Dissertations   Student Research

8-2019

Effects of Two Types of Creatine Feeding and Doxorubicin on Skeletal Muscle Apoptotic Markers

Zoltan A. Torok

Follow this and additional works at: https://digscholarship.unco.edu/dissertations

UNIVERSITYOF NORTHERN COLORADO

Greeley, Colorado

The Graduate School

EFFECTS OF TWO TYPES OF CREATINE FEEDING
AND DOXORUBICIN ON SKELETAL MUSCLE
APOPTOTIC MARKERS

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

Zoltan A. Torok

College of Natural and Health Sciences
School of Sport and Exercise Science
Exercise Physiology
August 2019

This Dissertation by: Zoltan A. Torok Entitled: *Effects of Two Types of Creatine Feeding and Doxorubicin on Skeletal Muscle Apoptotic Markers*

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

Accepted by the Doctoral Committee

_____

David S. Hydock Ph.D., Research Advisor

_____

Laura K. Stewart Ph.D., Committee Member

_____

Patrick Burns Ph.D., Committee Member

_____

James Haughian Ph.D., Faculty Representative

Date of Dissertation Defense: June 25, 2019

Accepted by the Graduate School

_____

Linda L. Black, Ed.D.

Associate Provost and Dean
Graduate School and International Admissions
Research and Sponsored Projects

ABSTRACT

Torok, Zoltan A. *Effects of Two Types of Creatine Feeding and Doxorubicin on Skeletal Muscle Apoptotic Markers.* Published Doctor of Philosophy Dissertation, University of Northern Colorado, 2019.

Doxorubicin (Dox) is a widely used anthracycline antibiotic used to treat a number of hematological and solid tumor cancers. Dosage however, is limited due to its toxic effects in healthy tissues. Negative consequences include myotoxicity in skeletal muscle, which may limit mobility and activities of daily living. Doxorubicin increases the formation of reactive oxygen species, which triggers apoptosis in the tumor and healthy tissue. Skeletal muscle undergoes apoptosis in response to Dox treatment, via the activation of the cleaved caspase-3 and its downstream target, poly(ADP-ribose) polymerase. Creatine monohydrate (Cr), a naturally occurring chemical, when taken exogenously, can improve athletic performance. Recently, it has been shown that Cr can positively impact several diseases and act as a antioxidant, capable of scavenging free radicals and reducing apoptosis. **Purpose:** To determine the effect of two types of Cr feeding and Dox administration on skeletal muscle apoptotic proteins at two separate time points. **Methods:** Ten week old male Sprague-Dawley rats were randomly assigned to one of six groups: control + saline (Con-Sal, n=10), control + doxorubicin (Con-Dox, n=10), creatine 1 + saline (C1-Sal, n=10), creatine 1 + doxorubicin (C1-Dox, n=10), creatine 2 + saline (C2-Sal, n=10), creatine 2 + doxorubicin (C2-Dox, n=10). Animals were fed for four weeks and were injected with a bolus 15 mg/kg i.p. injection of Dox or Sal and sacrificed either 1-day or 3-days post injection. Post injection, the extensor digitorum longus (EDL), soleus (SOL), and diaphragm (DIA) were excised and Western blotting for expression of cleaved PARP, caspase-3, and cleaved caspase-3 was

conducted. Creatine concentration was also analyzed using a commercially available assay kit. **Results:** A main drug effect was observed, with increased levels of apoptosis observed in the 1-day EDL, SOL, and in the 3-day EDL. Equally, a main drug effect was observed, with decreased caspase-3 levels in the 3-day SOL, and cleaved PARP levels in the 3-day DIA. There was a diet effect, with increased cleaved PARP levels in the 1-day EDL, SOL, and cleaved caspase-3 levels in the 3-day EDL. An equal diet effect was detected, with decreased caspase-3 levels in the 1-day EDL and cleaved caspase-3 levels in the 1-day SOL and DIA. The 1-day SOL had lower creatine concentration with Dox administration and the 3-day EDL had decreased Cr concentrations with both Dox administration and Cr feeding. **Conclusion:** Doxorubicin had the largest impact on apoptosis in the EDL, suggesting Dox's effects on apoptosis may differ between tissue. Creatine concentration in the EDL was lower in animals receiving Dox, and this was not observed in the DIA, supporting the idea that Dox toxicity may differ between tissue.

TABLE OF CONTENTS

LIST OF TABLES

TABLE

LIST OF FIGURES

FIGURE

CHAPTER I

INTRODUCTION TO THE STUDY

Background

Cancer is a group of diseases characterized by the uncontrolled growth and spread of abnormal cells. If this abnormal growth is left uncontrolled, it can result in death. According to Siegel, Miller, and Jemal (2019), as of January 1, 2016, more than 15.5 million Americans have a history of cancer. About 1.7 million new cancer cases are expected to be diagnosed in 2018, with about 609,640 Americans dying from the disease (Siegel et al., 2019). The overall age-adjusted cancer death rate has improved, decreasing from 215 deaths per 100,000 in 1991 to 159 deaths per 100,000 in 2015 which is a 26% decrease (Siegel et al., 2019). The improvement in age-adjusted death is largely due to advanced screening, increased awareness, and improved treatments (Siegel et al., 2019). Improvement in age-adjusted deaths should be interpreted with caution as they do not always indicate progress against cancer. In fact, cancer is still one of the leading causes of death and disease in the US, costing Americans $80.2 billion dollars in 2015 (Siegel et al., 2019).

Currently, there are three types of cancer treatment options available: surgery, radiation, and pharmacological therapy. These treatments can be used alone or in most cases, in combination. Surgery is the removal of a tumor and can be used to diagnose cancer, determine how far it has spread, stop growth and ultimately, the primary method to "cure" the patient. Radiation is the use of high energy waves/particles to destroy or

severely damage a tumor. Pharmacological therapy is the use of medication to destroy or slow growth of a tumor. Medications can include chemotherapy, targeted therapy, immunotherapy, hormone therapy, and other supportive medications.

Pharmacological therapy, specifically chemotherapy, is often used as part of a patient's cancer treatment. Doxorubicin (Dox), or Adriamycin, is an anthracycline antibiotic chemotherapy drug synthesized from the soil fungus Streptomyces. Doxorubicin is commonly used to treat a variety of cancers including breast, ovarian, bladder, lung, thyroid, and stomach cancers. Doxorubicin dosing is dependent on a patient's height, weight, general health, type of cancer, and has a lifetime maximal dose. A lifetime maximal dose means a patient can only receive a set amount of Dox in their lifetime and exceeding that dose can result in death from causes other than cancer. Doxorubicin is typically administered via intravenous injection (although in some cancers, such as peritoneal tumors, an intraperitoneal injection is used) and exerts its anti-cancer effects by halting the cell cycle in the tumor, preventing division. Doxorubicin halts the cell cycle by three main mechanisms, (1) intercalation into DNA disrupting replication, (2) disruption of topoisomerase-II-mediated DNA repair, and (3) generation of free radicals, which damages cellular membranes, DNA and proteins (Thorn et al., 2011). Along with its tumor damaging capabilities, Dox has the capacity to damage health tissue, specifically (to the research conducted in our lab), cardiac tissue and skeletal muscle.

Doxorubicin administration may result in chronic cardiotoxicity, with an estimated incidence of about 1.7% (Von Hoff et al., 1979) and is usually evident within 30 days of administration. Doxorubicin cardiotoxicity is a result of increased oxidative

stress (increased levels of reactive oxygen species and lipid peroxidation) (Takemura &

Fujiwara, 2007), decreased levels of antioxidants and sulfhydryl groups (Singal, Li,

Kumar, Danelisen, & Illiskovic, 2000), inhibition of nucleic acid and protein synthesis,

release of vasoactive amines (Bristow et al., 1980), altered adrenergic function, and

decreased expression of cardiac-specific genes (Tong, Ganguly, & Singal, 1991).

In skeletal muscle, Dox treatment decreased maximal twitch force in the extensor

digitorum longus and soleus, as well as, increased fatigue in the soleus (Gilliam et al.,

2012; Hydock, Lien, Jensen, Schneider, & Hayward, 2011; van Norren et al., 2009).

Equally, intraperitoneal injections of Dox decreased fiber bundle diameter and absolute

force production in the murine diaphragm (Bonifati et al., 2000). Further, rodents

injected with Dox showed loss of myofibrillar organization with interstitial edema

(Doroshow, Tallent, & Schechter, 1985), nucleolar segregation, and altered distribution

of the perinucleolar chromatin in hindlimb skeletal muscle (Merski, Daskal, & Busch,

1978). The increased skeletal muscle fatigue and loss of skeletal muscle size described

above resulted from increased apoptotic activation (Hilder, Carlson, Haystead, Krebs, &

Graves, 2005), mediated through increased Dox-induced ROS formation (Timolati et al.,

2006) and activation of caspases (Wang et al., 2004).

Currently there is a push to uncover nutraceutical interventions that can mitigate

Dox-induced myotoxicity. Studies have shown that exercise mitigates Dox-induced

myotoxcity through the impedance of the Dox-induced ROS production and protease

activation (Smuder, Kavazis, Min, & Powers, 1985), preservation of maximal twitch

force, attenuation of skeletal muscle fatigue (Bredahl, Pfannenstiel, Quinn, Hayward, &

Hydock, 2016) and reduction in chemotherapy-induced muscle fatigue (Fairman, Hyde,

& Focht, 2016; Van Moll, Schep, Vreugdenhil, Savelberg, & Husson, 2016). However, exercise as an intervention is problematic because adherence significantly decreases in patients that are in treatment and in those who have just finished treatment (Courneya et al., 2007; Markes, Brockow, & Resch, 2006; McNeely et al., 2006; Mutrie et al., 2007). Equally, research has shown that exercise preconditioning is protective against Dox-induced weakness and fatigue (Kavazis, Smuder, & Powers, 1985; Smuder et al., 1985) however, it has been reported that 30-40% of newly diagnosed cancer patients meet the minimum recommendations for physical activity, which makes this intervention not a viable option. This leads to a further search for a nutraceutical intervention that can mitigate Dox-induced myotoxicity and have a high rate of adherence in patients whom exercise adherence is typically low. Supplementing the diet with creatine as a nutraceutical intervention to mitigate Dox-induced myotoxicity is a possibility.

Creatine monohydrate (Cr) is perhaps one of the most widely used nutraceuticals ingested in an attempt to improve athletic performance. A large amount of research has been done on Cr supplementation, in efforts to delineate its sport-specific effects. It is hypothesized that Cr acts through a number of pathways and appears to be most effective in short-term, high-intensity physical activities. However, the past decade has showed that the use of Cr for therapeutic purposes has received increasing attention (Gualano, Artioli, Poortmans, & Lancha Junior, 2009). Creatine supplementation is beneficial in a large number of muscular, neurological, and cardiovascular diseases (Bender et al., 2007; Felber et al., 2000; Gordon et al., 1995; Holtzman et al., 1999; Matthews et al., 1999; Mazzini et al., 2001; Neubauer et al., 1999; Sharov et al., 1987; Stout, Eckerson, May, Coulter, & Bradley-Popovich, 2001; Tarnopolsky et al., 2004; Tarnopolsky, 2007;

Vorgerd et al., 2000; Wyss & Schulze, 2002;); however, less documented are the effects of Cr on Dox-induced skeletal muscle dysfunction, and there is a lack of research on possible mechanisms leading to improvements in Dox-induced skeletal muscle dysfunction.

To the author's knowledge, Bredahl and Hydock (2017) published the only study to examine the effects of Cr on Dox-induced skeletal muscle dysfunction. In this *ex vivo* study, Bredahl and Hydock (2017) found that incubating the soleus and extensor digitorum longus in creatine monohydrate prior to Dox treatment attenuated Dox-induced fatigue in both the soleus and the extensor digitorum longus. Although mechanisms contributing to the results were not analyzed in the aforementioned study, it is postulated that Cr acted as an antioxidant, decreasing ROS formation (Lawler & Powers, 1998; Sestili et al., 2006; Sestili et al., 2009), and preserving muscle function. In light of the above findings, and given that Dox treatment increases apoptosis, it was imperative to examine what effect Cr supplementation had on the apoptosis in Dox treated animals, specifically the apoptotic markers cleaved poly ADP ribose polymerase (PARP) and cleaved caspase-3, in skeletal muscle from Dox treated animals. It is important to begin to elucidate the benefits Cr supplementation has on Dox-induced skeletal muscle toxicity.

Statement of Purpose

The purpose of the current study was to examine the effects doxorubicin and two types of creatine monohydrate feeding, had on cleaved (PARP), caspase-3, and cleaved caspase-3, at 1-day post injection and 3-days post injection, in the extensor digitorum longus (EDL), soleus (SOL), and diaphragm (DIA).

Research Hypotheses

H1   Dox alone will increase apoptosis in the SOL, EDL, and DIA.

H2   Both types of Cr supplemented diets alone will have no effect on, apoptosis,at either 1-day or 3-day post-injection time points.

H3   Cr will attenuate Dox-induced apoptosis, with a higher percent Cr feeding having a greater attenuation than a lower percent Cr feeding in the SOL, EDL, and DIA.

H4   Dox alone will increase apoptosis more at the 3-days post injection time point, than at 1-day post injection time point in the SOL, EDL, and DIA.

H5   The 4% Cr supplemented diet will have a greater impact on attenuating Dox-induced increases in apoptosis, than the 2% Cr supplemented diet, at the 3-days post injection time point in the SOL, EDL, and DIA.

H6   Both types of Cr feeding will attenuate Dox-induced increases in apoptosis to a greater extent in the EDL, than to either the SOL or DIA at both the 1-day and 3-days post injection time points.

H7   Free Cr and phosphocreatine levels will be greater in the 4% Cr supplemented diet, than the 2% Cr supplemented diet, in the SOL, EDL, and DIA, at both 1-day and 3-days post injection time points.

Need for Study

It is well documented that chemotherapy treatment increases muscle fatigue and weakness leading to decreases in activities of daily living (ADL). Specifically, patients treated with Dox exhibit disrupted skeletal muscle function resulting in debilitating muscle weakness (Gilliam et al., 2012). Any treatment or intervention that can reduce or mitigate chemotherapy-induced muscle fatigue and weakness will improve a patients ADL, and overall quality of life. Animal studies have shown that exercise can mitigate chemotherapy-induced muscle fatigue, weakness, and cardiovascular dysfunction (Fairman, et al., 2016; Van Moll et al., 2016); however, adherence to exercise programs

during and post chemotherapy is very low, and a low percentage of cancer patients are active upon entering a treatment program (Courneya et al., 2007; Markes et al., 2006; McNeely et al., 2006; Mutrie et al., 2007). Therefore, it is important to find interventions that improve chemotherapy-induced muscle fatigue, weakness, and ADL and have the potential for greater adherence. Creatine supplementation has the potential to be such an intervention. Creatine can be taken daily, either mixed in liquid or food, and is generally well tolerated. Although not researched in human populations receiving chemotherapy, in healthy populations, creatine supplementation has been shown to increase aerobic performance and anaerobic capacity (Chwalbinska-Moneta, 2003). In light of these findings, there was rationale for investigating what effect creatine may have on muscular fatigue and weakness in patients receiving chemotherapy as a way of improving ADL's and attenuating Dox myotoxicity.

Preliminary Findings

Recent research was conducted on twenty rodents that were randomly assigned to receive either a bolus 15 mg/kg Dox injection (Dox, n=10) or an equivalent sterile saline injection (Con, n=10) and sacrificed 1-day post injection. Animals were anesthetized with sodium pentobarbital (50 mg/kg). After the rodent was completely anesthetized (i.e. no response with a tail pinch) the left soleus (SOL), left extensor digitorum longus (EDL) and diaphragm (DIA) were removed, trimmed, weighed, flash frozen in liquid nitrogen, and stored at -80^0C for biochemical analysis. The tissues were homogenized, and Western blotting analysis was conducted on the SOL, EDL, and DIA for the presence of cleaved PARP and caspase-3.

One-day Extensor Digitorum Longus
Apoptotic Protein Levels

In the EDL (Figure 1), an unpaired t-test revealed no significant effects in cleaved

PARP levels, $p>0.05$ (Figure 1A). There was a significant drug effect, with the Dox

group exhibiting lower caspase-3 levels, $p<0.05$ (Figure 1B).

One-day Soleus Apoptotic
Protein Levels

In the SOL (Figure 2) no main effects were observed ($p>0.05$) for cleaved PARP

(Figure 2A) and caspase-3 (Figure 2B).

One-day Diaphragm Apoptotic
Protein Levels

In the DIA (Figure 3) no main effects were observed ($p>0.05$) for cleaved PARP

(Figure 3A) and caspase-3 (Figure 3B).

A

B
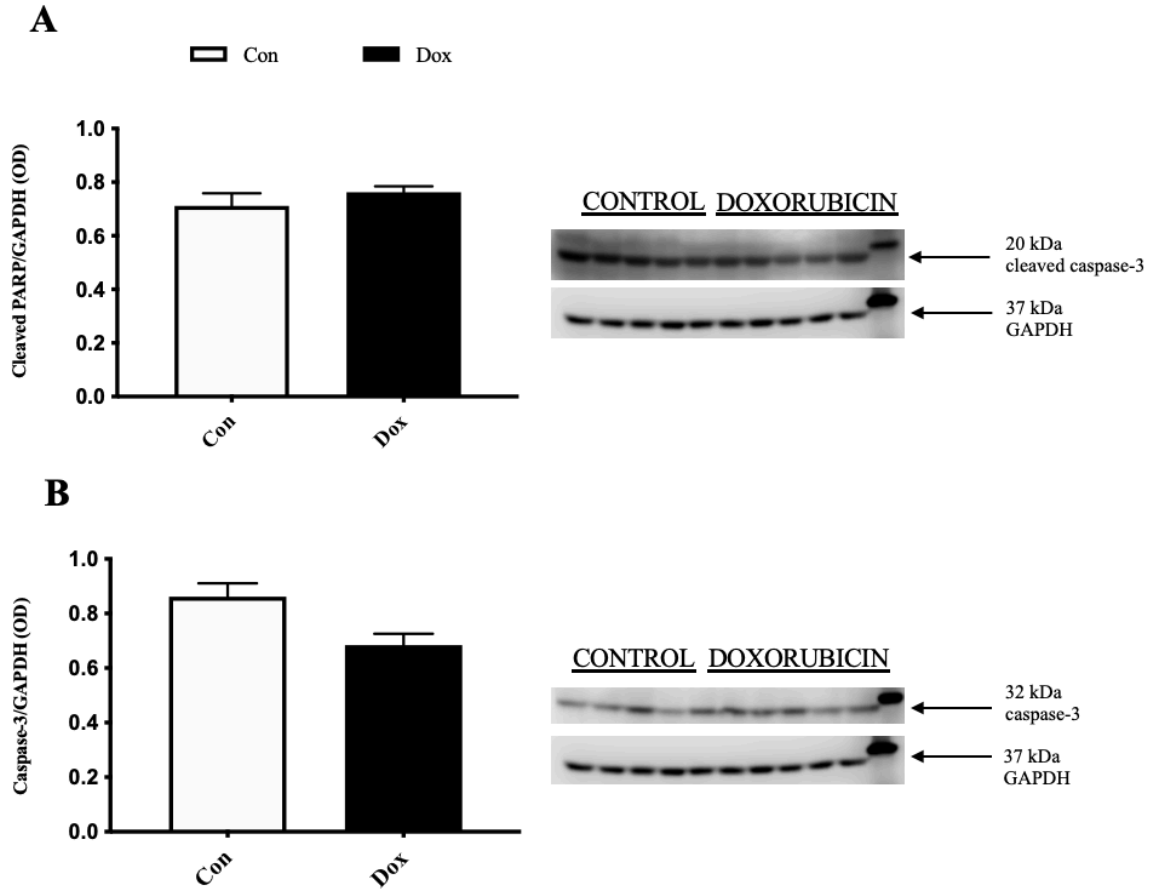
*Figure 1.*1-day EDL apoptotic protein levels, (A) cleaved PARP and (B) caspase-3. EDL = extensor digitorum longus, kDa = kilodalton, OD = optical density, Con = control saline, n=10; Dox = control doxorubicin, n=10; Values are mean ± SEM. (B) significant drug effect (*p<0.05)*
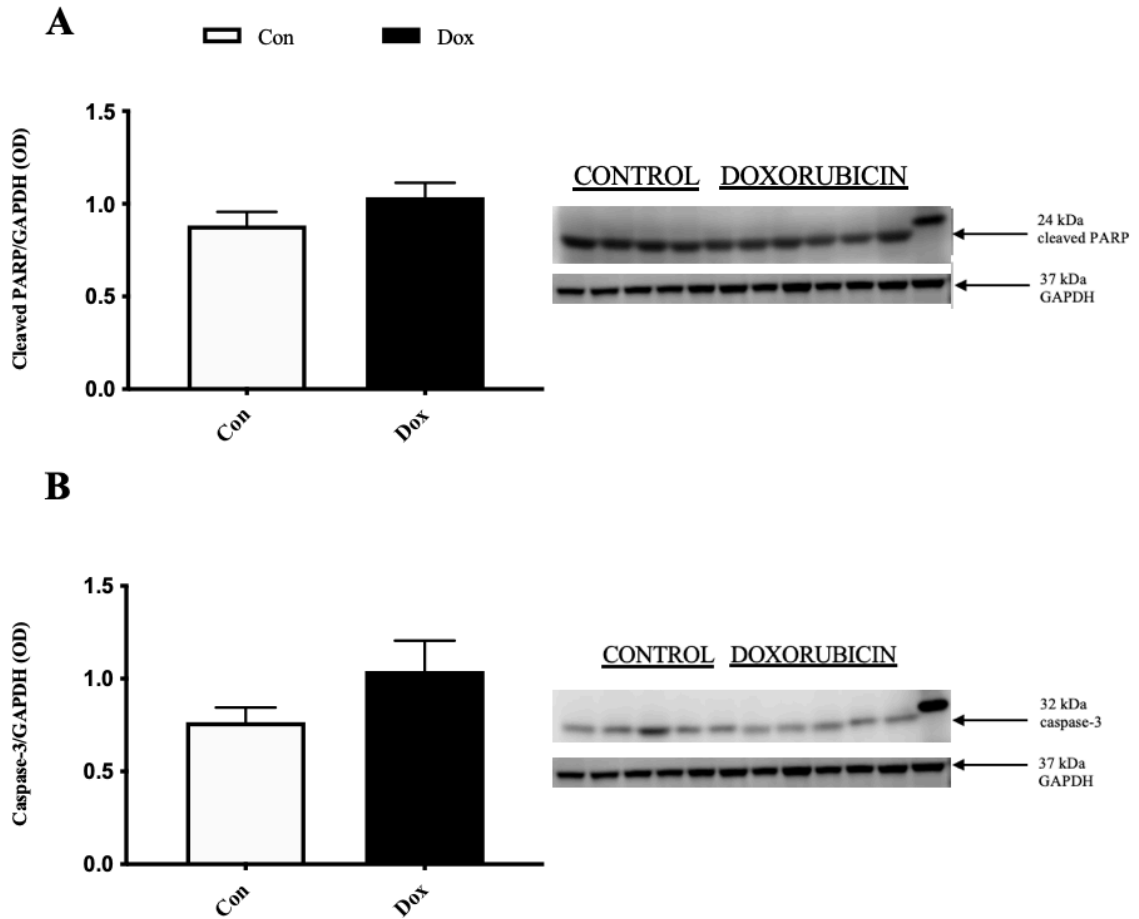
Figure 2. 1-day SOL apoptotic protein levels, (A) cleaved PARP and (B) caspase-3. SOL = soleus, kDa = kilodalton, OD = optical density, Con = control saline, n=10; Dox = control doxorubicin, n=10; Values are mean ± SEM.
No significant effects (*p>0.05)*
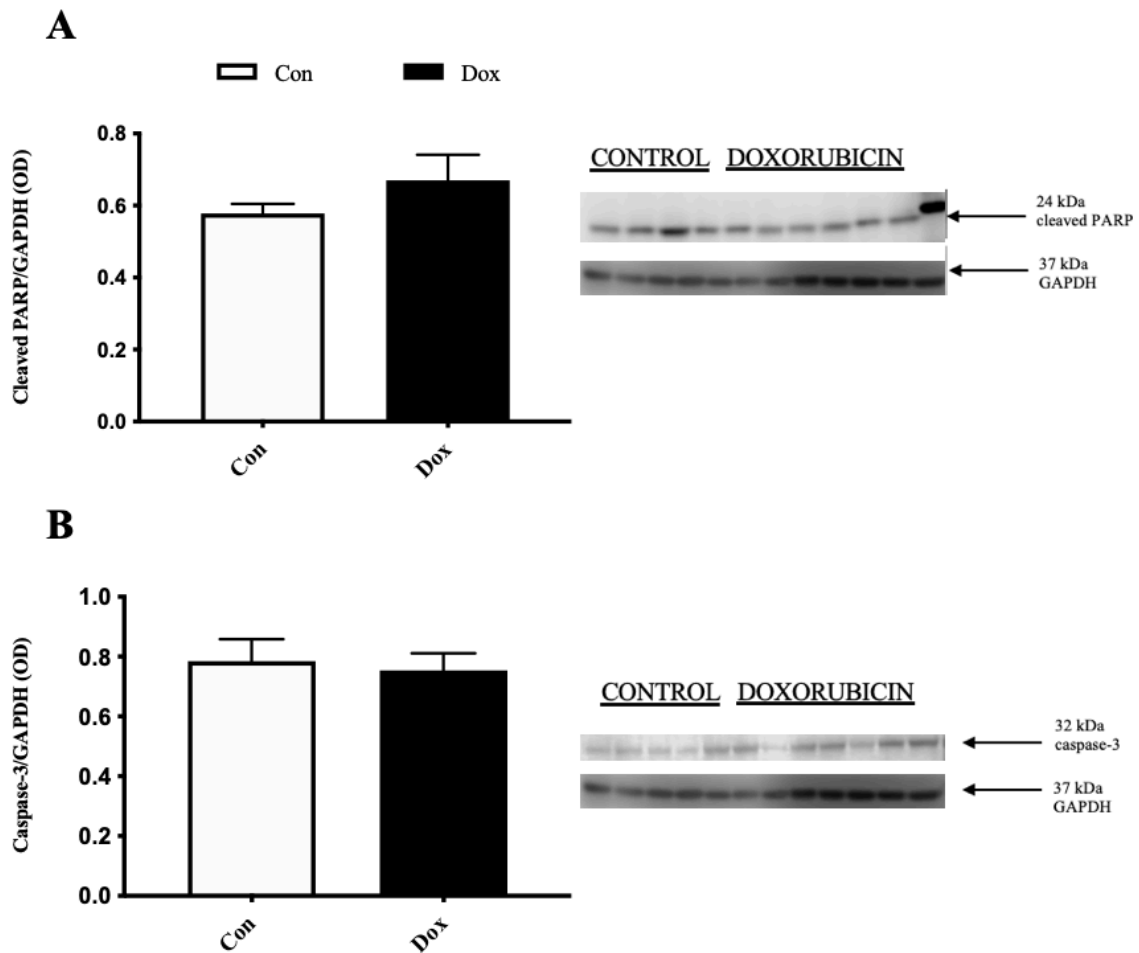
Figure 3. 1-day DIA apoptotic protein levels, (A) cleaved PARP and (B) caspase-3.
DIA = diaphragm, kDa = kilodalton, OD = optical density, Con = control saline, n=10;
Dox = control doxorubicin, n=10; Values are mean ± SEM.
No significant effects (*p>0.05)*

Discussion

Figure 1B showed that Dox treatment significantly affected caspase-3 levels in the EDL. Equally, although not significant, Figure 1B shows a trend for higher cleaved PARP levels with Dox administration. The changes in caspase-3 and cleaved PARP levels suggest an increase in apoptosis in the EDL with Dox. In the SOL and DIA, although not significant, there were increased levels of cleaved PARP with Dox administration, once again, suggesting that apoptosis may be increased in the SOL and DIA with Dox. These results suggest that in the EDL, SOL, and DIA, Dox administration may increase apoptosis.

