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Effects of Creatine Monohydrate Supplementation on Cytochrome C and Caspase-9 Expression in Skeletal Muscles from Doxorubicin Treated Rats

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EFFECTS OF CREATINE MONOHYDRATE SUPPLEMENTATION ON CYTOCHROME *C* AND CASPASE-9 EXPRESSION IN SKELETAL MUSCLES FROM DOXORUBICIN TREATED RATS

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ABSTRACT

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Doxorubicin (DOX) is an effective chemotherapeutic drug for many types of cancer. However, the deleterious effect of DOX is also observed in heart and skeletal muscles. Doxorubicin-induced reactive oxygen species (ROS) in mitochondria causes muscle dysfunction and atrophy leading to the release of cytochrome *c* (cyt *c*) and activation of caspase-9 (casp-9) which cause apoptosis, a programmed cell death in skeletal muscles. Previous studies have proposed that creatine (Cr) possesses antioxidant capacity by reducing ROS in oxidative environment. Creatine supplements have shown to reduce mitochondrial ROS production and decrease apoptotic enzyme activities in oxidative skeletal muscles; however, Cr's effects on DOX-induced apoptosis are not yet fully understood. **PURPOSE:** To investigate the effects of two different Cr supplementation protocols on cyt *c* and casp-9 expression in skeletal muscles from rats treated with DOX at two different time points. **METHODS:** Male Sprague Dawley rats (n=120) were randomly assigned to one of the three different diet treatments: control (CON, n=40), 2% Cr for four-week (2% Cr, n=40), and the Cr loading consisting of 4% Cr for one-week followed by three-week of 2% Cr (4% Cr, n=40). Then, each group was randomly assigned to two different time points for the drug administration: one-day prior to the sacrifice (1, n=20) and three-day prior to the sacrifice (3, n=20). Lastly, each group

was randomly assigned to two different drug treatments: 3 mL of saline (SAL, n=10) and 15 mg/kg of DOX (DOX, n=10). The left soleus (SOL) and left extensor digitorum longus (EDL) were collected after anesthesia and homogenized. A western blot assay was used to determine protein expression of cyt *c* and casp-9. **RESULTS:** No significant effects of Cr supplementation and DOX administration on cyt *c* and casp-9 expressions were observed in both the SOL and EDL ($p>0.05$). The interaction between the Cr supplementation and the DOX injection was also not significant ($p>0.05$). A trend toward a decrease in cyt *c* and casp-9 expression was observed in the 4% Cr group of 3-SOL and 1-EDL with the DOX injection, but there were no significant differences ($p>0.05$). **CONCLUSIONS:** Even though there were no significant main effects of Cr diet treatment, DOX administration and no interactions, a potential trend indicated that the Cr loading protocol may prevent apoptosis by attenuating the expressions of cyt *c* and casp-9 in skeletal muscles. Further research with measuring oxidative stress level, protein synthesis and degradation, and amount of Cr supplementation consumed needs to be conducted in order to fully understand the mechanism of the Cr supplement and the DOX administrations in skeletal muscles.

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CHAPTER I

INTRODUCTION

Chemotherapy is one of the major cancer treatments, along with surgery, radiation therapy, and immunotherapy. The effect of chemotherapy was originated from World War I when mustard gas was used and found to possess the "anti-carcinogenic" effect. (Berenblum, 1929). Since then, scientists investigated the effect of chemotherapy for cancer treatment, and it was introduced to clinics in the early 20th century (Papac, 2001). Chemotherapy utilizes one or more chemotherapeutic agents to prolong life, reduce symptoms of cancer, and potentially cure certain types of cancer. Ideally, chemotherapeutic agents should specifically target neoplastic cells, also called tumors, and decrease the size of tumors by poisoning the inside of the cell, leading to an inhibition of mitosis, or cell division. Anti-cancer drug-induced cytotoxicity also has been observed in normal cells due to a lack of specificity and increased the side effects of chemotherapeutic drugs including hair loss, anemia, fatigue, and weight loss (Johnstone, Ruefli, & Lowe, 2002; Coates et al., 1983).

