Effects of Creatine Supplementation and Doxorubicin Treatment of Myostatin and Muscle Ring-Finger Protein-1 Expression in the Rat Diaphragm

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EFFECTS OF CREATINE SUPPLEMENTATION AND DOXORUBICIN TREATMENT ON MYOSTATIN AND MUSCLE RING-FINGER PROTEIN-1 EXPRESSION IN THE RAT DIAPHRAGM

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science

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Exercise Physiology

August 2019
This Thesis by: Addison M. Shepard

Entitled: *Effects of Creatine Supplementation and Doxorubicin Treatment on Myostatin and Muscle RING-Finger Protein-1 Expression in the Rat Diaphragm*

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

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ABSTRACT


Doxorubicin (DOX) is a highly potent chemotherapy drug that impacts the entire body rather than a targeted treatment area. While DOX treatment assists in the disruption and prevention of certain types of cancer growth, it damages many physiological processes in the body that were functioning normally and otherwise healthy prior to its administration. Damage induced by DOX on organ functioning can lead to other malignancies in the body, such as cachexia, cardiotoxicity, and myotoxicity, among others. Skeletal muscle relies on myogenic regulatory factors (MRFs), transcription factors expressed by satellite cells, for positive regulation of phases of muscle development and response to damage. In skeletal muscle, DOX administration has caused decreased MRF levels as well as increased myostatin and ubiquitin ligase expression, which are negative regulators of muscle mass. Previous findings have reported altered regulatory protein expression and shifts in muscle fiber characteristics of the diaphragm (DIA), as well as decline in patient respiratory function, following DOX treatment. Creatine monohydrate (Cr) supplementation prior to DOX administration has protected skeletal muscle against DOX-induced dysfunction, damage, and decline in MRF expression. However, the effects of DOX treatment in combination with Cr supplementation on the expression of proteins that negatively regulate muscle mass, such as myostatin and MuRF-1, in the DIA
have yet to be investigated. **PURPOSE:** To investigate the effects of *in vivo* DOX administration, following the completion of two Cr supplementation protocols, on the expression of negative regulators of skeletal muscle mass, myostatin and MuRF-1, in the DIA tissue of rats sacrificed one- and three-days post-injection (*N*=115). **METHODS:** Male Sprague-Dawley rats were fed a diet supplemented with a standard Cr dosage of 2% for four weeks (CrS), a Cr loading dosage of 4% for one week and 2% for three weeks (CrL), or a standard control (CON) diet for four weeks. Based on random assignment, animals received an intraperitoneal injection of DOX (15 mg/kg) or saline (SAL) of the same volume and underwent anesthesia one- or three-days post-injection. Following DIA removal, Western blotting was used to quantify the protein expression of myostatin and MuRF-1. **RESULTS:** In the DIA, there was no significant diet effect, drug effect, or interaction at the one-day timepoint relating to myostatin expression (*p* = 0.255, *p* = 0.412, *p* = 0.770, respectively). There was also no diet effect, drug effect, or interaction pertaining to myostatin expression in the DIA at the three-day timepoint. (*p* = 0.710, *p* = 0.935, *p* = 0.566, respectively). In the DIA, there was no significant diet effect, drug effect, or interaction at the one-day timepoint pertaining to the expression of MuRF-1 (*p* = 0.772, *p* = 0.248, *p* = 0.137, respectively). There also was no diet effect, drug effect, or interaction relating to MuRF-1 expression in the DIA at the three-day timepoint (*p* = 0.826, *p* = 0.931, *p* = 0.941, respectively). **CONCLUSION:** There were no significant diet effects, drug effects, or interactions at either timepoint among myostatin and MuRF-1 protein expression in the DIA as determined by two-way ANOVA (*p* > 0.05). These findings suggest that mediators and mechanisms unrelated to myostatin and MuRF-1
protein expression may contribute to the decline in respiratory function commonly observed in individuals undergoing chemotherapy treatment.
DEDICATED TO KAEDEN R. CLICK

In acknowledgement of your unwavering and unconditional love and support which have carried me through the past eighteen years. I am grateful to know such an extraordinary person who happens to be my nephew. Thank you for being my one true constant.

The sky is the limit with you.
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CHAPTER I
INTRODUCTION

In 2018, there were nearly two million new cases of cancer in North America alone (American Cancer Society: Global Cancer Facts & Figures 4th Edition, 2018), and it is anticipated that over half a million individuals in the United States will die of cancer in 2019 (American Cancer Society: Cancer Facts & Figures 2019, 2019). The cancer death rate in North America has steadily declined for almost 30 years due to greater understanding of cancer mechanisms, improved screening techniques, and increased mindfulness of cancer-causing materials and products.

Chemotherapy is a commonly utilized method of cancer treatment that affects several tissues in the body rather than a localized area. Anthracyclines are a class of drugs often used in chemotherapy due to their high success rates in the treatment of a majority of cancers (Weiss, 1992). Doxorubicin (DOX), an anti-tumor chemotherapy antibiotic belonging to the anthracycline drug class, was first administered to patients in Milan, Italy, in 1968 (Bonadonna, Monfardini, de Lena, & Fossati-Bellani, 1969). However, DOX was not approved by the Food and Drug Administration for use in the United States until 1974 (Cassinelli, 2016).

A key limitation of DOX administration is the harm it inflicts on organs and tissues in the body that were not intended treatment targets. This may cause other damage to the body in the form of cachexia, cardiotoxicity, cytotoxicity, myotoxicity, muscle
fatigue, and muscle dysfunction. The severity of DOX-induced damage can be predicted based on the total administered dose (Thorn et al., 2011), which indicates that the prescribed dosage should be sensibly considered in each case (Carvalho et al., 2009).

Treatment with DOX has led to feelings of whole-body and skeletal muscle fatigue, as well as muscle atrophy, weakness, and dysfunction. At least seven out of every ten individuals with cancer have reported symptoms of fatigue (Miller, Maguire, and Kearney, 2007). While there are several proposed mediators of skeletal muscle dysfunction following DOX treatment, an increased production of reactive oxygen species (ROS) is of particular interest. If antioxidant protections are weakened as a result of cancer treatment, for example, an overabundance of ROS may be created and cause oxidative stress. Furthermore, Gilliam et al. (2012) determined that mitochondrial ROS aided DOX in the initiation of skeletal muscle degradation. Although the exact mechanisms that lead to muscle damage remain unclear, several studies have investigated the effects of DOX administration on components of cardiac, EDL, and SOL muscle functioning.

It has been observed that DOX exerts its effects in dose-, time-, and tissue-dependent manners. van Norren et al. (2009) reported that incubation of the extensor digitorum longus (EDL) muscle in DOX for two hours led to a decline in ex vivo maximal force, as well as decreased contraction and relaxation velocity in mice. Similarly, Hydock, Lien, Jensen, Schneider, and Hayward (2011) observed that maximal twitch force, rate of force production, and rate of force decline were significantly decreased five days following administration of a 15 mg/kg dose of DOX. Fatigue rate was unaltered in the EDL from DOX administration at doses of 10 mg/kg, 12.5 mg/kg,
and 15 mg/kg, but was significantly hindered by all three doses in the soleus (SOL). These findings suggest that skeletal muscle function in rats is impaired based on the dose of DOX administered.

Additionally, Hydock et al. (2011) explored the effects of DOX treatment on myotoxicity in rats and compared the severity of the damage to that of cardiotoxicity. Proper functioning of the heart was diminished in a dose-dependent manner and declined to the same degree as that of SOL muscle. Therefore, Hydock et al. (2011) were the first to report that cardiotoxicity induced by DOX treatment, as well as direct effects of DOX on skeletal muscle, contributed to the decline of skeletal muscle function in rats. Moreover, Gilliam, Moylan, Callahan, Sumandea, & Reid (2011) found that DOX administration led to a decline in contractile force and reduced cross-sectional area of muscle fibers in the DIA muscle of rats. Based on these observations, Gilliam, Moylan, Callahan, et al. (2011) were the first to report evidence that DOX treatment can lead to respiratory dysfunction.

A time-dependent decline in both cardiac and skeletal muscle function in rats was observed by Hayward et al. (2013) five days following a 15 mg/kg dose of DOX. Accumulation of DOX was greatest in cardiac muscle, although all muscular tissues demonstrated comparable dysfunction severity. Similarly, Yamada, Sugiyama, Kosaka, Hayakawa, and Ozawa (1995), injected rats with a total cumulative dose of 15 mg/kg of DOX, partially to investigate DOX accumulation in the heart, liver, and diaphragm (DIA) tissue. Yamada et al. (1995) concluded that DOX accumulated in the DIA at a greater rate 6 and 24 hours following the last DOX injection in comparison to in the heart and liver. Based on these findings, DOX accumulates at different rates depending on the type
of tissue, which may explain variances in DOX-induced damage. Specific characteristics of the muscle tissue, such as the type of fiber composition, may also influence the severity of DOX-induced muscle dysfunction (Hydock et al., 2011).

In order for muscular contraction to take place, energy must be obtained from either adenosine triphosphate (ATP) or phosphocreatine (PCr) (Banerjee et al., 2010). The phosphorylated form of creatine, PCr, is synthesized in a reaction catalyzed by the enzyme creatine kinase. Creatine is a substance that is naturally formed in the body, but its skeletal muscle stores are most effectively increased from oral consumption (Riesberg, Weed, McDonald, Eckerson, & Drescher, 2016). The use of creatine as an ergogenic aid gained popularity following its reported success from athletes in improving performance during the 1992 Summer Olympics (Close, Hamilton, Philp, Burke, & Morton, 2016). Dietary creatine supplementation also became more common in the early 1990s in response to Harris, Söderlund, and Hultman (1992) whose findings supported the testimonies of the Olympic athletes.

In addition to enhancing athletic performance, creatine supplementation has lessened the severity of muscle-wasting disease symptoms, diminished negative effects of corticosteroid and chemotherapy treatment on skeletal muscle function, as well as modified regulatory protein expression in favor of protection against muscle atrophy. Four months of creatine supplementation in boys with Duchenne muscular dystrophy (DMD) improved body composition and grip strength of the dominant hand (Tarnopolsky et al., 2004). Menezes, Sobreira, Neder, Rodrigues-Júnior, and Martinez (2007) demonstrated that creatine supplementation lessened corticosteroid-induced respiratory dysfunction and muscle atrophy in the DIA of rats. Additionally, ex vivo creatine
treatment in the SOL and EDL muscles of rats prior to DOX incubation protected against DOX-induced force decline in the SOL muscle and dysfunction of both the EDL and SOL muscles (Bredahl and Hydock, 2017). Following hypoxia-induced damage in the DIA of spiny mice, Cannata et al. (2010) observed doubled concentrations of myostatin and muscle RING-finger protein-1 (MuRF-1) mRNA. Maternal creatine supplementation reduced the regulatory protein mRNA elevations, which indicated that creatine loading prior to birth may have protected the DIA against hypoxia-induced impairments.

In summary, it is well-established that inhibited muscle growth and increased muscle atrophy are present in a variety of tissues as a result of DOX administration. Specifically, these unfavorable outcomes may be accompanied by muscular dysfunction and fatigue in skeletal muscle. The DIA, the primary inspiratory muscle, is not spared from the negative consequences of DOX on skeletal muscle. This observation pertaining to the impact of DOX on the DIA may be of particular importance, because dyspnea is a widely reported side effect of chemotherapy treatment. Harmful outcomes of DOX administration on protein composition and functioning of skeletal muscle, including that of the DIA, have been alleviated in past research as a result of supplementation with creatine. Overall, this research may provide a greater understanding of mechanisms pertaining to DIA myotoxicity, based on myostatin and MuRF-1 protein expression, following DOX administration and pretreatment with creatine monohydrate (Cr) in rats. Due to the involvement of the DIA muscle in respiration, these findings may also provide insight regarding future treatment of dyspnea and related side effects of chemotherapy treatment.
Purpose Statement

The primary purpose of this study was to investigate the effects of *in vivo* DOX administration, following the completion of two dietary Cr supplementation protocols, on the expression of myostatin and MuRF-1 in the DIA tissue of rats sacrificed at one- and three-day time points after DOX injection.

Research Hypotheses

H1  DOX administration at a dose of 15 mg/kg will increase myostatin and MuRF-1 protein expression in the DIA tissue of rats.

H2  Both Cr supplementation protocols will attenuate DOX effects on myostatin and MuRF-1 expression in the DIA tissue of rats at both time points.

H3  The Cr loading protocol, consisting of 4% Cr supplementation for one week, followed by 2% Cr supplementation for three weeks, will attenuate the effects of DOX on myostatin and MuRF-1 protein expression in the DIA tissue of rats to the greatest extent at both time points.
CHAPTER II
REVIEW OF LITERATURE

Cancer

According to the American Cancer Society, there were approximately 17 million new cases of cancer worldwide in 2018, with more than 1.9 million of those cases in North America alone (American Cancer Society: Global Cancer Facts & Figures 4th Edition, 2018). In 2019, an estimated 606,880 people are predicted to die of cancer in the United States (American Cancer Society: Cancer Facts & Figures, 2019). The cancer death rate in the United States reached its highest point in 1991 after being fueled for many years by the widespread use of tobacco products. Advancements in cancer screening and treatment, as well as increased awareness of cancer-causing agents, such as tobacco, have fostered a gradual decline in the cancer death rate since its peak in the early 1990s.

Cancer is treated using a variety of methods, such as chemotherapy, hormone therapy, immunotherapy, radiation therapy, and surgery. While many negative side-effects are reported by patients undergoing cancer treatment, fatigue is the most prevalent (Morrow, Andrews, Hickok, Roscoe, & Matteson, 2002). Symptoms of cancer-related fatigue (CRF) have been reported by 50-90% of cancer patients worldwide (Mohandas, Jaganathan, Mani, Ayyar, & Rohini Thevi, 2017). It has been suggested that CRF may affect patients specifically undergoing chemotherapy and radiation therapy to the greatest
extent (Irvine, Vincent, Bubela, Thompson, & Graydon, 1991). Some of the most common symptoms experienced by patients with CRF are physical fatigue, mental fatigue, inability to concentrate, pain, stress, and nausea, which can all negatively impact quality of life, daily activities, and self-sufficiency (Mohandas et al., 2017).