To further explore the effects of Dox treatment on apoptotic protein expression, adding the protein cleaved caspase-3 to the study will be important to see if the decrease in whole caspase-3 expression (observed DIA and EDL in the pilot study) is coupled with an increase in cleaved caspase-3 expression. In light of these findings, further exploration is warranted to examine what effect different doses of creatine monohydrate may have on cleaved PARP, caspase-3, and cleaved caspase-3 in the SOL, EDL, and DIA. Equally, adding an additional time point (3-days post injection) will broaden our understanding of how Dox may impact apoptosis, via measuring the changes in cleaved PARP, caspase-3, and cleaved caspase-3 levels. The data gathered will be useful in beginning to illuminate what role creatine monohydrate may have in reducing Dox-induced myotoxicity.

CHAPTER II

REVIEW OF THE LITERATURE

Doxorubicin

Doxorubicin (Dox) or Adriamycin, and sometimes Rubex, is an Anthracycline antibiotic, synthesized from the soil fungus Streptomyces. Doxorubicin is among the most commonly used drugs for treating a wide range of human cancers, including leukemias, Wilms tumor, neuroblastoma, soft tissue and bone sarcomas, breast carcinoma, ovarian carcinoma, transitional cell bladder carcinoma, thyroid carcinoma, gastric carcinoma, Hodgkin's disease, malignant lymphoma, and lung cancer. Doxorubicin is administered via intravenous injection through a central line or peripheral venous line over several minutes and is given in 21-day intervals (Johnson-Arbor & Dubey, 2017). Doxorubicin dosing is calculated using body surface area derived from the Du Bois and Du Bois formula published in 1916 (Pouliquen et al., 2011), and due to its induction of cardiotoxicity and heart failure (Chatterjee, Zhang, Honbo, & Karliner, 2010), has a maximum lifetime dose not to exceed 450 mg/m^2 (Rahman, Yusuf, & Ewer, 2007).

Although not completely elucidated, the proposed mechanisms that explain Dox's tumor killing activity are redox cycling in the mitochondria, topoisomerase inhibition, oxidative stress, and apoptosis signaling (Wang et al., 2004). By far the most damaging activity of Dox is its redox cycling, and will be furthered explained in the section, 'Doxorubicin – Redox Cycling'.

Reactive Oxygen Species

The utilization of molecular oxygen by mammals leads to the formation of reactive oxygen species (ROS). Reactive oxygen species are small molecules that are short-lived and highly reactive (Halliwell, 2011). ROS can be generated endogenously during mitochondrial oxidative phosphorylation, or via interactions with exogenous sources. The main ROS consist of hydrogen peroxide (H_2O_2), hydroxyl radical ($HO\bullet$), nitric oxide ($NO^\bullet$), peroxyl radical ($ROO\bullet$), peroxynitrite anion ($ONOO^-$), and superoxide anion ($O_2^{\bullet-}$) and consist of a radical and non-radical oxygen species formed by the partial reduction of oxygen. Reactive oxygen species can be generated by both exogenous and endogenous stimuli. Endogenous production occurs when electrons leak during mitochondrial electron transport chain activity (Dickinson & Chang, 2011). Superoxide is produced in complexes I and III of the electron transport chain, by NADPH oxidases, and via enzymatic activation of cytochrome P450 reductases (Bae, Oh, Rhee, & Yoo, 2011). The powerful oxidant peroxynitrite anion ($ONOO^-$) is formed when the superoxide anion ($O_2^{\bullet-}$) reacts with the reactive nitrogen species (RNS) nitric oxide ($NO^\bullet$) (Dickinson & Chang, 2011). The superoxide anion ($O_2^{\bullet-}$) can also be dismutated into oxygen (O_2) and hydrogen peroxide (H_2O_2) by the antioxidant superoxide dismutase (SOD). Once formed, H_2O_2 can be used in the inflammatory process or form a hydroxyl radical ($OH^\bullet$) (Kehrer & Klotz, 2015). The hydroxyl radical is highly reactive, and the cell avoids formation by detoxifying H_2O_2 via the antioxidant enzymes catalase and glutathione peroxidase (West & Marnett, 2006). generation of ROS in cells exist in equilibrium with a wide variety of antioxidant defenses.

The antioxidant defenses include the enzymatic scavenger's superoxide dismutase (SOD), catalase, glutathione peroxidase and peroxiredoxins (Halliwell, 2011). There are also non-enzymatic scavengers including vitamins C and E, glutathione (GSH), lipoic acid, carotenoids and iron chelators (Halliwell, 2011). Normal ROS production is important and essential for the regulation of normal physiological functions, including, cell cycle progression and proliferation, differentiation, migration, and cell death (Covarrubias, Hernandez-Garcia, Schnabel, Salas-Vidal, & Castro-Obregon, 2008). Equally, ROS play a role in the immune system and in maintenance in redox balance (Zhang et al., 2016). Lastly, ROS have been implicated in the activation of various cellular signaling pathways and transcription factors (Zhang et al., 2016). If this control tightly ROS, antioxidant system becomes dysregulated, oxidative stress results.

Halliwell (2011) defined oxidative stress as "a serious imbalance between the generation of ROS and antioxidant defenses in favor of ROS, causing excessive oxidative damage". Oxidative stress or excess levels of cellular ROS can cause fragmentation of amino acids in proteins, alteration of DNA bases, and lipid peroxidation to membranes and organelles such as the mitochondria (Halliwell, 2011). Equally, oxidative stress has been implicated in the development of pathologies, (Trachootham, Alexandre, & Huang, 2009), neurodegeneration (Andersen, 2004; Shukla, Mishra, & Pant, 2011), atherosclerosis, diabetes (Paravicini & Touyz, 2006), aging (Haigis & Yankner, 2010), and the promotion of tumor metastasis through gene activation (Ishikawa et al., 2008). Relevant to the current study, is the effect that ROS have on the activation of apoptotic signaling pathways.

Apoptosis

Apoptosis is a specific distinct form of cell death, that occurs normally during development and aging and as a homeostatic mechanism to maintain cell populations in tissues. There are a wide variety of both physiological and pathological stimuli that can trigger apoptosis, and not all cells will necessarily die at the same rate or as a result of the same stimuli. For example, chemotherapy drugs or radiation treatment may damage DNA in some cells leading to apoptosis via the p53-dependent pathway, where other cells do not share the same fate (Yang, Niepel, Mitchison, & Sorger, 2010).

Although, it is not the focus of this dissertation study, it is worth noting that an alternative to apoptosis is necrosis, the degradative process that occurs after cell death, which some do not consider necrosis to be a viable form of cell death. However, there is some overlap in the apoptotic/necrotic pathways, with the distinction depending on the nature of the cell death signal, tissue type, developmental stage of the tissue, and the physiologic milieu (Fiers, Beyaert, Declercq, & Vandenabeele, 1999; Zeiss, 2003).

Apoptosis can be triggered via two main pathways, the extrinsic and intrinsic pathways, and in some instances the two pathways can influence one another (Igney & Krammer, 2002). There is an additional pathway that involves T-cell mediated cytotoxicity and perforin-granzyme-dependent killing of the cell (Kaspar et al., 2001). As an overview of apoptosis, regardless of the pathway signaled (extrinsic, intrinsic, T-cell), they all converge on the same terminal which is initiated by the cleavage of caspase-3. Caspase-3 cleavage results in DNA fragmentation, degradation of cytoskeletal/nuclear proteins, cross linking of proteins, formation of apoptotic bodies, expression of ligands

for phagocytic cell receptors and then the uptake of the degraded parts by phagocytic cells (Elmore, 2007).

The extrinsic apoptotic pathway involves transmembrane receptor-mediated interactions, specifically the tumor necrosis factor receptor gene family death receptors Fas receptor or FasR (Locksley, Killeen, & Lenardo, 2001). The binding of the Fas ligand (FasL) to FasR, initiates the extrinsic apoptotic pathway, transmitting the death signal from the cell surface into the intracellular signaling pathway. Fas ligand is a transmembrane protein that binds to it receptor FasR. Fas ligands are formed when they are cleaved by matrix metalloproteinase (MMP-7) and enter circulation. Fas ligands then binds to the FasR on the cell surface which results in the binding of the adapter protein Fas-associated death domain (FADD) to the death domain of Fas, via its own death domain. FADD then associates, via dimerization, with the death domain of procaspase-8 (Wajant, 2002). This forms the death-inducing signaling complex (DISC), which activates procaspase-8 (Kischkel et al., 1995). Activated caspase-8 will trigger the execution phase of apoptosis (Kischkel et al., 1995).

Unlike the extrinsic apoptotic pathway that is initiated by ligand/receptor interaction, the intrinsic apoptotic pathway is triggered by non-receptor-mediated stimuli that produces an intracellular signal that acts directly on targets directly within the cell. The non-receptor-mediated stimuli can either act in a positive or negative fashion. A negative stimulus is a withdrawal of factors that suppress the pathway, thereby triggering the pathway. A positive stimulus increases the apoptotic pathway, an example being free radical production, which is relevant to the current study. Regardless of the non-receptor-mediated stimuli (positive or negative) the result are changes in the inner

mitochondrial membrane: opening of the mitochondrial permeability pore, loss of transmembrane potential and release of two groups of normally sequestered pro-apoptotic proteins from the intermembrane space to the cytosol (Saelens et al., 2004).

The first group of pro-apoptotic proteins that are released from the intermembrane space via non-receptor-mediated stimuli are cytochrome *c*, Smac/DIABLO, and the serine protease HtrA2/Omi (Du, Fang, Li, Y., Li, L., & Wang, 2000; Garrido et al., 2006). The second group of pro-apoptotic proteins are apoptosis-inducing factor, endonuclease G and CAD, which are released later than the first group of pro-apoptotic proteins, after the cell has committed to die. The above mentioned apoptotic mitochondrial events (i.e., the mitochondrial release of the pro-apoptotic proteins) are controlled and regulated by Bcl-2 family of proteins and the tumor suppressor p53 regulation of Bcl-2, with the mechanisms not being entirely understood (Cory & Adams, 2002; Schuler & Green, 2001). The Bcl-2 family of proteins is quite large, containing 25 genes that have been identified in the Bcl-2 family. The Bcl-2 proteins govern mitochondrial permeability and determine if the cell will commit to apoptosis or abort (Cory & Adams, 2002).

The last apoptotic pathway to briefly discuss is the T-cell mediated and involves the cytotoxic T lymphocytes (CTLs). Upon binding to the target cell, the CTLs secrete the transmembrane pore-forming molecule perforin, which then releases its cytoplasmic granules (granzyme A and granzyme B) through the pore into the target cell (Pardo et al., 2004; Trapani & Smyth, 2002). Once inside, granzyme A and B act as proteases, which end up activating procaspase-10, directly activating caspase-3 and signaling the induction of the execution phase of apoptosis (Sakahira, Enari, & Nagata, 1998).

All of the pathways (extrinsic, intrinsic, T-cell), once signaled, end with triggering of the execution pathway, which is the final apoptotic pathway. In the execution pathway, the execution caspases are activated, which activate cytoplasmic endonucleases that begin the process of nuclear and cytoskeletal protein degradation, nuclear material breakdown, cytoskeletal reorganization, chromatin condensation, and disintegration of the cell into apoptotic bodies (Sakahira et al., 1998; Slee, Adrain, & Martin, 2001). The main execution caspases (or sometimes referred to as effector caspases) are caspase-3, caspase-6, and caspase-7, which cleave various substrates such as poly (ADP-ribose) polymerase (PARP), alpha fodrin, NuMA, and others, which cause the morphological changes described above (Slee et al., 2001).

Of all the execution caspases, caspase-3 is considered to be imperative for apoptosis to occur. When there is a disruption in mitochondrial function (loss of transmembrane potential, permeability transition and release of cytochrome c), as with Dox therapy, the result is activation of apoptosis-activating factor 1 (AIF) and caspase -9, leading to the processing of pro-caspase-3 (Kluck et al., 1997; Li et al., 1997; Pan, O'Rourke, & Dixit, 1998; Reed, 1997; Zou, Henzel, Liu, Lutschg, & Wang, 1997). Worth noting is that in caspase-9 knockout mice, caspase-3 fails to activate in the absence of caspase-9, giving evidence that caspase-9 is a critical upstream activator of caspase-3 (Cheng et al., 1997). Once caspase-3 is activated, downstream death substrates are cleaved, including poly (ADP-ribose) polymerase (PARP) (Janicke, Ng, Sprengart, & Porter, 1998). Caspase-3 can also amplify the upstream death cascade, increasing cytochrome c release by cleaving Bcl-2 converting it from an anti-apoptotic to a pro-apoptotic protein (Cheng et al., 1997). In closing, apoptosis is a fundamental and

complex biological process that enables an organism to kill and remove unwanted cells during animal development, normal homeostasis and disease (Jacobson, Weil, & Raff, 1997; Thompson, 1995).

Reactive Oxygen Species and Apoptosis

As stated in the previous section, ROS are small molecules that are short-lived and highly reactive (Halliwell, 2011). They can be oxygen derived like superoxide anion ($O_2^{\bullet-}$) and the hydroxyl radical ($OH^\bullet$), or non-radical molecules like hydrogen peroxide (H_2O_2). As stated earlier, apoptosis is a tightly regulated and highly conserved process of cell death during which a cell undergoes self-destruction (Kerr, Wyllie, & Currie, 1972). It is also worth mentioning that apoptosis is an essential process that multicellular organisms use to eliminates damaged cells, neutralize cells with DNA damage, and protect against carcinogenesis (Fulda, Gorman, Hori, & Samali, 2010; Pallepati & Averill-Bates, 2012).

Normal, low dose ROS production has been linked to a cell survival response, via the ROS activation of the tumor suppressor protein p53 (Carafoli & Lehninger, 1971). p53, a key protein that regulates the cellular stress response, either inducing cell cycle arrest for cell repair, or if damage is beyond repair, triggering cell death via apoptosis (Guerrero-Hernandez, Gallegos-Gomez, Sanchez-Vazquez, & Lopez-Mendez, 2014). At rest, p53 is maintained at low cellular levels and rises during cell stress (Guerrero-Hernandez et al., 2014). As p53 levels rise during elevated cellular stress and if the cellular damage is too severe, p53 can regulate apoptosis transcriptionally by downregulating pro-survival proteins and upregulating pro-apoptotic proteins (Guerrero-Hernandez et al., 2014). The activation of the pro-apoptotic proteins can also induce the

activation of both the intrinsic apoptotic pathway (i.e., changes to the mitochondrial membrane) and the extrinsic apoptotic pathway (i.e., death receptor activation).

Reactive oxygen species stimulation of the intrinsic apoptotic pathway results in the formation of the permeability transition pore, and produces mitochondrial outer membrane permeabilization, resulting in the mitochondria-to-cytosol translocation of cytochrome c, AIF, or Smac/DIABLO, that trigger the caspase cascade that results in cell death (Redza-Dutordoir & Averill-Bates, 2016). ROS can trigger the intrinsic pathway via a number of mechanisms include directly oxidizing mitochondrial proteins, inducing lipid peroxidation, and damaging mitochondrial DNA (mtDNA) (Redza-Dutordoir & Averill-Bates, 2016). The mtDNA damage can results in decreased transcription of proteins essential in the electron transport chains. The decrease in protein transcription disrupts respiratory chain function, which further increases ROS generation. This leads to a disruption in mitochondrial membrane potential and impaired ATP synthesis; all of which will lead to apoptosis (Orrenius, Gogvadze, & Zhivotovsky, 2015). Equally, ROS, particularly H_2O_2, can oxidize protein components of the mitochondrial permeability transition pore (MPTP), which will cause its formation of a pore in the mitochondrial membrane. The formation of the pore causes mitochondrial membrane hyperpolarization, mitochondrial translocation of Bax and Bad, cytochrome c release, and the formation of the executioner caspase-3, 6, and 7 (Nie et al., 2008).

There is a reported link between the ROS production and the extrinsic apoptotic pathway. More specifically there seems to be a link between ROS production and TNF-α activation, although this relationship is not completely understood. TNF-α is an important regulator of the signaling network that promote either cell-survival or cell

death by apoptosis (Mahmood & Shukla, 2010). ROS are important in regulation of the

TNF-R1 signaling pathway, and at high doses, ROS can trigger the TNF-R1 pathway to

drive cell death (Mahmood & Shukla, 2010). The extrinsic apoptotic death receptor

Fas/FasL pathway can also be activated by the ROS H_2O_2 (Pallepati & Averill-Bates,

2011). Pallepati and Averill-Bates, (2011) found that H_2O_2 caused up-regulation of FasL,

and FADD translocation to the plasma membrane and caspase-8 activation in the HeLa

cell line. Equally in endothelial cells, human leukemia cells, and intestinal epithelial

cells, H_2O_2 has been shown to increase mRNA levels of FasL and Fas and direct

activation of caspase-8 and caspase-3 (Denning et al., 2002; Suhara et al., 1998; Zhuang,

Demirs, & Kochevar, 2000). Lastly, not well understood is the capacity for ROS to

activate Fas and TNF-α which causes lipid raft formation, recruitment and activation of

Nox. Activation of the Nox family of transmembrane proteins are an important source of

ROS, and this increase in Nox-mediated ROS can feedback and further stimulate the

death receptors Fas and TNF-α, furthering Nox family activation and ROS production

(Zhang et al., 2007).

In closing, ROS at lower doses is important for proper cell survival responses,

where they can induce cell cycle arrest to promote DNA repair and survival. However, at

higher doses ROS activate the death process such as apoptosis. At these higher doses,

ROS can directly activate the intrinsic mitochondrial apoptotic pathway through

disrupting permeability of the mitochondrial membrane and through the extrinsic death

receptor apoptotic pathway by activating certain death receptors. Both ROS activated

apoptotic pathways, intrinsic and extrinsic, lead to the activation of the execution

pathway and cell death. Doxorubicin, through the process of redox cycling, creates a

large quantity of ROS, and will increase the signaling of the intrinsic and extrinsic apoptotic pathways, increasing the rate of cell death in all cells where doxorubicin undergoes redox cycling.

Doxorubicin and Redox Cycling

Oxidative stress is a widely recognized mechanism of Dox-induced toxicity (Wang et al., 2004). The development of oxidative stress with Dox treatment is driven by two mechanisms, increased levels of ROS following Dox treatment and the downregulation of antioxidants enzymes following Dox administration. The first mechanism, increased ROS levels, may result from different mechanisms including 1) decreased cellular antioxidant defenses, 2) disruption of the mitochondrial electron transport chain with subsequent electron leakage, and 3) the ability of Dox to undergo redox cycling and produce ROS (Zhao et al., 2010). Dox redox cycling leading to ROS production is the most widely recognized mechanism by which oxidative stress increases with Dox administration.

Redox cycling typically involves the reduction of a quinone molecule to form a semiquinone radical species (Hopkins, 2016) and occurs in the cytochrome P450 enzyme system and the mitochondrial electron transport chain. The creation of ROS with Dox administration occurs when the semiquinone radical donates one of its electrons to molecular oxygen. This donation reduces molecular oxygen to superoxide anion radical, and the semiquinone radical is oxidized back to the original quinone molecule. This one-electron reduction and oxidation cycle will continue and persist in the production of superoxide anion radicals and secondary reactive species such as hydrogen peroxide, hydroxyl radical, and peroxynitrite (Hopkins, 2016). Excessive ROS production can lead

to significant cellular damage. Doxorubicin is a quinone compound and has been proposed to undergo redox cycling, specifically in complex I of the mitochondrial electron transport chain. During this process Dox may go through redox activation to a semiquinone intermediate (Dox$^-$) which generates superoxide radical upon the one-electron reduction of O_2 (Dox$^-$ + O_2 $\rightarrow$ Dox + O_2^-) (Zhu et al., 2016). Early research from Davies and Doroshow (1986) demonstrated that the quinone moiety of anthracyclines (doxorubicin) is reduced by one electron at complex I of the mitochondrial electron transport chain. This results in the one-electron transfer to molecular oxygen to form superoxide anion, hydroxyl radical, and hydrogen peroxide (Davies & Doroshow, 1986).

Doxorubucin has a strong capacity to trigger apoptosis, autophagy, and morphology in skeletal muscle. In mice Dox administration resulted in a trend towards a reduction in body weight in overall body weight and a significant increase in caspase-3, caspase-9 and calpain activity (Mitchell, Merkel, & Quadrilatero, 2015). Equally, Dox administration has been shown to elevate Bax, caspase-3 activity, and apoptotic DNA fragmentation in aged mice skeletal muscle and C57BL/6 mice increasing apoptosis (Sin et al., 2016; Yu et al., 2014). In cardiac tissue one of the mechanisms that leads to myocardial toxicity and heart failure following Dox administration is ROS-triggering of apoptosis (Childs, Phaneuf, Dirks, Phillips, & Leeuwenburgh, 2002; Singal et al., 2000; Takemura & Fujiwara, 2007).

Although not completely understood, Dox administration may produce ROS from a different mechanism. It is well understood that a molecular target of Dox is topoisomerase IIα, which is found in proliferating cells and required for DNA replication

(Zhu et al., 2016). However, this would not be relevant for cell that are not actively proliferating, such as skeletal muscle cells or cardiomyocytes. Doxorubicin has also been shown to target topoisomerase IIβ, which is present in the mitochondria of all quiescent cells (Vejpongsa & Yeh, 2014). It has been suggested when topoisomerase IIβ is target by Dox, resulting in DNA double strand breaks, defective mitochondrial biogenesis, and increased ROS accumulation (Zhang et al., 2016). Although not completely understood, the theory is, Dox inhibition of topoisomerase IIβ may cause p53 activation via the mitochondrial DNA damage response, repression of PPARγ, PGC1α, PGC1β, leading to mitochondrial dysfunction, increased electron leakage, superoxide formation, and subsequent further activation of p53 (Sahin & DePinho, 2012). Regardless of the mechanism, whether direct ROS production through redox cycling, or increased topoisomerase IIβ leading to ROS production, Dox administration leads to increased mitochondrial superoxide production, and damage to tissue.

Creatine

Creatine monohydrate (Cr) is one of the most widely used supplements ingested in an attempt to improve high intensity athletic performance. A large amount of research has been done on Cr supplementation in efforts to delineate its sport-specific effects. It has been hypothesized that Cr can act through a number of pathways and appears to be most effective in short-bout, high-intensity physical activities. However, over the past decade Cr used for therapeutic and chemo preventive purposes has received increasing attention (Gualano et al., 2009). Research has showed that Cr supplementation is beneficial in a large number of muscular, neurological, and cardiovascular diseases. Sharov et al. (1987) demonstrated that in the presence of exogenous phosphocreatine, a

rat heart that had been made ischemic for 35 minutes regained almost all heart function, phosphocreatine content, and 61% of adenosine triphosphate content, whereas only 33% recovery of heart function was observed in control. Three days of subcutaneous creatine injections significantly reduced the rate of hypoxic seizures in 5-to-30-day-old rabbit pups (Holtzman et al., 1999). Creatine supplement in the diets of Sprague Dawley rats was significantly protective against malonate and 3-nitropropionic acid induced neurotoxicity (Matthews et al., 1999). In Duchenne muscular dystrophy, orally administered creatine monohydrate given to 9-year-old patients improved muscle performance (Felber et al., 2000). Mazzini et al. (2001) demonstrated that creatine supplementation significantly increased maximal isometric power in 10 different muscle groups, in 28 patients with definite Amyotrophic Lateral Scelerosis. Stout et al. (2001) supplemented a single patient with myasthenia gravis, 5 grams of Cr and 3 times per week resistance exercise for 15 weeks. Stout et al. (2001) found that after creatine supplementation and training body weight, fat free mass, upper body strength, lower body strength and peak strength increased, without any negative effects on blood chemistry. Creatine supplementation has been investigated in four transgenic mouse model of Huntington's disease, resulting in a 19% improvement in survival, delayed onset of weight loss, slowed the development of motor symptoms, of brain atrophy, and of atrophy of striatal neurons, lessened the formation of intranuclear inclusions, and delayed the onset of diabetes (Wyss & Schulze, 2002). Tarnopolsky et al. (2004) investigated oral Cr supplementation in seven patients with mitochondrial cytopathies and found that Cr supplementation increased high intensity anaerobic and aerobic performance. Tarnopolsky (2007) showed that Cr supplementation (5-10 g/day for 5

days) all indices of muscle strength in 102 patients with neuromuscular disease. Lastly Bender et al. (2007) investigated the effect of oral Cr supplementation on aging in 162 C57BI/6J and showed that supplementation increased median life span by 9%, improved performance on neurobehavioral tests, reduced ROS, lowered accumulation of "aging pigment" lipofuscin, upregulated genes implicated in neuronal growth, neuroprotection and learning.