Doxorubicin (DOX) is one of many chemotherapeutic drugs and frequently used to treat many types of cancers, including leukemia, lymphomas, solid tumors, and sarcomas (Lipshult et al., 1991). The mechanism of DOX is that the production of reactive oxygen species (ROS) through its metabolism damages cell membranes, proteins, and deoxyribonucleic acid (DNA) resulting in cell death (Minotti, Menna, Salvatorelli, Cairo, & Gianni, 2004). DOX treatment for cancer has been shown to be

effective; however, DOX-induced toxicity also has been observed to cause critical damage in normal tissues including heart and skeletal muscles (Hydock, Lien, Jensen, Schneider, & Hayward, 2011). In skeletal muscle, DOX treatment decreased force production and the level of contractile proteins and initiated cell death because of DOX-induced ROS production (Min et al., 2015). Therefore, the usage of DOX treatment has been limited in the clinics due to its toxicity in healthy tissues.

Reactive oxygen species (ROS) are molecules that contain oxygen and are produced by the metabolism of oxygen (Devasagayam et al., 2004). Reactive oxygen species are often taken up by the antioxidant defense enzyme such as superoxide dismutase (SOD) to prevent ROS from reacting with other molecules. (Valko et al., 2007). The accumulation of ROS, known as oxidative stress, damages macromolecules such as carbohydrates, lipids, protein, and DNA and may initiate apoptosis (Birben, Sahiner, Sackesen, Erzurum, & Kalayci, 2012). It is essential to tightly regulate oxidative stress because it can cause many pathological conditions including neurodegenerative disease, gene mutations, inflammatory disease, chronic fatigue syndrome, and cancer (Sies, Stahl, & Sundquist, 1992).

Apoptosis is a tightly regulated programmed cell death, and it is a normal event as a homeostatic mechanism in tissues (Alberts et al., 2008). Apoptosis is often triggered by physical or pathological conditions such as DNA damage and the overproduction of ROS (Norbury, & Hickson, 2001; Yuan, Murrell, Trickett, & Wang, 2003). This programmed cell death is often initiated by the release of apoptosis-inducing factors (AIF) such as cytochrome *c* and the activation of proapoptotic proteins including caspase-9 and caspase-3. Preventing the initiation of apoptosis is critical especially when there is too

much apoptosis because it can cause neurodegenerative diseases, ischemic damage, and atrophy in tissues (Elmore, 2007).

Creatine (Cr) is an organic compound that is produced primarily in the liver and consumed through a regular diet (Walker, 1979). The ergogenic effect of Cr as a supplement is well known to enhance exercise performance such as power, strength, and speed with exercise training (M. Bemben, D. Bemben, Loftiss, & Knehans, 2001; Skare, Skadberg, & Wisnes, 2001). Recently, researchers have proposed a direct antioxidant capacity of Cr. Creatine as a supplement has shown to scavenge free radicals, specifically superoxide anion ($^{\bullet}O^{-}_{2}$) and peroxynitrite ($OONO^{-}$) and reduce the level of free radicals induced by exercise in skeletal muscle (Deminice & Jordao, 2012; Guimarães-Ferreira et al., 2012; Lawler, Barnes, Wu, Song, & Demaree, 2002). This direct antioxidant capacity of Cr has been debatable due to unclear exact mechanisms; however, the creatine supplement seemed to activate an intracellular signaling pathway that regulates mitochondrial function in oxidative skeletal muscle and reduce apoptotic enzyme activities by activating the anti-apoptotic pathway in ischemic heart tissue (Ceddia & Sweeney, 2004; Caretti et al., 2010).

