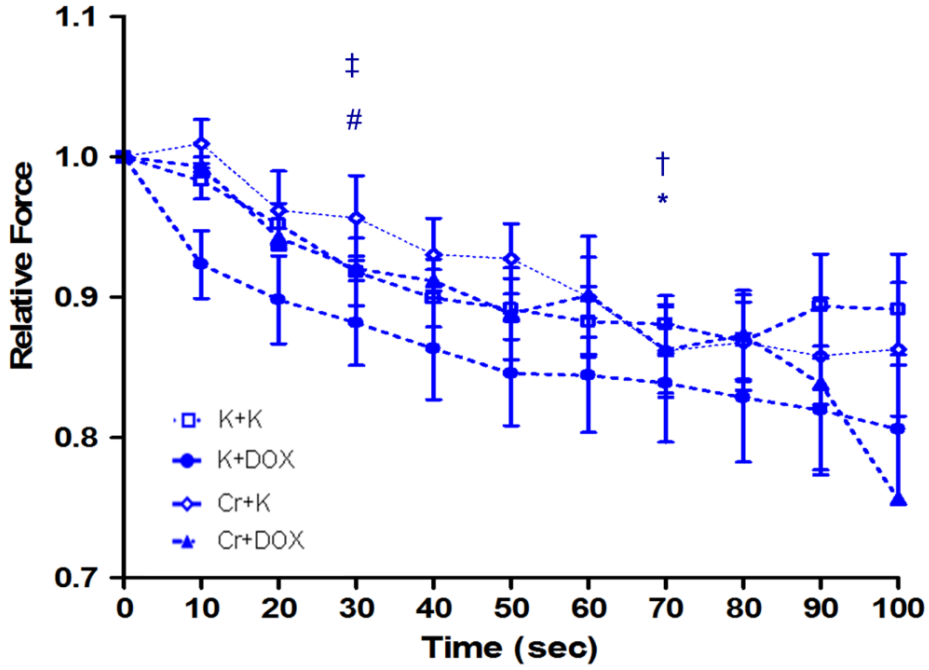


Figure 3. Soleus fatigue. † (K)+K, significantly lower than baseline (P<0.05), ‡ K+DOX, significantly lower than baseline (P<0.05), * Cr +K significantly lower than baseline (P<0.05), and # Cr+DOX, significantly lower than baseline (P<0.05).

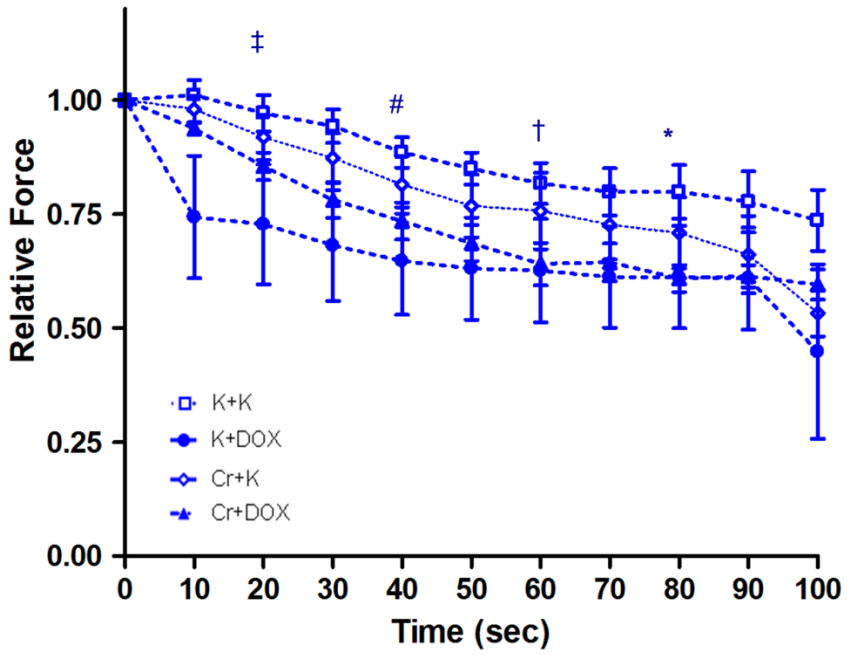


Figure 4. Extensor digitorum longus Fatigue. † K+K, significantly lower than baseline (P<0.05), ‡ K+DOX, significantly lower than baseline (P<0.05), * Cr+K significantly lower than baseline (P<0.05), and # Cr+DOX, significantly lower than baseline (P<0.05).