Creatine Metabolism

Creatine, or N-aminoiminomethyl-N-methylglycine, is an endogenously formed amino acid that is taken up in diets containing fresh meat or fish. Biosynthesis of creatine takes place in the liver and begins with the transfer of the amindino group of arginine (synthesized in the kidneys) to glycine to yield L-ornithine and guanidinoacetic acid, catalyzed by L-arginine:glycine amindinotransferase. The enzyme S-adenosyl-L-methionine:N-guanidinoacetate methyltransferase then methylates the amidino group on guanidinoacetic acid and forms creatine. Worth noting and still largely accepted, is that creatine biosysnthesis first occurs in the kidneys with the formation of guanidinoacetate, which is then transported through the blood to the liver where guanidinoacetate is methylated to form creatine (Wyss & Kaddurah-Daouk, 2000). Regardless of the route, creatine is then transported out of the liver, into the bloodstream, transported into the tissue via the sarcolemma bound creatine transporter (Murphy et al., 2001) and stored in the tissue (brain, brown adipose tissue, intestine, seminal vesicles fluid, endothelial cells, and marcrophages) with the about 95% stored in skeletal muscle (Cooper, Naclerio, Allgrove, & Jimenez, 2012). Once in skeletal muscle, Cr undergoes what is referred to as the phosphocreatine (PCr) 'shuttle' system. The PCr shuttle system occurs in the

mitochondria, where a high-energy phosphate is transferred from ATP formed in oxidative phosphorylation, to the guanidine group on Cr, via the enzyme creatine kinase mitochondria (CKmito). Phosphocreatine then diffuses out of the mitochondria into the cytoplasm, where the creatine kinase cytosolic isoform catalyzes the reversible transfer of the N-phosphoryl group from PCr to ADP to regenerate ATP. The ATP is then localized to areas of need, and the Cr returns to the interior of the mitochondria (Wyss & Kaddurah-Daouk, 2000). A constant fraction of the body creatine (1.1%) and PCr (2.6%) are spontaneously and nonenzymatically converted to creatinine, with this reaction being both pH and temperature dependent (Wyss & Kaddurah-Daouk, 2000). Creatinine will then diffuse out of the cell, into the blood, and is excreted by the kidneys into the urine. The converted creatinine then has to be replaced by Cr from the diet or from de novo biosynthesis (Walker, 1979).

Creatine and Apoptosis

As described in the section above, apoptosis is a morphologically distinct and important mode of 'programmed' cell death that occurs in mammalian cells. As stated earlier, creatine supplementation can benefit a large number of muscular, neurological, and cardiovascular diseases. One such mechanism may be creatine's ability to modulate apoptosis.

Rahimi, Mirzaei, Rahmani-Nia, and Salehi (2015) examined the effects of creatine monohydrate supplementation on apoptotic markers and found that creatine monohydrate supplementation prevented exercise induced apoptosis, as measured by decreases in p53 concentration. It is well known that p53 plays a significant role in regulating apoptosis by affecting the expression of apoptotic peptidase activating factor-1

and by altering mitochondrial membrane potential (Haldar, Negrini, Monne, Sabbioni, & Croce, 1994; Moll & Zaika, 2001). Rahimi (2011) suggested that the increase in oxidative stress with aerobic exercise signal p53, which in turn triggered the p53 dependent apoptotic pathway to preserve genomic intensity and cellular homeostasis. Although not directly measured, Rahimi et al. (2015) suggest that Cr may act as an antioxidant, scavenging ROS. This then reduces the ROS-induced signaling of p53, which was one of the findings of the study, reduced serum p53 concentration with Cr supplementation. Sheikholeslami-Vatani and Faraji (2018) equally found that creatine supplementation reduced apoptotic markers with exercise, reducing caspases (-3 and -9), p53, and Bax when compared to placebo.

Sheikholeslami-Vatani and Faraji (2018) suggest that although not directly measured, the reduction in the apoptotic markers may have been due to the ability for Cr supplementation to reduce ROS production which reduced the signaling of the apoptotic caspases (-3 and -9) and p53. Lastly, in exercise-induced apoptosis, Cr supplementation reduced lipid peroxidation and glutathione peroxidase activity, and attenuated oxidative DNA damage, with the suggested mechanisms being the potential ROS scavenging capabilities (Basta, Skarpan'ska-Steinborn, & Pilaczyn'ska-Szczes'niak, 2006; Mirzaei, Rahmani-Nia, Salehi, & Rahimi, 2013; Rahimi, 2011). Although not completed in skeletal muscle, Zhu et al. (2004) showed that in focal cerebral ischemia, Cr supplementation either directly or indirectly inhibited cytochrome *c* release and downstream caspase-3 activation. Equally, Cr supplementation has been shown to reduce caspase-3/7 activation in HL-1 cardiomyocytes (Santacruz et al., 2015). In summary, Cr supplementation can reduce markers of apoptosis in a variety of conditions. Although the

mechanisms are not elucidated, it is postulate that Cr acts an antioxidant, scavenging free radicals, and reducing their quantity which ultimately reduces apoptosis.

Creatine as an Antioxidant

Creatine is the most widely used supplement advocated as an ergogenic aid (Kraemer & Volek, 1999). The literature claims creatine supplementation increases lean body mass, skeletal muscle strength, muscle power, and endurance (Dangott, Schultz, & Mozdziak, 2000; Demant & Rhodes, 1999; Ingwall, 1976; Jones, Atter, & Georg, 1999; Reid, Stokic, Koch, Khawli, & Leis, 1994). Recently, it has been suggested that creatine could possess an antioxidant effect.

As mentioned, creatine is a downstream product of the amino acids glycine and arginine. Arginine is a substrate in the formation of the free radical nitric oxide which is involved in modulating metabolism, contractility of smooth and skeletal muscle, and skeletal muscle glucose uptake (Lawler & Powers, 1998; Reid, 2001). It has been demonstrated that amino acids such as histidine, methionine, and cysteine are susceptible to free radical oxidation, and it is possible, that due to the amino acid makeup of creatine, it may be susceptible to free radical oxidation as well. In fact, Vergnani et al. (2000) showed a protective role of arginine against oxidative stress in endothelial cells. Equally, Wu and Meininger (2000), indicated that arginine may be able to quench free radicals such as superoxide anions (O_2^-). Matthews et al. (1998) first proposed that creatine supplementation provides direct or indirect antioxidant protection against metabolic damage in a Huntington's disease mouse model. Lawler, Barnes, Wu, Song, and Demaree (2002) showed that creatine has the ability to act as a direct antioxidant, capable of removing 3 charged free radical and ROS, superoxide anions (O_2^-), peroxynitrite

(OONO⁻), and 2,2'-azino-bis (3-ethylbenzothiaxoline-6-sulfonic acid) (ABTS⁺).

Important to note Lawler et al. (2002) observed that it was creatine, not creatine

phosphate, that was capable of scavenging charged radicals and the study was conducted

in an acellular setting.

Sestili et al. (2006) expounded on Lawler's findings and investigated the

antioxidant effects of creatine on three mammalian cell lines and found Cr preloading to

be mildly but significantly cytoprotective in a dose related manner. In a follow up study,

Sestili et al. (2007) using HPLC, in an attempt to understand whether the cytoprotective

effects of Cr depend on antioxidant or an energy-related mechanism, found that tissues

accumulated free Cr at different levels, and that protection occurred only when free Cr

levels reached a certain threshold. The aforementioned study identified that the cells

(U937, HUVEC, and C2C12) pretreated with Cr and treated with H_2O_2 formed an

oxidation product with a molecular weight of 136amu and 150amu, with these structures

still needing to be better defined by means of infrared spectroscopy and mass-mass

spectrometry (Sestili et al., 2007). In a separate study, Sestili et al. (2009) showed that Cr

pre-treatment significantly protected mitochondrial DNA from oxidative damage, with

respect to H_2O_2-treated, Cr unsupplemented samples, and again, that the protective

effects were Cr dose dependent.

Doxorubicin has been shown to act as both an alkylating and an oxidizing agent,

damaging RNA (Fimognari, Sestili, Lenzi, Cantelli-Forti, & Hrelia, 2009). Cr

supplementation was able to reduce the RNA damage in Jurkat T-leukemia cells

incubated in H_2O_2 and Dox (Fimognari et al., 2009). Lastly, Cr supplementation

significantly and dose-dependently attenuated the cytotoxic impact of H_2O_2 to differentiating C2C12 myoblasts (Sestili et al., 2009).

In closing, it is well established that Cr supplementation can improve athletic performance (Earnest, Snell, Rodriguez, Almada, & Mitchell, 1995). More recently, research has demonstrated that Cr supplementation may beneficial in a large number of muscular, neurological, and cardiovascular diseases (Bender et al., 2007; Felber et al., 2000; Gordon et al., 1995; Holtzman et al., 1999; Matthews et al., 1999; Mazzini et al., 2001; Neubauer et al., 1999; Sharov et al., 1987; Stout et al., 2001; Tarnopolsky et al., 2004; Tarnopolsky, 2007; Vorgerd et al., 2000; Wyss & Schulze, 2002).   Free Cr, not Cr bound to a phosphate, can reduce apoptosis in various types Dox treated cells, possible through its proposed antioxidant capacity. Overall, Cr supplementation is well tolerated in humans, with the most common side effect being diarrhea and bloating.   Equally, Cr supplementation is easy to ingest, whether mixed in a liquid or taken as a pill and can be taken with or without food. In light of the aforementioned, Cr supplementation may be valuable nutraceutical treatment to reduce Dox-induced muscle fatigue that affects so many cancer patients receiving Dox treatment.

CHAPTER III

METHODOLOGY

Experimental Design

The current study examined the effects of a nutritional intervention, using two types of creatine feeding interventions: 2% creatine feeding for four weeks (C1) and 4% creatine feeding for one week followed by 2% creatine feeding for three more weeks (C2). Equally, the study examined a drug effect, with rats receiving either a 15 mg/kg injection of Dox or an equivalent sterile saline injection (Sal). Lastly, animals were sacrificed either 1-day post injection or 3-days post injection. Western blotting technique measured the apoptotic markers, cleaved poly ADP ribose polymerase (PARP), cleaved caspase-3, and caspase-3 in the fast, type II extensor digitorum longus (EDL), the slow, type I soleus (SOL) and the diaphragm (DIA). Upon sacrifice, tissues were harvested, flash frozen in liquid nitrogen, and stored at -80°C for biochemical analysis.

Subjects

All protocols used for the study were approved by the University of Northern Colorado Institutional Animal Care and Use Committee and followed the Animal Welfare Act guidelines. Male Sprague Dawley rats (120 total animals; 60 animals x 1 day and 60 animals x 3 day) were purchased from Envigo and housed at the University of Northern Colorado Research Facility. All animals were housed individually in a temperature-controlled facility with a 12-hour light/12-hour dark cycle. Animals were provided their nutritional intervention and water *ad libitum*.

At 10 weeks of age, the animals were assigned to one of two, four-week creatine feeding interventions or control feeding referred to below (See Table 1.). The first feeding protocol (creatine feeding protocol 1 or C1) attempted to duplicate human dosing of creatine, which is typically, 10 grams per day. Creatine feeding protocol 1 (C1) consisted of a rodent chow supplemented with 2% creatine and was feed *ad libitum* to the rodents for 4 weeks. The second feeding protocol (creatine feeding protocol 2 or C2) attempted to duplicate the human equivalent of a loading phase, which is taking 5 grams of Cr, 4 times per day, for 7 days, then to a maintenance dose of 10 grams a day for the duration of the supplement period. Creatine feeding protocol 2 (C2) consisted of a rodent chow supplemented with 4% creatine for one week, followed by rodent chow supplemented with 2% creatine for three weeks (creatine feeding protocol 2, C2). Animals assigned to the control group were fed standard rodent chow (Teklad Global) which is 16.4% protein, 4% fat, 48.5% carbohydrate, and 3.3% fiber, made predominantly from ground wheat, ground corn, and wheat middlings, for four weeks.

At the end of four-week nutritional intervention, the animals were randomly assigned to receive either an intraperitoneal bolus 15 mg/kg Dox injection or an equivalent sterile saline injection (Sal). The final volume of the Doxorubicin injection was calculated by first multiplying the animals body mass by 15mg/kg, then dividing by the 2mg/ml Doxorubicin concentration. Following injections, the animals were assigned to be sacrificed either 1-day post injection or 3-days post injection, and continued diet.

Animals were anesthetized with sodium pentobarbital (50 mg/kg). After the rodent was completely anesthetized (i.e., no response with a tail pinch) the left soleus (SOL), left extensor digitorum longus (EDL), and diaphragm (DIA) were removed,

trimmed, weighed, flash frozen in liquid nitrogen, and stored at -80^{0}C for biochemical

analysis.

Table 1

Experimental design for treatments.

Group	Diet	Drug	Sacrifice Time
Con-Sal	4 wks Con Diet	Sal	1 day (n=10), 3 day (n=10)
Con-Dox	4 wks Con Diet	Dox	1 day (n=10), 3 day (n=10)
C1-Sal	4 wks 2% Cr Diet	Sal	1 day (n=10), 3 day (n=10)
C1-Dox	4 wks 2% Cr Diet	Dox	1 day (n=10), 3 day (n=10)
C2-Sal	1 wk 4% Cr Diet - 3 wks 2% Cr Diet	Sal	1 day (n=10), 3 day (n=10)
C2-Dox	1 wk 4% Cr Diet - wks 2% Cr Diet	Dox	1 day (n=10), 3 day (n=10)

Note. Con-Sal = control saline, 1 day n=10, 3 day n=10; Con-Dox = control doxorubicin, 1 day n=10, 3 day n=10; C1-Sal = creatine 1 saline, 1 day n=10, 3 day n=10; C1-Dox, creatine 1 doxorubicin, 1 day n=10, 3 day n=10; C2-Sal, creatine 2 saline, 1 day n=10, 3 day n=10; C2-Dox, creatine 2 doxorubicin, 1 day n=10, 3 day n=10.

Biochemical Analyses

Homogenate Preparation

The SOL, EDL and DIA were homogenized in a cocktail of

radioimmunoprecipitation (RIPA) buffer at a 1:10 weight:volume ratio and 10 µl protease

enzyme inhibitor (Santa Cruz Biotechnology). The tissue samples (between 0.7-1.0

grams) were manually homogenized using a Cole-Parmer PTFE tissue grinder (Cole-

Parmer: Vernon Hills, IL), sonicated using 10 x 1 second bursts (Fischer Scientific, Sonic

Dismembrator, Model 100), and then transferred to a 2 ml eppendorf tube. The

homogenate was centrifuged for 10 minutes at 10,000 g, the pellet discarded, and the

supernatant transferred to a new 2 ml Eppendorf. A Bradford Assay was performed to

assess total protein concentration using a Genesys 20 photo spectrometer

(ThermoSpectronic: Rochester, NY) at 595 nanometers. Radioimmunoprecipitation assay

buffer (RIPA) and Laemmli Sample Buffer (Sigma-Aldrich) was pipetted into each

sample to standardize the concentration of each sample.

Western Blotting

Western blot analysis was conducted on the, SOL, EDL, and DIA for the presence

of cleaved PARP (24 kilodalton), caspase-3 (32 kilodalton), and cleaved caspase-3 (17

kilodalton), and used the 35.8 kilodalton, glyceraldehyde 3-phosphate dehydrogenase

(GAPDH) as the loading control. Prepared samples were boiled in water for 2 minutes,

chilled for 5 minutes on ice, with 15 µl loaded onto 4 - 20% Tris-Glycine (Invitrogen)

precast gels, along with 10µl SeeBlue protein standard (Thermo Fisher Scientific) and 10

µl of MagicMark XP western protein standard (Thermo Fisher Scientific). Gels were run

at a constant 125V at 4 mA in an Xcell II blot module (ThermoFisher Scientific) until

tracking dye reaches bottom of the gel. Once the protein migration was complete, the proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane at 25 volts and100 mA for 90 minutes. Membranes were blocked using 10 ml WesternBreeze ™ Superblocker Solution (ThermoFisher Scientific) for 30 minutes, washed twice in water for 5 minutes and then incubated overnight at room temperature in 10 mL of primary antibody solution. Membranes were then washed four times in 10 mL of WesternBreeze Wash Solution (ThermoFisher Scientific) and incubated for 1 hour in the species-specific secondary antibody conjugated with horseradish peroxidase (HRP). Membranes were then washed 4 more times with WesternBreeze Wash Solution and prepared for protein band detection.

Protein band detection involved incubating the membranes in Enhancedchemiluminescence (ThermoFisher Scientific, ECL) solution containing peroxide buffer and luminol substrate, mixed in a 2 mL microcentrifuge tube (concentration 1:1) and poured onto the center of the opened C-Digit imager (Li-Cor: Lincoln, NE). Membranes were placed on top of the substrate face down for 5 minutes. A cellophane sheet was then placed on top of the membrane, the lid closed, and the C-Digit imager captured the image. Optical density of the protein bands was quantified using Image J (NIH: Bethesda, MD). The ratio of cleaved PARP was quantified and this value was divided by the optical density of GAPDH for the final optical density for cleaved PARP, with the same process completed for caspase-3 and cleaved caspase-3.

Table 2

Characteristics of antibodies used for Western blotting.

Antibody name	Dilution ratio	Species specificity	Source	Supplier	Catalog No.
GAPDH	1:1000	Rabbit	Mouse mAB	Santa Cruz Bio	Sc-32233
Caspase-3	1:200	Human	Mouse mAB	Santa Cruz Bio	Sc-5603
PARP-1	1:200	Human	Mouse mAB	Santa Cruz Bio	Sc-8007
M-IgGk BP-HRP	1:1000	Anti-mouse		Santa Cruz Bio	Sc-516102

Note. GAPDH = glyceraldehyde-3-phosphate dehydrogenase, PARP-1 = poly (ADP-ribose) polymerase-1, M-IgGk BP-HRP = mouse IgG kappa binding protein conjugated to Horseradish Peroxidase, mAB = monoclonal antibody

Creatine Assay

Creatine and phosphocreatine levels were analyzed in the SOL, EDL, and DIA at both the 1-day and 3-days post injection time points using the commercially available creatine assay kit (Abcam). Skeletal muscle samples (0.7-1.0 g) were homogenized in radioimmunoprecipitation assay buffer (RIPA buffer), centrifuged for 10 minutes at 10,000 x g, then the supernatant transferred to a clean tube and the pellet discarded. The reagents were then prepared using the materials supplied by the Creatine Assay Kit. Prior to beginning the equipment was equilibrated and the reagents brought to room temperature and all standards, controls, and samples duplicate. The creatine standard wells were filled with 25 µl of the creatine standard, and 25 µl of the reaction mix. The sample wells were filled with 8 µl of tissue supernatant, 17 µl of creatine assay buffer, and 25 µl of reaction mix. The wells were mixed for 2 minutes and incubated in the dark at 37°C for 60 minutes. Measurement of the wells were done on a microplate reader at 570 nm and expressed as the molar concentration of creatine per whole tissue.

Statistical Analysis

Data was presented as means ± standard error (mean±SEM). A two-way (diet x drug) analysis of variance (ANOVA) was used at each time point to determine main diet effect, drug effect, diet x drug, of the dependent variables (apoptotic markers: cleaved PARP, caspase-3, cleaved caspase-3, and creatine concentration). When ANOVA significant main effects and/or interaction were observed, and a Tukey's post hoc testing was performed to identify where the differences existed. Significance was set at the $\alpha=0.05$ level.

CHAPTER IV

RESULTS

The purpose of this study was to determine the effects of two separate creatine feeding, and acute Dox administration on apoptosis in skeletal muscle. This study investigated the expression of cleaved PARP, caspase-3, and cleaved caspase-3 at two separate time points following a bolus Dox injection. Equally, the concentration of total Cr was measured in EDL, SOL, and DIA. This chapter presents the findings of the study.

General Observations

Table 3 presents the animal characteristics at the time of injection and at the 1-day sacrifice time point. At the time of injection, there were no main effects observed in body mass between groups, $p>0.05$. At the time of sacrifice, a drug effect was observed with Dox animals exhibiting significantly lower body mass, $F(1, 54) = 6.91$, $p<0.05$. In the EDL, there was a significant drug effect, with Dox animals having significantly lower EDL mass, $F(1, 50) = 4.24$, $p<0.05$. In the SOL there was a significant drug effect, $F(1, 54) = 5.759$ and diet x drug interaction, $F(2, 54) = 5.12$, $p<0.05$, with Dox animals having significantly lower SOL mass. *Post hoc* tests revealed SOL mass to be significantly lower in the Con-Dox than Con-Sal. In the DIA, no significant main effects were observed and no interactions were detected, $p>0.05$.

Table 3

1-day animal characteristics.

	Con-Sal	Con-Dox	C1-Sal	C1-Dox	C2-Sal	C2-Dox
Injection Mass (g)	390 ± 7	394 ± 10	400 ± 5	395 ± 9	387 ± 9	380 ± 14
Sacrifice Mass (g) [‡]	388 ± 7	374 ± 9	397 ± 5	377 ± 9	386 ± 9	361 ± 13
EDL Mass (mg) [‡]	172 ± 12	143 ± 5	166 ± 8	140 ± 18	154 ± 12	148 ± 6
SOL Mass (mg) [‡+]	148 ± 5	134 ± 4 [*]	147 ± 4	147 ± 3	144 ± 5	143 ± 6
DIA Mass (mg)	261 ± 29	261 ± 21	271 ± 18	249 ± 24	232 ± 21	266 ± 14

Note. Con-Sal = control saline, n=10; Con-Dox = control doxorubicin, n=10; C1-Sal = creatine 1 saline, n=10; C1-Dox, creatine 1 doxorubicin, n=10; C2-Sal, creatine 2 saline, n=10; C2-Dox, creatine 2 doxorubicin, n=10. Values are means ± SEM.
[‡] = signficant drug effect ($p<0.05$)
[+] = significant interaction ($p<0.05$)
[*] = significantly different than Con-Sal

Biochemical Analyses

Western Blotting

Expression of the apoptic proteins, cleaved PARP, caspase 3, and cleaved caspase 3, were measured in the SOL, EDL, and DIA homogenates at the 1 and 3 day time points to evaluate the influence of creatine monohydrate and Dox on their expression. Forty six μg of protein from the 1 day and 3 day, SOL, EDL, and DIA homogenates were added to 4-20% Tris-glycine precast gels and run through SDS-PAGE. Cleaved PARP, caspase-3, and cleaved caspase-3 levels were assessed by enhanced chemiluminescence and are expressed relative to GAPDH as a loading control. No signficiant GAPDH main effects or interactions ($p > 0.05$) were observed, suggesting that the drug and diet treatments did not affect the loading control.

One-day extensor digitorum longus apoptotic protein levels. In the EDL,

cleaved PARP levels were higher with the administration of Dox, $F (1, 52) = 28.12$,

$p<0.05$ (Figure 4A). *Post hoc* tests revealed that Con-Dox, C1-Dox, C2-Sal, and C2-Dox

expressed significantly higher levels of cleaved PARP than C1-Sal. *Post hoc* tests also

revealed that C2-Dox expressed significantly higher levels of cleaved PARP than Con-

Sal (Figure 4A). A 2-way ANOVA revealed a significant diet effect, with creatine groups

exhibiting lower levels of caspase-3, $F (2, 53) = 4.142$, $p<0.05$ (Figure 4B). No

significant main effects or interaction was detected for cleaved caspase-3 (Figure 4C).

Figure 4. 1-day EDL apoptotic protein levels, (A) cleaved PARP, (B) caspase-3, (C) cleaved caspase-3.

EDL = extensor digitorum longus, kDa = kilodalton, OD = optical density, Con-Sal = control saline, n=10; Con-Dox = control doxorubicin, n=10; C1-Sal = creatine 1 saline, n=10; C1-Dox = creatine 1 doxorubicin, n=10; C2-Sal = creatine 2 saline, n=10; C2-Dox = creatine 2 doxorubicin, n=10; Values are mean ± SEM.

(A) significant drug and diet effect ($p<0.05$)

(B) significant diet effect ($p<0.05$)

* = significantly different from C1-Sal

+ = significantly different from Con-Sal

One-day soleus apoptotic protein levels. For cleaved PARP, a significant drug effect was observed, with Dox groups exhibiting higher levels of cleaved PARP, $F(1, 50) = 6.30$, $p<0.05$ (Figure 5A). A diet effect was observed, with Cr groups expressing higher levels of cleaved PARP, $F(2, 20) = 5.41$, $p<0.05$. No significant interaction was detected. *Post hoc* tests also showed that Con-Sal, C1-Sal, C1-Dox, and C2-Sal expressed significantly lower levels of cleaved PARP than C2-Dox (Figure 5A). No significant main effects were observed and no interaction was detected for caspase-3 (Figure 5B). Cleaved caspase-3 levels were lower with diet, $F(2, 52) = 3.43$, $p<0.05$ (Figure 5C), and a drug effect was not observed.
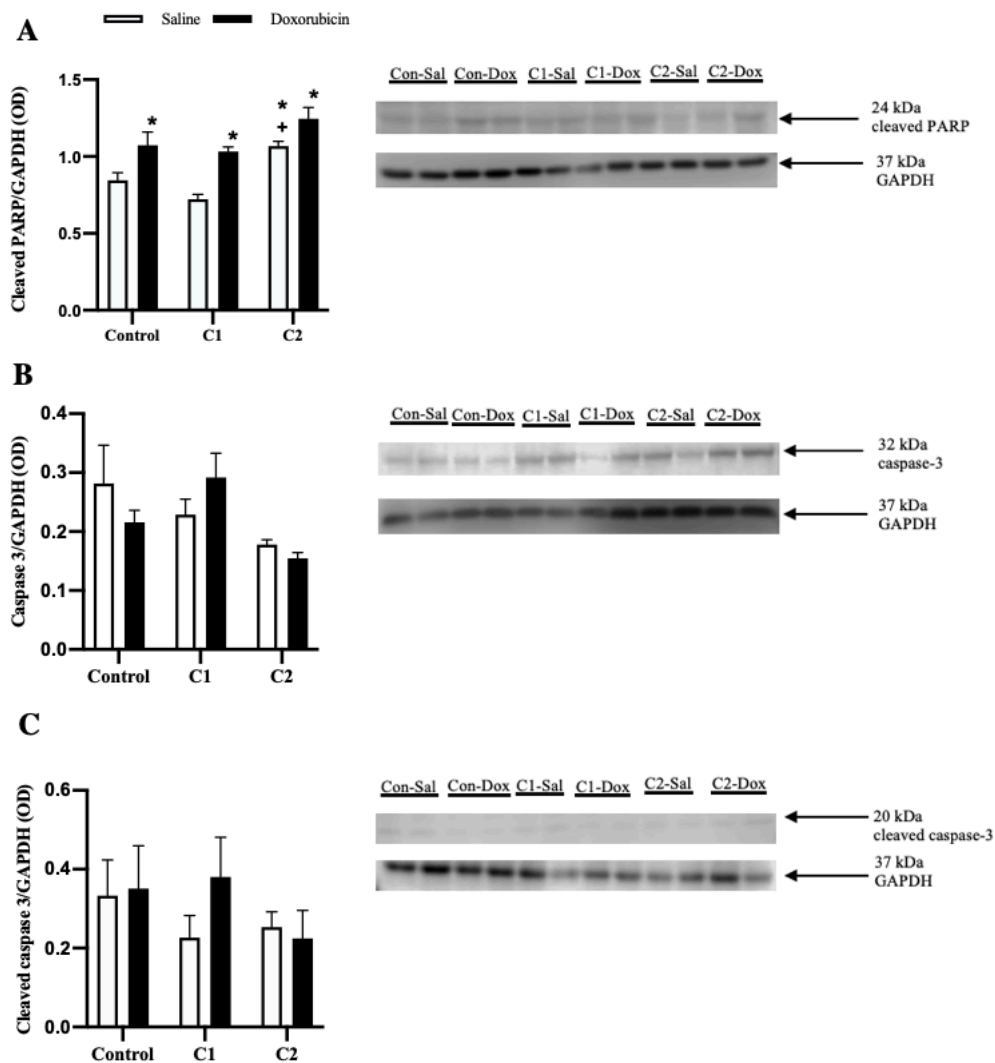
A

B

C

Figure 5. 1-day SOL apoptotic protein levels, (A) cleaved PARP, (B) caspase-3, (C) cleaved caspase-3.