**Chemotherapy and Doxorubicin (DOX)**

Chemotherapy is a type of cancer treatment that affects the entire body rather than a localized area, as radiation therapy does. This attribute is ideal for individuals with metastasized cancers and for patients whose objective is to eradicate, control the spread of, or alleviate symptoms caused by the cancer (American Cancer Society: Chemotherapy- What It Is, How It Helps, 2018). Anthracyclines are among the most successful chemotherapeutic drugs due to their efficacy in treating most cancers (Weiss, 1992). Doxorubicin (DOX), also known as Adriamycin, and daunorubicin (DNR) are two anthracycline anti-tumor antibiotics that were first isolated in 1967 from the *Streptomyces peucetius* bacterium species (Bonadonna et al., 1969). The single molecular variance between DOX and DNR lies in the side chain, by which the actions of the two drugs are differentiated (Minotti, Menna, Salvatorelli, Cairo, & Gianni, 2004). While DNR treatments are utilized primarily for fighting certain leukemias, DOX treatments are ideal for combatting a variety of cancers such as lymphoma, leukemia, and neuroblastoma, as well as lung, thyroid, ovarian, bladder, and stomach cancer. Based on its intended target, DOX can be injected intravenously or intraperitoneally for the treatment of various cancers.

Although the exact mechanisms remain unclear, there are two probable ways in which DOX acts in the body to disrupt cancer growth: 1) DOX acts in the nuclei of cells
to prevent an enzyme, topoisomerase-II, from disentangling deoxyribonucleic acid (DNA) strands, which ultimately leads to DNA damage and cell death (Thorn et al., 2011) and 2) DOX presence in the cell causes free radical formation, which leads to an imbalance in the ability of the cell to detoxify intermediates, disruptions in cell signaling, and damage to the cell membrane (Gewirtz, 1999). While treatment with DOX can be helpful in disrupting and preventing certain types of cancer growth and is sometimes necessary for survival, it can harm and inflict damage to many physiological processes in the body that were functioning normally and healthily prior to treatment.

**Doxorubicin (DOX)-Induced Toxicities**

While DOX treatment may provide anti-cancer benefits over a wide range of action, a major shortcoming of DOX treatment is the toxicity inflicted on the patient. DOX-mediated toxicity has a direct relationship to the treatment dose and can impair functioning of the heart, brain, liver, and kidneys (Carvalho et al., 2009). This damage may lead to other malignancies in the body, such as cachexia, myotoxicity, cytotoxicity, muscle fatigue, muscle dysfunction, and cardiotoxicity, among others. The standard used to predict the degree of toxicity from DOX treatment is the total dose administered to the patient (Thorn et al., 2011). For this reason, the dose administered to each individual undergoing treatment should be methodically and vigilantly considered (Carvalho et al., 2009).

One of the primary motives for the dose-limited administration of DOX is its ability to induce long-lasting and severe cardiotoxic effects (Carvalho et al., 2009). The heart is especially susceptible to DOX-induced toxicity for several reasons, but one of the most prominent is that superoxide production is promoted in the mitochondria of the
heart (Nohl, 1987). This generation of ROS is compounded because cardiomyocytes have a higher density of mitochondria per unit volume in comparison to other tissues (Berthiaume & Wallace, 2007) in combination with lower levels of antioxidant enzymes (Odom, Hatwig, Stanley, & Benson, 1992). Amelioration of cells following DOX-induced damage is problematic because only half of cardiomyocytes are renewed throughout the lifespan (Carvalho & de Carvalho, 2010). It has also been suggested by Damiani et al. (2016) that damage induced from DOX may be caused by an interaction of the drug and iron, which leads to increased ROS generation and the eventual impairment of mitochondria. Moreover, Damiani et al. (2016) highlighted that treatment with DOX causes an interference with redox cycling, which has the potential to influence other physiological processes and lead to heart failure.

Cachexia

Cachexia is a complex metabolic condition that is associated with underlying diseases, illnesses, or medical conditions such as chronic obstructive pulmonary disease (COPD), multiple sclerosis, chronic heart failure, and cancer, among others (Aoyagi, Terracina, Raza, Matsubara, & Takabe, 2015). Cancer cachexia is characterized by a progressive and involuntary loss of lean body mass, with or without fat mass loss, that cannot be completely ameliorated with nutritional interventions (Fearon et al., 2011). Some evidence suggests that chemotherapy drugs, such as DOX, may play a role in promoting cachexia development, but the exact mechanisms leading to this association are yet to be fully elucidated (Pin, Barreto, Couch, Bonetto, & O’Connell, 2019). Pin et al. (2019) utilized two murine models of cancer-induced and chemotherapy-induced cachexia to investigate the pathways through which cachexia is developed and sustained.
For the first time, it was demonstrated that there are differences in metabolic derangements based on the cause of cachexia, which indicated that therapeutic treatments should highlight these variances. For example, there was a significant increase in low-density lipoprotein particles only in the cancer-induced cachexia model, while elevated skeletal muscle ROS were observed in both the cancer- and chemotherapy-induced cachexia models.

Several factors other than those pertaining to nutrition have been explored as mediators of skeletal muscle atrophy in cachexia, because cachexia may occur even in circumstances of normal energy intake and expenditure (Tisdale, 1999). Some of the investigated factors, such as cytokines and elements of tumors, have the ability to mobilize amino acids from skeletal muscle tissue and fatty acids from adipose tissue. This may account for the dramatic losses in muscle and, sometimes, fat mass that are observed in individuals with cachexia. Barreto et al. (2016) reported that chemotherapy in mice may have instigated changes in mitochondrial functioning and expression, which may have contributed to the muscle wasting observed following treatment. Similarly, Gilliam et al. (2013) observed that skeletal muscle mitochondria were a main source of oxidants and that treatment with DOX in rats disrupted mitochondrial functioning. It was suggested that there was an increase in ROS as a result of this disturbance, which offered a potential explanation for the decreased energy expenditure and respiratory capacity that was noted by Gilliam et al. (2013). Furthermore, Fabris and MacLean (2018) were the first to report that DOX administration affects amino acid concentrations in skeletal muscle, the vasculature, and the space between the skeletal muscle and vasculature. A possible dose-dependent effect of DOX on amino acid transport mechanisms from the
skeletal muscle into the vasculature was reported. These findings indicated that amino acid metabolism may be a factor involved in cachexia, in relation to protein synthesis and degradation and should be a future therapeutic target in chemotherapy patients.

Attempts have been made in several studies to prevent chemotherapy-induced cachexia by blocking certain mechanisms of the pathways that allow for skeletal muscle loss. Hulmi et al. (2018) were the first to compare the effects of blocking activin receptor type IIB (ACVR2B-Fc), by treatment with a soluble ligand binding domain, on components of cachexia in skeletal and cardiac muscle tissue following DOX administration. Blocking ACVR2B-Fc ligands led to a significant decrease in skeletal muscle wasting, but only slightly decreased cardiac muscle atrophy. While cardiac and skeletal muscles exhibited similar wasting due to DOX treatment, changes in transcriptional factors were most noticeable in skeletal muscle. One plausible explanation for this dissimilarity may be related to the gene expression of myostatin, which was 18 times higher in the skeletal muscle compared to cardiac muscle. Gene expression of myostatin, an ACVR2B ligand, can directly affect regulator factors of the cell cycle, such as the inhibitory protein p21 (Patel, A., Tripathi, Patel, U., Shah, & Joshi, 2014).

**Fatigue and Dysfunction**

In addition to cachexia, treatment with DOX has caused both generalized and skeletal muscle fatigue, muscle dysfunction, and damage to a variety of tissues. According to Miller et al. (2007), over half of a sample of chemotherapy patients reported generalized fatigue that lingered for weeks following treatment. Moreover, skeletal muscle atrophy has been correlated with increased rates of disease and death among patients (Powers, Lynch, Murphy, Reid, & Zijdewind, 2016). These realities signify the
importance of understanding the mechanisms responsible for the presentation of fatigue and atrophy of individuals undergoing chemotherapy treatments.

Increased fatigability and atrophy of skeletal muscle following DOX administration has been reported in both animals and humans. For instance, *ex vivo* maximal force, rate of contraction, and relaxation velocity were compromised in the EDL muscle of mice following two hours of incubation with DOX (van Norren et al., 2009). Also, van Norren et al. (2009) observed that the calcium (Ca2+) response of C2C12 myotubes previously incubated with DOX was altered after stimulation with caffeine or ATP. Stimulation of the C2C12 myotubes *in vitro* with caffeine and ATP caused marked increases in Ca2+ influx, which demonstrated that DOX treatment influences Ca2+ handling and, therefore, impacts muscular contraction. As reported by Nguyen et al. (2016), coadministration of DOX with Ca2+ resulted in a drastically higher rate of cell survival compared to cells treated only with DOX. Also, Ca2+ presence played a role in the suppression of DOX cytotoxicity in human breast cancer cells. This finding is especially relevant to individuals treated with DOX who are simultaneously administered another drug or dietary supplement that alters Ca2+ levels. For example, Vitamin D supplementation may increase Ca2+ concentrations in the body (Lips, 2012) and may, therefore, influence the efficacy of DOX treatment.

Skeletal muscle dysfunction is a common consequence of chemotherapy treatment, as observed in animals and humans, and occurs through a variety of mechanisms and mediators. One potential mediator of muscle contractile dysfunction is tumor necrosis factor-alpha (TNF-α), a cytokine that promotes inflammation. Gilliam et al. (2009) noted that treatment with DOX in mice resulted in decreased maximal specific
and absolute force production as well as earlier exhaustion of the EDL muscle under a fatigue protocol. Additionally, elevated concentrations of TNF-α were observed 72 hours following injection with DOX. To investigate whether the observed dysfunction was mediated by TNF-α and TNF-α receptor 1, the same experiment was repeated with mice lacking ample amounts of TNF-α receptor 1. While time to fatigue was not significantly altered, the decrease in maximal specific force production caused by DOX injection was averted in mice with the TNF-α receptor 1 deficiency. Based on these results, Gilliam et al. (2009) were the first to suggest targeting the TNF-α and TNF-α receptor 1 signaling pathway as a potential therapeutic intervention for chemotherapy patients to attenuate muscle dysfunction.

It is unclear if the degree of skeletal muscle dysfunction and myotoxicity commonly observed following DOX treatment is related to that of cardiotoxicity. Hydock et al. (2011) were the first to investigate the effects of three different doses of DOX on EDL and SOL muscle dysfunction in rats five days following injection and compared the outcomes to those of DOX on cardiac dysfunction. Treatment with all three doses of DOX led to a fatigue pattern where the SOL muscle, which is composed primarily of slow-twitch fibers, contracted with significantly less force compared to baseline measures at roughly 40 to 50 seconds earlier than control. However, DOX treatment did not cause the same decline in fatigue resistance in EDL muscle, comprised mostly of fast-twitch fibers, where contraction force was only significantly less than baseline 20 seconds earlier than control at most. There was variation between DOX doses in contraction force of both SOL and EDL muscles under the fatigue protocol, which indicated that DOX-induced muscle dysfunction was dependent on the dose administered. Muscle function
was impaired in the heart to the same extent as SOL muscle, which highlighted the relationship between cardiac dysfunction and DOX treatment in contributing to the decline in skeletal muscle function.

While the dose-dependent relationship of DOX administration on skeletal and cardiac muscle dysfunction was identified by Hydock et al. (2011), a time-dependent gradual increase in muscle dysfunction in rats was observed by Hayward et al. (2013) one-, three-, and five-days post-injection with DOX. Accumulation of DOX was different in smooth, skeletal, and cardiac tissue, but was the greatest in cardiac tissue one-day post-treatment and steadily declined over the course of the five-day study period. Decline in contractile function of the SOL and EDL muscles was similar even though there was a significant difference in DOX accumulation. Moreover, this was the first study to quantify DOX in vascular tissue as well as describe changes in accumulation over time. Taken together, these findings indicated that interactions between physiological factors, rather than DOX accumulation alone, may influence muscle dysfunction, such as those between cardiac muscle, the vasculature, and skeletal muscle (Hayward et al., 2013).

**Cardiotoxicity**

The effects of DOX administration on physiological processes in the human body have been widely investigated, especially those pertaining to cardiotoxicity. Cardiac dysfunction is the most severe side effect of DOX treatment and can ultimately lead to heart failure (Zhang, Shi, Li, & Wei, 2009). Specifically, long-term toxicity in the heart leads to dysfunction of the left ventricle, which can progress to congestive heart failure (Slørdal & Spigset, 2006). The risk for developing congestive heart failure following treatment with a cumulative DOX dose of 700 mg/m² is between 18 and 48% (Senkus &
Jassem, 2011), but the dose-response relationship pertaining to cardiotoxicity may fluctuate between individuals (Khakoo & Yeh, 2008). Potential mechanisms of DOX cardiotoxicity have been explored but are yet to be fully understood. He et al. (2018) created a physiologically-based pharmokinetic (PBPK) model to measure the outcomes of DOX administration in as broad of a context as its effects on physiological systems to a narrow focus on its effects in organelles of the cell. The PBPK model utilized by He et al. (2018) pointed to oxidative stress as the principal mechanism for DOX-induced cardiotoxicity in humans.

**Oxidative Stress and Reactive Oxygen Species (ROS)**

Oxidative stress occurs when there is a disruption in the balance between ROS creation and antioxidant protections. The stability of molecules is decreased if they possess unpaired electrons, which is a defining quality of free radicals (Betteridge, 2000). This attribute causes free radicals to be highly reactive, with some possessing potencies higher than others. While oxidative stress cannot be measured directly, it can be measured through the quantification of oxidative damage products, such as those resulting from protein oxidation, lipid peroxidation, and DNA oxidation (Betteridge, 2000).

Skeletal muscle proteolytic pathways are highly influenced by the presence of ROS, which partially encourage protein breakdown in muscle by activating protein degradation systems, such as the ubiquitin-proteasome pathway. One of the main sources of ROS is nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, also known as NOX (Panday, Sahoo, Osorio, & Batra, 2015). The enzyme NOX is comprised of
numerous subunits that constitute elements of the cell membrane and cytosol and is partially responsible for ROS production.