APPENDIX B

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL

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 C

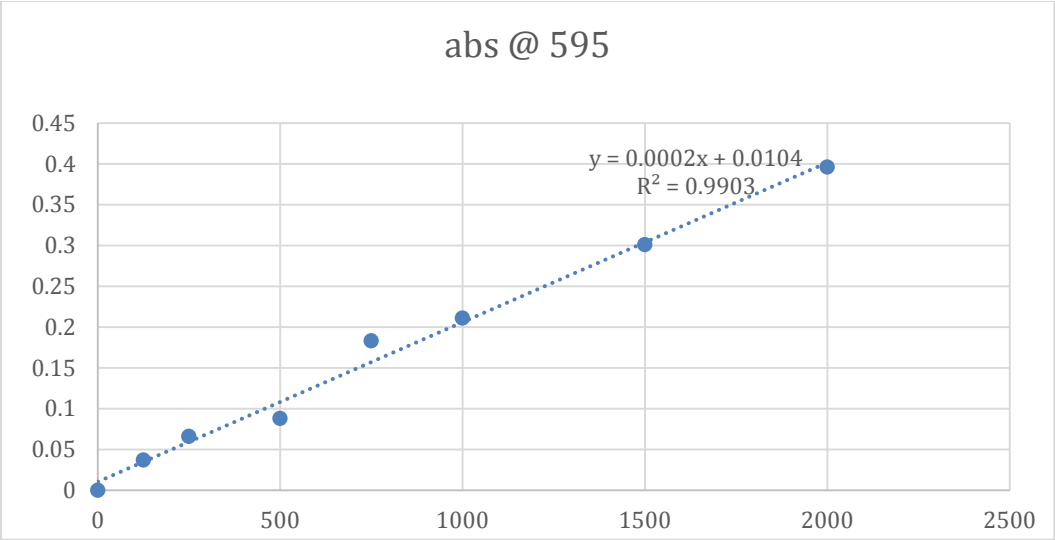
TISSUE MASSES

Tissue Masses

Group	R-SOL (g)	R-EDL (g)	L-SOL (g)	L-EDL (g)
SED-K-K	0.2241±0.02	0.1820±0.03	0.2109±0.01	.0.1975±0.03
SED-Cr-K	0.2606±0.03	0.2106±0.03	0.1992±0.03	0.1942±0.01
SED-Cr-DOX	0.2300±0.02	0.2006±0.03	0.1976±0.01	0.1919±0.01
SED-K-DOX	0.2085±0.01	0.1940±0.02	0.1772±0.02	0.1743±0.04
RT-K-K	0.2708±0.04	0.2359±0.02	0.2496±0.07	0.2227±0.04
RT-Cr-K	0.2532±0.04	0.2690±0.04	0.2332±0.023	2329±0.01
RT-Cr-DOX	0.2666±0.05	0.2309±0.03	0.2345±0.02	0.2295±0.02
RT-K-DOX	0.2604±0.06	0.2160±0.02	0.2334±0.02	0.2329±0.03