SOL = soleus, kDa = kilodalton, OD = optical density, Con-Sal = control saline, n=10; Con-Dox = control doxorubicin, n=10; C1-Sal = creatine 1 saline, n=10; C1-Dox = creatine 1 doxorubicin, n=10; C2-Sal = creatine 2 saline, n=10; C2-Dox = creatine 2 doxorubicin, n=10. Values are mean ± SEM.

(A) significant drug ffect ($p<0.05$)

(C) significant diet effect ($p<0.05$)

* = significantly different from C2-Dox

One-day diaphragm apoptotic protein levels. In the DIA, a significant diet effect was observed, with Cr groups exhibiting lower levels of cleaved caspase 3, $F_{(2, 52)} = 5.01$, $p<0.05$ (Figure 6C). No main effects or interaction observed for cleaved PARP and caspase-3 (Figures 6A and 6B).
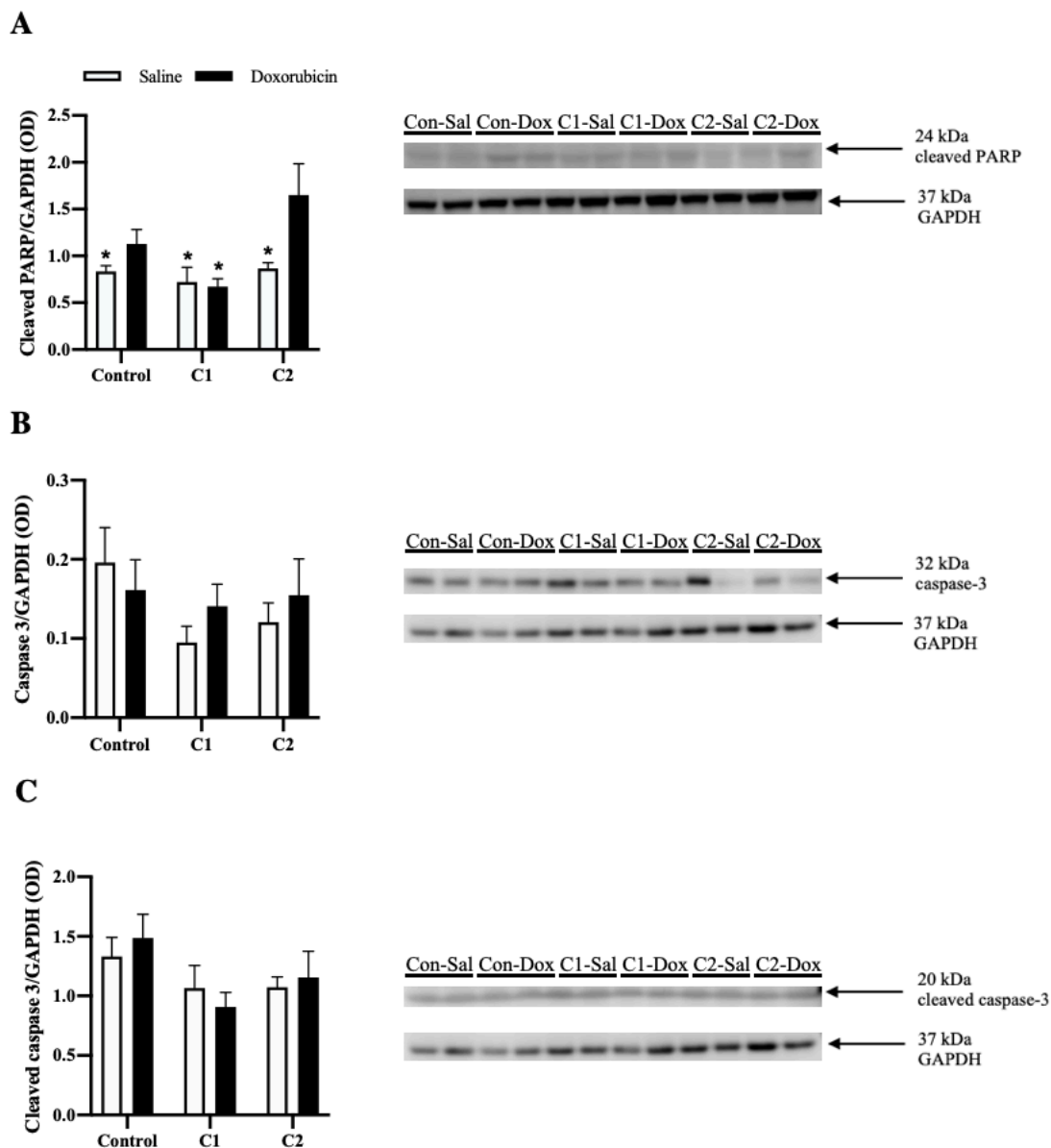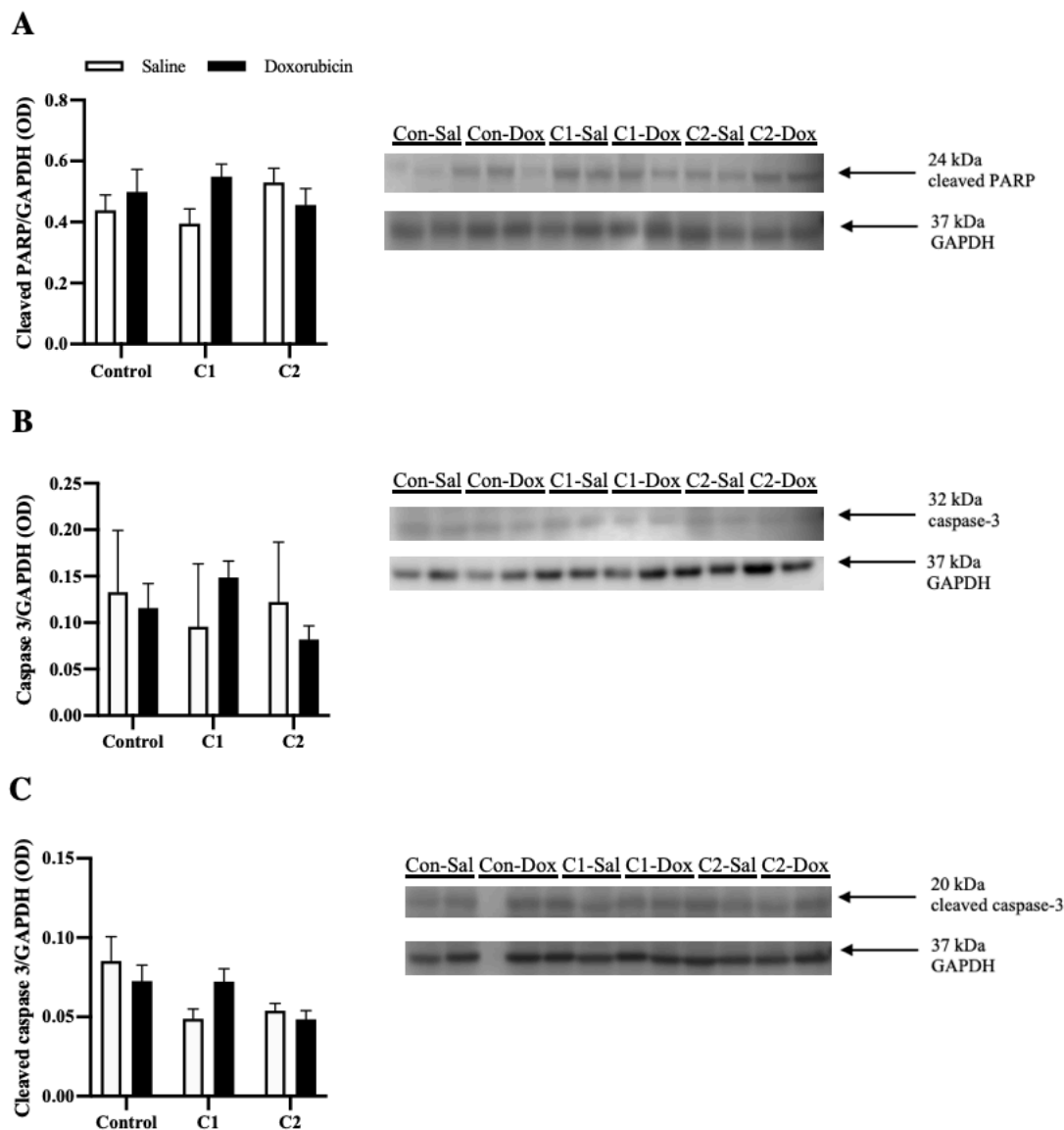
Figure 6. 1-day DIA apoptotic protein levels, (A) cleaved PARP, (B) caspase-3, (C) cleaved caspase-3.

DIA = diaphragm, kDa = kilodalton, OD = optical density, Con-Sal = control saline, n=10; Con-Dox = control doxorubicin, n=10; C1-Sal = creatine 1 saline, n=10; C1-Dox = creatine 1 doxorubicin, n=10; C2-Sal = creatine 2 saline, n=10; C2-Dox = creatine 2 doxorubicin, n=10. Values are mean ± SEM.

(C) significant diet effect ($p<0.05$)

Table 4 presents the animal characteristics at the 3 day sacrifice time point. At the time of injection there were no significant difference in body mass between groups, p>0.05. At the time of sacrifice, a main effect was observed in animal body mass, $p<0.05$. A drug effect was observed with Dox animals exhibiting significantly lower body mass, $F(1, 54) = 11.34$, $p<0.05$.

There was a main effect in EDL, SOL, and DIA mass, $p<0.05$. In the EDL, there was a drug effect, with Dox animals exhibiting significantly lower tissue mass, $F(1, 51) = 11.72$, $p<0.05$. *Post hoc* testing revealed EDL mass to be significantly lower in the C1-Dox than Con-Sal. In the SOL, there was a drug effect, with Dox animals exhibiting significantly lower tissue mass, $F(1, 51) = 4.39$, $p<0.05$. Lastly, in the DIA there was a significant drug effect, with Dox animals exhibiting significantly higher tissue mass, $F(1, 51) = 4.38$, $p<0.05$.

Table 4

3-day animal characteristics.

	Con-Sal	Con-Dox	C1-Sal	C1-Dox	C2-Sal	C2-Dox
Injection Mass (g)	386 ± 13	381 ± 4	373 ± 5	387 ± 10	375 ± 6	392 ± 6
Sacrifice Mass (g) ‡	387 ± 13	351 ± 9	374 ± 5	353 ± 13	378 ± 6	352 ± 8
EDL Mass (mg) ‡	160 ± 7	142 ± 4	153 ± 7	127 ± 8 *	154 ± 6	134 ± 11
SOL Mass (mg) ‡	158 ± 9	134 ± 3	137 ± 8	137 ± 5	153 ± 7	141 ± 3
DIA Mass (mg) ‡	229 ± 21	285 ± 43	243 ± 32	299 ± 23	245 ± 16	277 ± 14

Note. Con-Sal = control saline, n=10; Con-Dox = control doxorubicin, n=10; C1-Sal = creatine 1 saline, n=10; C1-Dox, creatine 1 doxorubicin, n=9; C2-Sal, creatine 2 saline, n=10; C2-Dox, creatine 2 doxorubicin, n=10. Values are means ± SEM.
Significant drug effect in Sacrifice Mass ($p < 0.05$).
‡ = signficant drug effect ($p < 0.05$)
* = significantly different than Con-Sal

Three-day extensor digitorum longus apoptotic protein levels. No main effects or interaction was observed for cleaved PARP and caspase-3 (Figures 7A and 7B). A 2-way ANOVA revealed a significant diet effect, with Cr groups exhibiting higher levels of cleaved capase-3, $F(2, 49) = 17.96$, $p<0.05$ (Figure 7C). Cleaved caspase-3 levels were higher in the Dox groups, $F(1, 49) = 0.1880$, $p<0.05$ (Figure 7C). Lastly, an interaction was observed, with the highest cleaved caspase-3 levels observed in the C1-Dox group, $F(2, 49) = 7.230$, $p<0.05$ (Figure 7C). *Post hoc* tests revealed that C1-Sal, C1-Dox, and C2-Sal produced significantly higher levels of cleaved caspase-3 than Con-Sal. Equally, *post hoc* tests exposed C1-Dox and C2-Sal expressed significantly higher levels of cleaved caspase-3 than Con-Dox. Lastly, *post hoc* tests showed C2-Dox expressed significantly lower levels of cleaved caspase-3 than C1-Dox (Figure 7C).

A

B

C
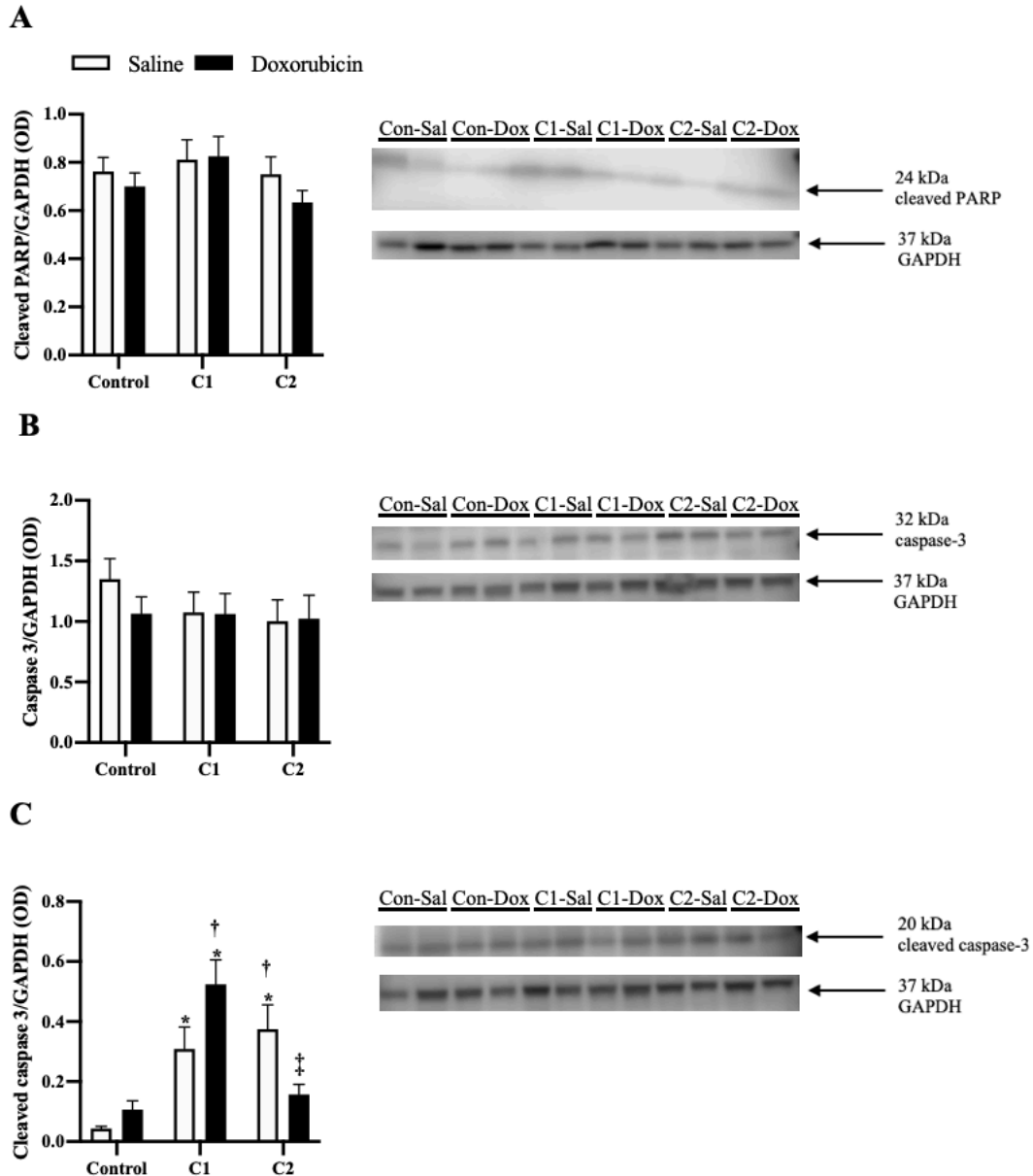
Figure 7. 3-day EDL apoptotic protein levels, (A) cleaved PARP, (B) caspase-3, (C) cleaved caspase-3.

EDL = extensor digitorum longus, kDa = kilodalton, OD = optical density, Con-Sal = control saline, n=10; Con-Dox = control doxorubicin, n=10; C1-Sal = creatine 1 saline, n=10; C1-Dox = creatine 1 doxorubicin, n=10; C2-Sal = creatine 2 saline, n=10; C2-Dox = creatine 2 doxorubicin, n=10. Values are mean ± SEM.

(C) significant drug and diet effect ($p<0.05$)

(C) significant interaction ($p<0.05$)

* = significantly different from Con-Sal

† = significantly different from Con-Dox

‡ = significantly different from C1-Dox

Three-day soleus apoptotic protein levels. No main effects or interaction was observed for cleaved PARP (Figure 8A). A 2-way ANOVA revealed a significant drug effect, with Dox groups exhibiting lower levels of capase-3, $F(1, 52) = 4.776$, $p<0.05$ (Figure 8B). There were no main effects or interaction observed for cleaved caspase-3 (Figure 8C).

A

B

C
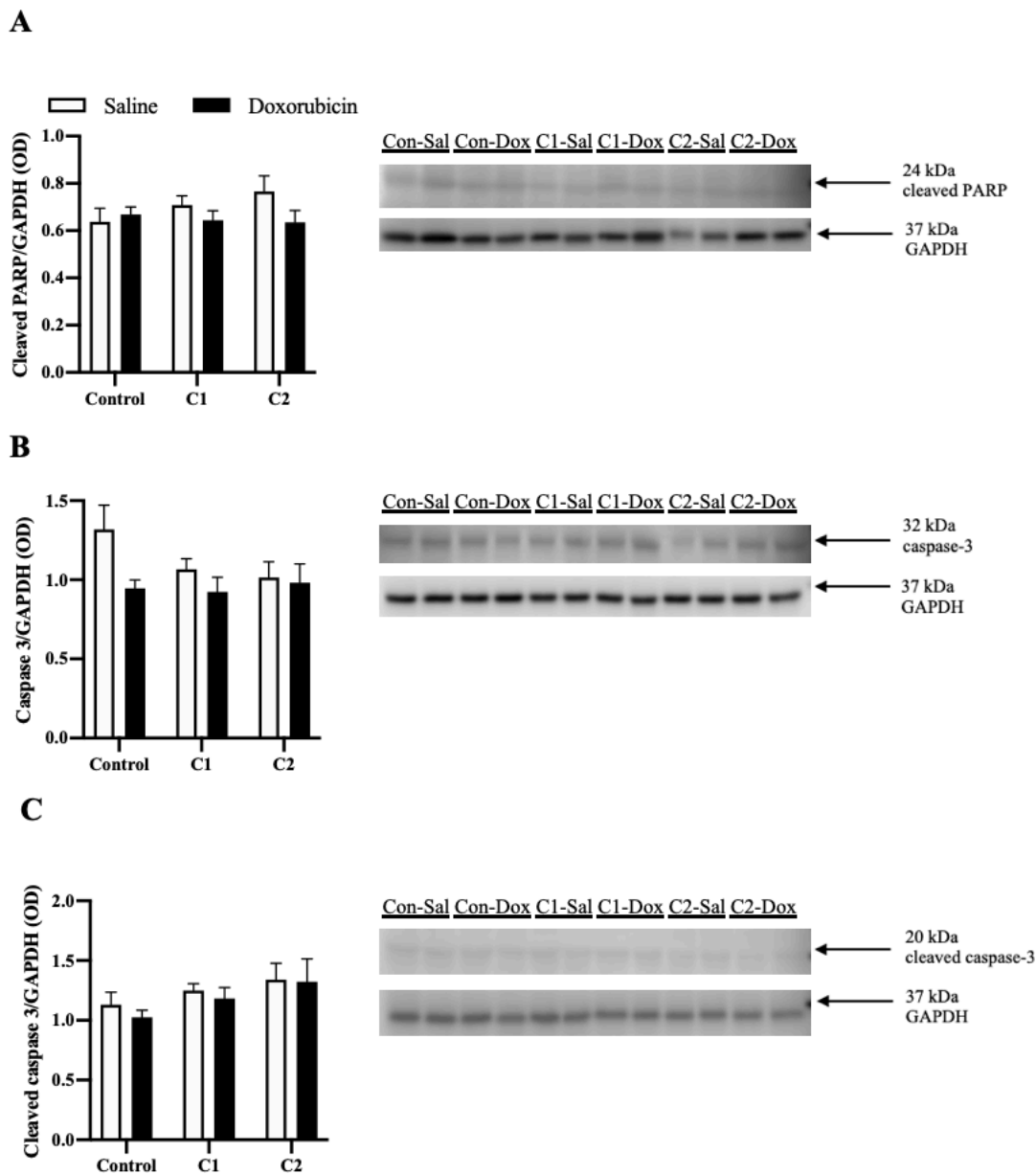
Figure 8. 3-day SOL apoptotic protein levels, (A) cleaved PARP, (B) caspase-3, (C) cleaved caspase-3.

(A) cleaved PARP, (B) caspase-3, (C) cleaved caspase-3; SOL = soleus, kDa = kilodalton, OD = optical density, Con-Sal = control saline, n=10; Con-Dox = control doxorubicin, n=10; C1-Sal = creatine 1 saline, n=10; C1-Dox = creatine 1 doxorubicin, n=10; C2-Sal = creatine 2 saline, n=10; C2-Dox = creatine 2 doxorubicin, n=10. Values are mean ± SEM.

(B) significant drug effect ($p<0.05$)

Three-day diaphragm apoptotic protein levels. A 2-way ANOVA revealed a significant drug effect, with Dox groups exhibiting lower levels of cleaved PARP F (1, 54) = 11.24, $p<0.05$ (Figure 9A). *Post hoc* tests revealed that Con-Dox expressed significantly lower levels of cleaved PARP than Con-Sal (Figure 9A). No significant main effects or interaction was detected for caspase-3 (Figure 9B) and cleaved caspase-3 (Figure 9C).
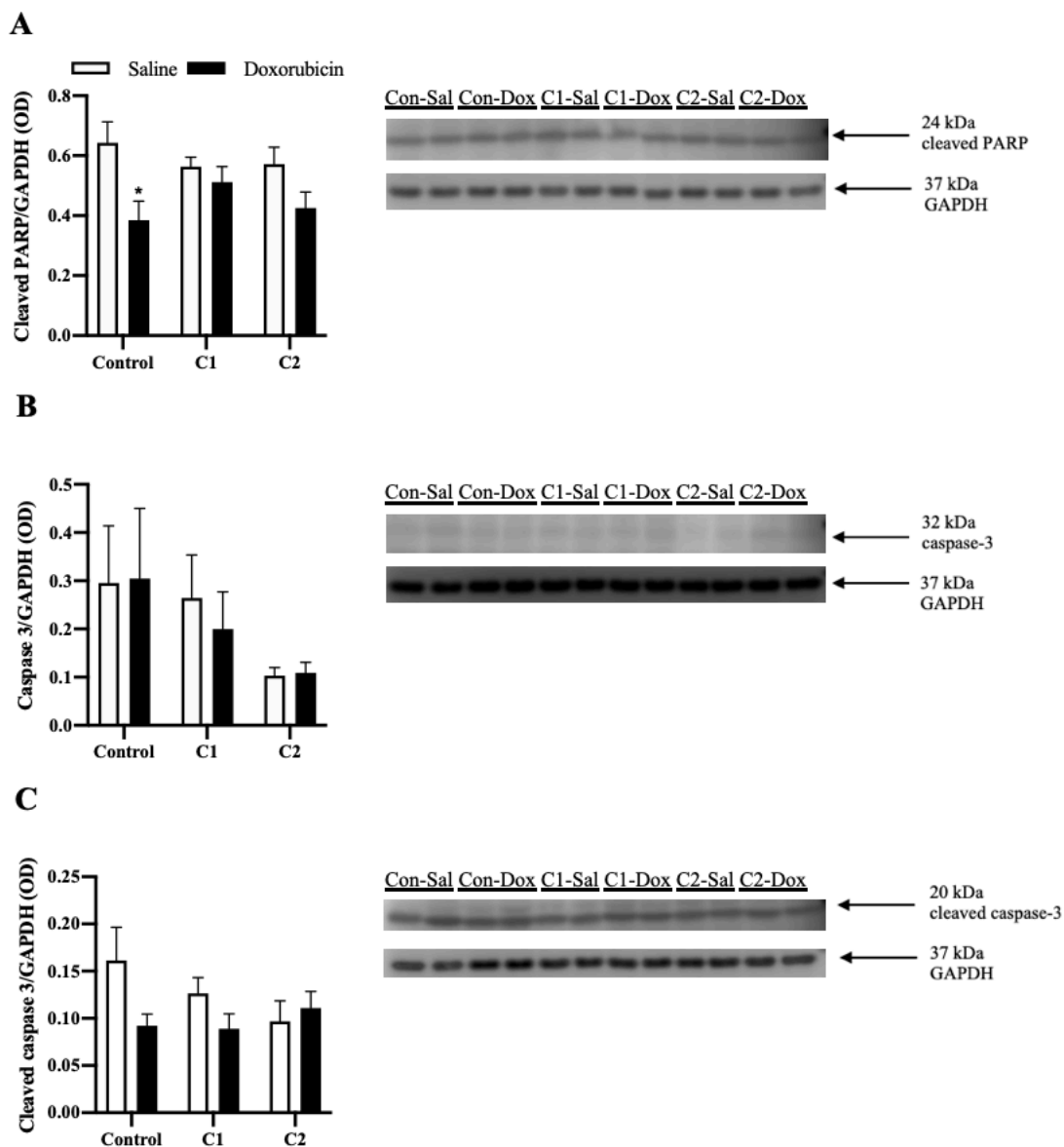
Figure 9. 3-day DIA apoptotic protein levels, (A) cleaved PARP, (B) caspase-3, (C) cleaved caspase-3.