Therefore, doxorubicin treatment is well-known to be effective in many types of cancers; however, the mechanism of DOX induces toxicity in heart and skeletal muscles by producing an excessive amount of ROS. Doxorubicin-induced damage in skeletal muscles can lead to decrease force production and contractile proteins, causing apoptosis. The direct antioxidant effect of Cr supplement can be potentially one of preventive interventions for ROS-induced apoptosis in skeletal muscles treated with DOX.

Statement of Purpose

The main purpose of this study was to investigate the effects of *in vivo* DOX administration at two different time points prior to rat sacrifice (one- and three-day) on the expressions of the apoptotic initiator proteins cytochrome *c* and caspase-9 in type I and type II skeletal muscle fibers. The second purpose of this study was to explore the effects of two different Cr supplementations, 2% Cr supplementation for four-week and Cr loading treatment consisting of 4% Cr supplementation for one-week then followed by 2% Cr supplementation for three-week, on cytochrome *c* and caspase-9 expressions in type I and type II skeletal muscle fibers. The third purpose of this study was to examine the interaction between the Cr supplementation and the DOX administration in type I and type II skeletal muscle fibers.

Research Hypotheses

H1 DOX administration at 1-day and 3-day prior to rat sacrifice will increase cytochrome *c* and caspase-9 expressions in both the soleus (SOL) and extensor digitorum longus (EDL).

H2 Both Cr supplementation treatments, 2% Cr and 4% Cr, will attenuate the increased cytochrome *c* and caspase-9 protein expressions in both the SOL and EDL.

H3 Both Cr supplementations treatment, 2% Cr and 4% Cr, will attenuate the effects of DOX on cytochrome *c* and caspase-9 expressions in both the SOL and EDL.

H4 The Cr loading treatment, one week of 4% Cr supplementation followed by three weeks of 2% Cr supplementation, will provide the greatest degree of protection against the effects of DOX on cytochrome *c* and caspase-9 expressions in both the SOL and EDL.

CHAPTER II

REVIEW OF LITERATURE

Doxorubicin (DOX)

Doxorubicin (DOX) is an anthracycline chemotherapeutic agent used to treat leukemia, lymphomas, and solid tumors such as breast, lung, gastric, and soft tissue sarcomas (Lipshult et al., 1991). Doxorubicin is composed of daunosamine and an addition of hydroxyl group at carbon 14 position (Takemura & Fujiwara, 2007). The main metabolic pathway for DOX is going under redox cycling where the reduction of two electrons yields doxorubicinol, a secondary alcohol, or one electron reduction with oxidoreductases including cytosolic nicotinamide adenine dinucleotide phosphate hydrogen, (NAD(P)H), xanthine oxidase, and synthases lead to form a doxorubicin-semiquinone radical. Due to its structure, the doxorubicin-semiquinone is an unstable metabolite so that it converts back to doxorubicin immediately. During this process, it releases reactive oxygen species (ROS), which can lead to lipid peroxidation, the oxidative degradation of fatty acids. Reactive oxygen species-induced lipid peroxidation can lead to membrane damage, DNA damage, oxidative stress, and eventually, it can continue to apoptotic pathways of cell death (Doroshow, 1986). The DOX chemotherapy has been effective in fighting against cancer by inducing DNA damage with the inhibition of topoisomerase II, an enzyme which manages DNA tangles and supercoils and by increasing oxidative stress which will lead to ROS-induced apoptosis (Gewirtz, 1999; Minotti et al., 2004; Momparler, Karon, Siegel, & Avila, 1976; Wang et al., 2004).

While the DOX chemotherapy is highly effective in DNA damaging for cancer cells, it also can damage healthy cells, especially in heart tissue with an increase in DOX-induced ROS causing cardiotoxicity (L'Ecuyer et al., 2006; Yoshida, Shiojima, Ikeda, & Komuro, 2009). Therefore, DOX treatment has been limited to decrease the damage in normal healthy tissues.