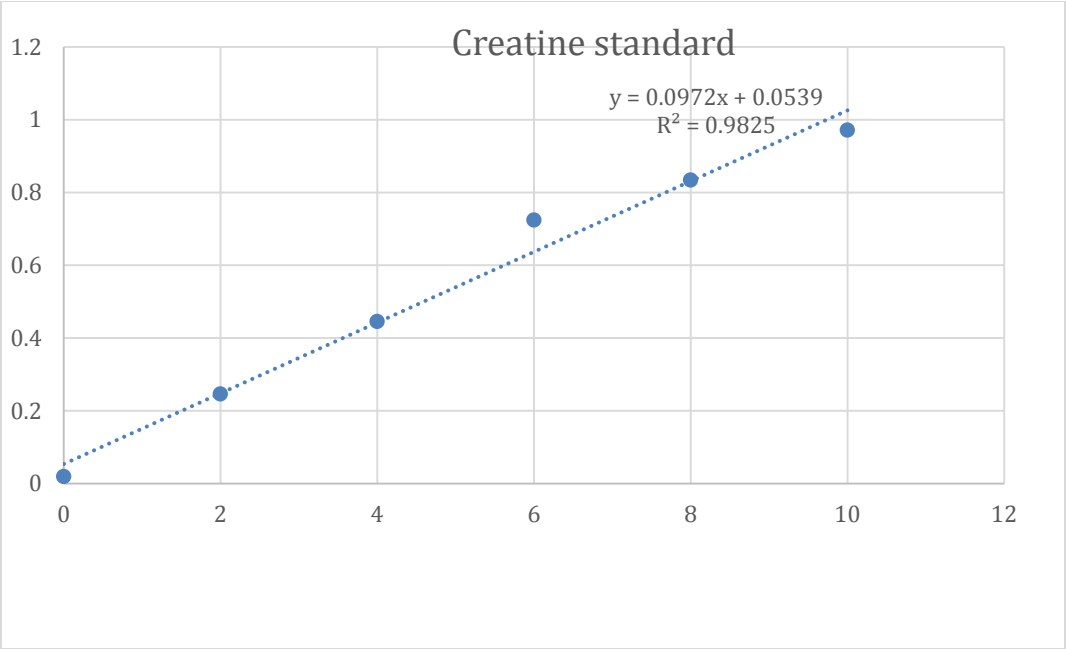
APPENDIX D

BRADFORD STANDARD CURVE


abs @ 595
y = 0.0002x + 0.0104
R² = 0.9903
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
500
1000
1500
2000
2500

APPENDIX E

CREATINE STANDARD CURVE


Creatine standard
y = 0.0972x + 0.0539
R² = 0.9825
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
12

APPENDIX F

CREATINE ASSAY INSTRUCTIONS

SIGMA-ALDRICH®

sigma-aldrich.com

3050 Spruce Street, St. Louis, MO 63103 USA
Tel: (800) 521-8956 (314) 771-5765 Fax: (800) 325-5052 (314) 771-5757
email: techservice@sial.com sigma-aldrich.com

Product Information

Creatine Assay Kit

Catalog Number **MAK079**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description
Creatine is a nitrogenous compound that acts as a high-energy reservoir for the rapid regeneration of ATP. Approximately 95% of creatine is found in skeletal muscle, primarily as phosphocreatine. Creatine can be acquired through dietary consumption or formed from L-arginine, glycine, and L-methionine in a multi-step reaction that occurs in the kidneys and liver. Creatine is then transported to muscle tissue. Creatine supplementation is used for the enhancement of sports performance, primarily by increasing muscle mass. Creatine is also being investigated as a treatment of neuromuscular diseases, where it may aid in neuroprotection and by improving the cellular bioenergetic state.

In this assay, Creatine concentration is determined by a coupled enzyme reaction, which results in a colorimetric (570 nm)/fluorometric (λ_{ex} = 535/λ_{em} = 587 nm) product, proportional to the Creatine present.

Components
The kit is sufficient for 100 assays in 96 well plates.

Creatine Assay Buffer Catalog Number MAK079A	25 mL
Creatine Probe, in DMSO Catalog Number MAK079B	0.2 mL
Creatinase Catalog Number MAK079C	1 vl
Creatine Enzyme Mix Catalog Number MAK079D	1 vl
Creatine Standard, 10 µmole Catalog Number MAK079E	1 vl

Reagents and Equipment Required but Not Provided.