DIA = diaphragm, kDa = kilodalton, OD = optical density, Con-Sal = control saline, n=10; Con-Dox = control doxorubicin, n=10; C1-Sal = creatine 1 saline, n=10; C1-Dox = creatine 1 doxorubicin, n=10; C2-Sal = creatine 2 saline, n=10; C2-Dox = creatine 2 doxorubicin, n=10. Values are mean ± SEM.

(A) significant drug effect ($p<0.05$)

* = significantly different from Con-Sal

Creatine Assay

Creatine levels were measured in the SOL, EDL, and DIA at the 1 and 3-day time points to evaluate the effects of Cr feeding and Dox administration on the concentration of creatine monohydrate in skeletal muscle. Eight μL of the supernatant from the 1-day and 3-day, SOL, EDL, and DIA homogenates were added to 17 uL of creatine assay buffer, in a 96 well plate. Twenty-five μL of the creatine reaction mix was then added to each well, and was incubated at 37°C for 1 hour. The plates were then measured at 570 nm using a microplate reader. Results were expressed as molar (M) creatine concentration per whole skeletal muscle (g).

One-day creatine concentration. In the 1-day EDL, a 2-way ANOVA revealed no significant effects or interaction (Figure 10). In the SOL, a drug effect was observed, with Dox groups displaying lower creatine concentrations, $F(1, 50) = 8.005$, $p<0.05$ (Figure 11). There were no significant main effects or interaction in the DIA (Figure 12).

Figure 10. 1-day EDL creatine concentration.
EDL = extensor digitorum longus; Con-Sal = control saline, n=10; Con-Dox = control doxorubicin, n=10; C1-Sal = creatine 1 saline, n=10; C1-Dox = creatine 1 doxorubicin, n=10; C2-Sal = creatine 2 saline, n=10; C2-Dox = creatine 2 doxorubicin, n=10. Values are mean ± SEM.
No significant effects ($p>0.05$)

Figure 11. 1-day SOL creatine concentration.
SOL = soleus; Con-Sal = control saline, n=10; Con-Dox = control doxorubicin, n=10; C1-Sal = creatine 1 saline, n=10; C1-Dox = creatine 1 doxorubicin, n=10; C2-Sal = creatine 2 saline, n=10; C2-Dox = creatine 2 doxorubicin, n=10. Values are mean ± SEM. Significant drug effect ($p<0.05$).

Figure 12. 1-day DIA creatine concentration.

DIA = diaphragm; Con-Sal = control saline, n=10; Con-Dox = control doxorubicin, n=10; C1-Sal = creatine 1 saline, n=10; C1-Dox = creatine 1 doxorubicin, n=10; C2-Sal = creatine 2 saline, n=10; C2-Dox = creatine 2 doxorubicin, n=10. Values are mean ± SEM. No significant effects (p>0.05)

Three-day creatine concentration. In the EDL, a 2-way ANOVA revealed a significant diet effect $F_{(2, 53)} = 4.668$, $p<0.05$; drug effect $F_{(1, 53)} = 29.71$, $p<0.05$ (Figure 13). *Post hoc* test revealed that Con-Dox, C1-Sal, C1-Dox, and C2-Dox had significantly lower Cr concentration than Con-Sal (Figure 13). Additionally, *post hoc* test revealed C2-Dox had significantly lower Cr concentration than C2-Sal (Figure 13). No significant main effects were observed and no interaction was detected in the SOL (Figure 14) and the DIA (Figure 15).
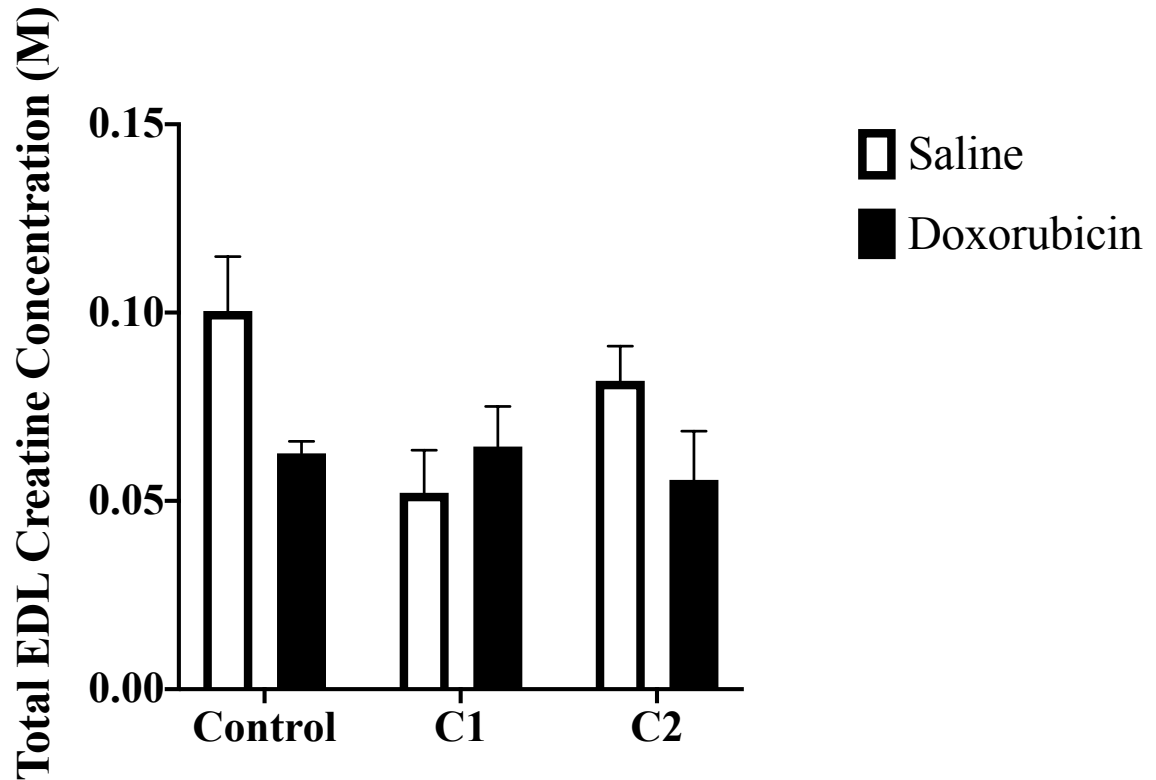
Figure 13. 3-day EDL creatine concentration.
EDL = extensor digitorum longus; Con-Sal = control saline, n=10; Con-Dox = control doxorubicin, n=10; C1-Sal = creatine 1 saline, n=10; C1-Dox = creatine 1 doxorubicin, n=10; C2-Sal = creatine 2 saline, n=10; C2-Dox = creatine 2 doxorubicin, n=10. Values are mean ± SEM.
Significant diet effect ($p<0.05$)
Significant drug effect ($p<0.05$)
* = significantly different than Con-Sal ($p<0.05$)
† = significantly different than C2-Sal ($p<0.05$)
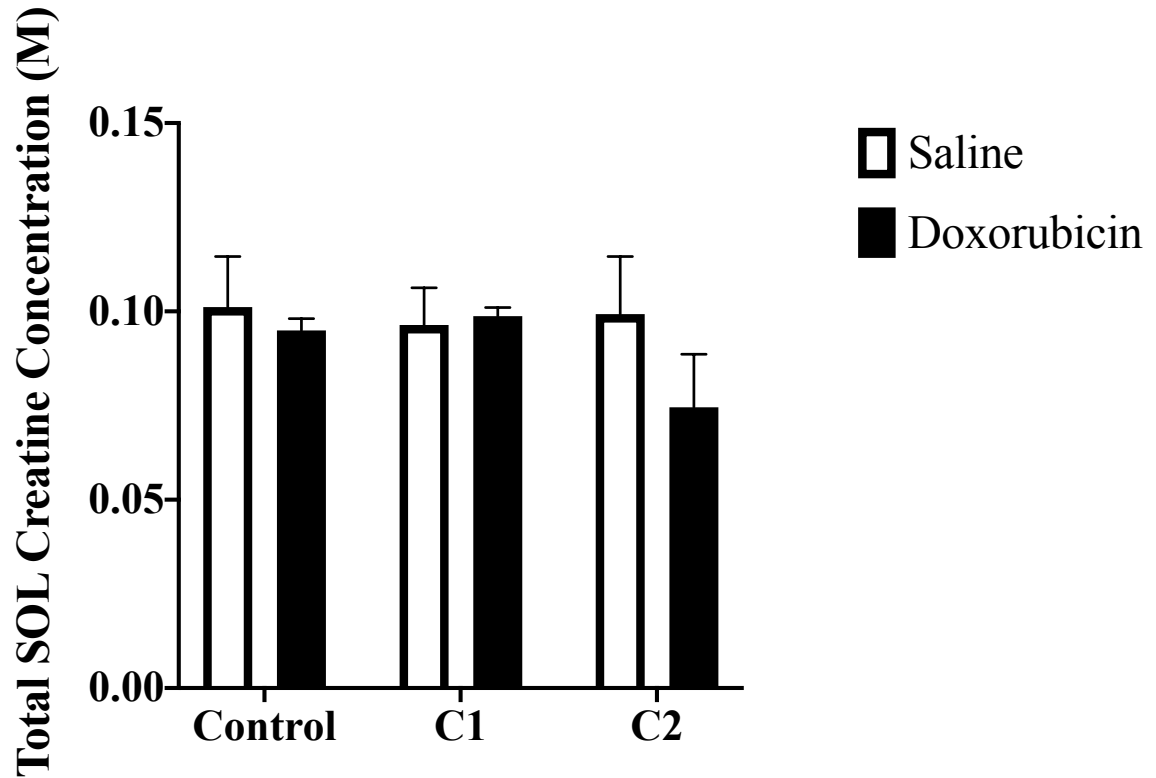
Figure 14. 3-day SOL creatine concentration.
SOL = soleus; Con-Sal = control saline, n=10; Con-Dox = control doxorubicin, n=10;
C1-Sal = creatine 1 saline, n=10; C1-Dox = creatine 1 doxorubicin, n=10; C2-Sal =
creatine 2 saline, n=10; C2-Dox = creatine 2 doxorubicin, n=10. Values are mean ± SEM.
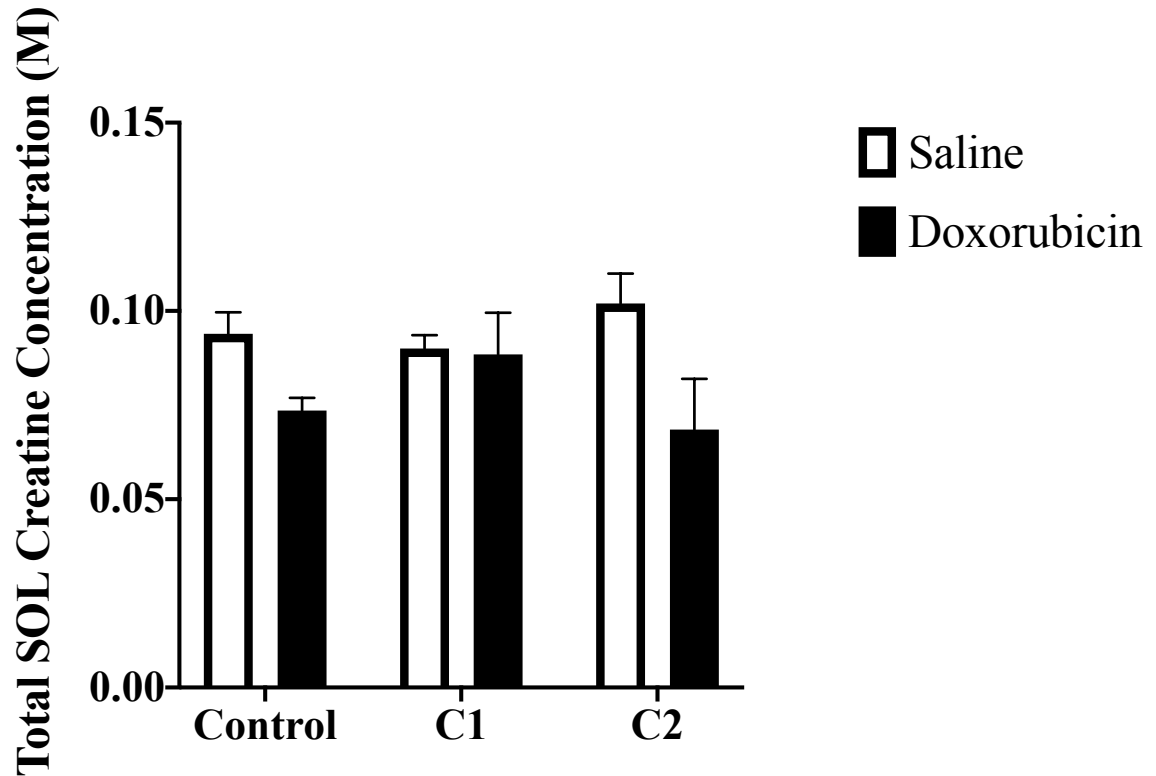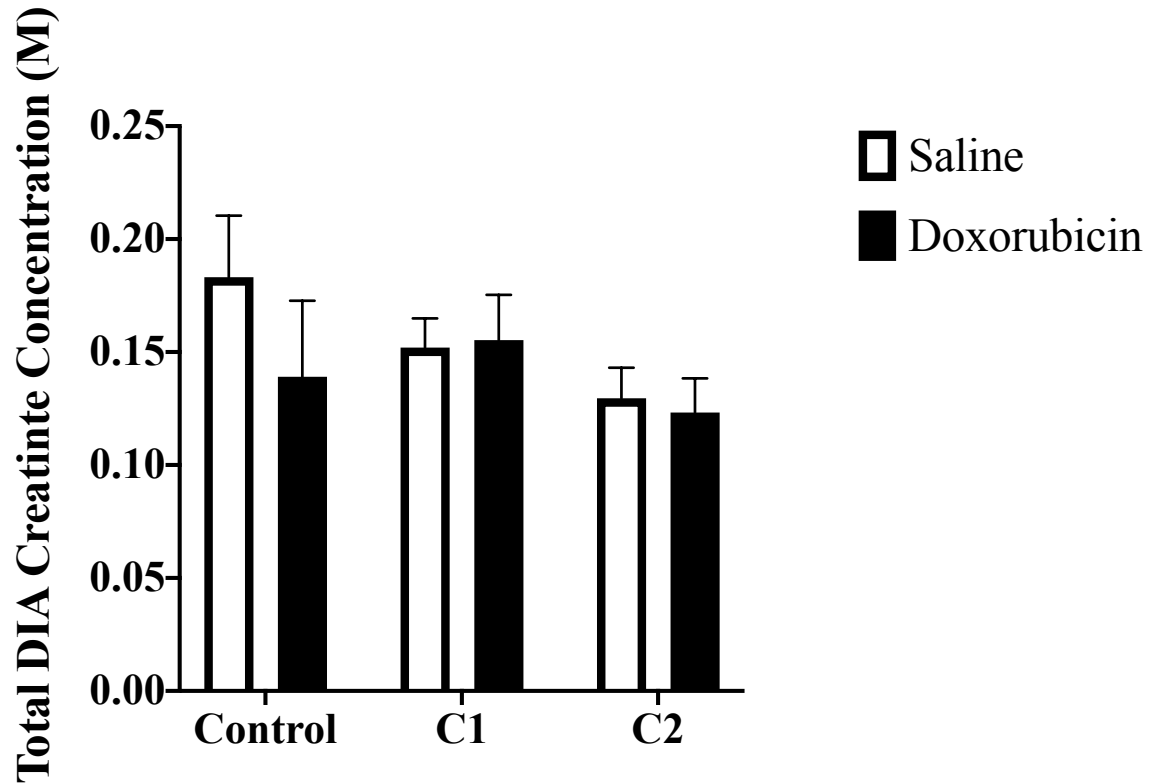No significant effects ($p > 0.05$)

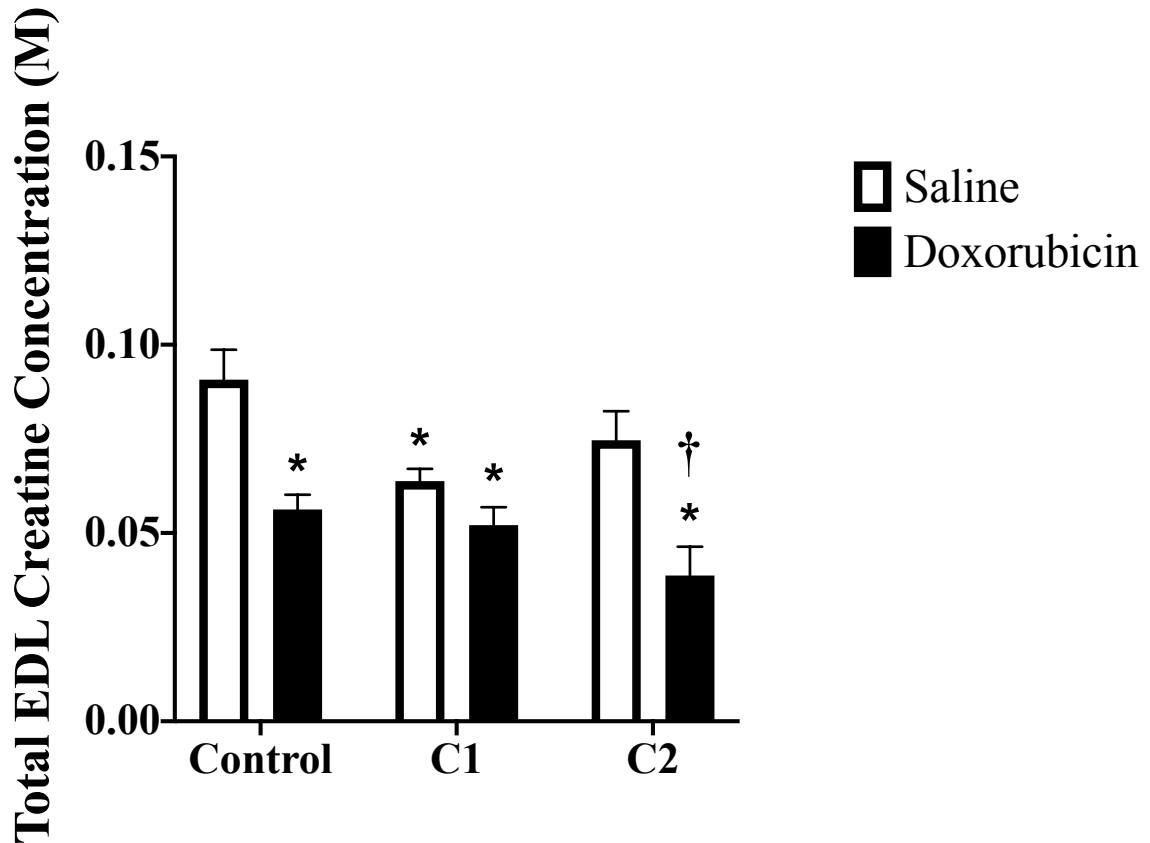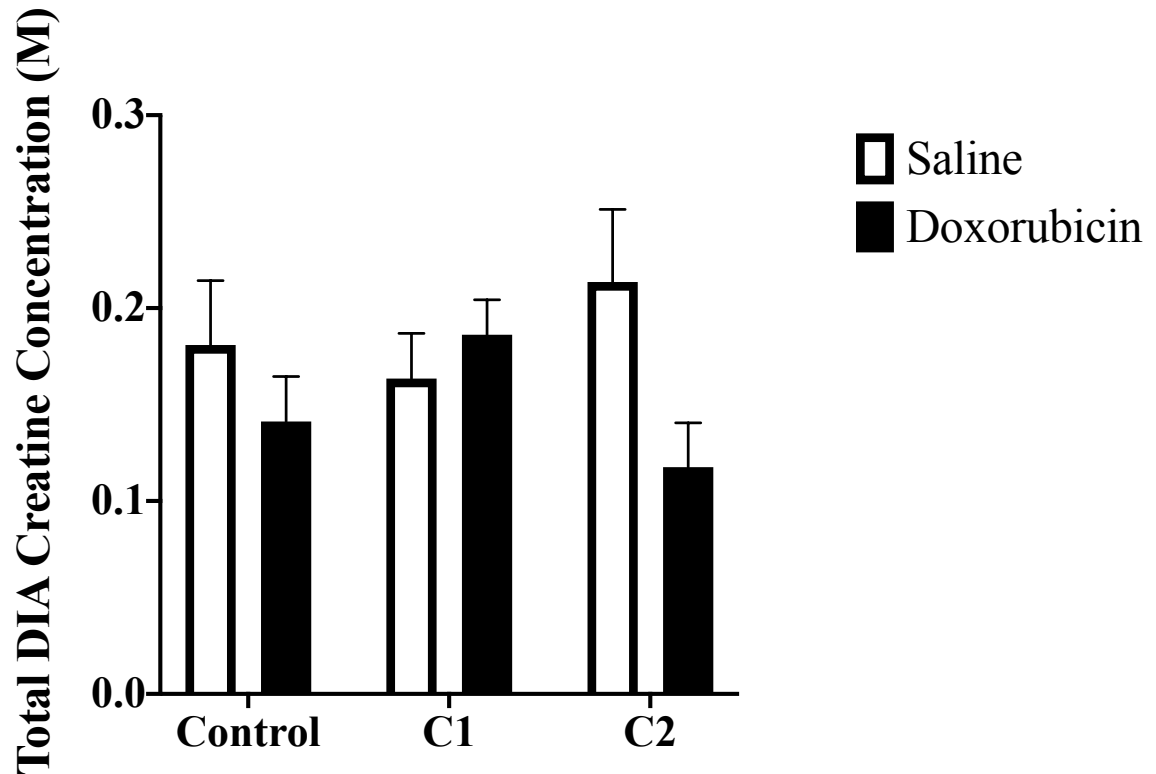*Figure 15.* 3-day DIA creatine concentration.
DIA = diaphragm; Con-Sal = control saline, n=10; Con-Dox = control doxorubicin, n=10; C1-Sal = creatine 1 saline, n=10; C1-Dox = creatine 1 doxorubicin, n=10; C2-Sal = creatine 2 saline, n=10; C2-Dox = creatine 2 doxorubicin, n=10. Values are mean ± SEM. No significant effects ($p > 0.05$)

Summary

At the time of injection, at both the 1 and 3-day time points, main effects were not observed in animal body mass. There was a significant drug effect in the sacrifice body mass at both the 1 and 3-day time points, with Dox treated animals displaying lower body masses. In both the 1 and 3-day EDL and SOL, there was a significant drug effect in tissue mass with the Dox groups having lower tissue mass. Equally, the 3-day DIA experienced a significant decrease in tissue mass. No mass differences were seen in the 1-day DIA.

Cleaved PARP levels were increased with Dox and Cr in both the 1-day EDL and SOL and decreased in the 3-day DIA. Cleaved PARP levels were not significantly different in the 1-day DIA or 3-day EDL or SOL. Caspase-3 levels were lower in the 1-day EDL Cr groups and were higher in the 3-day SOL Dox gropus. There was not a significant change in caspase-3 levels in the 1 or 3-day DIA, nor the 1-day SOL and 3-day EDL. Cleaved caspase-3 levels were lower in both the 1-day SOL and DIA Cr groups, but not in the 1-day EDL. Cleaved caspase-3 levels were lower in both Cr, Dox groups and showed an interaction in the 3-day EDL, with no main effects observed in the 3-day SOL or DIA.

Total Cr concentration was lower in the 1-day SOL and 3-day EDL with Dox administration. Total Cr concentration was overall lower in the 3-day EDL Cr groups. There were no main effects or interaction observed in the 1-day EDL and DIA; 3-day SOL and DIA.

CHAPTER V

DISCUSSION AND CONCLUSIONS

This is the first study to assess two types of Cr diets and *in vivo* Dox treatment, at two separate time points on apoptosis in skeletal muscle. It was hypothesized that Dox would increase levels of the apoptotic proteins cleaved PARP, cleaved caspase-3, and decrease levels of caspase-3 at both time points in all tissues and that Cr feeding would attenuate these effects. The major findings are that 1) Dox did not increase cleaved PARP and cleaved caspase-3 levels in all muscles analyzed and only affected cleaved caspase-3 levels in one condition, 2) Cr feeding did not attenuate the effects of Dox on these apoptotic proteins.

**Doxorubicin and Creatine's Effect
on Animal Characteristics**

Dox animals had significantly lower sacrifice body masses at both time points. Equally, when the 1 and 3-day EDL and SOL were excised and weighed, the Dox animals had significantly lower tissue mass when compared to non-Dox treated groups. This was the same for the 3-day DIA, but not the 1-day DIA. These findings are supported by previous research demonstrating that Dox injections decrease muscle mass in mice (Doroshow et al., 1985). Creatine feeding had no effect on sacrifice body or skeletal muscle mass, which indicates Cr was not effective at reducing skeletal muscle loss in Dox treated animals.