It is well-established that the over-expression of transforming growth beta factor (TGF-β), a regulator of inflammation and fibrosis in several tissues, contributes to muscle degradation and decline of myosin and actin, contractile proteins. Narola, Pandey, Glick, and Chen (2013) demonstrated that an over-expression of transforming growth factor beta 1 (TGF-β1) caused skeletal muscle atrophy in mice, with the degree of muscle wasting being directly related to the total TGF-β1 and endogenous TGF-β1 expression. Mendias et al. (2011) measured the contractility of the EDL muscle in mice in vitro five days following injection with TGF-β. Following injection, whole muscle and individual muscle fiber cross-sectional area was significantly decreased together with maximum isometric force production. TGF-β treatment also increased atrogin-1 expression in the tibialis anterior muscles, which was anticipated.

While it is well-known that TGF-β causes skeletal muscle atrophy, the exact mechanisms of this relationship remain undetermined. However, Abrigo, Rivera, Simon, Cabrera, and Cabello-Verrugio (2016) were the first to state that TGF-β exposure stimulated NOX-induced ROS production. This finding helped to establish a component of the mechanism for skeletal muscle atrophy. Moreover, markers of muscle atrophy elicited by TGF-β1 were lessened when cells were treated with NOX inhibitors or antioxidants. Taken together, these findings provide insight as to the potential mechanism of skeletal muscle atrophy induced by TGF-β presence in skeletal muscle tissue and offer targets for therapeutic intervention in individuals with diseases that induce skeletal muscle atrophy.
If antioxidant defenses are weakened as a result of cancer treatment, for example, tissue damage may occur. Drugs typically used in cancer treatments, such as DOX, alter the structure of myosin and impair mitochondria while concurrently increasing ROS production (Carvalho et al., 2009). In addition to structure being altered, a decrease in overall myosin expression in C2C12 murine myotube cell cultures has been observed following DOX treatment (Guigni et al., 2018). Co-administration of DOX with MitoQ, an antioxidant that targets mitochondria, inhibited the reduction of myosin expression, loss of mitochondria, and creation of ROS previously observed in murine C2C12 myotubes by Guigni et al. (2018). Similarly, Gilliam et al. (2012) treated C2C12 differentiated myotubes with DOX and observed an approximate 50% decrease in myosin and actin expression. Oxidant activity in the cytosol of myotubes was elevated only hours following DOX treatment and expression of ubiquitin ligases, MuRF-1 and MAFbx, was significantly increased 24 hours post-treatment. Involvement of mitochondria in this process was verified by the administration of a peptide antioxidant, capable of permeating the cell, that concentrated in the inner membrane of the mitochondria and improved mitochondrial functioning, similar to the technique used by Zhao et al. (2004).

In agreement with Gilliam et al. (2012), Min et al. (2015) reported that treatment with a specific peptide in the mitochondria is capable of inhibiting DOX-induced dysfunction. Moreover, increased creation of oxidants by mitochondria encourages the development of, and is necessary for, skeletal and cardiac muscle dysfunction (Min et al., 2015). Treatment of the myotubes with the antioxidant by Gilliam et al. (2012) also prevented expression of the ubiquitin ligases, which indicated that DOX utilized ROS in the mitochondria to induce muscle degradation.
Negative Regulators of Muscle Growth

Myostatin

TGF-β is a multifunctional cytokine and main inhibitor of myogenesis (Hodgson, Mafi, R., Mafi, P., & Khan, 2018). Myostatin belongs to the TGF-β superfamily of cell regulatory proteins and is essential to the proper regulation of skeletal muscle hypertrophy in mammals (Ríos, Fernández-Nocelos, Carneiro, Arce, & Devesa, 2004). Also known as growth differentiation factor-8 (GDF-8), myostatin is created and released by myocytes that negatively regulate skeletal muscle mass. Unlike other members of the TGF-β family, myostatin is confined solely to the skeletal muscle tissue during development (Ríos, Carneiro, Arce, & Devesa, 2002).

Components of muscle atrophy, such as muscle denervation and disuse, have been associated with an increase in myostatin expression in skeletal muscle tissue (Delfino et al., 2013). McPherron, Lawler, and Lee (1997) first identified GDF-8 and determined its expression was specific to both developing and adult skeletal muscle. Following myostatin disruption in mice, McPherron et al. (1997) also concluded that a combination of muscle cell hypertrophy and hyperplasia encouraged the dramatic increase in skeletal muscle mass. Certain genetic mutations have been identified in mice and cattle where myostatin was essentially non-existent or non-functioning in the muscle tissue. These circumstances resulted in muscle doubling and unregulated muscle growth (Kambadur, Sharma, Smith, & Bass, 1997). Zimmers et al. (2002) were able to induce a condition in adult mice similar to cachexia, where significant losses in fat and muscle mass were detected following injections of circulating myostatin into the thighs. This observation provided insight about the function of myostatin in cachexia and genetic conditions.
Similar to other regulatory proteins belonging to the TGF-β family, myostatin signals to the muscle cell with the assistance of a receptor complex comprised of the activin type I and type II receptors (Ríos et al., 2004). Myostatin shares the receptor pair with other members of the TGF-β family, which suggests that there is a mechanism that restricts myostatin actions to the muscle cell. The signaling pathway utilized by myostatin involves the binding of myostatin directly to one of the activin type II receptors (Rodriguez et al., 2014). This binding leads to the activation of the type I receptors and eventual activation of Smad proteins. Following activation, Smad proteins translocate into the nuclei of cells, prompting gene transcription and interactions with promoter DNA sequences (Derynck, Zhang, & Feng, 1998). Moreover, Taylor et al. (2001) observed that myostatin partially controls muscle mass by preventing growth and regeneration through the inhibition of DNA and protein synthesis. Overall, myostatin acts through several interactions with other proteins and signaling pathways to regulate skeletal muscle growth.

Joulia et al. (2003) provided data which indicated that MyoG, one of the MRFs, is likely a main target of endogenous myostatin. An overexpression of myostatin influenced myogenic protein factor levels, which signified the potential for myogenin transcription influence. However, Ríos et al. (2002) found that an overexpression of myostatin specifically downregulates messenger ribonucleic acid (mRNA) levels of positive regulatory factors of muscle mass, thus inhibiting muscle growth. Furthermore, Joulia et al. (2003) determined that p21 is an additional target of myostatin. This conclusion was in agreement with that of Thomas et al. (2000), who suggested that an increase in p21 expression was initiated by myostatin signaling.
It has been well-documented that myostatin directly regulates the formation of excess fibrous connective tissue in skeletal muscle, which can lead to scarring and hardening of the tissue. Muscle tissue fibrosis is attributed to an excess deposition of collagen, along with other extracellular matrix (ECM) components, by muscle fibroblasts (Wynn, 2008). Li, Kollias, and Wagner (2008) observed that myostatin induced the production of ECM proteins, such as collagen, both in vitro and in vivo, and that myostatin directly regulated muscle fibroblasts and myocyte growth. This finding offered support for myostatin knockout models as future research interests to treat diseases associated with skeletal muscle fibrosis.

Increasing muscle mass and strength, which has been demonstrated after the inhibition of myostatin activity, could be an effective method for researching the potential treatment of several degenerative muscle diseases (Tobin & Celeste, 2005). Several studies have utilized myostatin knockout models and myostatin inhibitory models to research prospective treatments for individuals with degenerative muscle diseases. For the first time, Whittemore et al. (2003) demonstrated that skeletal muscle mass and grip strength were increased following treatment with an inhibitory antibody to myostatin. Lin et al. (2002) were the first to report that an inhibition of ligands of the myostatin family, using two models of cancer cachexia, significantly increased muscle mass in adult mice. There was as much as a 40% increase in the muscle mass of the myostatin-family ligand-inhibited mice compared to control mice. This result offered support for the future use of cancer cachexia models in the continued exploration of therapeutic treatments for individuals with muscle wasting diseases.
In a study which utilized the *mdx* mouse model of DMD, an inherited disease of progressive muscle degeneration, myostatin inhibition *in vivo* attenuated the dystrophic phenotype (Bogdanovich et al., 2002). Over a period of three months, myostatin inhibition was completed by means of intraperitoneal injections of blocking antibodies. This resulted in a decrease in muscle breakdown and an increase in body weight, absolute muscle strength, muscle mass, and muscle hypertrophy. In a similar limb-girdle muscular dystrophy model by Bogdanovich, McNally, and Khurana (2008), myostatin blockade resulted in gains in muscle mass, size, and absolute force. However, there were no significant improvements in the histology of the skeletal muscle tissue. These observations indicated that limitations of therapeutic interventions exist depending on the severity of the degenerative muscle disease (Bogdanovich et al., 2008).

The myostatin signaling pathway may be a desired target for therapeutic intervention, because gene inactivation and drug administration have successfully modulated the pathway in favor of reducing the negative effects of cancer cachexia. The influence of TNF-α, a cachectic factor, on the myostatin signaling pathway was investigated by Costelli et al. (2008). Levels of myostatin and follistatin, an endogenous myostatin inhibitor, were elevated seven days following tumor transplantation into the gastrocnemius muscle of rats. Pentoxifylline (PTX), an anti-inflammatory drug that inhibits TNF-α, was administered to the tumor-bearing rats, which resulted in decreased myostatin expression after four days and increased follistatin activity after seven days. Similarly, Klimek et al. (2010) systemically administered Activin receptor extracellular domain/Fc fusion protein, ACVR2B-Fc, in an attempt to impede myostatin expression in a mouse model of cancer cachexia. Tumor growth and adipose tissue stores were
unaffected while muscle atrophy was significantly prevented. Moreover, Gallot et al. (2014) employed myostatin gene inactivation in tumor-bearing mice, which prevented the expression of MuRF-1 and muscle atrophy F-box (MAFbx), two ubiquitin ligases. Proteolytic pathway activation was hindered due to the inhibition of the ubiquitin ligases, which encouraged further investigation of these pathways.

**Ubiquitin Ligases**

The ubiquitin-proteasome system is the primary regulatory mechanism of protein degradation in skeletal muscle and is highly influenced by muscle-specific ubiquitin-ligase enzymes, such as MuRF-1 and MAFbx, also known as atrogin-1. Expression of MuRF-1 and MAFbx has been upregulated in at least 13 models of skeletal muscle atrophy and is a notable component of many physiological conditions and chronic diseases pertaining to muscle disuse (Clarke et al., 2007). Common human pathologies associated with muscle atrophy, and its correlation to MuRF-1 and MAFbx expression, are chronic kidney disease, diabetes, cancer cachexia, chronic obstructive pulmonary disease, and age-related muscle loss (Rom & Reznick, 2016).

MuRF-1 and MAFbx levels are promptly activated in several models of muscle atrophy, which denotes that these proteins may play a role in encouraging muscle wasting (Foletta, White, Larsen, Léger, & Russell, 2011). In animal models, muscle remodeling has been closely associated with the expression of MuRF-1 and MAFbx, which are influenced by numerous lifestyle factors, such as alcohol consumption, physical activity, smoking, and diet (Rom & Reznick, 2016). Several agents have demonstrated an ability to lessen the severity of muscle atrophy through the inhibition of MuRF-1 or MAFbx expression via two pathways: the p38 mitogen-activated kinase (MAPK) pathway and the
nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway (Rom & Reznick, 2016). Oxidative stress and inflammatory cytokines, such as interleukin-1 (IL-1), have demonstrated an ability to activate the p38 MAPK and NF-κB pathways. Moreover, TNF-α and IL-1 activate the NF-κB pathway, which prompts skeletal muscle degradation via the transcriptional upregulation of MuRF-1. Both TNF-α and IL-1 activate the p38 MAPK pathway, which stimulates MAFbx expression (Glass, 2005).

While the role of ubiquitin ligases in skeletal muscle atrophy has been well-established, their involvement in cancer cachexia remains largely unexplored. Mechanisms of muscle atrophy were studied by Yuan et al. (2015) in a colon cancer cachexia mouse model and C2C12 cells were also treated with TNF-α. Expression of MuRF-1 and atrogin-1 in the skeletal muscle of individuals with malignant and benign disease were analyzed. Ubiquitin ligases were upregulated during cancer cachexia in the mouse model, while a statistically significant excess of atrogin-1 expression was observed in the C2C12 cells. A knockdown of atrogin-1 by small interfering RNA demonstrated a protective effect on the C2C12 cells from TNF-α, which indicated that atrogin-1 may be a therapeutic target for treating muscle atrophy in individuals with cancer cachexia and warrants further investigation (Yuan et al., 2015).

Myogenic Regulatory Factors (MRFs)

Approximately 50% of adult human body mass is comprised of skeletal muscle tissue (Zanou & Gailly, 2013). Skeletal muscle has the ability to regenerate in the event of damage. This tissue damage ranges in severity from small tears, due to daily activity, to acute muscle loss in cases of degenerative muscle disease or trauma. Regeneration takes place with the assistance of satellite cells, which are skeletal muscle stem cells that
activate in response to stimuli, such as damage (Zanou & Gailly, 2013). When prompted, satellite cells express transcription factors belonging to the MRF family: Myogenic Factor 5 (MYF5), Myogenic Differentiation (MyoD), Myogenin (MyoG), and Myogenic Regulatory Factor (MRF4) (Zammit, 2017). These positive regulators of muscle mass play a key role in muscle tissue growth by controlling phases of development and guiding myoblast proliferation and differentiation into multinucleated single muscle cells (Zanou & Gailly, 2013).

**Insulin-like Growth Factor 1 (IGF-1)**

Insulin-like growth factor (IGF-1) is a protein that encourages muscle growth in childhood and induces anabolic effects in the skeletal muscle tissue of adults. Myogenic differentiation, a process that results in mature skeletal muscle formation, is primarily regulated by myostatin and IGF-1 (Retamales, et al. 2015). Insulin-like growth factors (IGFs), alongside MRFs, are essential to muscle repair (Zanou & Gailly, 2013). Furthermore, expression of IGFs is controlled, in part, by MRFs and stimulation of IGFs induces MRF activity.