- 96 well flat-bottom plate – It is recommended to use black plates with clear bottoms for fluorescence assays and clear plates for colorimetric assays.
- Fluorescence or spectrophotometric multiwell plate reader
- 10 kDa Molecular Weight Cut-Off (MWCO) Spin Filter

Precautions and Disclaimer
This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions
Briefly centrifuge vials before opening. Use ultrapure water for the preparation of reagents. To maintain reagent integrity, avoid repeated freeze/thaw cycles.

Creatine Assay Buffer – Allow buffer to come to room temperature before use.

Creatine Probe – Thaw at room temperature to melt solution prior to use. Aliquot and store protected from light and moisture at –20 °C. Upon thawing, the Creatine Probe is ready-to-use in the colorimetric assay.

For the fluorescence assay, dilute an aliquot of the Creatine Probe Solution 5 to 10-fold with Creatine Assay Buffer, just prior to use. This will reduce the background of the fluorescence assay.

Creatinase and Creatine Enzyme Mix – Reconstitute each with 220 µL of Creatine Assay Buffer. Mix well by pipetting, then aliquot and store, protected from light, at –20 °C. Use within 2 months of reconstitution and keep cold while in use.

Creatine Standard – Reconstitute in 100 µL of water to generate a 100 mM (100 nmole/µL) Creatine Standard solution. Mix well by pipetting, then aliquot and store at –20 °C. Use within 2 months of reconstitution and keep cold while in use.

Storage/Stability
The kit is shipped on wet ice. Storage at –20 °C, protected from light, is recommended.

Procedure
All samples and standards should be run in duplicate.

Creatine Standards for Colorimetric Detection
Dilute 10 µL of the 100 mM (100 nmole/µL) Creatine Standard Solution with 990 µL of Creatine Assay Buffer to prepare a 1 mM (1 nmole/µL) standard solution. Add 0, 2, 4, 6, 8, and 10 µL of the 1 mM Creatine standard solution into a 96 well plate, generating 0 (blank), 2, 4, 6, 8, and 10 nmole/well standards. Add Creatine Assay Buffer to each well to bring the volume to 50 µL.

Creatine Standards for Fluorometric Detection
Dilute 10 µL of the 100 mM (100 nmole/µL) Creatine Standard Solution with 990 µL of Creatine Assay Buffer to prepare a 1 mM (1 nmole/µL) standard solution. Dilute 10 µL of the 1 mM standard solution with 90 µL of Creatine Assay Buffer to generate a 0.1 mM (0.1 nmole/µL) standard solution. Add 0, 2, 4, 6, 8, and 10 µL of the 0.1 mM Creatine standard solution into a 96 well plate, generating 0 (blank), 0.2, 0.4, 0.6, 0.8, and 1.0 nmole/well standards. Add Creatine Assay Buffer to each well to bring the volume to 50 µL.

Sample Preparation
Tissue (10 mg) or cells (2×10^6) should be rapidly homogenized in 4 volumes of cold Creatine Assay buffer. Centrifuge at 13,000 × *g* for 10 minutes at 4 °C to remove insoluble material. High concentrations of proteins may interfere with the assay and should be removed with a 10 kDa MWCO spin filter. The soluble fraction may be assayed directly.

Serum samples may be deproteinized with a 10 kDa MWCO spin filter. The soluble fraction may be assayed directly.

Bring samples to a final volume of 50 µL with Creatine Assay Buffer.

For unknown samples, it is suggested to test several sample dilutions to ensure the readings are within the linear range of the standard curve.

Sarcosine present in the sample can generate background. To control for sarcosine background, include a blank sample for each sample by omitting the Creatinase in the Reaction Mix.

Assay Reaction
1. Set up the Reaction Mixes according to the scheme in Table 1. 50 µL of the appropriate Master Reaction Mix is required for each reaction (well).