Reactive Oxygen Species (ROS)

Reactive oxygen species are atoms or a group of atoms that have one or more unpaired electrons such as superoxide ($^{\bullet}O^{-}_{2}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$), and hydroperoxyl (HO_2). They are endogenously generated in the process of mitochondrial oxidative phosphorylation, or the interaction of exogenous factors can lead to the accumulation of ROS. When there is an imbalance between the production of ROS and the cellular antioxidant defenses, oxidative stress occurs and causes ROS-mediated damage in nucleic acids, proteins, and lipids which can directly or indirectly result in cancer, diabetes, neurodegeneration, and atherosclerosis (Ray, Huang, & Tsuji, 2012).

Reactive oxygen species are often produced by oxidoreductases such as NAD(P)H, and xanthine oxidase in cell membranes, mitochondria, and endoplasmic reticulum. Mitochondrial ROS are generated in the process of oxidative phosphorylation where mitochondria convert energy into a form of adenosine triphosphate (ATP) as a product of citric acid cycle, fatty acid oxidation, and amino acid oxidation (Muller, 2000). The process of ATP synthesis in mitochondria involves the transport of hydrogen ions (H^+) across the inner mitochondrial membrane utilizing an enzymatic series of electron donors and acceptors, called the electron transport chain (ETC). The first electron acceptor, called complex I, accepts electrons from NADH, which is an electron

carrier from the citric acid cycle. The electrons get passed down to coenzyme Q, which also accepts electrons from complex II. Coenzyme Q continues to pass down electrons to complex III, then to cytochrome *c*. The final complex is complex IV, where oxygen is reduced to water (H_2O) (Han, Williams, & Cadenas, 200; Muller, 2000). In the process of passing electrons to oxygen, a small number of electrons do not complete the whole series which results in converting oxygen into the free radical, superoxide ($^{\bullet}O^{-}_{2}$) by NADPH and xanthine oxidase (Muller, Lustgarten, Jang, Richardson, & Van Remmen, 2007). Superoxide is mostly generated during complex I and III and initiates lipid peroxidation when it converts to hydrogen peroxide (H_2O_2) (Li et al., 2013). Due to the toxicity of $^{\bullet}O^{-}_{2}$ at high concentration, the antioxidant defense enzyme, superoxide dismutase (SOD), efficiently catalyzes superoxide into H_2O_2 (Valko et al., 2007). H_2O_2 can be converted to hydroperoxyl (·OH) which is highly reactive and can lead to damage of macromolecules, including nucleic acids, carbohydrate, lipids, and amino acids. To prevent the accumulation of ·OH, H_2O_2 can also be quickly decomposed by the peroxisomal enzyme, called catalase, into oxygen and water (Birben et al., 2012; Reiter et al., 1995).

Apoptosis

Apoptosis is defined as a form of programmed cell death which is a highly regulated and controlled process in multicellular organisms unlike necrosis, a form of traumatic cell death from acute cell injuries (Alberts et al., 2008). Apoptosis is a normal event during development and aging as a homeostatic mechanism in tissues. This programmed cell death can act as an immunity defense system when cells are damaged by disease, or physiological and pathological conditions can trigger apoptotic cell death.

Apoptosis can be induced by direct exposure of cells to ROS, including H_2O_2 and ·OH (Buttke & Sandstrom, 1994; Norbury & Hickson, 2001). Also, chemotherapeutic drugs result in DNA damage and can lead to a p53-dependent apoptotic pathway in cancer cells. Either too little or too much of apoptosis is critical in many human conditions, including neurodegenerative diseases, ischemic damage, autoimmune disorders, atrophy, and many types of cancers (Elmore, 2007).