A variety of growth factors are released during the repair of muscle tissue. Among these growth factors, IGFs solely encourage myocyte growth and differentiation (Zanou & Gailly, 2013). IGF-1 activates the phosphatidyl-inositol 3-kinase (PI3K)-Akt pathway, which prompts skeletal muscle hypertrophy and is necessary during tissue repair (Glass, 2003). In addition to activating the PI3K-Akt pathway, IGF-1 also inhibits specific factors that negatively impact muscle growth and encourage skeletal muscle wasting. Stitt et al. (2004) were the first to display that IGF-1 functions to impede pathways associated
with MuRF-1 and MAFbx. Glass (2005) agreed that transcriptional upregulation of the ubiquitin ligases, MURF-1 and MAFbx, may be inhibited by IGF-1.

The findings of Retamales et al. (2015) provided evidence of cross-talk between IGF-1 and myostatin signaling pathways. Incubation of skeletal myoblasts with myostatin resulted in a significant increase in the ratio of phosphorylated Smad3 (pSmad3) to Smad3. This effect was prevented when myostatin was incubated with a Smad3 inhibitor. Myoblasts incubated with myostatin expressed a significantly lower MyoD expression and decreased fusion of myoblasts, which indicated a dependence of signaling on Activin receptor-like kinase/Smad3 (Retamales et al., 2015). Furthermore, pre-incubation of skeletal myoblasts with IGF-1 prevented the increased ratio of pSmad3:Smad3 that was demonstrated following myostatin incubation. Pre-incubation with IGF-1 also impeded the previously observed inhibitory effect of myostatin on MyoD levels. Lastly, in myoblasts preincubated with IGF-1 and treated with myostatin, the interaction of Smad3 and Akt was greater than the interaction observed following IGF-1 preincubation alone. Taken together, the results of Retamales et al. (2015) indicate that there is cross-talk between growth proteins and that the IGF-1/PI3K/Akt pathway prevents myostatin signaling during myoblast differentiation.

Forkhead Box O (FoxO)

Forkhead box O (FoxO) transcription factors are involved in numerous signaling pathways and play key roles in regulation, cell fate decisions, and tumor suppression (Fu & Tindall, 2008). Regulated by growth factors and stress, FoxO transcription factors are a subfamily of the fork head transcription factor family and are also involved in muscle atrophy (Farhan et al., 2017). Following the addition of IGF-1 to two cell models of
muscle atrophy, Sandri et al. (2004) found that atrogin-1 transcription was curbed and phosphorylated FoxO1, FoxO3, and FoxO4 concentrations were elevated. This suppression of atrogin-1 in the cell models was controlled by the PI3K/Akt pathway. Sandri et al. (2004) also determined that atrogin-1 expression in the skeletal muscle tissue of adult mice was subdued by Akt activity, which involved the inactivation of FoxO transcription factors. Moreover, Stitt et al. (2004) stated that FoxO inhibition was necessary for the IGF-1/PI3K/Akt pathway to prevent the expression of the ubiquitin ligases, MuRF-1 and MAFbx, and thus, block atrophy activity. FoxO3 is negatively regulated by the NF-κB pathway, which is triggered by the presence of inflammatory cytokines (Schiaffino, Dyar, Ciciliot, Blaauw, & Sandri, 2013). There is a highly-coordinated feedback loop between FoxO proteins and reactive oxygen species (ROS), also known as free radicals (Farhan et al., 2017). Inhibition of FoxO transcription factors, therefore, may be a practical approach to encourage anti-atrophy activity.

**Dexamethasone (DEX)**

Glucocorticoids are steroid hormones that mediate muscle loss in several catabolic states. Dexamethasone (DEX), a synthetic glucocorticoid, may be used by individuals to control inflammation resulting from a variety of conditions (Assali, Shawahna, Dayyeh, Shareef, & Alhimony, 2018). For example, DEX is sometimes administered following chemotherapy to curb the negative side effects of treatment, such as inflammation and nausea.

High doses and chronic use of glucocorticoids can lead to losses in skeletal muscle strength and mass due to increases in myofibrillar protein degradation and decreases in protein synthesis (Kayali, Young, & Goodman, 1987). The deletion of
MuRF-1 and MAFbx in a mouse model has spared muscle mass specifically following muscle denervation, but the role of the ubiquitin ligases in atrophy-inducing conditions is less certain (Baehr, Furlow, & Bodine, 2011). Baehr et al. (2011) determined that mice with a MuRF-1 deletion had more muscle mass sparing compared to control mice following two weeks of DEX treatment. This result indicates that the principal role of MuRF-1 may be something other than regulating protein degradation. However, there was no evidence that the MAFbx deletion attenuated muscle mass losses when compared to control mice. These findings support the concept that MuRF-1 and MAFbx may function dissimilarly under different atrophy models (Baehr et al., 2011).

The role of myostatin in the skeletal muscle tissue in potentially aiding muscle atrophy following treatment with DEX has also been investigated. Allen & Loh (2011) demonstrated that myostatin mRNA levels were doubled in fast-twitch skeletal muscle fibers in mice following DEX injection in vivo. Similarly, Gilson et al. (2007) demonstrated that there was an increase in the mRNA of enzymes involved in protein breakdown following DEX treatment in mice. Moreover, DEX treatment did not significantly impact mRNA levels in myostatin knockout mice, which indicated that myostatin omission prevents skeletal muscle atrophy in mice treated with DEX. Administration of DEX has also been associated with an upregulation of myostatin gene expression in mice (Qin et al., 2013).

Myosin heavy chain (MHC), the motor protein of the thick filaments in skeletal muscle, has multiple isoforms with different characteristics utilized in muscle function modulation (Wells, Edwards, & Bernstein, 1996). It is well established that DEX treatment impacts MHC expression. Chromiak and Vandenburgh (1992) first concluded
that DEX treatment prompted a loss of MHC protein in avian fast-twitch skeletal myofibers in vitro that was attenuated by mechanical stimulation. Research pertaining to the effects of DEX treatment on MHC was advanced by Clarke et al. (2007), where C2C12 myotubes were treated with the synthetic glucocorticoid DEX for one day. There was a loss of MHC protein and an increase in MuRF-1, as well as evidence that MHC physically associates with MuRF-1 in the skeletal muscle. Therefore, MHC protein expression was preserved through the inhibition of MuRF-1 in mice treated with DEX (Clarke et al., 2007). Castillero, Alamdari, Lecker, and Hasselgren (2013) provided evidence for a compensatory mechanism that regulates the ubiquitin ligases, since suppression of atrogin-1 elicited augmented expression of MuRF-1, and vice versa, following DEX treatment. Furthermore, the findings of Castillero et al. (2013) provided support for the hypothesis that both MuRF-1 and atrogin-1 expression must be inhibited to prevent the muscle atrophy and protein degradation that occur as a result of DEX treatment.

**Creatine Supplementation**

Creatine is a substance that is naturally synthesized in the human body by the liver and plays an important role in facilitating energy provision during muscular contraction (Fairman et al., 2019). In addition to being formed in the body, creatine can be obtained in the diet from certain meats and fish. Dietary supplementation is the most efficient way to increase creatine stores in both skeletal muscle and the brain (Riesberg et al., 2016). Roughly 95% of creatine is located in the skeletal muscle tissue and the remaining approximate 5% is distributed amongst the brain, kidneys, and testes (Poortmans, Rawson, Burke, Stear, & Castell, 2010). Moreover, nearly two-thirds of
creatine in the skeletal muscle tissue is in the form of PCr, while the other approximate one-third is in the form of free creatine (Wyss & Kaddurah-Daouk, 2000).

Energy for muscular contractions is acquired from PCr, a phosphorylated creatine molecule, and ATP, an organic chemical (Banerjee et al., 2010). At rest, ATP is produced in the mitochondria through oxidative phosphorylation. However, short-duration, high-intensity resistance exercises rely on the phosphagen system for ATP production in maximal efforts lasting less than 10 seconds, while anaerobic glycolysis is utilized for energy production between 10 and 30 seconds (Hall & Trojian, 2013). In a reaction catalyzed by phosphoryl creatine kinase (CK), ATP is used to convert creatine to PCr in the sarcoplasm of the muscle cell (Andres et al., 2017). The reverse of this reaction is utilized to provide additional ATP in situations that necessitate a higher ATP demand, such as the muscular contraction required during exercise or physical activity (Poortmans et al., 2010). Based on these mechanisms, creatine may also function to uphold a pool of PCr to be utilized for energy needs.

Creatine supplementation is successfully used as an ergogenic aid for activities that involve repeated bouts of high-intensity exercise. This is not only due to the increase in PCr stores available to be utilized, but also possibly because of increased rates of PCr resynthesis during rest and recovery (Lemon, 2002). Furthermore, the onset of fatigue may be delayed by increasing PCr stores through creatine supplementation, which may enhance performance during maximal-effort exercises. Several studies have reported that total creatine and PCr stores in skeletal muscle tissue were increased by supplementing with oral creatine for five to seven days with a dose of approximately 20 to 25 grams (g)
per day (Hall & Trojan, 2013). Moreover, uptake of creatine into the muscle tissue was highest two days following the start of supplementation in a study by Harris et al. (1992).

The most effective dosing for creatine supplementation involves a brief loading period followed by several weeks of a maintenance period. Although loading doses are the most constructive means of supplementation, they are not required to increase stores of creatine in skeletal muscle tissue (Hall & Trojan, 2013). According to Hultman, Söderlund, Timmons, Cederblad, and Greenhaff (1996), creatine loading could be accomplished in the average adult male by consuming a loading dose of 0.3 g of creatine per kilogram (kg) of body mass per day for between five and six days. An adequate maintenance dose following the loading period was 0.03 g of creatine per kg of body mass per day. Maintenance dosing is typically upheld for a period for four to six weeks depending on the intended goal of the individual (Hall & Trojan, 2013). Furthermore, supplementation with 2–5 g per day of pure creatine has been recommended in adults and older individuals as a preventative health measure. This is especially important in older and elderly adults, because these populations tend to eat less meat compared to the general population (Wallimann, Tokarska-Schattner, & Schlattner, 2011). The largest and most long-term reported dose of creatine that has safely been consumed by healthy individuals, and even patient populations, of all ages was a maximum dose of 30 g per day for five years (Kreider et al., 2017).

Aguiar, Januário, et al. (2013) were the first to exclusively examine long-term creatine supplementation in combination with 12 weeks of resistance training in healthy older females. Long-term creatine supplementation paired with resistance training resulted in improved submaximal strength functional abilities and maximal strength.
Likewise, Candow et al. (2014) compared the outcomes of creatine supplementation in healthy older males and females before versus after supervised resistance training sessions for 12 weeks and was the first study to do so. Improvements in lean tissue mass, limb muscle hypertrophy, and muscular strength were comparable between groups, which indicated that timing of supplementation may not be as important in older individuals as in younger populations. However, Candow, Vogt, Johannsmeyer, Forbes, and Farthing (2015), who also utilized an older untrained participant population, concluded that resistance training elicited the greatest improvements in lean tissue mass when creatine was ingested immediately after training.

As a potential therapeutic treatment, the effect of oral creatine supplementation on muscular strength in individuals with Duchenne muscular dystrophy (DMD) was investigated by Banerjee et al. (2010). Creatine supplementation led to a statistically significant increase in the ratio of phosphocreatine to inorganic phosphate, which indicated that there was an improvement in cellular energetics in individuals with DMD who received a standard dose of creatine once a day for eight weeks. Tarnopolsky et al. (2004) also investigated the effects of creatine supplementation in boys with DMD and found that four months of supplementation led to increases in fat-free mass. There was also a statistically significant increase in hand grip strength in the dominant hand of boys with DMD, but it is unclear if these results could be replicated in older individuals with DMD.

Creatine supplementation has also shown to attenuate the adverse effects of damage caused by DOX treatment, cancer cachexia, and chemicals in a range of tissues. Santacruz et al. (2015) exhibited that cardiomyocytes treated in cell culture with DOX
were protected from damage following creatine supplementation. Specifically, ROS production was significantly decreased, which implied that oxidative stress was also reduced. Relatedly, oxidative stress was prevented in tumor-bearing rats following supplementation with creatine in a study by Deminice et al. (2016). Progression of cancer cachexia was also inhibited following creatine supplementation, as determined by an approximate 30% average reduction in tumor weight compared to tumor-bearing controls. Campos-Ferraz et al. (2016) also reported an estimated 30% average decrease in tumor growth in tumor-bearing rats supplemented with creatine for 40 days compared to control rats. Moreover, there was also a trend toward a reduction in TNF-α in tumor-bearing rats treated with creatine for 15 days compared to controls, similar to the findings of Cella et al. (2019).

Norman et al. (2006) explored the effects of creatine supplementation on quality of life and muscle function in individuals undergoing chemotherapy treatment for colorectal cancer. Self-reported quality of life and muscle function were not improved following supplementation with creatine, but bioimpedance markers, such as capacitance, demonstrated favorable outcomes. Relatedly, a creatine loading protocol was utilized by Jatoi et al. (2017) to investigate its potential impacts on the severity of cancer anorexia and weight loss syndromes experienced by patients with terminal malignancies. Creatine supplementation did not have a protective effect in patients, as rates of unfavorable intervention-related events were comparable between both groups of patients.

Creatine supplementation prior to treatment with DOX has protected skeletal muscle chemically- and DOX-induced damage and dysfunction. Chemically-induced damage to the EDL muscle of rats was inflicted by bupivacaine, an anesthetic, in research
by Cooke, Rybalka, Stathis, and Hayes (2018), which focused on the ability of creatine supplementation to attenuate the damaging effects of this medication. An intramuscular injection of bupivacaine typically causes the breakdown of nearly all fibers in the muscle, which may be permanent depending on the dosage (Rosenblatt & Woods, 1992). Cooke et al. (2018) found that creatine-supplemented EDL muscles exhibited a greater ratio of intact (non-damaged) fibers and greater cross-sectional areas of non-damaged fibers compared to controls seven days post-injection. Creatine-supplemented EDL muscles also produced higher magnitudes of absolute force in comparison to controls at fourteen days post-injection. Taken together, these findings indicated that creatine supplementation had a protective effect on EDL muscle following intramuscular injection with bupivacaine in rats, which may have clinical implications in treating various muscle diseases. Similarly, treatment with creatine prior to DOX administration ex vivo in rat skeletal muscle attenuated the muscle dysfunction in the EDL and SOL muscles typically caused by DOX treatment, as reported by Bredahl and Hydock (2017). Creatine pretreatment extended time-to-fatigue of DOX-treated SOL muscle and lessened the severity of force decline in DOX-treated EDL muscle.