Doxorubicin's Effect on Apoptotic Proteins

Dox-induced oxidative stress is one of the most pronounced mechanisms by which toxicity and eventual apoptosis is triggered in cells (Wang et al., 2004). The development of oxidative stress with Dox treatment is propelled by two mechanisms, increased levels of ROS following Dox treatment and the downregulation of antioxidant enzymes following Dox administration. The first, increased ROS levels, may result from different mechanisms, 1) decreased cellular antioxidant defenses, 2) disruption of the mitochondrial electron transport chain with subsequent electron leakage, and 3) the ability of Dox to undergo redox cycling and produce ROS (Zhao et al., 2010). Doxorubicin redox cycling leading to ROS production is the most widely recognized mechanism by which oxidative stress increases with Dox administration.

Reactive oxygen species, particularly H_2O_2, generated by Dox administration, causes formation of a pore in the mitochondrial membrane, activation of caspase 9, and ultimately the formation of executioner caspase-3, 6, and 7 (Kluck et al., 1997; Li et al., 1997; Nie et al., 2008; Pan et al., 1998; Reed, 1997; Zou et al., 1997). Once caspase-3 is activated, downstream death substrates are cleaved, including poly (ADP-ribose) polymerase (PARP) (Janicke et al., 1998).

In the current study, rats were administered a 15 mg/kg bolus interaperitoneal injection of Dox and sacrificed 1-day or 3-day post-injection. Doxorubicin administration only affected cleaved caspase-3 levels in 3-day EDL, caspase-3 levels in the 3-day SOL, and cleaved PARP in the 1-day EDL and SOL, and 3-day DIA. Doxorubicin administration had no other significant effect on these particular apoptotic

proteins in any other condition. These results agree with the original hypothesis that Dox administration will increase levels of cleaved PARP, cleaved caspase-3, and decrease levels of caspase-3. The literature, clearly demonstrates that Dox administration increases caspase-3 activation in cell culture, rodent cardiomyocytes and roden tcardiac tissue, resulting in activation of cleaved caspase-3 and eventually cleaved PARP (Asakura et al., 1999; Ueno et al., 2006). Doxorubicin also had a greater effect on 3-day apoptotic proteins, than 1-day apoptotic proteins. Research has demonstrated that Dox accumulation and effects differ depending on the tissue and on the length of time spent in the tissue (Chenard, Fabris, & MacLean, 2012). Chenard et al. (2012) found Dox accumulation is time dependent, which could explain why Dox administraiton had the greatest impact on apoptotic protein levels in the 3-day tissues. Equally, Hayward et al. (2013), examined Dox accumulation in cardiac and skeletal muslce tissue and found that Dox accumulation is highest in cardiac tissue and accumulation differs in the EDL and SOL. Hayward et al. (2013) postulated that the accumulation differed due to Dox's affinity to accumulate in the mitochondria, and tissues with higher mitochondria content have higher levels of Dox accumulation. Interestingly, Dox effects on cleaved PARP did not follow this rationale of a greater Dox effect on 3-day tissue as compared to 1-day tissue. Cleaved PARP levels increased in 1-day EDL and SOL, and not on 3-day EDL or SOL. Even more confusing is the decrease in cleaved PARP levels observed in the 3-day DIA. Additionally, previous research has demonstrated that cleaved caspase-3 is a direct activator of cleaved PARP, cleaving PARP and activating it (Slee et al., 2001). It would follow then, that if Dox administration increased cleaved PARP levels, a subsequent increase in cleaved caspase-3 and decrease in procaspase-3 would follow in that tissue.

This was not the case, there was not an increase in cleaved PARP and cleaved caspase-3 levels and a subsequent decrease in caspase-3, in any condition.

One explanation for the seemingly uncharacteristic response of cleaved PARP to Dox administration is the PARP-1 gene is expressed constitutively. Its activation cannot be surmised from increased protein expression, but rather by measuring the consumption of the substrates used in its catalytic reaction (Shah et al., 2011). According to Shah et al. (2011), to accurately measure PARP-1 activation *in vivo*, assessment of the formation of pADPr, depletion of the substrate NAD^+, or formation of protons resulting in the rapid and reversible intracellular acidification, is the the most accurate and sensitive methods. Presently, it is possible, that the results for cleaved PARP do not agree with the research, not because Dox administration had no effect, but rather because the laboratory technique used (Western blot) is not sensitive enough to accurately detect the activation of PARP-1.

In summary, if research by Shah et al. (2011) is correct, and in the current study the laboratory technique used to measure cleaved PARP was not sensitive enough and we interpret the results with caution, then what we presently observe is Dox administration only affected apoptotic proteins in 3-day tissue, and only affected certain apoptotic proteins in certain tissues. Doxorubicin decreased caspase-3 leveles in the 3-day SOL, and increased cleaved caspase-3 levels in the 3-day EDL.

Creatine's Effect on Apoptotic Proteins

Creatine monohydrate (Cr) is perhaps one of the most widely used nutraceuticals ingested in an attempt to improve high intensity athletic performance. A large amount of research has been done on Cr supplementation in efforts to delineate its sport-specific effects. It is hypothesized that Cr acts through a number of pathways and appears to be

most effective in short-term, high-intensity physical activities. However, the past decade has shown that the use of Cr for therapeutic purposes has received increasing attention (Gualano et al., 2009). Creatine supplementation is beneficial in a large number of muscular, neurological, and cardiovascular diseases (Bender et al., 2007; Felber et al., 2000; Gordon et al., 1995; Holtzman et al., 1999; Matthews et al., 1999; Mazzini et al., 2001; Neubauer et al., 1999; Sharov et al., 1987; Stout et al., 2001; Tarnopolsky et al., 2004; Tarnopolsky, 2007; Vorgerd et al., 2000; Wyss & Schulze, 2002), however, less documented are the effects of Cr on Dox-induced skeletal muscle dysfunction, and there is a lack of research on possible mechanisms leading to improvements in Dox-induced skeletal muscle dysfunction. One such mechanism may be creatine's ability to modulate apoptosis.

Rahimi et al. (2015) examined the effects of creatine monohydrate supplementation on apoptotic markers and found that creatine monohydrate supplementation prevented exercise induced apoptosis, as measured by decreases in p53 concentration. It is well known that p53 plays a significant role in regulating apoptosis by affecting the expression of apoptotic peptidase activating factor-1 and by altering mitochondrial membrane potential (Haldar et al., 1994; Moll & Zaika, 2001). Rahimi et al. (2015) suggested that the increase in oxidative stress with aerobic exercise signals p53, which in turn triggered the p53 dependent apoptotic pathway to preserve genomic intensity and cellular homeostasis. Although not directly measured, Rahimi et al. (2015) suggest that Cr may act as an antioxidant, scavenging ROS. This then reduces the ROS-induced signaling of p53, which was one of the findings of that particular study, reduced serum p53 concentration with Cr supplementation. Sheikholeslami-Vatani and Faraji

(2018) also found that creatine supplementation reduced apoptotic markers with exercise, reducing caspases (-3 and -9), p53, and Bax when compared to placebo.

As with Rahimi et al. (2015), Sheikholeslami-Vatani and Faraji (2018) suggest that although not directly measured, the reduction in the apoptotic markers may have been due to the ability for Cr supplementation to reduce ROS production which reduced the signaling of the apoptotic caspases (-3 and -9) and p53. Lastly, in exercise-induced apoptosis, Cr supplementation reduced lipid peroxidation and glutathione peroxidase activity, and attenuated oxidative DNA damage with the suggested mechanisms being the potential ROS scavenging capabilities (Basta et al., 2006; Mirzaei et al., 2013; Rahimi, 2011). Although not examined in skeletal muscle, Zhu et al. (2004) showed that in focal cerebral ischemia, Cr supplementation either directly or indirectly inhibited cytochrome *c* release and downstream caspase-3 activation. Equally, Cr supplementation has been shown to reduce caspase-3/7 activation in HL-1 cardiomyocytes (Santacruz et al., 2015). In summary, Cr supplementation can reduce markers of apoptosis in a variety of conditions. Although the mechanisms are not elucidated, it is postulate that Cr acts an antioxidant, scavenging free radicals, and reducing their quantity which ultimately reduces apoptosis.

In the current study, rats were fed one of two experimental diets, C1 and C2. Creatine 1 was a 2% Cr supplemented diet for four weeks, and Creatine 2 was one week of a 4% Cr supplemented diet, followed by three weeks of a 2% Cr supplemented diet. Based on human studies examining Cr supplementation (Casey & Greenhaff, 2000; Greenhaff, Bodin, Soderlund, & Hultman, 1994; Stout et al., 2001), it has been determined that Cr supplementation has to occur for long enough to raise Cr

concentration levels enough for Cr to exert its ergogenic effects. In light of this research, the current study employed a Cr feeding protocol that was hoped to be long enough (four weeks) to raise Cr levels in the skeletal muscle of the animals. Equally, the current study implemented a loading phase, with 4% Cr feeding for a week, followed by 2% Cr feeding for three weeks. Lastly, the C2 diet attempted to increase the amount of free Cr stores (i.e., Cr not bound to a phosphate). Research has shown that Cr can function as an antioxidant, decreasing the free radical load on the cell (Basta et al., 2006; Mirzaei et al., 2013; Rahimi et al., 2015). In these studies, it is free Cr that can function as an antioxidant, and the current study attempted to increase free Cr stores via the C2 diet.

The overall findings were that Cr feeding, had the greatest impact on 1-day apoptotic proteins. At the 1-day time point, Cr increased cleaved PARP levels in the EDL and SOL, decreased caspase-3 levels in the EDL, and decrease cleaved caspase-3 levels in the SOL and DIA. The only effect observed at the 3-day time point was Cr increased cleaved caspase-3 levels in the EDL.

Cleaved PARP levels increased with Cr feeding in the EDL and SOL. A search of the literature failed to show that Cr supplementation would have an affect on cleaved PARP. An increase in cleaved PARP means the cell is going to be killed, via apoptosis, and as mentioned, there is no research suggesting Cr triggers apoptosis. Specifically, the highest levels of cleaved PARP were in the C2-Dox group (although not significantly higher than in the Con-Dox group) and C2-Sal group had significantly higher cleaved PARP levels than in the C1-Sal group. As described in previous sections, the current method used to measure cleaved PARP may not be sensitive enough and may be impacting our assessment of the effects of Cr feeding on cleaved PARP in the EDL, SOL,

and DIA. Therefore, the current results for cleaved PARP should be interpreted with caution.

In the 1-day EDL, Cr feeding decreased caspase-3 levels, with the highest level observed in the C1-Dox and lowest level in the C2-Dox. A higher level of caspase-3 suggests that apoptosis is not very active, with lower levels of caspase-3 indicating that caspase-3 is being cleaved and converted to cleaved caspase-3 which is a strong driver of apoptosis in the cell. The highest caspase-3 values were observed in the C1-Dox condition, suggesting that the C1 feeding may have protected the cell from Dox-induced cleaving of caspase-3. As stated with cleaved PARP, research does not indicate that Cr supplementation by itself, would be a stimulator of apoptosis in the cell, and possilby, in a condition where there is an increase in free radicials, Cr supplementation may decrease cleaved caspase-3 levels in the cell. Based on this, one would postulate that cleaved capase-3 levels, an indicator that apoptosis is occurring in the cell, would stay the same with Cr feeding. This was the case in the 1-day SOL and DIA, where Cr feeding decreased cleaved caspase-3 levels. However, in the 3-day EDL, Cr feeding had the greatest effect on cleaved caspase-3 levels.

In the 3-day EDL, cleaved caspase-3 levels were significantly higher in C1-Sal, C1-Dox, and C2-Sal when compared to Con-Sal. Surprising is the significant increase in cleaved caspase-3 levels in the C1-Sal and C2-Sal when compared to Con-Sal. This suggests that Cr feeding somehow increased cleaved caspase-3 levels, and potentially apoptosis in the cells. These results do not agree with the literature, as Cr has not been shown to drive apoptosis, especially in tissues treated with a substance similar to Dox.

In the 3-day EDL, cleaved caspase-3 levels were significantly higher in the C1-Dox and C2-Sal groups when compared to Con-Dox. Again, this suggests that Cr feeding increased cleaved caspase-3 levels and may indicate that Cr increases apoptosis, which is not supported in the literature.

Lastly, there was an interaction in the 3-day EDL. Cleaved caspase-3 levels were observed to be higher in the C1-Dox group when compared to the C2-Dox group. This agrees with the research that shows free Cr can act as an antioxidant, scavenging free radicals that Dox administration has been shown to increase, decreasing the triggering of apoptosis and subsequent increase in caspase-3 activation to cleaved caspase-3 (Rahimi et al., 2015; Sheikholeslami-Vatani & Faraji, 2018).

Overall, there are several findings in the current study that do not agree with the hypothesis or research that demonstrate that Dox drives apoptosis in cells. Doxorubicin administration had very little effect on apoptotic proteins, especially in the 3-day tissue, with a drug effect only being observed in 3 conditions (cleaved PARP, 3-day DIA; caspase-3, 3-day SOL; cleaved caspase, 3-day EDL), and the decrease in cleaved PARP levels in the 3-day DIA do not agree with the literature. Equally, Cr feeding seemed to increase levels of apoptotic proteins, suggesting that it may somehow drive or trigger cell damage, which is nowhere supported in the literature. Regarding cleaved PARP, it is likely that the Western blot technique used to detect PARP activity in the current study, may have not been the most sensitive method to tease out the possibly subtle changes in PARP with Cr feeding and Dox administration.

The PARP gene is expressed constitutively, meaning it is expressed at a constant level in the cell, and measuring expression of mRNA or protein will not accurately assess

its activation.   Research has shown that the response of eukaryotic cells to DNA damage

is the catalytic activation of PARP. When activated PARP splits nicotinamide adenine

dinucleotide (NAD^+) to produce ADP-ribose, nicotinamide, and a proton (Affar, Shah,

Dallaire, Castonguay, & Shah, 2002). The then activate PARP strings together the ADP-

ribose (forms polymer of ADP-ribose or pADPr) which binds and modifies cellular

proteins (Rouleau, Patel, Hendzel, Kaufmann, & Poirier, 2010).   Based on this, the most

accurate and sensitive method to detect the catalytic activation of PARP is by

demonstrating the consequences of its catalytic reaction. The catalytic reaction results in

the consumption of the substrate nicotinamide adenine dinucleotide (NAD^+) and

formation of polymer of ADP-ribose (pADPr or PAR), nicotinamide, and protons.

As an alternative to the techniques used in the current study to quantify changes in

PARP levels, techniques employed by Ueno et al. (2006) may be a more appropriate

option. Ueno et al. (2006) suggests Western blotting of pADPr modified proteins, which

have a signal of 110 to 250 kDa are to be probed. The method for preparing the tissue

differs, in that urea-SDS buffer is used in place of RIPA buffer. Along with Western

blot, quantification of NAD^+ levels should be used to assess PARP activation. The

lowering of NAD^+ substrate levels in response to DNA damage is an accurate way to

represent the activation of PARP (Vodenicharov, Ghodgaonkar, Halappanavar, Shah, R.

G., & Shah, G. M., 2005). In closing, the PARP results in the current study may not

reveal the entire findings. The PARP results may be limited by the use of a less than

ideal laboratory technique and had the techniques described by Ueno et al. (2006) been

employed, additional drug and diet effects may had been uncovered.

The current study demonstrated very little drug effect on cleaved caspase-3 in any tissue, no matter the time point. The caspases are collectively a family of proteases and are the central executioners of the apoptotic pathway. The main methods designed to detect activation of caspases are (1) measure specific synthetic substrates designed to contain flurogenic or chromogenic leaving groups after the aspartate residue; (2) Western blot using an antibody specific to the active caspase; (3) Western blot caspase-specific cleaved products (PARP, cytokeratin-18, and lamin A); (4) immunocyto-chemical localization of an activated caspase using antibodies specific to the caspase; (5) fluorochrome-labeled inhibitors of caspases as affinity ligands that bind to the active center of the active caspase; and (6) colored fluorescent protein covalently linked to a small peptide that target a caspase (Darzynkiewicz, Pozarowski, Lee, & Johnson, 2011; Tawa, Tam, Cassady, Nicholson, & Xanthoudakis, 2001). Based on the research above, unlike the case of cleaved PARP, the Western blot method has been shown to be an effective method for quantifying cleaved caspase-3 expression. Another possible reason for not seeing elevated levels of cleaved caspase-3 in Dox treated animals is that when the EDL, SOL, and DIA are extracted, they are flash frozen in liquid nitrogen and then stored in a -80°C freezer. This essentially freezes the cellular state of the tissue in time. Research shows that caspase levels can vary in the cell, rising and falling, and that this undulation can differ based on tissue and on the type of caspase (Liu et al., 2017). It is possible that what was observed in the current study is not the actual level of apoptotsis in the muscle, but the levels of these caspases in the moment of time we extracted the tissue. The caspase levels could differ if we extracted the tissues earlier or later, and their levels may not accurately represent what effect Cr and Dox have on their expression.

Equally, muscles from the animals were not all extracted at the exact same moment, and this could explain why the caspase expression fluctuated from tissue to tissue and had no disernable pattern from 1-day to 3-day. Worth mentioning is the effect Dox has on other types of muscle tissue, specifically cardiac muscle tissue.

Doxorubicin's major adverse effect is cardiotoxicity, which is the number one factor that limits its use, and can be fatal (Jordon, 2002; Takemura & Fujiwara, 2007). Doxorubicin accumulation is tissue dependent, with higher rates of accumulation observed in cardiac tissue when compared to skeletal muscle tissue (Hayward et al., 2013). This is possible due to Dox accumulating primarily in the mitochondria and cardiac tissue has higher amounts of mitochondria when compared to both type I and type II skeletal muscle fibers (Hayward et al., 2013). The higher the Dox dose, the greater the incident of cardiotoxicity (Lefrak, Pitha, Rosenheim, & Gottlieb, 1973; Hayward & Hydock, 2007). The main effects Dox treatment produces in the heart is cardiomyopathy, referred to as doxorubicin cardiomyopathy. Interestingly, the mechanisms of Dox-cardiotoxicity differ from Dox-tumor toxicity, in that the cardiotoxicity results from increased oxidative stress, as evident from increased reactive oxygen species and lipid peroxidation (Singal, Deally, & Weinberg, 1987). There is significant evidence that Dox induces apoptosis of cardiomyocytes (Wang, Ma, Markovich, Chen, & Wang, P. H., 1998; Kotamraju, Konorev, Joseph, & Kalyanaraman, 2000). The increase in oxidative stress increases formation of hydogren peroxide and superoxide, which activates p53-induced apoptosis and ultimately Dox-cardiomyopathy (Wang et al., 1998; Kotamraju et al., 200).

Lastly, the animal characteristic data showed a statistically significant drug effect in both the 1-day and 3-day EDL, SOL, and DIA tissue mass. This illustrates that the Dox treated animals (both 1-day and 3-day) lost tissue mass, which means some form of cell death was occurring, which means cleaved caspase-3 and cleaved PARP levels would increase. Based on this observation, I conclude the laboratory method for measuring PARP levels and that the tissues extraction markers a point in time, are the best explanations to somewhat describe the drug effects results of the current study.

The diet effects results are equally hard to explain. The most confusing are: Cr feeding increased cleaved PARP levels in the 1-day EDL and SOL, and increased cleaved caspase-3 levels in the 3-day EDL. As stated in multiple sections, research has not demonstrated Cr supplementation to increase cleaved PARP or cleaved caspase-3 levels in the tissue, cell culture. Interestingly, research has showed that Cr supplementation is beneficial in a large number of muscular, neurological, and cardiovascular diseases (Felber et al., 2000; Holtzman et al., 1999; Matthews et al., 1999; Mazzini et al., 2001; Sharov et al., 1987; Stout et al., 2001). Equally, the animal characteristic data showed no significant diet effect on EDL, SOL, or DIA tissue mass at either the 1-day or 3-day time point. Based on previous Cr research and the animal characteristic data, an explanation for the current findings that Cr supplementation may increase apoptosis in the EDL and SOL has remained elusive.

**Doxorubicin and Creatine's Effect on
Creatine Concentration**

Creatine, or methylguanidine-acetic acid, is a naturally occurring compound synthesized from arginine, glycine, and methionine in humans in the liver and pancreas (Bloch & Schoenheimer, 1941; Walker, 1979). Creatine is found in meat and found

principally in skeletal muscle, and in its free and phosphorlayted form plays a pivotal role in the regulation and homeostasis of skeletal muscle energy metabolism (Bessman & Fonyo, 1966; Bessman & Geiger, 1981; Meyer, Sweeney, & Kushmerick, 1984; Walliman, Wyss, Brdiczka, Nicolay, & Eppenberger, 1992).   In an average 70 kg adult, the total creatine pool in the body amounts to about 120 g and is continuously degraded to creatinine, and excreted in the urine at a rate of about 2 g/day (Walker, 1979). Creatine replishment is a combination of dietary intake and endogenous systhesis and most of the total creatine pool is contained in skeletal muscle, with 65% in a phosphorylated form called phosphocreatine (Casey, Constantin-Teodosiu, Howell, Hultman, & Greenhaff, 1996a; Hunter, 1922; Walker, 1979).

Human skeletal muscle is composed of several fiber types that differ substantially in functional and metabolic characteristics. Fiber types range from type I, slow-twitch fiber (example, soleus and diaphragm), to the type II, fast-twitch fiber (e.g., extensor digitorum longus). Specifically, creatine metabolism differs between fiber type, with type II fibers utilizing phosphocreatine (thus reducing free Cr to resynthesize pCr) at greater rates than type I fibers (Greenhaff, Nevill, & Soderlund, 1994; Soderlund, Greenhaff, & Hultman, 1992; Tesch, Thorsson, & Fujitsuka, 1989).   In rodents, total creatine concentration of type II, fast-twitch muscle is 45% greater than that of type I, slow-twitch muscle, and that the increase in phosphocreatine concentration after creatine supplementation is greater in type II fibers with free creatine concentration greater in type II fibers (Casey, Constantin-Teodosiu, Howell, Hultman, & Greenhaff, 1996b; Greenhaff, Bodin, & Casey, 1996).

It has been shown that the ingestion of creatine can increase the total creatine (free creatine + phosphocreatine) levels in skeletal muscle.   Harris, Soderlund, and Hultman (1992) found that 5 g creatine taken 4-6 times/day for several consecutive days increased the total creatine concentration of human skeletal muscle by an average of 25 mmol/kg dry mass, with 30% of which occurred in phosphorylated form as phosphocreatine. Equally, Greenhaff et al. (1993), found that ingestion of 20-30 g Cr/day for several days can lead to a >20% increase in human skeletal muslce total Cr content. However, in human studies, the data illustrate that there is a large variation in creatine uptake between individuals after creatine supplementation, from 6 – 38 mmol/kg dry mass (Casey et al., 1996a).   It has been suggested that a maintenance dose of 2 g/day after the 20-30 g/day for several days, will maintain a high total creatine concentration in the muscle for a period of 28 days (Hultman, Soderlund, Timmons, Cederblad, & Greenhaff, 1996). Lastly, in humans, supplementing with dose larger than 30 g/day shows no evidence of any potentiating effect on muslce creatine uptake (Harris et al., 1992; Hultman et al., 1996). In fact, a consistent finding from several studies is that there appears to be a definable upper limit to the intramuscular total creatine concentration of 160 mmol/kg dry mass, and once this limit is reached, further supplementation will only result in increased excretion of creatine in the urine (Casey et al., 1996b; Harris et al., 1992).

The assay used in the current study quantified total Cr concentration in the samples. This includes free Cr (creatine not bound) and Cr bound to a phosphate. This is a limitation, in that there is no way of determining if the drug and/or diet interventions effected the concentrations of the unbound Cr or Cr bound to a phosphate. Presently, Cr

concentration did not increase in the EDL, SOL, or DIA, at either time point. In fact, the only diet effect was observed in the 3-day EDL, with there being an overall lower Cr concentration with Cr feeding. Equally, there was a drug effect in the 3-day EDL, with lower Cr concentrations observed in rodents administered Dox. Taken together, the diet effect observed in the 3-day EDL could be influenced by the fact that the Cr-Dox animals had significantly lower Cr concentrations. The decrease in Cr concentration in the Cr-Dox animals could impact the overall observation that diet decreases Cr concentration. The only other drug effect was observed in the 1-day SOL, with Dox administration decreasing Cr concentration levels. There was neither a drug or diet effect observed in the DIA at either time point. Equally, no main effects were observed in the 1-day EDL or 3-day SOL. The decrease in total Cr concentration with Dox administration is not surprising, although, the exact mechanism(s) that may have caused this is speculative. One potential mechanism could be a reduction in creatine transport into all three skeletal muscles via reduced quantity of cell membrane creatine transporter.

Creatine, which is synthesized in the kidneys, must be transported into tissue (e.g., skeletal muscle) via creatine transporter which is embedded into the plasma membrane of the cell (Guerrero-Ontiveros & Wallimann, 1998). Creatine transporters are found in rat cardiac, skeletal muscle (quadriceps, soleus, and gastrocnemius), kidney, and brain (Neubauer et al., 1999). Additionally, the highest expression of the creatine transporter is found in the heart, and skeletal muscle, specifically in the soleus (Guerrero-Ontiveros & Wallimann, 1998; Murphy et al., 2001). Research by Darrabie et al. (2012), found a significant and irreversible decrease in creatine transport after an incubation with 50 – 100 nmol/l doxorubicin. Similar studies have shown Dox to decrease

phosphocreatine (pCr) and ATP, as much as 70% (Maslov et al., 2010; Ohhara, Kanaide, & Nakamura, 1981; Seraydarian, Artaza, & Goodman, 1977). Interestingly, kinetic analysis showed that the decrease in creatine transport was not due to Dox-induced increased cell death, but rather to a decrease in Cr V_{max}, K_m, and creatine transporter protein content (Darrabie et al., 2012). These results indicate that Dox may alter the affinity of the creatine transporter for Cr and/or the stability of the creatine transporter due to potential oxidative damage of sensitive amino acids in the transporter (Darrabie et al., 2012). In the current study the drug effect in the 1-day SOL and 3-day EDL could be due to creatine transporter dysfunction resulting in decreased Cr transport from the blood into the EDL and DIA. As stated previously (Murphy et al., 2001), creatine transporter expression is higher in the SOL (when compared to the EDL and DIA) and this may have led to higher level of Cr transport into the SOL. This could be an explanation as to the decrease in Cr concentration observed in the EDL and not the SOL. In regards to the 1-day EDL, SOL and DIA, it is possible there was not enough time for Dox to damage the creatine transporter to a significant level to affect creatine transport and subsequent Cr concentration in the 1, 3-day DIA, 1-day EDL, and 3-day SOL

Of most interest was the effects of Dox and Cr on Cr concentration levels in the 1-day EDL, 3-day SOL, and 1, 3-day DIA. No effects were observed in these muscle tissues. This means Cr feeding did not increase or decrease creatine concentration, nor did Dox. Research has shown Cr concentration to differ with fiber type, with free creatine concentration greater in type II fibers (Casey et al., 1996b; Greenhaff et al., 1996). The DIA, which is a mixture of type I and type II fibers, had similar effects as the type II EDL. It could be that in the above mentioned skeletal muscles there was an

increased level of Dox-induced free radical production and free Cr was acting as an antioxidant, scavenging free radicals in these muscles. This could have resulted in lower amounts of free Cr in these skeletal muscles, thus decreasing total Cr concentration observed in these muscles, and not reflecting a statistical change in Cr concentration with Cr feeding. To further support this, cleaved PARP and cleaved caspase-3 levels did not change with Dox treatment in the 3-day SOL, and cleaved caspase-3 levels decreased with Dox in the 3-day EDL. This could suggest that at the 3-day time point Dox may effect the type II EDL differently than the type I SOL, resulting in higher Dox-toxicity in the EDL than SOL. This possibly increased activity could have lead to increased use of free Cr as an antioxidant, resulting in the observed reduction in total Cr concentration in the 3-day EDL. Lastly, the lack of observable increase in total Cr concentration with Cr feeding in the EDL, SOL, and DIA in the C1-Sal, and C2-Sal groups at either time point remains unknown.