Apoptosis can be activated by one of two main apoptotic pathways: the extrinsic/death receptor pathway and the intrinsic/mitochondrial pathway. The extrinsic pathway involves death receptors that are members of the tumor necrosis factor (TNF) receptor gene superfamily (Locksley, Killeen, & Lenardo, 2001). The TNF receptor family contains a cytoplasmic "death domain" that transmits the death signal to the intracellular signaling pathways from the cell surface. Once extracellular ligands including FasL and TNF-α bind to the TNF receptors with the death domain, a death-inducing signaling complex (DISC) is formed, leading to the activation of caspases, essential protease enzymes in apoptosis. The cleaved caspases (activation form of caspase) can activate other procaspases (an inactivated form of caspase) and allow to initiate the caspase cascade leading to apoptosis (Elmore, 2007; Peter & Krammer, 1998).

The intrinsic apoptosis pathway is initiated in response to non-receptor-mediated stimuli including radiation, hypoxia, increased intracellular concentration of free fatty acids, and ROS (Cotran & Kumar, 1998; Hardy et al., 2003; Mattson & Chan, 2003). These stimuli that initiate the intrinsic pathway produce intracellular signals which can act directly on targets of a cell and are the events initiated by mitochondria. *p53*-mediated apoptosis is an example of the intracellular signaling apoptotic pathway. The tumor

suppressor gene *p53* is a transcription factor that regulates the cell cycle. The apoptotic stimuli such as DNA damage at the G_1/S phase of the cell cycle activate *p53* and initiate the apoptosis. The activation of *p53* regulates Bcl-2 family of the proteins which play a role in mitochondrial membrane permeability. The proteins of the Bcl-2 family can be either pro-apoptotic or anti-apoptotic. For example, Bax and Bak are the pro-apoptotic proteins of the Bcl-2 family and formed an oligomeric pore in the outer mitochondrial membrane.

The anti-apoptotic proteins Bcl-2 and Bcl-x physically block the oligomeric pore of Bax and Bak and prevents the release of cyt *c* from mitochondria to the cytoplasm. The activation of *p53* by DNA damage recognition leads to a conformational change in Bax/Bak and Bcl-2/Bcl-x resulting in the release of cytochrome *c* (cyt *c*) from inner mitochondria to the cytoplasm (Cory & Adams, 2002; Elmore, 2007; Schuler & Green, 2001). Then, the mitochondria-mediated apoptosis occurs when the release of cyt *c* activates apoptotic protease activating factor-1 (Apaf-1) as well as procaspase-9 (Saelens et al., 2004). Cleaved caspase-9 is an initiator caspase of caspase cascade and leads to activating the effector caspases such as caspase-3, -6, and -7 resulting in apoptosis (Rai, Tripathi, Sharma, & Shukla, 2005). Additionally, Smac/Diablo, the secondary mitochondria-derived activator of caspases, binds to inhibitor of apoptosis proteins and enhances caspases activation (Susin et al., 1999).

In addition, calcium (Ca^{2+}) plays an essential role in the initiation of the intrinsic apoptotic pathway. The homeostasis of Ca^{2+} is tightly regulated in mitochondria. The concentration of Ca^{2+} in mitochondria can be increased by a Ca^{2+} uniporter, transporting Ca^{2+} from the cytosol into mitochondria, or pathological conditions. The Ca^{2+}-mediated

initiation of apoptosis is associated with an opening of mitochondrial permeability transition (MPT) pore. The large accumulation of Ca^{2+} in mitochondria can lead to osmotic swelling, and then it ends up with bursting the mitochondrial membrane. The rupture of the membrane can release cyt *c* into the cytosol through the opening of MPT (Orrenius, Gogvadze, & Zhivotovsky, 2015).

Additionally, Ca^{2+} can dephosphorylate the Bcl-2 family, including Bax and Bak by the protein phosphate calcineurin which is often activated by Ca^{2+}. This dephosphorylation of Bax and Bak triggers the unblock of Bcl-2 and Bcl-x on Bax/Bak on the outer mitochondrial membrane and leads to the release of cyt *c* to the cytoplasm (Wang, Lin-Shiau, & Lin, 1999). The Ca^{2+}-mediated activation of calpain in the mitochondria can initiate apoptosis in the cell as well. The entry of Ca^{2+} into the inner mitochondria can directly activate mitochondrial calpain. Then calpain promotes the cleavage of cardiolipin, which anchors apoptosis-inducing factors (AIF) including cyt *c* in the inner mitochondrial membrane. The release of AIF via cleavage of cardiolipin into the cytosol can cause DNA fragmentation and initiate cell death (Orrenius et al., 2015).