Additionally, the effects of creatine supplementation in the DIA muscle have been researched in a variety of contexts. Menezes et al. (2007) investigated the effects of creatine supplementation on body mass, exercise performance, respiratory function, and DIA muscle fiber diameter and weight in rats following treatment with DEX. Simultaneous treatment with both creatine and DEX lessened the severity of the impairments, such as those on maximal oxygen consumption and maximum treadmill speed able to be maintained during treadmill training, that were caused by the use of
DEX alone. Tidal volume, the volume of air displaced between inhalation and exhalation, was elevated due to DEX treatment alone. Simultaneous treatment with creatine led to a significant decrease in the elevation, which indicated that there was a protective effect of creatine on respiratory function. Administration of DEX alone led to significant DIA muscle mass losses in comparison to controls, while treatment with creatine alone caused muscle mass gains in comparison to controls. Moreover, the simultaneous use of creatine with DEX lessened the severity of the DIA muscle weight losses caused by DEX. The findings of Menezes et al. (2007) show that creatine monohydrate supplementation attenuated the harmful effects of high-dose DEX on maximal exercise performance and DIA muscle mass.

The effects of supplementing the diet of spiny mice with creatine during pregnancy on DIA muscle functioning in pups after birth was investigated by LaRosa et al. (2016) and Cannata et al. (2010). LaRosa et al. (2016) compared the effects of birth asphyxia on DIA muscle structure and function in pups from mothers supplemented with creatine during pregnancy to controls. There was a significant increase in the proportion of fast-twitch glycolytic fibers and a decrease in cross-sectional area of all muscle fiber types in the DIA following birth asphyxia in pups without maternal creatine supplementation. These outcomes were not observed in pups from mothers supplemented with creatine. Similarly, Cannata et al. (2010) explored the effects of maternal creatine supplementation on DIA muscle fiber contractile function and structure in pups, along with mRNA concentrations of the ubiquitin ligases, myostatin and MuRF-1. Cannata et al. (2010) reported a nearly 20% average decrease in cross-sectional area of muscle fibers following hypoxia-induced damage of the DIA, comparable to the findings of LaRosa et
al. (2016). This DOX-induced outcome pertaining to muscle fiber cross-sectional area was also attenuated by maternal creatine supplementation. Furthermore, myostatin and MuRF-1 mRNA levels were doubled following hypoxia-induced damage and maternal creatine supplementation lessened the severity of this consequence.

Likewise, Saremi et al. (2010) and Cella et al. (2019) focused on the effects of creatine supplementation on ubiquitin ligase expression. Following eight weeks of resistance training, serum myostatin levels in males were significantly decreased. Creatine supplementation in combination with resistance training led to a greater decrease in serum myostatin, which offers an explanation for the increased muscle mass also noted by Saremi et al. (2010). Cella et al. (2019) observed that creatine supplementation in tumor-bearing rats prevented the increase in MuRF-1 and atrogin-1 levels shown in rats not treated with creatine. Additionally, the creatine treatment promoted lower levels of TNF-α, which implies that the negative consequences of the pro-inflammatory environment caused by tumor presence, such as ROS formation, were reduced.

The effects of creatine supplementation on the musculoskeletal system have been thoroughly researched, but there is much less understanding of its possible influence on other physiological systems (Riesberg et al. 2016). A small amount of research has focused on the impact of creatine in neurological and immune system functioning, but its regulatory mechanisms have yet to be fully determined. Lawler, Barnes, Wu, Song, and Demaree (2002) were the first to report that creatine exhibited a statistically significant ability to act as a direct antioxidant scavenger against ROS and free radical ions. Lawler et al. (2002) conducted one of the first studies to associate creatine with potential
therapeutic interventions for individuals with neuromuscular diseases and similar syndromes.

Furthermore, there is evidence to suggest that creatine may activate genes in the skeletal muscle tissue that are involved in several physiological processes (Safdar, Yardley, Snow, Melov, and Tarnopolsky, 2008). Safdar et al. (2008) were the first to report that a short-term creatine supplementation loading protocol activated genes that influenced osmosensing, metabolism, signal transduction, cell repair, and satellite cell proliferation and differentiation in the skeletal muscle tissue of young adult males. This observation may indicate that creatine influences regulatory proteins in skeletal muscle at a molecular level, especially since these effects were independent of any additional intervention pertaining to resistance training or diet. Taken together, these findings may provide insight as to the function of creatine in other physiological processes and influence future therapeutic treatments for individuals with physiological disorders and injuries.

**Exercise**

It is well-known that exercise stimulates the uptake of creatine into the skeletal muscle for utilization during muscular contraction (Wyss & Kaddurah-Daouk, 2000). This process occurs in all individuals at the onset of exercise, regardless of creatine supplementation status, and acts to prepare the body for performance. While the onset of physical activity causes creatine to be taken up by the skeletal muscle, exercise also induces the release of myokines from skeletal muscle into the circulation (Piccirillo, 2019). These proteins are of particular importance because they assist in the regulation of muscle hypertrophy and are functionally able to lessen the severity of muscle wasting in
cases of heart failure and cancer. Myokines act in an autocrine, endocrine, or paracrine fashion and demonstrate protective effects in other organs or within the muscle fiber itself (Pedersen & Febbraio, 2012). While the release of exercise-induced myokines has beneficial potential for individuals suffering from muscle atrophy, there are additional protective mechanisms and effects of exercise that have been explored. For instance, the advantageous effects of exercise on DOX-induced skeletal and cardiac muscle dysfunction, oxidative stress, and mitochondrial toxicity have been extensively investigated.

Exercise has influenced the expression of negative regulators of muscle mass in skeletal muscle tissue, which is another protective mechanism against muscle wasting. Chen, Mou, Yang, Wang, and Zhao (2011), were the first to report that decreased muscle wasting observed following exercise training in individuals with diabetes may be related to an inhibition of oxidative stress-induced mechanisms. According to Chen et al. (2011) oxidative stress was decreased in rats with diabetes following exercise training. This reduction in oxidative stress was correlated with a significant decrease in MuRF-1 expression, which may have prevented the breakdown of both slow and fast MHC. These findings were similar to that of Clarke et al. (2007), where MHC expression in C2C12 myotubes was maintained due to MuRF-1 inhibition following administration of DEX. Smuder, Kavazis, Min, and Powers (2011) also investigated the impact of exercise on skeletal muscle following DOX administration, but focused specifically on markers of autophagy, which is a protein degradation system that can be triggered by oxidative stress. Expression of autophagy genes was elevated following DOX treatment, but the implementation of an exercise protocol protected skeletal muscle from increased...
autophagy activity. Similarly, Marques-Aleixo et al. (2015) utilized two models of long-term exercise, treadmill endurance training and free-wheel exercise, to investigate the impact of DOX on mitochondrial oxidative stress and damage. The use of both exercise models resulted in overall improved mitochondrial functioning, and it is possible that this was a consequence of prevented oxidative damage and stress in the mitochondria.

Comparably, exercise has employed a protective effect on several physiological components other than oxidative stress-induced mechanisms following DOX administration. One such mechanism is that the activity of MRFs in the skeletal muscle tissue increases due to exercise (Aguiar, Vechetti-Júnior, et al., 2013). Quinn and Hydock (2018) were the first to specifically investigate the effects of short-term endurance exercise prior to treatment with DOX on MRF expression in slow- and fast-twitch skeletal muscle in rats. No significant effects were observed in EDL muscle, which is primarily composed of fast-twitch fibers. However, exercise significantly increased Myf5 expression in SOL muscle, which is almost exclusively comprised of slow-twitch fibers. Enhanced MRF expression in SOL muscle in response to exercise may act as a protective mechanism against muscle atrophy and dysfunction by providing the muscle with the means to repair damage induced by DOX (Bredahl, Pfannenstiel, Quinn, Hayward, & Hydock, 2016).

Different types of exercise training protocols associated with DOX treatment have been investigated to determine which protocol elicits the best protective outcomes on skeletal muscle function. Bredahl et al. (2016) compared the differences in resistance and endurance exercise protocols in diminishing DOX-induced skeletal muscle dysfunction by measuring maximal twitch force, rate of force production, and rate of force decline.
Both the resistance and treadmill training protocols improved certain components of skeletal muscle function in the SOL muscle, but neither protocol improved any measure of EDL muscle function following DOX treatment. These findings indicate that specific protective effects of exercise on muscle dysfunction following DOX treatment may depend on the type of exercise performed as well as fiber type of the muscle. For example, the resistance protocol prevented maximal twitch force and rate of force decline, while the treadmill protocol improved the rate of decline in force production in the SOL muscle only. Based on these findings, resistance and treadmill training protocols may be utilized in a single therapeutic exercise plan to elicit the most protective effects on skeletal muscle function.

Rather than compare the effectiveness of different types of exercise in protecting skeletal muscle tissue from damage and dysfunction, Dickinson et al. (2017) assessed the timing of an exercise protocol in relation to DOX treatment in rats. Animals completed a treadmill interval training protocol five days per week, starting five days prior to the first DOX injection, and maintained this frequency throughout the remaining 40-day study duration. Two additional injections with DOX were administered at two- and four-weeks following the first injection. Animals in the exercise group exhibited less damage to MHC I and MHC IIa fibers in the SOL muscle compared to controls. This finding suggested that exercise before and during long-term DOX administration may be used as a strategy to lessen the severity of damage to skeletal muscle that is primarily oxidative, such as DIA muscle.
Diaphragm (DIA)

It is well-known that the DIA muscle acts not only as the primary inspiratory muscle, but also as a component of several physiological functions throughout the body (Kocjan, Adamek, Gzik-Zroska, Czyżewski, & Rydel, 2017). The DIA is comprised of a non-contractile tendon and peripheral muscle that conjoin to form a dome-shaped partition between the thoracic and abdominal cavities (Downey, 2011). The location of the DIA allows for its involvement in the information exchange and signaling in the body (Kocjan et al., 2017). The DIA is a skeletal muscle that is mostly under involuntary control, but can be voluntarily controlled when necessary (Kokatnur & Rudrappa, 2019) and is comprised of a blend of both slow- and fast-twitch fibers (Quinn & Hydock, 2018). Meznaric and Cvetko (2016) found that postmortem human costal DIA, the part of the muscle that attaches to the ribs, was comprised of approximately 50% slow-twitch and 47% fast-twitch fibers on average. Also, the cross-sectional area of muscle fibers in the DIA was much smaller in comparison to that of other skeletal muscles, such as the vastus lateralis muscle. It was suggested that slow-twitch fibers in the DIA encourage fatigue resistance, not only due to their aerobic nature, but also because of the reduced distance required for the diffusion of oxygen (McKenzie, 2006).

One primary role of the DIA is to enlarge the chest cavity during inhalation to allow air to enter. The DIA is of great importance for respiration (Zhou et al., 2018), seeing as approximately 80% of the work required for respiration is accomplished by the DIA (Kocjan et al., 2017). In non-cancer populations, respiratory function assessments have displayed variations related to the degree of dyspnea (Bruera, Schmitz, Pither, Neumann, & Hanson, 2000). Dyspnea is defined as a self-reported experience of labored
breathing (Campbell, 2017) while at rest or during activities requiring exertion, such as
exercise. In cancer patients, fatigue is most commonly associated with other physical
symptoms, such as dyspnea (Stone et al., 1999). It is unclear if there is a common
mechanism of dyspnea in cancer patients, especially when dyspnea is most intense
(Travers et al., 2008).

Another key function of the DIA is to block the space where the distal esophagus
and proximal stomach join in order to protect against reflux (Downey, 2011). Other roles
of the DIA include assistance with trunk stabilization, childbirth, swallowing, vomiting,
defecation, and urination, as well as involvement in lymphatic and cardiac functioning.
The DIA acts as a lymphatic pump during deep breathing, with nearly two-thirds of all
lymph nodes in the body positioned directly underneath the DIA (Kocjan et al., 2017).
Furthermore, DIA functioning is related to the modulation of cardiovascular components
such as stroke volume and cardiac output, which indicates that cardiac and DIA
dysfunction are interrelated.

**Weakness and Dysfunction**

Numerous studies have investigated DIA muscle dysfunction induced by
sustained mechanical ventilation in humans as well as DIA muscle weakness following
periods of hypoxic stress in murine models. Powers et al. (2011) were the first to identify
that prevention of mitochondrial ROS production in the DIA, through the use of an
antioxidant targeted to the mitochondria, protected the DIA from ventilator-induced
weakness in rats. Based on this observation, Powers et al. (2011) were also the first to
report that mitochondria are a key source of ROS in the DIA in circumstances of
extended mechanical ventilation and that disruption of this ROS production attenuated DIA weakness following ventilation.

An additional protective mechanism against decline in DIA functioning induced by prolonged mechanical ventilation was identified by Zambelli et al. (2016). The administration of Angiotensin (1–7) (Ang-(1–7)), a peptide involved in processes of the renin-angiotensin system, inhibited atrophy of the DIA muscle in mechanically-ventilated rats without improving force generation. Levels of MuRF-1 and atrogin-1 were elevated following mechanical ventilation, but rats in the Ang-(1–7) administration group exhibited a reduction in expression of both ubiquitin ligases. Based on this observation, it was suggested that Ang-(1–7) acted in the DIA by suppressing MuRF-1 and atrogin-1 expression, which offered an explanation for the prevention of DIA muscle wasting.

Furthermore, O'Leary, Drummond, Edge, and O'Halloran (2018) demonstrated the protective effects of N-acetyl cysteine, an antioxidant, on DIA function following eight hours of hypoxic stress. Pretreatment with N-acetyl cysteine inhibited dysfunction in the DIA induced by hypoxia, as shown by an increase in specific force generation following hypoxia in comparison to the control group. Oxidative stress in the DIA was also prevented, as indicated by meaningfully lower concentrations of lipid peroxidation byproducts compared to those of hypoxia and control groups.