Creatine Concentration and Apoptotic Proteins

In the current study Dox had the greatest effect on decreasing Cr concentration in the 1-day SOL and 3-day EDL. Subsequently, the only effect observed in the 3-day EDL was increased cleaved caspase-3 levels with both Dox and Cr. The only effect observed in the 3-day DIA was a decrease in cleaved PARP with Dox. Creatine feeding only decreased Cr concentration in the 3-day EDL. In the 1-day EDL, drug and diet increased cleaved PARP levels; diet decreased caspase-3 levels, and there was no change in cleaved caspase-3 levels. The 1-day SOL, in which there was a drug or diet effect on Cr concentration observed, had an increase in 1-day cleaved PARP levels with drug and diet,

and a decrease in cleaved caspase-3 with Cr feeding. Equally, caspase-3 levels were lower in the 3-day Sol with Dox.

In closing, this is the first report examining the effects of two types of Cr feeding and Dox on the apoptotic marker; cleaved PARP, caspase-3, and cleaved caspase-3 in three separate skeletal muscles, at two different time points. Both Cr feeding and Dox administration affected Cr concentration differently depending on the time point and tissue. Apoptotic protein levels were affected by both Cr and Dox, and levels varied depending on the protein, tissue, and time point. Overall, Dox administration and Cr feeding affect Cr concentration and apoptotic protein levels in the EDL, SOL, and DIA at both the 1-day and 3-day time points.

Moving forward, the results obtained indicate that Cr has some effect on skeletal muscle treated with Dox. The current study examined the apoptotic pathway, and previous research our lab has examined myogenic regulatory factors, and these findings only begin to attempt to explain how Cr may be affecting Dox-induced myotoxicity. In response, it is important to continue investigating Cr and other nutraceutical supplements that may help reduce the negative impacts of chemotherapy on cancer patients. It is equally important to find alternative treatments that have low side effects and are safe to take along side traditional cancer treatments. The current findings are encouraging, that there are compound (such as Cr) that exist that are cheap, safe, and easy to ingest, that may improve the quality of life for cancer patients, or possibly the quality of life for patients being treated for other diseases. In light of this, as research to find better treatments increase (with potentially greater unpleasant, or deadly side effects), so should research for treatments to combat the side effects of the new treatments increase. This

approach would ultimately lead to a better quality of life for the patient and possibly

better over treatment outcomes.

References

Affar, E. B., Shah, R. G., Dallaire, A. K., Castonguay, V., & Shah, G. M. (2002). Role of poly(ADP-ribose) polymerase in rapid intracellular acidification induced by alkylating DNA damage. *Proceedings of the National Academy of Sciences of the United States of Amercia, 99*(1), 245-250.

Andersen, J. K. (2004). Oxidative stress in neurodegeneration: cause of consequence? *Nature Medicine,* Supplemental, S18-S25.

Asakura, T., Sawai, T., Hashidume, Y., Ohkawa, Y., Yokoyama, S., & Ohkawa, K. (1999). Caspase-3 activation during apoptosis caused by glutathione-doxorubicin conjugate. *British Journal of Cancer, 80*(5-6), 711-715.

Bae, Y. S., Oh, H., Rhee, S. G., & Yoo, Y. D. (2011). Regulation of reactive oxygen species generation in cell signaling. *Molecular Cell, 32*, 491-509.

Basta, P., Skarpan′ska-Stejnborn, A., & Pilaczyn′ska-Szczes′niak, L. (2006). Creatine supplementation and parameters of exercise-induced oxidative stress after a standard rowing test. *Studies in Physical Culture and Tourism, 13*, 17-23.

Bender, A., Beckers, J., Schneider, I., Holter, S. M., Haack, T., Ruthsatz, T., … Klopstock, T. (2007). Creatine improves health and survival of mice. *Neurobiology of Aging, 29*, 1401–1411.

Bessman, S. P., & Fonyo, A. (1966). The possible role of mitochondrial bound creatine kinase in regulation of mitochondrial respiration. *Biochemical and Biophysical Research Communications, 22,* 597-602.

Bessman, S. P., & Geiger, P. J. (1981). Transport of energy in muscle. The phosphorylcreatine shuttle. *Science Washington DC, 211*, 448-452.

Bloch, L., & Schoenheimer, R. (1941). The biological precursors of creatine. *Journal of Biological Chemistry, 138*, 167-194.

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

Bredahl, E. C., & Hydock, D. S. (2017). Creatine supplementation and doxorubicin-induced skeletal muscle dysfunction: an ex vivo investigation. *Nutrition and Cancer, 69*(4), 607-615.

Bredahl, E. C., Pfannenstiel, K. B., Quinn, C. J., Hayward, R., & Hydock, D. S. (2016). Effects of exercise on doxorubicin-induced skeletal muscle dysfunction. *Medicine and Science in Sports and Exercise, 48*(8), 1468-1473.

Bristow, M. R., Sageman, W. S., Scott, R. H., Billingham, M. E., Bowden, R. E., Kernoff, R. S., … Daniels, J. R. (1980). Acute and chronic cardiovascular effects of doxorubicin in the dog: the cardiovascular pharmacology of drug-induced histamine release. *Journal of Cardiovascular Pharmocology, 2*(5), 487-515.

Carafoli, E., & Lehninger, A. L. (1971). A survey of the interaction of calcium ions with mitochondria from different tissues and species. *Biochemical Journal, 122*, 681-690.

Casey, A., Constantin-Teodosiu, D., Howell, S., Hultman, E., & Greenhaff, P. L. (1996a). Metabolic response of type I and II muscle fibres during repeated bouts of maximal exercise in humans. *American Journal of Physiology, 271*, E38-E43.

Casey, A., Constantin-Teodosiu, D., Howell, S., Hultman, E., & Greenhaff, P. L. (1996b). Creatine ingestion favorably affects performance and muscle metabolism during maximal exercise in humans. *American Journal of Physiology, 271*, E31-E37.

Casey, A., & Greenhaff, P. L. (2000). Does dietary creatine supplementation play a role in skeletal muscle metabolism and performance? *American Journal of Clinical Nutrition, 72*(suppl), 607S-617S.

Chatterjee, K., Zhang, J., Honbo, N., & Karliner, J. S. (2010). Doxorubicin cardiomyopathy. *Cardiology, 115,* 155-162.

Chenard, P., Fabris, S., & MacLean, D. (2012). Tissue concentrations of Doxorubicin and Doxorubicinol 24 and 96 hours following injection in the rat. *The FASEB Journal, 26*(1), 1530-6860.

Cheng, E. H. Y., Kirsch, D. G., Clem, R. J., Ravi, R., Kastan, M. B., Bedi, A., … Hardwick, J. M. (1997). Conversion of Bcl-2 to bax-like death effector by caspases. *Science, 278*, 1966-1968.

Childs, A. C., Phaneuf, S. L., Dirks, A. J., Phillips, T., & Leeuwenburgh, C. (2002). Doxorubicin treatment *in vivo* causes cytochrome *c* release and cardiomyocyte apoptosis, as well as increased mitochondrial efficiency, superoxide dismustase activity, and Bcl-2:Bax ratio. *Cancer Research*, 62, 4592-4598.

Chwalbinska-Moneta, J. (2003). Effect of creatine supplementation on aerobic performance and anaerobic capacity in elite rowers in the course of endurance training. *International Journal of Sport Nutrition and Exericse Metabolism, 13*(2), 173-183.

Cooper, R., Naclerio, F., Allgrove, J., & Jimenez, A. (2012). Creatine supplementation with specific view to exercise/sports performance: an update. *Journal of the International Society of Sports Nutrition, 9*(33), 1-11.

Cory, S., & Adams, J. M. (2002). The Bcl2 family: regulators of the cellular life-or-death switch. *Nature Reviews Cancer, 2*, 647-656.

Courneya, K. S., Segal, R. J., Gelmon, K., Reid, R. D., Mackey, J. R., Friedenreich, C. M., … McKenzie, D. C. (2007). Six-month follow-up of patient-rated outcomes in a randomized controlled trial of exercise training during breast cancer chemotherapy. *Cancer Epidemiology, Biomarkers & Prevention, 16*(12), 2572-2578.

Covarrubias, L., Hernandez-Garcia, D., Schnabel, D., Salas-Vidal, E., & Castro-Obregon, S. (2008). Function of reactive oxygen species during animal development: passive or active? *Developmental Biology, 320*, 1-11.

Dangott, B., Schultz, E., & Mozdziak, P. E. (2000). Dietary creatine monohydrate supplementation increases satellite cell mitotic activity during compensatory hypertrophy. *International Journal of Sports Medicine, 21*, 13-16.

Darrabie, M. D., Arciniegas, A. J. L., Mantilla, J. G., Mishra, R., Vera, M. P., Santacruz, L., & Jacobs, D. O. (2012). Exposing cardiomylocytes to subclinical concentrations of doxorubicin rapidly reduces their creatine transport. *American Journal of Physiology-Heart and Circulatory Physiology, 303*, H539-H548.

Darzynkiewicz, Z., Pozarowski, P., Lee, B. W., & Johnson, G. L. (2011). Fluorochrome-labeled inhibitors of caspases: convenient in vitro and in vivo markers of apoptotic cells for cytometric analysis. *Methods in Molecular Biology, 682,* 103-114.

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

Demant, T. W., & Rhodes, E. C. (1999). Effects of creatine supplementation on exercise performance. *Sports Medicine, 28*, 49-60.

Denning, T. L., Takaishi, H., Crowe, S. E., Boldogh, I., Jevnikar, A., & Ernst, P. B. (2002). Oxidative stress induces the expression of Fas and Fas ligand and apoptosis in   murine intestinal epithelial cells. *Free Radical Biology and Medicine, 33,* 1641-1650.

Dickinson, B. C., & Chang, C. J. (2011). Chemistry and biology of reactive oxygen species in signaling or stress responses. *Nature Chemical Biology, 7,* 504-511.

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

Du, C., Fang, M., Li, Y., Li, L., & Wang, X. (2000). Smac, a mitochondrial protein that promotes cytochrome *c*-dependent caspase activation by eliminating IAP inhibition. *Cell,102*, 33-42.

Earnest, C. P., Snell, P. G., Rodriguez, R., Almada, A. L., & Mitchell, T. L. (1995). The effect of creatine monohydrate ingestion on anaerobic power indices, muscular strength and body composition. *Acta Physiologica Scandinavica, 153,*207–209.

Elmore, S. (2007). Apoptosis: a review of programmed cell death. *Toxicologic Pathology, 35*(4), 494-516.

Fairman, C. M., Hyde, P. N., & Focht, B. C. (2016). Resistance training interventions across the cancer control continuum: a systematic review of the implementation of resistance training principles. *British Journal of Sports Medicine, 51*(8), 677-685.

Felber, S., Skladal, D., Wyss, M., Kremser, C., Koller, A., & Sperl, W. (2000). Oral creatine supplementation in Duchenne muscular dystrophy: a clinical and 31P magnetic resonance spectroscopy study. *Neurological Research, 22,* 145–150.

Fiers, W., Beyaert, R., Declercq, W., & Vandenabeele, P. (1999). More than one way to die: apoptosis, necrosis and reactive oxygen damage. *Oncogene, 18*, 7719-7730.

Fimognari, C., Sestili, P., Lenzi, M., Cantelli-Forti, G., & Hrelia, P. (2009). Protective effect of creatine against RNA damage. *Mutation Research, 670*, 59-67.

Fulda, S., Gorman, A. M., Hori, O., & Samali, A. (2010). Cellular stress response: cell survival and cell death. *International Journal of Cell Biology, 10*, 1-23.

Garrido, C., Galluzzi, L., Brunet, M., Puig, P. E., Didelot, C., & Kroemer, G. (2006). Mechanisms of cytochrome *c* release from mitochondria. *Cell Death and Differentiation, 13*, 1423-1433.

Gilliam, L. A., Moylan, J. S., Patterson, E. W., Smith, J. D., Wilson, A. S., Rabbani, Z., & Reid, M. B. (2012). Doxorubicin acts via mitochondrial ROS to stimulate catabolism in C2C12 myotubes. *American Journal of Physiology. Cell Physiology, 302*(1), C195-C202.

Gordon, A., Hultman, E., Kaijser, L., Krisjansson, S., Rolf, C. J., Nyquist, O., & Sylven, C. (1995). Creatine supplementation in chronic heart failure increases skeletal muscle creatine phosphate and muscle performance. *Cardiovascular Research, 30*, 413-418.

Greenhaff, P. L., Bodin, K., & Casey, A. (1996). Dietary creatine supplementation and fatigue during high intensity exercise in man. *Biochemistry of Exercise IX.* Champaign, IL: Human Kinetics Publishers, 219-242.

Greenhaff, P. L., Bodin, K., Soderlund, K., & Hultman, E. (1994). Effect of oral creatine supplementation on skeletal muscle phosphocreatine resynthesis. *American Journal of Physiology, 266*, E725-E730.

Greenhaff, P. L., Casey, A., Short, A. H., Harris, R., Soderlund, K., & Hultman, E. (1993). Influence of oral creatine supplementation on muscle torque during repeated bouts of maximal voluntary exercise in man. *Clinical Science, 84*, 565-571.

Greenhaff, P. L., Nevill, M. E., & Soderlund, K. (1994). The metabolic responses of human type I and II muscle fibres during maximal treadmill sprinting. *Journal of Physiology, 478*, 149-155.

Gualano, B., Artioli, G. G., Poortmans, J. R., & Lancha Junior, A. H. (2009). Exploring the therapeutic role of creatine supplementation. *Amino Acids, 38*, 31-44.

Guerrero-Hernandez, A., Gallegos-Gomez, M. L., Sanchez-Vazquez, V. H., & Lopez-Mendez, M. C. (2014). Acidic intracellular Ca stores and caveolae in Ca signaling and diabetes. *Cell, 56*, 323-331.

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. *Molecular and Cellular Biochemistry, 184*, 427-437.

Haigis, M. C., & Yankner, B. A. (2010). The aging stress response. *Molecular Cell, 40*(2), 333-344.

Haldar, S., Negrini, M., Monne, M., Sabbioni, S., & Croce, C. M. (1994). Down-regulation of bcl-2 by p53 in breast cancer cells. *Cancer Research, 54*, 2095-2097.

Halliwell, B. (2011). Free radicals and antioxidants – quo vadis? *Trends in Pharmacological Sciences, 32*, 125-130.

Harris, R. C., Soderlund, K., & Hultman, E. (1992). Elevation of creatine in resting and exercised muscle of normal subjects by creatine supplementation. *Clinical Science London, 83*, 367-374.

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

Hayward, R., & Hydock, D. S. (2007). Doxorubicin cardiotoxicity in the rat: an in vivo characterization. *Journal of the American Association for Laboratory Animal Science, 46*(4), 20-32.

Hilder, T. L., Carlson, G. M., Haystead, T. A., Krebs, E. G., & Graves, L. M. (2005). Caspase-3 dependent cleavage and activation of skeletal muscle phosphorylase b kinase. *Molecular and Cellular Biochemistry, 275*(1-2), 233-242.

Holtzman, D., Khait, I., Mulkern, R., Allred, E., Rand, T., Jensen, F., & Kraft, R. (1999). In vivo development of brain phosphocreatine in normal and creatine-treated rabbit pups. *Journal of Neurochemistry, 73*, 2477– 2484.

Hopkins, R. Z. (2016). Superoxide in biology and medicine: an overview. *Reactive Oxygen Species, 1*(2), 99-109.

Hultman, E., Soderlund, K., Timmons, J., Cederblad, G., & Greenhaff, P. L. (1996). Muscle creatine loading in man. *Journal of Applied Physiology, 811*, 232-237.

Hunter, A. (1922). The physiology of creatine and creatinine. *Physiological Reviews-American Journal of Physiology, 2*, 586-626.

Hydock, D. S., 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 Research, 31*(6), 2023-2028.

Igney, F. H., & Krammer, P. H. (2002). Death and anti-death: tumor resistance to apoptosis. *Nature Reviews Cancer, 2*, 277-288.

Ingwall, J. S. (1976). Creatine and control of muscle specific protein synthesis in cardiac and skeletal muscle. *Circulation Research, 38*, I1115-I1123.

Ishikawa, K., Takenaga, K., Akimoto, M., Koshikawa, N., Yamaguchi, A., Imanishi, H., … Hayashi, J. (2008). ROS-generating mitochondrial DNA mutations can regulate tumor cell metastasis. *Science, 320*(5876), 661-664.

Jacobson, M. D., Weil, M., & Raff, M. C. (1997). Programmed cell death in animal development. *Cell, 88*, 347-354.

Janicke, R. U., Ng, P., Sprengart, M. L., & Porter, A. G. (1998). Caspase-3 is required for cleavage of α-fodrin but dispensable for cleavage of other death substrates in apoptosis. *Journal of Biological Chemistry, 273*, 15540-15545.

Johnson-Arbor, K., & Dubey, R. (2017). Doxorubicin. StatPearls Publishing, Jan 2018.

Jones, A. M., Atter, T., & Georg, K. P. (1999). Oral creatine supplementation improves multiple sprint performance in elite ice-hockey players. *The Journal of Sports Medicine and Physical Fitness, 39*, 189-196.

Jordon, M. A. (2002). Anti-cancer agents. *Current Medical Chemotherapy, 2*, 1-17.

Kaspar, A. A., Okada, S., Kumar, J., Poulain, F. R., Drouvalakis, K. A., Kelekar, A., … Krensky, A. M. (2001). A distinct pathway of cell-mediated apoptosis initiated by granulysin. *Journal of Immunology, 167(1), 350-356.*

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

Kehrer, J. P., & Klotz, L. O. (2015). Free radicals and related reactive species as mediators of tissue injury and disease: implications for health. *Critical Reviews in Toxicology, 45*, 765-798.

Kerr, J. F., Wyllie, A. H., & Currie, A. R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implication in tissue kinetics. *British Journal of Cancer, 26*, 239-257.

Kischkel, F. C., Hellbardt, S., Behrmann, I., Germer, M., Pawlita, M., Krammer, P. H., & Peter, M. E. (1995). Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. *EMBO Journal, 14*, 5579-5588.

Kluck, R. M., Martin, S. J., Hoffman, B. M., Zho, J. S., Green, D. R., & Newmeyer, D. D. (1997). Cytochrome *c* activation of CPP32-like proteolysis plays a critical role in a *Xenopus* cell-free apoptosis system. *EMBO Journal, 16*, 4639-4649.

Kotamraju, S., Konorev, E. A., Joseph, J., & Kalyanaraman, B. (2000). Doxorubicin-induced apoptosis in endothelial cells and cardiomyocytes is ameliorated by nitrone spin traps and ebselen. Role of reactive oxygen and nitrogen species. *The Journal of Biological Chemistry, 275,* 33585-33592.

Kraemer, W. J., & Volek, J. S. (1999). Creatine supplementation. Its role in human performance. *Clinics in Sports Medicine, 18*, 651-666.

Lawler, J. M., Barnes, W. S., Wu, G., Song, W., & Demaree, S. (2002). Direct antioxidant properties of creatine. *Biochemical and Biophysical Research Communications, 290*(1), 47-52.

Lawler, J. M., & Powers, S. K. (1998). Oxidative stress, antioxidant capacity, and the contracting diaphragm. *Canadian Journal of Applied Physiology, 23*, 23-55.

Lefrak, E. A., Pitha, J., Rosenheim, S., & Gottlieb, J. A. (1973). A clinicopathologic analysis of Adriamycin cardiotoxicity. *Cancer, 32*, 302-314.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnermi, E. S., & Wang, X. (1997). Cytochrome *c* and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell, 91*, 479-489.

Liu, P. F., Hu, Y. C., Kang, B. H., Tseng, Y. K., Wu, P. C., Liang, C. C., … Shu, C. W. (2017). Expression levels of cleaved caspase-3 and caspase-3 in tumorigenesis and prognosis of oral tongue squamos cell carcinoma. *PLoS ONE, 12*(7), 1-14.

Locksley, R. M., Killeen, N., & Lenardo, M. J. (2001). The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell, 104*, 487-501.

Mahmood, Z., & Shukla, Y. (2010). Death receptors: targets for cancer therapy. *Experimental Cell Research, 316,* 887-899.

Markes, M., Brockow, T., & Resch, K. L. (2006). Exercise for women receiving adjuvant therapy for breast cancer. *The Cochrane Database of Systematic Reviews, 18*(4), CD005001.

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. *American Journal of Physiology-Heart and Circulatory Physiology, 299*, H332-H337.

Matthews, R. T., Ferrante, R. J., Klivenyi, P., Yang, L., Klein, A. M., Mueller, G., … Beal, M. F. (1998). Neuroprotective effects of creatine and cyclocreatine in animal models of Huntington's disease. *Journal of Neuroscience, 18*, 165-163.

Matthews, R. T., Ferrante, R. J., Klivenyi, P., Yang, L., Klein, A. M., Mueller, G., … Beal, M. F. (1999). Creatine and cyclocreatine attenuate MPTP neurotoxicity. *Exploratory Neurology, 157*, 142–149.

Mazzini, L., Balzarini, C., Colombo, R., Mora, G., Pastore, I., De Ambrogio, R., & Caligari, M. (2001). Effects of creatine supplementation on exercise performance and muscular strength in amyotrophic lateral sclerosis: preliminary results. *Journal of Neurological Science, 191*, 139–144.

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. *Canadian Medical Association Journal, 175*(1), 34-41.

Merski, J., Daskal, Y., & Busch, H. (1978). Comparison of Adriamycin-induced nucleolar segregation in skeletal muscle, cardiac muscle, and liver cells. *Cancer Treatment Reports, 62*(5), 771-778.

Meyer, R. A., Sweeney, H. L., & Kushmerick, M. J. (1984). A simple analysis of the "phosphocreatine shuttle." *American Journal of Physiology, 246, C*365-C377.

Mirzaei, B., Rahmani-Nia, F., Salehi, Z., & Rahimi, R. (2013). Effects of creatine monohydrate supplementation on oxidative DNA damage and lipid peroxidation induced by acute incremental exercise to exhaustion in wrestlers. *Kinesiology, 45*, 30-40.

Mitchell, A., Merkel, L., & Quadrilatero, J. (2015). Examining the effect of Doxorubicin on apoptotic signaling in skeletal muscle of ARC KO mice. *The FASEB Journal,* LB623.

Moll, U. M., & Zaika, A. (2001). Nuclear and mitochondrial apoptotic pathways of p53. *FEBS Letters, 493,*65-69.

Murphy, R., McConell, G., Cameron-Smith, D., Watt, K., Ackland, L., Walzel, B., … Snow, R. (2001). Creatine transporter protein content, localization, and gene expression in rat skeletal muscle. *American Journal of Physiology-Cell Physiology, 280*, C415-C422.

Mutrie, N., Campbell, A. M., Whyte, F., McConnachie, A., Emslie, C., Lee, L., … Ritchie, D. (2007). Benefits of supervised group exercise programme for women being treated for early stage breast cancer: pragmatic randomized controlled trial. *British Medical Journal, 334*(7592), 517.

Neubauer, S., Remkes, H., Spindler, M., Horn, M., Wiesmann, F., Prestle, J., … Wallimann, T. (1999). Downregulation of the Na(+)creatine cotransporter in failing human myocardium and in experimental heart failure. *Circulation, 100*, 1847–1850.

Nie, C., Tian, C., Zhao, L., Petit, P. X., Mehrpour, M., & Chen, Q. (2008). Cysteine 62 of Bax is critical for its conformational activation and its proapoptotic activity in response to H2O2-induced apoptosis. *The Journal of Biological Chemistry, 283*, 15359-15369.

Ohhara, H., Kanaide, H., & Nakamura, M. (1981). A protective effect of coenzyme Q10 on the adriamycin-induced cardiotoxicity in the isolated perfused rat heart. *Journal of Molecular and Cellular Cardiology, 13*, 741-752.

Orrenius, S., Gogvadze, V., & Zhivotovsky, B. (2015). Calcium and mitochondria in the regulation of cell death. *Biochemical and Biophysical Research Communications, 460*(1), 72-81.

Pallepati, P., & Averill-Bates, D. A. (2011). Mild thermotolerance induced at 40 degrees C protects HeLa cells against activation of death receptor-mediated apoptosis by hydrogen peroxide. *Free Radical Biology and Medicine, 50*, 667-679.

Pallepati, P., & Averill-Bates, D. A. (2012). Reactive oxygen species, cell death signaling and apoptosis. *Free Radical Biology and Medicine, 48*(6), 749-762.

Pan, G., O'Rourke, K., & Dixit, V. M. (1998). Caspase-9, Bcl-X$_L$, and Apaf-1 form a ternary complex. *Journal of Biological Chemistry, 273*, 5841-5845.

Paravicini, T. M., & Touyz, R. M. (2006). Redox signaling in hypertension. *Cardiovascular Research, 71*(2), 247-258.

Pardo, J., Bosque, A., Brehm, R., Wallich, R., Naval, J., Mullbacher, A., … Simon, M. M. (2004). Apoptotic pathways are selectively activated by granzyme A and/or granzyme B in CTL-mediated target cell lysis. *Journal of Cell Biology, 167*(3), 457-468.