Table 1.
Reaction Mixes

Reagent	Sample Blank	Samples and Standards
Creatine Assay Buffer	46 µL	44 µL
Creatinase	–	2 µL
Creatine Enzyme Mix	2 µL	2 µL
Creatine Probe	2 µL	2 µL

2. Add 50 µL of the appropriate Reaction Mix to each of the wells. Mix well using a horizontal shaker or by pipetting, and incubate the reaction for 60 minutes at 37 °C. Protect the plate from light during the incubation.

3. For colorimetric assays, measure the absorbance at 570 nm (A_{570}). For fluorometric assays, measure fluorescence intensity (λ_{ex} = 535/λ_{em} = 587 nm).

Results

Calculations

The background for the assays is the value obtained for the 0 (blank) Creatine Standard. Correct for the background by subtracting the 0 (blank) value from all readings. Background values can be significant and must be subtracted from all readings. Use the values obtained from the appropriate Creatine standards to plot a standard curve.

Note: A new standard curve must be set up each time the assay is run.

Subtract the blank sample value from the sample reading to obtain the corrected measurement. Using the corrected measurement, the amount of Creatine present in the sample may be determined from the standard curve

Concentration of Creatine

$$S_a/S_v = C$$

S_a = Amount of Creatine in unknown sample (nmole) from standard curve

S_v = Sample volume (µL) added into the wells

C = Concentration of Creatine in sample

Creatine molecular weight: 131.13 g/mole

Sample Calculation

Amount of Creatine (S_a) = 5.84 nmole (from standard curve)

Sample volume (S_v) = 50 µL

Concentration of Creatine in sample

$$5.84 \text{ nmole}/50 \text{ µL} = 0.1168 \text{ nmole/µL}$$

$$0.1168 \text{ nmole/µL} \times 131.13 \text{ ng/nmole} = 15.32 \text{ ng/µL}$$

Troubleshooting Guide

Problem	Possible Cause	Suggested Solution
Assay not working	Ice Cold Assay Buffer	Assay Buffer must be at room temperature
	Omission of step in procedure	Refer and follow Technical Bulletin precisely
	Plate reader at incorrect wavelength	Check filter settings of instrument
	Type of 96 well plate used	For colorimetric assays, use clear plates
Samples with erratic readings	Samples prepared in different buffer	Use the Assay Buffer provided or refer to Technical Bulletin for instructions
	Cell/Tissue culture samples were incompletely homogenized	Repeat the sample homogenization, increasing the length and extent of homogenization step.
	Samples were not deproteinized	Use a 10 kDa MWCO spin filter to deproteinize samples
	Samples used after multiple freeze-thaw cycles	Aliquot and freeze samples if samples will be used multiple times
	Presence of interfering substance in the sample	If possible, dilute sample further
	Use of old or inappropriately stored samples	Use fresh samples and store correctly until use
Lower/higher readings in samples and standards	Improperly thawed components	Thaw all components completely and mix gently before use
	Use of expired kit or improperly stored reagents	Check the expiration date and store the components appropriately
	Allowing the reagents to sit for extended times on ice	Prepare fresh Master Reaction Mix before each use
	Incorrect incubation times or temperatures	Refer to Technical Bulletin and verify correct incubation times and temperatures
	Incorrect volumes used	Use calibrated pipettes and aliquot correctly
Non-linear standard curve	Use of partially thawed components	Thaw and resuspend all components before preparing the reaction mix
	Pipetting errors in preparation of standards	Avoid pipetting small volumes
	Pipetting errors in the Reaction Mix	Prepare a Master Reaction Mix whenever possible
	Air bubbles formed in well	Pipette gently against the wall of the tubes
	Standard stock is at incorrect concentration	Refer to the standard dilution instructions in the Technical Bulletin
	Calculation errors	Recheck calculations after referring to Technical Bulletin
	Substituting reagents from older kits/lots	Use fresh components from the same kit
Unanticipated results	Samples measured at incorrect wavelength	Check the equipment and filter settings
	Samples contain interfering substances	If possible, dilute sample further
	Sample readings above/below the linear range	Concentrate or dilute samples so readings are in the linear range

MF,LS,MAM 02/14-1