While an ample amount of research pertaining to MRFs and proteins that negatively regulate muscle mass in EDL and SOL muscles has been conducted, less has been explored regarding protein expression and signaling in the DIA muscle of animals and humans with chronic conditions. Of the research that has been conducted regarding positive and negative regulatory protein expression in the DIA muscle, most is relative to
individuals with COPD or critically-ill individuals with respiratory dysfunction. Testelmans et al. (2010) investigated the signaling pathways of atrophy and hypertrophy, protein expression, and muscle fiber characteristics in the DIA muscle of patients with COPD in comparison to patients without COPD, but with other chronic conditions. MyoD protein expression and mRNA levels as well as cross-sectional area of slow-twitch, type I, and fast-twitch, type II, muscle fiber types were significantly decreased in individuals with COPD. Conversely, binding activity of the NF-κB pathway, MAFbx mRNA concentrations, and myostatin protein expression were drastically increased. Moreover, the proportion of type I fibers was significantly higher while the proportion of type II fibers was reduced in the DIA muscle of COPD patients compared to controls. These findings indicate that the signaling pathways of atrophy and hypertrophy in the DIA muscle of COPD patients, specifically the ubiquitin-proteasome and NF-κB pathways, are modified in comparison to controls. This alteration in activity, along with the shift of DIA muscle fibers to a slower profile, may contribute to DIA dysfunction in individuals with COPD and other chronic conditions that affect respiration.

In a similar model of COPD in rats by Zhou et al. (2018), myostatin expression in the DIA muscle was significantly greater in COPD animals than controls, which was comparable to the findings of Testelmans et al. (2010). However, Zhou et al. (2018) also investigated markers of apoptosis in the DIA of COPD animals and observed an increase correlated with myostatin expression. Therefore, myostatin may be involved in the promotion of apoptosis in the DIA and contribute to its dysfunction in a variety of conditions. Zhou et al. (2018) also noted a significant decrease in DIA mass in COPD animals in comparison to controls, which was not a variable investigated by Testelmans
et al. (2010). Hoojiman et al. (2015) focused on the role of the ubiquitin-proteasome pathway in muscle wasting and contractile dysfunction in the DIA of critically ill individuals who were mechanically ventilated prior to undergoing lung resections. The expression of MuRF-1 doubled and MAFbx tripled in the DIA of critically ill individuals in comparison to controls, which signified an increased activation of the ubiquitin-proteasome pathway. Moreover, both slow- and fast-twitch fibers in the DIA exhibited a decrease in cross-sectional area and a decline in contractile force by approximately 50% in critically ill patients, which supported the results of Testelmins et al. (2010) regarding contractile function and muscle fiber characteristics in the DIA.

**Doxorubicin (DOX)-Induced Weakness and Dysfunction**

Several studies pertaining to cancer treatment have focused on the impacts of DOX administration in a variety of tissues, but its specific effects on DIA muscle function and atrophy have been less investigated. Gilliam, Moylan, Callahan, et al. (2011) focused on respiratory function in two murine models of chemotherapy, which represented the two methods by which cancer patients receive chemotherapy: intravenous (i.v.) and intraperitoneal (i.p.) injection. Both methods of administration led to a significant decrease in specific force, which was most severe following i.p. injection. As a result of i.p. injection with DOX only, oxidant activity in the cytosol, a marker of oxidative stress, was doubled in the DIA muscle of mice. Muscle weakness in the DIA resulted from both injection methods, which suggested that this loss of force generation may have been a potential mechanism for dyspnea in individuals undergoing DOX treatments. Moreover, inflammatory effects were only observed in mice following i.p.
injection with DOX, which highlighted differences in treatment outcomes between the two methods.

Gilliam, Moylan, Ferreira, and Reid (2011) reported another potential mechanism for DIA dysfunction related to an increase in TNFR-1 mRNA levels in mice following i.v. injection with DOX. Mice with a genetic deficiency of TNFR-1 did not exhibit DIA muscle weakness, as determined by a maintenance of specific force following DOX administration. This finding indicated that the signaling pathway of TNF-α and TNFR-1 regulated DOX-induced DIA weakness is likely a mechanism of DIA dysfunction. The observations of Gilliam et al. (2009) pertaining to the protection of the DIA muscle in TNFR-1 deficient mice are in agreement with those of Gilliam, Moylan, Ferreira, et al. (2011).

**Endurance Exercise**

Endurance exercise has demonstrated an assortment of impacts on MRF expression, muscle function, mitochondrial expression, and antioxidant production in the DIA, particularly in cases concerning DOX- and ventilator-induced damage. Quinn and Hydock (2018) also investigated the effects of exercise training prior to treatment with DOX on MRF expression in the DIA muscle. There were no main effects in the DIA muscle from DOX treatment, which supports the concept that DOX treatment influences skeletal muscle differentially based on muscle fiber type. This conclusion was in agreement with that of Bredahl et al. (2016), where DOX differentially affected EDL and SOL muscle.

The first evidence that an exercise protocol could provide protection against ventilator-induced damage was reported by Smuder et al. (2012). An endurance-based
treadmill protocol protected the DIA muscle of rats against ventilator-induced oxidative damage in the mitochondria. A potential mechanism behind this observed protection was investigated by Sollanek et al. (2017), by comparing the set of expressed proteins in the DIA muscle of sedentary rats to that of endurance-trained rats. In the mitochondria of the DIA, the endurance exercise protocol significantly increased the relative abundance of proteins associated with protection of the cytosol in other cell types. While the functions of these proteins in the DIA muscle are not fully understood, these observations provided evidence that these proteins may be necessary for, or at least encourage, exercise-induced protection against muscle wasting due to sedentary behavior.

These findings regarding exercise-induced protection in the DIA have also been applied to research involving DOX treatment. Two weeks of treadmill exercise preserved respiratory function, mitochondrial function, and DIA tissue following DOX treatment by reducing mitochondrial accumulation of DOX and ROS production according to Morton, Mor Huertas, et al. (2019). Moreover, Morton, Smuder, et al. (2019) hypothesized that mitochondrial antioxidant enzyme superoxide dismutase (SOD2) needed to be elevated in order for exercise to have a protective effect against DIA dysfunction induced by mechanical ventilation based on previously reported findings. Upon prevention of the exercise-induced increase in diaphragmatic SOD2, exercise-mediated protection from DIA atrophy was absent while protection against contractile dysfunction was only moderately lost. These observations signify that increased antioxidant, specifically SOD2, expression in the DIA is required to fully attain exercise-induced protection against ventilator-induced impairments in functioning.
While endurance exercise is known to increase muscle damage of \textit{mdx} mice, less is understood about the influence of sex on muscle wasting, particularly in the DIA. Hermes et al. (2018) compared the effects of exercise on DIA muscle response in male and female \textit{mdx} mice by measuring CK levels, quantifying regenerated DIA muscle fibers, and assessing alpha and beta estrogen receptors (ER) in comparison to controls. Female \textit{mdx} mice in the exercise group demonstrated the greatest alpha and beta ER expression. Male \textit{mdx} mice presented the highest quantification of regenerated fibers and the exercise group specifically exhibited increased CK levels. Therefore, DIA damage and inflammation may have been selectively prevented in \textit{mdx} mice based on ER expression and estrogen presence of the different sexes.

**Conclusion**

At least half of cancer patients worldwide have reported symptoms of fatigue associated with their condition (Mohandas et al., 2017). This cancer-associated fatigue likely affects patients receiving chemotherapy and radiation therapy to the greatest extent, due to their potency, in comparison to individuals receiving other forms of treatment (Irvine et al., 1991). DOX is a chemotherapy drug that can be administered intravenously or intraperitoneally to treat several types of cancer and affects the body systemically rather than locally. While DOX administration may provide anti-cancer benefits, a major limitation is that its toxicity impacts organs that are not the intended targets of treatment. Furthermore, the total dose of DOX administered is used to predict the severity of the toxicity that will be imposed on the patient (Thorn et al., 2011).

Cardiac and skeletal muscle dysfunction is a common outcome of chemotherapy treatment and transpires through many mechanisms and mediators. Interactions between
physiological variables, rather than just the accumulation of DOX, may impact proper muscle functioning (Hayward et al., 2013). Hydock et al. (2011) reported a dose-dependent relationship of DOX treatment on cardiac and skeletal muscle dysfunction, while Hayward et al. (2013) observed a time-dependent steady decline in muscle function one-, three-, and five-days following DOX administration.

Oxidative stress occurs when there is an imbalance of the production of ROS and the protection of antioxidants. DOX administration increases ROS creation (Carvalho et al., 2009), which may consequently weaken antioxidant defenses. Muscle degradation mediators and pathways, such as TGF-β and the ubiquitin-proteasome pathway, are influenced by ROS presence. Myostatin is a cytokine belonging to the TGF-β superfamily of proteins responsible for cell regulation (Ríos et al., 2004). Factors related to muscle atrophy, such as denervation and disuse of skeletal muscle tissue, have been observed under conditions of increased myostatin expression (Delfino et al., 2013).

Myostatin also directly regulates the creation of fibrous connective tissue in skeletal muscle, an excess of which can cause fibrosis. Moreover, increases in muscle mass and strength have been observed under conditions of inhibited myostatin activity (Tobin & Celeste, 2005). The ubiquitin-proteasome system is the main regulatory mechanism of skeletal muscle protein degradation. Levels of MuRF-1 and MAFbx, two ubiquitin ligases, play a role in muscle wasting, as demonstrated by their activation in numerous models of muscle atrophy (Foletta et al., 2011). Although the role of ubiquitin ligases in skeletal muscle atrophy has been well-established, the involvement of MuRF-1 and MAFbx in causing the symptoms specifically experienced by individuals undergoing chemotherapy remains largely uninvestigated.
The impact of DOX administration on a variety of tissues has been vastly explored, but DOX-induced effects on respiratory function have been less investigated. In order for respiration to occur, the DIA must perform more than three-fourths of the total amount of work needed (Kocjan et al., 2017). Dyspnea, characterized by uncomfortable and/or labored breathing at rest or during exertion, is a common outcome associated with chemotherapy treatment, but its exact mechanisms are unknown. A potential mechanism of the dyspnea experienced by patients receiving DOX treatment is the decline in specific force of the DIA muscle, which was observed in mice following both i.v. and i.p. injection with DOX (Gilliam, Moylan, Callahan, et al., 2011).

Creatine, a substance that is synthesized in the human body and able to be obtained from the diet, is involved in supplying energy for use during muscular contraction (Fairman et al., 2019). Professional athletes and individuals interested in physical fitness have effectively utilized creatine as an ergogenic aid for activities that require recurrent bouts of high-intensity exercise. Creatine supplementation has also lessened the severity of damage to the body caused by chemotherapy treatment, cachexia, and conditions characterized by muscle-wasting, such as muscular dystrophies. According to Bredahl and Hydock (2017), *ex vivo* incubation of the EDL and SOL muscles of rats with creatine prior to DOX incubation lessened the severity of DOX-induced muscle dysfunction. Time-to-fatigue was extended in DOX-treated SOL muscle and force decline was attenuated in the EDL muscle following incubation of the muscle in a creatine-supplemented organ bath. Similarly, Menezes et al. (2007) reported that creatine supplementation demonstrated a protective effect on respiration and DIA muscle wasting following administration of DEX.
This study potentially demonstrates the therapeutic benefits of two creatine supplementation protocols following DOX administration in lessening the severity of DOX-induced increases in negative regulatory protein expression in the DIA muscle at one- and three-day time points post-injection. Therefore, this research may provide a greater understanding of the mechanisms of negative regulatory protein expression in the DIA following DOX administration, and have implications for the possible future prevention of dyspnea in individuals undergoing chemotherapy treatment.
CHAPTER III

METHODS

Animals and Animal Care

All procedures and protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Northern Colorado and were performed in compliance with the requirements of the Animal Welfare Act. Male Sprague-Dawley rats \( (N = 120) \) were acquired from Envigo (Indianapolis, IN) and were singly housed in cages under a 12hr:12hr light:dark cycle in an environmentally managed facility. Animals were provided a standard rodent chow from Envigo (Indianapolis, IN) and water upon arrival at the facility, which were both available \textit{ad libitum}. Chow was consistently replaced to 200 g after 40 g or less was remaining.

Experimental Design

Male Sprague-Dawley rats (ten-week old; \( N = 120 \)) were randomly assigned to one of six groups: (1) CON+SAL (control chow, saline, \( n = 20 \)), (2) CON+DOX (control chow, doxorubicin, \( n = 20 \)), (3) CrS+SAL (standard creatine diet, saline, \( n = 20 \)), (4) CrS+DOX (standard creatine diet, doxorubicin, \( n = 20 \)), (5) CrL+SAL (creatinine loading diet, saline, \( n = 20 \)), (6) CrL+DOX (creatinine loading diet, doxorubicin, \( n = 20 \)). Injections with SAL or DOX were delivered at four weeks. Animals were sacrificed at one day (One-Day, \( n = 60 \)) or three days (Three-Day, \( n = 60 \)) post-injection. The experimental design for the One-Day and Three-Day animals is depicted by Figures 1 and 2, respectively.
Figure 1. Experimental Design of Treatments and Interventions for One-Day Animals.
Figure 2. Experimental Design of Treatments and Interventions for Three-Day Animals.
Creatine Monohydrate (Cr) Administration

Groups randomly assigned to creatine monohydrate supplementation were fed rodent chow which was supplemented with a standard creatine dosage (CrS) or a creatine loading dosage (CrL). The standard chow provided to the CrS groups was supplemented with 2% creatine monohydrate (Sigma-Aldrich®: St. Louis, MO) for four weeks. The standard chow provided to the CrL groups was supplemented with 4% Cr for one week, followed by 2% Cr for three weeks.

Doxorubicin (DOX) Treatment

Animals randomly assigned to DOX treatment groups received an intraperitoneal injection of DOX (Teva Pharmaceuticals USA: North Wales, PA) at a dosage of 15 mg per 1 kg bodyweight (15mg/kg) while animals in the SAL treatment groups received a saline injection (0.9% NaCl) of the same volume. Animals were randomly assigned to one-day or three-day sacrifice, which occurred one- and three-days following injection, respectively.

Biochemical Analyses

Tissue Preparation

Each animal was anesthetized via a single intraperitoneal sodium pentobarbital (50 mg/kg) injection in preparation for sacrifice, which occurred at the One-Day ($n=60$) or Three-Day ($n=60$) time point after SAL or DOX injection. A tail pinch was performed when each animal appeared to be visibly anesthetized following the injection. Failure of the animal to respond to the tail pinch indicated that the animal was fully anesthetized, which allowed for immediate sacrifice. The DIA of each animal was collected, weighed,
flash frozen in liquid nitrogen, and stored at a temperature of -80°C in preparation for the biochemical analyses of myostatin and MuRF-1 proteins.