Pouliquen, A. L., Escalup, L., Jourdan, N., Cottu, P., Faure, P., & Madelaine-Chambrin, I. (2011). Dose standardization of anticancer drugs. *International Journal of Clinical Pharmocology, 33,* 221-228.

Rahimi, R. (2011). Creatine supplementation decreases oxidative DNA damage and lipid peroxidation induced by a single bout of resistance exercise. *Journal of Strength and Conditioning Research, 25*, 3448-3455.

Rahimi, R., Mirzaei, B., Rahmani-Nia, F., & Salehi, Z. (2015). Effects of creatine monohydrate supplementation on exercise-induced apoptosis in athletes: a randomized, double-blind, and placebo-controlled study. *Journal of Research in Medical Science, 20*(8), 733-738.

Rahman, A. M., Yusuf, S. W., & Ewer, M. S. (2007). Anthracycline-induced cardiotoxicity an the cardiac-sparing effect of liposomal formulation. *International Journal of Nanomedicine, 2*(4), 567-583.

Redza-Dutordoir, M., & Averill-Bates, D. A. (2016). Activation of apoptosis signaling pathways by reactive oxygen species. *Biochimica et Biophysica Acta, 1863*(12), 2977-2922.

Reed, J. C. (1997). Cytochrome *c*: can't live with it – can't live without it. *Cell, 91*, 559-562.

Reid, M. B. (2001). Redox modulation of skeletal muscle contraction: what we know and what we don't. *Journal of Applied Physiology, 90*, 724-731.

Reid, M. B., Stokic, D. S., Koch, S. M., Khawli, F. A., & Leis, A. A. (1994). *N*-Acetylcysteine inhibits muscle fatigue in humans. *Journal of Clinical Investigation, 94*, 2468-2474.

Rouleau, M., Patel, A., Hendzel, M. J., Kaufmann, S. H., & Poirier, G. G. (2010). PARP inhibition: PARP1 and beyond. *Nature Reviews Cancer, 10*(4), 293-301.

Saelens, X., Festjens, N., Vande Walle, L., van Gurp, M., van Loo, G., & Vandenabeele, P. (2004). Toxic proteins released from the mitochondria in cell death. *Oncogene, 23*, 2861-2874.

Sahin, E., & DePinho, R. A. (2012). Axis of ageing: telomeres, p53 and mitochondria. *Nature Reviews Molecular Cell Biology, 13*(6), 397-404.

Sakahira, H., Enari, M., & Nagata, S. (1998). Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. *Nature, 391*, 96-99.

Santacruz, L., Darrabie, M. D., Mantilla, J. G., Mishira, R., Feger, B. J., & Jacobs, D. O. (2015). Creatine supplementation reduces doxorubicin-induced cardiomyocellular injury. *Cardiovascular Toxicology, 15*, 180-188.

Schuler, M., & Green, D. R. (2001). Mechanisms of *p53*-dependent apoptosis. *Biochemical Society Transactions, 29*, 684-688.

Seraydarian, M. W., Artaza, L., & Goodman, M. F. (1977). Adriamycin: effect on mammalian cardiac cells in culture. I. Cell population and energy metabolism. *Journal of Molecular and Cellular Cardiology, 9*, 375-382.

Sestili, P., Barbieri, E., Martinelli, C., Battistelli, M., Guescini, M., Vallorani, L., … Stocchi, V. (2009). Creatine supplementation prevents the inhibition of myogenic differentiation in oxidatively injured C2C12 murine myoblasts. *Molecular Nutrition and Food Research, 53*, 1187-1204.

Sestili, P., Martinelli, C., Bravi, G., Piccoli, G., Curci, R., Battistelli, M., … Stocchi, V. (2006). Creatine supplementation affords cytoprotection in oxidatively injured cultured mammalian cells via direct antioxidant activity. *Free Radical Biology and Medicine, 40*, 837-849.

Sestili, P., Martinelli, C., Ricci, D., Fraternale, D., Bucchini, A., Giamperi, L., … Stocchi, V. (2007). Cytoprotective effect of preparations from various parts of Punica granatum L. fruits in oxidatively injured mammalian cells in comparison with their antioxidant capacity in cell free systems. *Pharmacological Research, 56*, 18- 26.

Shah, G. M., Kandan-Kulangara, F., Montoni, A., Shah, R. G., Brind'Amour, J., Vodenicharov, M. D., & Affar, E. B. (2011). Approaches to detect PARP-1 activation in vivo, in situ, and in vitro. In: Tulin A. (eds.) Poly(ADP-ribose) Polymerase. *Methods in Molecular Biology (Methods and Protocols)*, vol 780. (3-34), Humana Press, Totowa, NJ

Sharov, V. G., Saks, V. A., Kupriyanov, V. V., Lakomkin, V. L., Kapelko, V. I., Steinschneider, A., & Javadov, S. A. (1987). Protection of ischemic myocardium by exogenous phosphocreatine. I. morphologic and phosphorus 31-nuclear magnetic resonance studies. *Journal of Thorac Cardiovascular Surgery, 94*, 749-761.

Sheikholeslami-Vatani, D., & Faraji, H. (2018). Influence of creatine supplementation on apoptosis markers after downhill running in middle-aged men: a crossover randomized, double-blind, and placebo-controlled study. *American Journal of Physical Medicine & Rehabilitation, 97*(11), 825-831.

Shukla, V., Mishra, S. K., & Pant, H. C. (2011). Oxidative stress in neurodegeneration. *Advances in Pharmacological Sciences, 2011*, 1-13.

Siegel, R. L., Miller, K. D., & Jemal, A. (2019). Cancer Statistics, 2019. *CA: A Cancer Journal for Clinicians, 69*(1), 7-34.

Sin, T. K., Tam, B. T., Yu, A. P., Yip, S. P., Yung, B. Y., Chan, L. W., … Siu, P. M. (2016). Acute treatment of resveratrol alleviates Doxorubicin-induced myotoxicity in aged skeletal muscle through SIRT1-dependent mechanisms. *Journals of Gerontology: Biological Sciences, 71*(6), 730-739.

Singal, P. K., Deally, C. M., & Weinberg, L. E. (1987). Subcellular effects of Adriamycin in the heart: a concise review. *Journal of Molecular and Cellular Cardiology, 19*, 817-828.

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

Slee, E. A., Adrain, C., & Martin, S. J. (2001). Executioner caspase-3, -6, and -7 perform distinct, non-redundant roles during the demolition phase of apoptosis. *The Journal of Cell Biology, 276*, 7320-7326.

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

Soderlund, K., Greenhaff, P. L., & Hultman, E. (1992). Energey metabolism in type I and type II human muscle fibres during short term electrical stimulation at different frequencies. *Acta Physiologica Scandinavica, 144*, 15-22.

Stout, J. R., Eckerson, J. M., May, E., Coulter, C., & Bradley-Popovich, G. E. (2001). Effects of resistance exercise and creatine supplementation on myasthenia gravis: a case study. *Medicine and Science in Sports and Exercise, 33*, 869–872.

Suhara, T., Fukuo, K., Sugimoto, T., Morimoto, S., Nakahashi, T., Hata, S., … Ogihara, T. (1998). Hydrogen peroxide induces up-regulation of Fas in human endothelial cells. *Journal of Immunology, 160*, 4042-4047.

Takemura, G., & Fujiwara, H. (2007). Doxorubicin-induced cardiomyopathy from the cardiotoxic mechanisms to management. *Progress in Cardiovascular Diseases, 49*(5), 330-352.

Tarnopolsky, M. A. (2007). Clinical use of creatine in neuromuscular and neurometabolic disorders. *Subcellular Biochemistry, 46*, 183–204.

Tarnopolsky, M. A., Mahoney, D. J., Vajsar, J., Rodriguez, C., Doherty, T. J., Roy, B. D., & Biggar, D. (2004). Creatine monohydrate enhances strength and body composition in Duchenne muscular dystrophy. *Neurology, 62*, 1771–1777.

Tawa, P., Tam, J., Cassady, R., Nicholson, D. W., & Xanthoudakis, S. (2001). Quantitative analysis of fluorescent caspase substrate cleavage in intact cells and identification of novel inhibitors of apoptosis. *Cell Death and Differentiation, 8*, 30-37.

Tesch, P. A., Thorsson, A., & Fujitsuka, N. (1989). Creatine phosphate in fiber types of skeletal muscle before and after exhaustive exercise. *Journal of Applied Physiology, 664*, 1756-1769.

Thompson, C. B. (1995). Apoptosis in the pathogenesis and treatment of disease. *Science, 267*, 1456-1462.

Thorn, C. F., Oshiro, C., Marsh, S., Hernandez-Boussard, T., McLeod, H., Klein, T. E., & Altman, R. B. (2011). Doxorubicin pathways: pharmacodynamics and adverse effects. *Pharmacogenet Genomics, 21*(7), 440-446.

Timolati, F., Ott, D., Pentassuglia, L., Giraud, M. N., Periard, J. C., Suter, T. M., & Zuppinger, C. (2006). Neuregulin-1 beta attenuates doxorubicin-induced alterations of excitation-contraction couplin and reduces oxidative stress in adult rat cardiomyocytes. *Journal of Molecular and Cellular Cardiology, 41*(5), 845-854.

Tong, J., Ganguly, P. K., & Singal, P. K. (1991). Myocardial adrenergic changes at two stages of heart failure due to Adriamycin treatment in rats. *American Journal of Physiology, 260*(3 Pt 2), H909-H916.

Trachootham, D., Alexandre, J., & Huang, P. (2009). Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach. *Nature Reviews Drug Discovery, 8*, 579-591.

Trapani, J. A., & Smyth, K. I. (2002). Functional significance of the perforin/granzyme cell death pathway. *Nature Reviews Immunology, 2*, 735-747.

Ueno, M., Kakinuma, Y., Yuhki, K., Murakoshi, N., Iemitsu, M., Miyauchi, T., & Yamaguchi, I. (2006). Doxorubicin induces apoptosis by activation of caspase-3 in cultured cardiomyocytes in vitro and rat cardiac ventricles in vivo. *Journal of Pharmacological Sciences, 101*(2), 151-158.

Van Moll, C. C., Schep, G., Vreugdenhil, A., Savelberg, H. H., & Husson, O. (2016). The effect of training during treatment with chemotherapy on muscle strength and endurance capacity: A systematic review. *Acta Oncologica, 55*(5), 539-546.

van Norren, K., van Helvoort, A., Argiles, J. M., van Tuijl, S., Arts, K., Gorselink, M., … van der Beek, E. M. (2009). Direct effect of doxorubicin on skeletal muscle contributes to fatigue. *British Journal of Cancer, 100*(2), 311-314.

Vejpongsa, P., & Yeh, E. T. (2014). Prevention of anthracycline-induced cardiotoxicity: challenges and opportunities. *Journal of the American College of Cardiology, 64*(9), 938-945.

Vergnani, L., Hatrick, S., Ricci, F., Passaro, A., Manzoli, N., Zuliani, G., … Malinski, T. (2000). Effect of native and oxidized low-density lipoprotein on endothelial nitric oxide and superoxide production: key role of L-arginine availability. *Circulation, 101*, 1147-1156.

Vodenicharov, M. D., Ghodgaonkar, M. M., Halappanavar, S. S., Shah, R. G., & Shah, G. M. (2005). Mechanism of early biphasic activation of poly(ADP-ribose) polymerase-1 in response to ultraviolet B radiation. *Journal of Cell Science, 118*, 589-599.

Von Hoff, D. D., Layard, M. W., Basa, P., Davis, H. L., Von Hoff, A. L., Rozencweig, M., & Muggia, F. M. (1979). Risk factors for doxorubicin-induced congestive heart failure. *Annals of Internal Medicine, 91*(5), 710-717.

Vorgerd, M., Grehl, T., Jager, M., Muller, K., Freitag, G., Patzold, T., … Malin, J. P. (2000). Creatine therapy in myophosphorylase deficiency (McArdle disease): a placebo-controlled crossover trial. *Archives of Neurology, 57*, 956–963.

Wajant, H. (2002). The Fas signaling pathway: more than a paradigm. *Science, 296*, 1635-1636.

Walker, J. B. (1979). Creatine: biosynthesis, regulation, and function. *Advances in Enzymology-and Related Areas of Molecular Biology, 50*, 177-242.

Walliman, T., Wyss, M., Brdiczka, D., Nicolay, K., & Eppenberger, H. M. (1992). Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: the "phosphocreatine circuit" for cellular energy homeostasis. *Biochemical Journal, 281*, 21-40.

Wang, L., Ma, W., Markovich, R., Chen, J. W., & Wang, P. H. (1998). Regulation of cardiomyocyte apoptotic signaling by insulin-like growth factor I. *Circulation Research, 83*, 516-522.

Wang, S., Konorev, E. A., Kotamraju, S., Joseph, J., Kalivendi, S., & Kalyanaraman, B. (2004). Doxorubicin induces apoptosis in normal and tumor cells via distinctly different mechanisms. *The Journal of Biological Chemistry, 279*(24), 25535-25543.

West, J. G., & Marnett, L. J. (2006). Endogenous reactive intermediates as modulators of cell signaling and cell death. *Chemical Research in Toxicology, 19*, 173-194.

Wu, G., & Meininger, C. J. (2000). Arginine nutrition and cardiovascular function. *Recent Advances in Nutritional Sciences, 130*, 2626-2629.

Wyss, M., & Kaddurah-Daouk, R. (2000). Creatine and creatinine metabolism. *Physiological Reviews, 80*(3), 1107-1213.

Wyss, M., & Schulze, A. (2002). Health implications of creatine: can oral creatine supplementation protect against neurological and atherosclerotic disease? *Neuroscience, 112*, 243–260.

Yang, R., Niepel, M., Mitchison, T. K., & Sorger, P. K. (2010). Dissecting variability in responses to cancer chemotherapy through systems pharmacology. *Clinical Pharmacology and Therapeutics, 88*(1), 34-38.

Yu, A. P., Pei, X. M., Sin, T. K., Yip, S. P., Yung, B. Y., Chan, L. W., … Siu, P. M. (2014). Acylated and unacylated ghrelin inhibit doxorubicin-induced apoptosis in skeletal muscle. *Acta Physiologica, 211*, 201-213.

Zeiss, C. J. (2003). The apoptosis-necrosis continuum: insights from genetically altered mice. *Veterinary Pathology, 40*, 481-495.

Zhang, A. Y., Yi, F., Jin, S., Xia, M., Chen, Q. Z., Gulbins, E., & Li, P. L. (2007). Acid sphingomyelinase and its redox amplification in formation of lipid raft redox signaling platforms in endothelial cells. *Antioxidants and Redox Signaling 9*, 817-828.

Zhang, J., Wang, X., Vikash, V., Ye, Q., Wu, D., Liu, Y., & Dong, W. (2016). ROS and ROS-mediated cellular signaling. *Oxidative Medicine and Cellular Longevity, 2016,* 4350965, 1-18.

Zhao, Y., McLaughlin, D., Robinson, E., Harvey, A. P., Hookham, M. B., & Shah, A. M. (2010). Nox2 NADPH oxidase promotes pathologic cardiac remodeling associated with doxorubicin chemotherapy. *Cancer Research, 97*(1), 77-87.

Zhu, H., Sarkar, S., Scott, L., Danelisen, I., Trush, M. A., Jia, Z., & Robert Li, Y. (2016). Doxorubicin redox biology: redox cycling, topoisomerase inhibition, and oxidative stress. *Reactive Oxygen Species, 1*(3), 189-198.

Zhu, S., Li, M., Figueroa, B. E., Liu, A., Stavrovskaya, I. G., Pasinelli, P., … Friedlander, R. M. (2004). Prophylatic creatine administration mediates neuroprotection in cerebral ischemia in mice. *Neurobiology of Disease, 24*(26), 5909-5912.

Zhuang, S., Demirs, J. T., & Kochevar, I. E. (2000). p38 mitogen-activated protein kinase mediated bid cleavage, mitochondrial dysfunction, and caspase-3 activation during apoptosis induced by singlet oxygen but not by hydrogen peroxide. *The Journal of Biochemistry, 275*, 25939-25948.

Zou, H., Henzel, W. J., Liu, X., Lutschg, A., & Wang, X. (1997). Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome *c*-dependent activation of caspase-3. *Cell, 90*, 405-413.

APPENDIX A

UNIVERSITY OF NORTHERN COLORAOD
INSTITUTIONAL ANIMAL CARE
AND USE COMMITTEE

UNIVERSITY OF
NORTHERN
COLORADO

IACUC Memorandum

    To: David Hydock

    From: Laura Martin, Director of Compliance and Operations

    CC: IACUC Files

    Date: December 28, 2017

    Re: IACUC Protocol Approval, 1711CE-DH-R-20

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 1711CE-DH-R-20.

The next annual review will be due before December 28, 2018.

Sincerely,

Laura Martin, Director of Compliance and Operations

APPENDIX B

APOPTOTIC PROTEIN LEVEL AND
CREATINE CONCENTRATION
TABLES

Table 5

1-day EDL apoptotic protein levels.

	Con-Sal	Con-Dox	C1-Sal	C1-Dox	C2-Sal	C2-Dox
Cleaved PARP [‡][†]	0.84 ± 0.05	1.07 ± 0.08 [*]	0.722 ± 0.03	1.03 ± 0.03 [*]	1.06 ± 0.03 [*+]	1.24 ± 0.07 [*]
Caspase-3 [†]	0.28 ± 0.06	0.21 ± 0.02	0.22 ± 0.02	0.29 ± 0.04	0.17 ± 0.01	0.15 ± 0.01
Cleaved caspase-3	0.33 ± 0.08	0.35 ± 0.10	0.22 ± 0.05	0.38 ± 0.10	0.25 ± 0.03	0.22 ± 0.07

Note. Con-Sal = control saline, n=10; Con-Dox = control doxorubicin, n=10; C1-Sal = creatine 1 saline, n=10; C1-Dox, creatine 1 doxorubicin, n=9; C2-Sal, creatine 2 saline, n=10; C2-Dox, creatine 2 doxorubicin, n=10. Values are means ± SEM.
‡ = signficant drug effect ($p<0.05$)
† = significant diet effect ($p<0.05$)
* = significantly different from C1-Sal
+ = significantly different from Con-Sal

Table 6

1-day SOL apoptotic protein levels.

	Con-Sal	Con-Dox	C1-Sal	C1-Dox	C2-Sal	C2-Dox
Cleaved PARP ‡	0.83 ± 0.05 *	1.12 ± 0.15	0.76 ± 0.15 *	0.67 ± 0.08 *	0.86 ± 0.06 *	1.64 ± 0.33
Caspase-3	0.19 ± 0.04	0.16 ± 0.02	0.09 ± 0.02	0.14 ± 0.02	0.12 ± 0.02	0.15 ± 0.04
Cleaved caspase-3 †	1.33 ± 0.15	1.48 ± 0.19	1.06 ± 0.19	0.90 ± 0.12	1.07 ± 0.08	1.15 ± 0.21

Note. Con-Sal = control saline, n=10; Con-Dox = control doxorubicin, n=10; C1-Sal = creatine 1 saline, n=10; C1-Dox, creatine 1 doxorubicin, n=9; C2-Sal, creatine 2 saline, n=10; C2-Dox, creatine 2 doxorubicin, n=10. Values are means ± SEM.
‡ = signficant drug effect ($p<0.05$)
† = significant diet effect ($p<0.05$)
* = significantly different from C2-Dox

Table 7

1-day DIA apoptotic protein levels.

	Con-Sal	Con-Dox	C1-Sal	C1-Dox	C2-Sal	C2-Dox
Cleaved PARP	0.43 ± 0.04	0.50 ± 0.07	0.39 ± 0.05	0.54 ± 0.04	0.52 ± 0.04	0.45 ± 0.05
Caspase-3	0.13 ± 0.02	0.11 ± 0.02	0.09 ± 0.02	0.14 ± 0.02	0.12 ± 0.02	0.08 ± 0.01
Cleaved caspase-3 [†]	0.08 ± 0.01	0.07 ± 0.01	0.04 ± 0.01	0.07 ± 0.01	0.05 ± 0.01	0.04 ± 0.01

Note. Con-Sal = control saline, n=10; Con-Dox = control doxorubicin, n=10; C1-Sal = creatine 1 saline, n=10; C1-Dox, creatine 1 doxorubicin, n=9; C2-Sal, creatine 2 saline, n=10; C2-Dox, creatine 2 doxorubicin, n=10. Values are means ± SEM.
[†] = significant diet effect ($p<0.05$)

Table 8

3-day EDL apoptotic protein levels.

	Con-Sal	Con-Dox	C1-Sal	C1-Dox	C2-Sal	C2-Dox
Cleaved PARP	0.76 ± 0.05	0.70 ± 0.05	0.81 ± 0.08	0.82 ± 0.08	0.75 ± 0.07	0.63 ± 0.05
Caspase-3	1.39 ± 0.16	1.06 ± 0.13	1.07 ± 0.17	1.06 ± 0.16	1.00 ± 0.17	1.02 ± 0.19
Cleaved caspase-3 [†‡]	0.04 ± 0.01	0.10 ± 0.03	0.31 ± 0.03 [*]	0.52 ± 0.08 [*+]	0.37 ± 0.08 [*+]	0.16 ± 0.03 [°]

Note. Con-Sal = control saline, n=10; Con-Dox = control doxorubicin, n=10; C1-Sal = creatine 1 saline, n=10; C1-Dox, creatine 1 doxorubicin, n=9; C2-Sal, creatine 2 saline, n=10; C2-Dox, creatine 2 doxorubicin, n=10. Values are means ± SEM.

[†] = significant diet effect ($p<0.05$)

[‡] = significant drug effect ($p<0.05$)

Significant interaction in the EDL cleaved caspase 3 ($p<0.05$)

[*] = significantly different from Con-Sal

[+] = significantly different from Con-Dox

[°] = significantly different from C1-Dox

Table 9

3-day SOL apoptotic protein levels.

	Con-Sal	Con-Dox	C1-Sal	C1-Dox	C2-Sal	C2-Dox
Cleaved PARP	0.63 ± 0.06	0.66 ± 0.03	0.70 ± 0.04	0.64 ± 0.04	0.76 ± 0.06	0.63 ± 0.05
Caspase-3 ‡	1.31 ± 0.15	0.94 ± 0.05	1.06 ± 0.06	0.92 ± 0.09	1.01 ± 0.10	0.98 ± 0.11
Cleaved caspase-3	1.13 ± 0.10	1.02 ± 0.06	1.24 ± 0.05	1.18 ± 0.09	1.34 ± 0.13	1.32 ± 0.19

Note. Con-Sal = control saline, n=10; Con-Dox = control doxorubicin, n=10; C1-Sal = creatine 1 saline, n=10; C1-Dox, creatine 1 doxorubicin, n=9; C2-Sal, creatine 2 saline, n=10; C2-Dox, creatine 2 doxorubicin, n=10. Values are means ± SEM.
‡ = significant drug effect ($p<0.05$)

Table 10

3-day DIA apoptotic protein levels.

	Con-Sal	Con-Dox	C1-Sal	C1-Dox	C2-Sal	C2-Dox
Cleaved PARP [‡]	0.64 ± 0.07	0.38 ± 0.06 [*]	0.56 ± 0.03	0.51 ± 0.05	0.57 ± 0.05	0.42 ± 0.05
Caspase-3	0.29 ± 0.11	0.30 ± 0.14	0.26 ± 0.08	0.20 ± 0.07	0.10 ± 0.01	0.10 ± 0.02
Cleaved caspase-3	0.16 ± 0.03	0.09 ± 0.01	0.12 ± 0.02	0.09 ± 0.01	0.09 ± 0.02	0.11 ± 0.02

Note. Con-Sal = control saline, n=10; Con-Dox = control doxorubicin, n=10; C1-Sal = creatine 1 saline, n=10; C1-Dox, creatine 1 doxorubicin, n=9; C2-Sal, creatine 2 saline, n=10; C2-Dox, creatine 2 doxorubicin, n=10. Values are means ± SEM.
[‡] = significant drug effect ($p<0.05$).
[*] = significantly different from Con-Sal

Table 11

1-day creatine concentration.

	Con-Sal	Con-Dox	C1-Sal	C1-Dox	C2-Sal	C2-Dox
EDL (M)	0.10 ± 0.04	0.06 ± 0.01	0.05 ± 0.03	0.06 ± 0.03	0.08 ± 0.02	0.05 ± 0.03
SOL (M) †	0.09 ± 0.01	0.07 ± 0.01	0.09 ± 0.01	0.08 ± 0.03	0.10 ± 0.02	0.06 ± 0.03
DIA (M) †	0.18 ± 0.08	0.13 ± 0.09	0.15 ± 0.04	0.15 ± 0.06	0.12 ± 0.04	0.12 ± 0.04

Note. EDL = extensor digitorum longus; SOL = soleus; DIA = diaphragm; Con-Sal = control saline, n=10; Con-Dox = control doxorubicin, n=10; C1-Sal = creatine 1 saline, n=10; C1-Dox, creatine 1 doxorubicin, n=9; C2-Sal, creatine 2 saline, n=10; C2-Dox, creatine 2 doxorubicin, n=10. Values are means ± SEM.
† = significant drug effect ($p < 0.05$)

Table 12

3-day creatine concentration.

	Con-Sal	Con-Dox	C1-Sal	C1-Dox	C2-Sal	C2-Dox
EDL (μM/μg) [†‡]	0.09 ± 0.02	0.05 ± 0.01 [*]	0.06 ± 0.01 [*]	0.05 ± 0.01 [*]	0.07 ± 0.02	0.03 ± 0.02 [*+]
SOL (μM/μg)	0.10 ± 0.04	0.09 ± 0.009	0.09 ± 0.03	0.09 ± 0.007	0.09 ± 0.04	0.07 ± 0.04
DIA (μM/μg)	0.18 ± 0.10	0.14 ± 0.06	0.16 ± 0.07	0.18 ± 0.05	0.21 ± 0.11	0.11 ± 0.06

Note. EDL = extensor digitorum longus; SOL = soleus; DIA = diaphragm; Con-Sal = control saline, n=10; Con-Dox = control doxorubicin, n=10; C1-Sal = creatine 1 saline, n=10; C1-Dox, creatine 1 doxorubicin, n=9; C2-Sal, creatine 2 saline, n=10; C2-Dox, creatine 2 doxorubicin, n=10. Values are means ± SEM.
[†] = significant diet effect ($p<0.05$).
[‡] = significant drug effect ($p<0.05$).
[*] = significantly different than Con-Sal ($p<0.05$).
[+] = significantly different than C2-Sal ($p<0.05$).