**Homogenate Preparation**

Individual DIA tissue samples were removed from the -80°C freezer and between 0.5 and 1.0 g of the tissue was placed in a glass tissue homogenizer. For each 1.0 µg sample, 10µL of radio-immunoprecipitation assay (RIPA) buffer (Santa Cruz Biotechnology: Santa Cruz, CA) was added to the homogenizing tube followed by 10µL of protease inhibitor (Sigma-Aldrich®: St. Louis, MO). Each sample was manually homogenized using a polytetrafluoroethylene (PTFE) tissue grinder (Cole Parmer: Vernon Hills, IL) and cell membranes were further disrupted using a Model 100 Sonic Dismembrator (Fisher Scientific: Hampton, NH) through ten, 1-second bursts. The contents of the glass tube were transferred to a 2.0 mL Eppendorf tube (Eppendorf: Hauppauge, NY) and were centrifuged at room temperature for 10 minutes at 10,000 g. After centrifugation, the supernatant was transferred to a new 2.0 mL Eppendorf tube and the remaining pellet was discarded.

The total protein concentration of each supernatant was quantified using the Bradford method (Bradford, 1976; Coomassie Plus Protein Assay Reagent, ThermoScientific: Rockford, IL). Spectrophotometry was performed using a Genesys 20 spectrophotometer (ThermoSpectronic: Rochester, NY) set at 595 nm. This process established a standard curve for protein concentration to absorbency, which was determined based on wavelength. Five standardized concentrations of BSA (Pre-diluted protein assay standards: Bovine serum albumin, ThermoScientific: Rockford, IL; 0.125 mg/mL, 0.25 mg/mL, 0.5 mg/mL, 1.0 mg/mL, and 2.0 mg/mL) were utilized to determine
the standard curve of protein concentration to wavelength. The linear equation provided from the standard curve, \( x = (y-b)/m \) (derived from \( y = mx+b \)), was used to predict the protein concentration of each 10 \( \mu \)L standard sample analyzed by the spectrophotometer. Based on each protein concentration value, a calculated amount of RIPA buffer (Santa Cruz Biotechnology: Santa Cruz, CA) was added to 100 \( \mu \)L of each supernatant to standardize the protein concentration. An equal amount of Laemmli buffer (Sigma-Aldrich®: St. Louis, MO) was added in order to decrease the concentration of protein by 50% and prepare the proteins for gel electrophoresis. Samples were stored at -80°C until the Western blot analysis was conducted.

**Western Blotting**

Western blot analysis was performed to quantify the protein expression of myostatin and MuRF-1 in DIA \((N=120)\). Samples were removed from the freezer, allowed to completely thaw, heated in boiling water for 2 minutes and chilled for 5 minutes on ice. Once cooled, 10 \( \mu \)L of each sample was loaded onto Tris-Glycine precast gels (4-20% gradient; Invitrogen™, Thermo Fisher Scientific; Waltham, MA) in addition to 10 \( \mu \)L of SeeBlue™ protein ladder and Magic Mark™ to track protein separation. The running buffer used in electrophoresis was Tris-Glycine SDS Running Buffer (ThermoScientific; Waltham, MA). Gels ran at a constant 125 V and 40 mA in an Xcell II™ blot module (Invitrogen™, Thermo Fisher Scientific; Waltham, MA) until the tracking dye migrated to the bottom of the gel, which usually occurred within 2 hours.

Proteins were then transferred to a polyvinylidene fluoride (PVDF) membrane (0.45 \( \mu \)m pore; Invitrolon™, Invitrogen™, Thermo Fisher Scientific; Waltham, MA) for approximately 90 minutes at a constant 25 V and 100 mA. During the transfer, proteins
were incubated in a transfer buffer which consisted of 40 mL of Tris-Glycine Transfer Buffer, 760 mL of deionized water, and 200 mL of methanol. Membranes were then blocked with 10 mL of Superblocker Solution (Thermo Fisher Scientific; Waltham, MA) for 30 minutes and washed in deionized water twice for five minutes. Once blocked, membranes were incubated with 10 mL of primary antibody solution for 12 to 18 hours. Following incubation with the primary antibody, membranes were washed four times in 20 mL of Wash Solution (WesternBreeze™; Thermo Fisher Scientific, Waltham, MA) for five minutes and incubated with the suitable secondary antibody (goat anti-mouse, 1:1000; Santa Cruz Biotechnology; Dallas, TX) for 60 minutes. Lastly, membranes were washed four times with 20 mL of Wash Solution (WesternBreeze™; Thermo Fisher Scientific; Waltham, MA) for five minutes and then rinsed with 20 mL of deionized water twice for five minutes in preparation for protein band detection and imaging.

Enhanced chemiluminescence (ECL) substrate (WesternSure® PREMIUM Chemiluminescent Substrate, LI-COR; Lincoln, NE) consisting of luminol and enhancer in a 2.0 mL Eppendorf tube (ratio 1:1 mL) was poured onto the testing surface of a Western blot scanner (C-Digit, LI-COR: Lincoln, NE). The membrane was incubated face-down in the ECL substrate for five minutes prior to the quantification of protein expression through the utilization of ImageJ software (NIH; Bethesda, MD).

The primary antibodies of focus were myostatin (anti-mouse, 1:500 dilution; Santa Cruz Biotechnology; Dallas, TX), also known as growth-differentiation factor 8 (GDF-8), and MuRF-1 (anti-mouse 1:100 dilution; Santa Cruz Biotechnology; Dallas, TX). The loading control used for each PVDF membrane was GAPDH (anti-mouse, 1:1000 dilution; Santa Cruz Biotechnology; Dallas, TX). The molecular weights of
proteins transferred to the PVDF membrane were verified based on a Magic Mark™ standard ladder (Invitrogen™, Thermo Fisher Scientific; Waltham, MA).

**Statistical Analyses**

Data were analyzed using GraphPad Prism 7 statistical software (GraphPad: La Jolla, CA) and are presented as means ± standard error of the mean (means ± SEM). A two-factor (diet x drug) analysis of variance (ANOVA) was completed to identify main diet effects, main drug effects, and diet x drug interactions at the one-day and three-day time points. If significant main effects were observed, Tukey’s *post hoc* testing was performed to determine where differences occurred between groups. Significance was established at $p < 0.05$. 
CHAPTER IV

RESULTS

Biochemical Analyses

Data are presented as means ± SEM. All animals were fed CON, CrS, or CrL diets and were anesthetized at either one day- or three days-post injection with DOX or SAL. The DIA of each animal (N=115) was analyzed to identify the potential effect(s) of four weeks of Cr supplementation, by means of a standard or loading protocol, in combination with DOX or SAL injection on the protein expression of myostatin and MuRF-1 at both time points. The DIA tissue samples of five animals were unavailable for analysis due to experimenter error during tissue collection or animal death prior to sacrifice (N=5).

General Observations

The effects of four weeks of CON, CrS, or CrL diets followed by intraperitoneal injection with DOX or SAL on percent change (± %) in myostatin and MuRF-1 protein expression in the DIA of rats under all conditions and both time points is depicted by Tables 1–4. Myostatin and MuRF-1 expression in the DIA of rats under all conditions and both time points is illustrated by Figures 3–6.
One-Day

**Myostatin**

There was no significant drug effect ($p = 0.225$), diet effect ($p = 0.412$), or interaction ($p = 0.770$) for myostatin protein expression one day post-injection in the DIA (See Figure 3). Myostatin expression was 11.6% greater in the DIA from CON+DOX in comparison to CON+SAL one day post-injection. Additionally, myostatin expression was 1.04% and 10.4% lower in the DIA from CrS+DOX and CrL+DOX respectively, in comparison to CON+DOX. Lastly, myostatin expression was 9.47% lower in the DIA from CrL+DOX when compared to CrS+DOX (See Table 1).

### Table 1

*Percent Change (±%) in Myostatin Expression in One-Day Rat DIA Tissue*

<table>
<thead>
<tr>
<th></th>
<th>CON+SAL</th>
<th>CON+DOX</th>
<th>CrS+SAL</th>
<th>CrS+DOX</th>
<th>CrL+SAL</th>
<th>CrL+DOX</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON+SAL</td>
<td>0.00</td>
<td>+11.6</td>
<td>+11.6</td>
<td>+10.5</td>
<td>−8.14</td>
<td>0.00</td>
</tr>
<tr>
<td>CON+DOX</td>
<td>-</td>
<td>0.00</td>
<td>0.00</td>
<td>−1.04</td>
<td>+21.5</td>
<td>−10.4</td>
</tr>
<tr>
<td>CrS+SAL</td>
<td>-</td>
<td>-</td>
<td>0.00</td>
<td>−1.04</td>
<td>−17.7</td>
<td>−10.4</td>
</tr>
<tr>
<td>CrS+DOX</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.00</td>
<td>+20.3</td>
<td>−9.47</td>
</tr>
<tr>
<td>CrL+SAL</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.00</td>
<td>+8.86</td>
</tr>
<tr>
<td>CrL+DOX</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.00</td>
</tr>
</tbody>
</table>

*Note.* DOX, doxorubicin; SAL, saline; CON, control; CrS, Standard creatine diet; CrL, Creatine loading diet; 1-Day, sacrifice one day post-injection ($N=58$).
Figure 3. Myostatin Expression in the DIA of One-Day Animals. There was not a significant diet effect, drug effect, or interaction (N=58). CON, control; CrS, Standard creatine diet; CrL, Creatine loading diet; DOX, doxorubicin; SAL, saline. Top bands represent myostatin. Bottom bands represent GAPDH as the loading control.
Muscle RING-Finger Protein-1 (MuRF-1)

No significant drug effect ($p = 0.772$), diet effect ($p = 0.248$), or interaction ($p = 0.137$) was observed for MuRF-1 protein expression one day post-injection in the DIA (See Figure 4). Although there were no detected interactions between diet and drug or main effects, MuRF-1 expression was 51.7% greater in the DIA from CON+DOX when compared to CON-SAL one day post-injection. Also, MuRF-1 expression was 14.3% and 13.2% lower in the DIA from CrS+DOX and CrL+DOX, respectively, in comparison to CON+DOX. Finally, MuRF-1 expression was 1.28% greater in the DIA from CrL+DOX when compared to CrS+DOX (See Table 2).

Table 2

Percent Change (± %) in MuRF-1 Expression in One-Day Rat DIA Tissue

<table>
<thead>
<tr>
<th></th>
<th>CON+SAL</th>
<th>CON+DOX</th>
<th>CrS+SAL</th>
<th>CrS+DOX</th>
<th>CrL+SAL</th>
<th>CrL+DOX</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON+SAL</td>
<td>0.00</td>
<td>+51.7</td>
<td>+43.3</td>
<td>+30.0</td>
<td>+23.3</td>
<td>+31.7</td>
</tr>
<tr>
<td>CON+DOX</td>
<td>-</td>
<td>0.00</td>
<td>+5.81</td>
<td>-14.3</td>
<td>+23.0</td>
<td>-13.2</td>
</tr>
<tr>
<td>CrS+SAL</td>
<td>-</td>
<td>-</td>
<td>0.00</td>
<td>-9.30</td>
<td>-14.0</td>
<td>-8.14</td>
</tr>
<tr>
<td>CrS+DOX</td>
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<td>-</td>
<td>0.00</td>
<td>+5.41</td>
<td>+1.28</td>
</tr>
<tr>
<td>CRL+SAL</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.00</td>
<td>+6.76</td>
</tr>
<tr>
<td>CRL+DOX</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Note. DOX, doxorubicin; SAL, saline; CON, control; CrS, Standard creatine diet; CrL, Creatine loading diet; 1-Day, sacrifice one day post-injection ($N=58$).
Figure 4. MuRF-1 Expression in the DIA of One-Day Animals. There was not a significant diet effect, drug effect, or interaction (N=58). CON, control; CrS, Standard creatine diet; CrL, Creatine loading diet; DOX, doxorubicin; SAL, saline. Top bands represent MuRF-1. Bottom bands represent GAPDH as the loading control.
Three-Day

Myostatin

There was no significant drug effect ($p = 0.710$), diet effect ($p = 0.935$), or interaction ($p = 0.566$) for myostatin protein expression three days post-injection in the DIA (See Figure 5). While there were no detected main effects or interactions between variables, myostatin expression was 15.0% greater in the DIA from CON+DOX when compared to CON+SAL three days post-injection. Moreover, myostatin expression was 11.3% and 10.4% lower in the DIA from CrS+DOX and CrL+DOX, respectively, in comparison to CON+DOX. Lastly, myostatin expression was 0.98% greater in the DIA from CrL+DOX when compared to CrS+DOX (See Table 3).

Table 3
Percent Change (±%) in Myostatin Expression in Three-Day Rat DIA Tissue

<table>
<thead>
<tr>
<th></th>
<th>CON+SAL</th>
<th>CON+DOX</th>
<th>CS+SAL</th>
<th>CrS+DOX</th>
<th>CrL+SAL</th>
<th>CrL+DOX</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON+SAL</td>
<td>0.00</td>
<td>+15.0</td>
<td>+25.0</td>
<td>+2.00</td>
<td>−9.00</td>
<td>+3.00</td>
</tr>
<tr>
<td>CON+DOX</td>
<td>-</td>
<td>0.00</td>
<td>−8.00</td>
<td>−11.3</td>
<td>+26.4</td>
<td>−10.4</td>
</tr>
<tr>
<td>CrS+SAL</td>
<td>-</td>
<td>-</td>
<td>0.00</td>
<td>−18.4</td>
<td>−27.2</td>
<td>−17.6</td>
</tr>
<tr>
<td>CrS+DOX</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.00</td>
<td>+12.1</td>
<td>+0.98</td>
</tr>
<tr>
<td>CrL+SAL</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.00</td>
<td>+13.2</td>
</tr>
<tr>
<td>CrL+DOX</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Note. DOX, doxorubicin; SAL, saline; CON, control; CrS, Standard creatine diet; CrL, Creatine loading diet; Three-Day, sacrifice three days post-injection ($N=57$).
Figure 5. Myostatin Expression in the DIA of Three-Day Animals. There was not a significant diet effect, drug effect, or interaction (N=57). CON, control; CrS, Standard creatine diet; CrL, Creatine loading diet; DOX, doxorubicin; SAL, saline. Top bands represent myostatin. Bottom bands represent GAPDH as the loading control.
**Muscle RING-Finger Protein-1 (MuRF-1)**

No significant drug effect \((p = 0.826)\), diet effect \((p = 0.931)\), or interaction \((p = 0.941)\) was observed for MuRF-1 protein expression three days post-injection in the DIA (See *Figure 6*). Although there were no detected main effects between variables or interactions between diet and drug, MuRF-1 expression was 8.53% greater in the DIA from CON+DOX in comparison to CON+SAL three days post-injection. Furthermore, MuRF-1 expression was 6.43% and 14.3% lower in the DIA from CrS+DOX and CrL+DOX, respectively, in comparison to CON+DOX. Finally, MuRF-1 expression was 8.40% lower in the DIA from CrL+DOX when compared to CrS+DOX (See Table 4).

**Table 4**

*Percent Change (±%) in MuRF-1 Expression in Three-Day Rat DIA Tissue*

<table>
<thead>
<tr>
<th></th>
<th>CON+SAL</th>
<th>CON+DOX</th>
<th>CrS+SAL</th>
<th>CrS+DOX</th>
<th>CrL+SAL</th>
<th>CrL+DOX</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON+SAL</td>
<td>0</td>
<td>+8.53</td>
<td>+4.65</td>
<td>+1.55</td>
<td>-5.43</td>
<td>-6.98</td>
</tr>
<tr>
<td>CON+DOX</td>
<td>-</td>
<td>0</td>
<td>+3.70</td>
<td>-6.43</td>
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<td>-14.3</td>
</tr>
<tr>
<td>CrS+SAL</td>
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<td>-</td>
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<td>-2.96</td>
<td>-9.63</td>
<td>-11.1</td>
</tr>
<tr>
<td>CrS+DOX</td>
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<td>-</td>
<td>-</td>
<td>0</td>
<td>+7.38</td>
<td>-8.40</td>
</tr>
<tr>
<td>CrL+SAL</td>
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<td>-</td>
<td>-</td>
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<td>-1.64</td>
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<tr>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
</tbody>
</table>

*Note.* DOX, doxorubicin; SAL, saline; CON, control; CrS, Standard creatine diet; CrL, Creatine loading diet; Three-Day, sacrifice three days post-injection \((N=57)\)
Figure 6. MuRF-1 Expression in the DIA of Three-Day Animals. There was not a significant diet effect, drug effect, or interaction ($N=57$). CON, control; CrS, Standard creatine diet; CrL, Creatine loading diet; DOX, doxorubicin; SAL, saline. Top bands represent MuRF-1. Bottom bands represent GAPDH as the loading control.
Summary

It was hypothesized that (1) DOX (15 mg/kg) administration would increase myostatin and MuRF-1 protein expression in the DIA tissue in comparison to SAL (0.9% NaCl), (2) Cr supplementation would attenuate the effects of DOX on myostatin and MuRF-1 expression at both time points, and (3) the creatine monohydrate loading (CrL) protocol would attenuate the effects of DOX to the greatest extent at both time points. There were no significant main diet effects, drug effects, or any diet x drug interactions at either time point among protein expression of myostatin and MuRF-1 in the DIA tissue as determined by two-way ANOVA ($p > 0.05$). Therefore, the hypotheses were not supported.
CHAPTER V
DISCUSSION

It is well-known that DOX administration leads to a decline in MRF expression in skeletal muscle, which disrupts muscle growth, causes atrophy, and potentially allows for increased activity of negative regulators of muscle mass, such as myostatin and MuRF-1. Skeletal muscle wasting and fatigue are common side effects of DOX administration, but increased skeletal muscle mass and strength, as well as decreased muscle atrophy, have been observed under conditions of inhibited myostatin and MuRF-1 activity (Tobin & Celeste, 2005; Rom & Reznick, 2016). Additionally, function and contractile strength of the DIA muscle are negatively impacted by DOX administration, which may be related to dyspnea experienced by patients undergoing chemotherapy treatment (Gilliam, Moylan, Callahan, et al., 2011). Therefore, it was hypothesized that DOX administration would lead to an increased expression of myostatin and MuRF-1 protein in the DIA.

Creatine is a widely used dietary supplement that has been shown to improve short-term, high-intensity athletic performance and offer potential therapeutic benefits to individuals with muscle wasting diseases and patients with chemotherapy-induced muscle dysfunction, weakness, and wasting. The most effective means of creatine supplementation is dietary (Riesberg et al., 2016) and involves a short-term loading period followed by a maintenance period (Kreider et al., 2017). However, stores of creatine can also be increased in skeletal muscle by standard supplementation protocols (Hall & Trojan, 2013). Therefore, it was hypothesized that Cr supplementation via the
loading, CrL, or standard, CrS, protocols would lessen the severity of DOX-induced changes in protein expression in comparison to control, CON. It was also hypothesized that the CrL protocol would elicit the most protective effects against DOX-induced alterations in myostatin and MuRF-1 protein expression in the DIA.

This was the first known study to analyze myostatin and MuRF-1 protein expression in the DIA of rats supplemented with Cr and later treated with a single intraperitoneal injection of DOX. The main focus of this research was to compare the effects of two Cr supplementation protocols, CrS and CrL, on protein expression in the DIA of rats sacrificed at one- and three-day time points following DOX administration. There were no statistically significant main effects on protein expression in the DIA as a result of Cr supplementation, DOX treatment, nor was there a diet x drug interaction.

While there were no significant diet effects, drug effects, or an interaction of diet and drug, potential trends demonstrating the potentially negative outcomes of DOX treatment and beneficial effects of Cr supplementation on DIA protein composition were observed. There was a potential trend in the CON+DOX group toward increased myostatin and MuRF-1 expression in the DIA in comparison to CON+SAL groups at the one-day time point by 11.6% (See Table 1) and 51.7% (See Table 2), respectively. Likewise, myostatin and MuRF-1 expression in the CON+DOX group was increased by 15.0% (See Table 3) and 8.53% (See Table 4), respectively, in comparison to CON+SAL groups at the three-day time point.

Based on the hypothesis that myostatin and MuRF-1 protein expression in skeletal muscle is associated with DOX presence in the tissue, the findings of Hayward et al. (2013) potentially suggest that myostatin and MuRF-1 protein expression should be
greatest one day post-injection. Hayward et al. (2013) did not implement a creatine supplementation protocol to be paired with DOX administration and reported that DOX accumulation in the EDL and SOL muscles was greatest one day after DOX injection. This finding seems to be in disagreement with the protein expression results of the current study, where myostatin expression was greater in the CON+DOX groups at the three-day time point in comparison to the one-day time point (See Figures 3 and 5). The expression of MuRF-1 was also higher in the CON+DOX groups at the three-day time point in comparison to the one-day time point (See Figures 4 and 6). Furthermore, the findings of Yamada et al. (1995) pertaining to DOX accumulation in the DIA are similar to those of Hayward et al. (2013) regarding DOX accumulation in the EDL and SOL muscles of rats. According to Yamada et al. (1995), DOX concentration in the DIA was highest six hours post-injection with DOX and declined by over 95% within one week. The observations of Hayward et al. (2013) and Yamada et al. (1995) do not appear to support the findings of the current study, where myostatin and MuRF-1 expression was greater at the three-day time point in comparison to the one-day time point. Based on these observations, DOX accumulation may differentially affect skeletal muscles, perhaps due to variations in the composition of fiber types. Additionally, these findings indicate that the initiation of mechanisms that contribute to myostatin and MuRF-1 protein expression in the DIA may occur more than one-day post-injection with DOX or other mechanisms may influence myostatin and MuRF-1 expression.

Supplementation with both Cr protocols in DOX groups potentially trended toward decreasing myostatin and MuRF-1 expression in the DIA at both time points when compared to CON-SAL. The expression of myostatin in CrS+DOX and CrL+DOX
groups was decreased by 1.10% and 11.6%, respectively, in comparison to the CON+DOX group at the one-day time point (See Table 1). MuRF-1 expression in CrS+DOX and CrL+DOX groups was decreased by 21.7% and 20.0%, respectively, in comparison to the CON+DOX group at the one-day time point (See Table 2). Similarly, myostatin expression in CrS+DOX and CrL+DOX groups was decreased by 13.0% and 12.0%, respectively, in comparison to the CON+DOX group at the three-day time point (See Table 3). The expression of MuRF-1 in CrS+DOX and CrL+DOX groups was decreased by 6.98% and 15.5%, respectively, in comparison to the CON+DOX group at the three-day time point (See Table 4).

Neither protocol was shown to be superior in decreasing the expression of both proteins at the one- and three-day time points, even though there was a potential trend toward decreasing myostatin and MuRF-1 protein expression in the DIA after both Cr supplementation protocols when compared to CON-SAL. For instance, myostatin expression was 10.5% lower and 1.00% higher in the CrL-DOX group at the one- and three-day time points, respectively, in comparison to the CrS-DOX group (See Tables 1 and 3). Moreover, MuRF-1 expression was 1.70% higher and 8.53% lower in the CrL-DOX group at the one- and three-day time points, respectively, in comparison to the CrS-DOX group (See Tables 2 and 4).

While a majority of the results of the present study were unanticipated, some were potentially explained by the outcomes of past research pertaining to regulatory protein expression in the DIA. The observed potential trend in decreased myostatin and MuRF-1 protein expression supports the conclusions of Cannata et al. (2010), where increased myostatin and MuRF-1 mRNA levels were lessened following DOX administration in
spiny rats due to maternal creatine supplementation. Additionally, creatine supplementation may have had a protective effect against DIA muscle atrophy even though markers of muscle atrophy were not specifically measured in the current study. For example, Testelmans et al. (2010) noted that atrophy was present even in cases of lower than average diaphragmatic myostatin mRNA levels. Based on this finding, myostatin may function to prevent muscle growth more so than to induce muscle wasting. Rather than solely focus on protein expression in the DIA, markers of muscle atrophy, such as muscle fiber cross-sectional area, should also be investigated in the future.

Overall, the hypotheses were not supported with statistically significant data. However, a trend toward an increase in myostatin and MuRF-1 expression in the DIA was observed at both time points in the CON-DOX group when compared to the CON-SAL group. Neither Cr supplementation protocol led to a statistically significant attenuation of DOX effects on myostatin and MuRF-1 expression at either time point. Further investigation is required to determine if creatine supplementation is directly related to myostatin and MuRF-1 expression in the DIA or indirectly associated with other mediators and pathways related to protein degradation and synthesis.

**Limitations**

One of the primary limitations of this research was that the sacrifice time of rats in the one-day and three-day groups was not exactly 24 hours and 72 hours post-injection, respectively, with DOX. Since the time of sacrifice following DOX administration may have varied slightly between animals in the same groups, protein expression may have been impacted. Additionally, a Western blot technique was utilized to quantify myostatin and MuRF-1 protein expression in the DIA, which may not have
been ideal. The accuracy of protein quantification throughout the DIA tissue samples may have been improved through the utilization of immunohistostaining methods. Lastly, the sample sizes utilized for each group were unequal due to experimenter error during DIA tissue removal and storage, as well as animal death from DOX-induced toxicity prior to sacrifice.

**Future Directions**

Future research should focus on the expression of proteins in the DIA other than myostatin and MuRF-1, as these findings suggest that other regulatory proteins and mechanisms may contribute to the respiratory function decline and dyspnea observed in individuals being treated with DOX. It may also be of interest to add an exercise component to the study design, since both endurance and resistance training prior to DOX administration in rats have been associated with an increased activity of mechanisms in the muscle that protect against skeletal muscle wasting and function decline (Bredahl et al., 2016). Furthermore, Marques-Aleixo et al. (2015) observed a potential reduction in oxidative stress and damage in the heart mitochondria of rats following short-term DOX treatment and the implementation of two exercise training protocols.

In order to obtain a greater understanding of mechanisms contributing to the decline of respiratory function in different sexes, the effects of Cr supplementation in both male and female rats should be investigated in DOX-treated animals. This may be relevant to human populations with degenerative muscle diseases or individuals undergoing chemotherapy treatment, because Hermes et al. (2018) suggested that estrogen offered a protective effect against DIA damage in female *mdx* mice submitted to
an exercise protocol. Furthermore, future researchers should measure the contractile force of DIA muscle from each group in order to investigate the potential relationship between DIA muscle dysfunction and the expression of specific proteins in the DIA. Contractile force of the DIA muscle should be measured using a force transducer in an experimental protocol, similar to the methods utilized by O’Leary et al. (2018), because past research has concluded that similar Cr supplementation and DOX administration protocols have impacted skeletal muscle functioning. For example, Bredahl and Hydock (2017) reported that isolated SOL and EDL muscles treated with Cr prior to incubation with DOX were protected from the fatigue typically induced by DOX. However, DIA muscle fatigue has not specifically been investigated under the same experimental conditions created by Bredahl and Hydock (2017), which may be of future interest.

**Conclusion**

The current study analyzed the effects of Cr treatment paired with DOX administration on myostatin and MuRF-1 protein expression in the DIA of rats at two time points following DOX injection. There were no significant diet effects, drug effects, or interactions at either timepoint among protein expression in the DIA ($p > 0.05$). However, there were trends toward decreased myostatin and MuRF-1 expression in the DIA. It is hoped that the use of Cr supplementation in individuals undergoing DOX treatment can attenuate the negative outcomes on respiratory and DIA functioning experienced by cancer patients, especially when paired with an appropriate exercise training protocol. Future research should highlight the expression of other regulatory proteins in the DIA, effects of DOX in the DIA at hourly time points following treatment, contractile function of the DIA, the effects of exercise in combination with Cr
supplementation and DOX administration, and the influence of sex on muscular damage in the DIA, in order to identify mechanisms contributing to respiratory and DIA dysfunction in individuals receiving chemotherapy.
REFERENCES


Zanou, N., & Gailly, P. (2013). Skeletal muscle hypertrophy and regeneration: Interplay between the myogenic regulatory factors (MRFs) and insulin-like growth factors (IGFs) pathways. *Cellular and Molecular Life Sciences, 70*(21), 4117-30.


APPENDIX A

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL
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