Cytochrome *c* (Cyt *c*)

Cytochrome *c* (cyt *c)* is a highly water-soluble hemeprotein involved in the ETC in the mitochondria. The heme group of cyt *c* accepts and transfers electrons to the complex IV, the final complex of ETC (Han, Williams, & Cadenas, 2001; Muller, 2000). Cyt *c* is also involved in the initiation of apoptosis. It binds to cardiolipin in the inner mitochondrial membrane, which prevents it from releasing cyt *c* out of mitochondria and initiating apoptosis when there is no intracellular apoptotic signal. Without apoptotic stimuli, cyt *c* is usually in a reduced form and is not capable of initiating the intrinsic

apoptotic pathway. When an apoptotic stimulus is present such as ROS produced by mitochondria, cyt *c* becomes oxidized by NAD(P)H and causes an opening of the MPT pore. Then the oxidized cyt *c* is detached from cardiolipin and released through the MPT pore into the cytoplasm (Matsuura, Canfield, Feng, & Kurokawa, 2016). Also, the release of cyt c is highly regulated by the Bcl-2 family via *p53* mediated apoptotic pathway. The *p53*-mediated conformational change of Bax/Bak makes an opening on mitochondrial membrane and allow cyt *c* to pass through the oligomeric pore of Bax/Bak from mitochondria to the cytoplasm (Meyer, Kim, & Penn, 2006). The release of cyt *c* triggers the formation of the apoptosome, which is assembled with a one-to-one ratio of cyt *c* and Apaf-1. The sevenfold symmetric structure of apoptosomes leads to an activation of procaspase-9, an initiator caspase of caspase cascade resulting in apoptosis (Ferraro, Fuoco, Strappazzon, & Cecconi, (2010).

Karpinich, Tafani, Rothman, Russo, and Farber (2002) showed the importance of cyt *c* in maintaining healthy cells has been shown in their study. The L929 mouse fibroblasts were treated with ten μM of etoposide, which is a topoisomerase II inhibitor and induces DNA damage. The etoposide-treated fibroblasts showed expression of cyt *c* in the cytoplasm by western blot assay after 24 hours. Also, the percentage of dead cells increased by 48.1 ± 2.4 % after 72 hours of etoposide treatment. This data suggests that the release of cyt *c* induced cell death to regulate fibroblasts with DNA damage induced by etoposide. In addition, oxidative stress by ROS can induce the cyt *c* release in the cytoplasm. Human tendon was treated with high concentration (50 μM) of H_2O_2 for 12 hours, and a significant increase of apoptotic cells (8%) was detected compared to the control group ($p<0.05$). Only after 4 hours of 50 μM of H_2O_2 exposure in tendon cells,

the release of cyt *c* was expressed by western blot assay while there was no detectable cyt *c* expression in control cultured human tendon cells. The oxidative stress-induced cyt *c* release resulted in a threefold increase in caspase-3 activity (Yuan et al., 2003).

Another study suggested that lipid peroxidation induces cyt *c* release from mitochondria by breaking the bond of cyt *c* and cardiolipin. The bovine heart was treated with oxygen gas overnight leading to an increase in the formation of a conjugated diene, the marker for lipid peroxidation. The lipid peroxidation led to a less effective activity between cardiolipin and cyt *c* resulting in release of cyt *c* to the cytoplasm from mitochondria (Shidoji, Hayashi, Komura, Ohishi, & Yagi, 1999). Additionally, it has been shown that the increase in the release of cyt *c* from mitochondria was induced by Ca^{2+}-mediated mitochondrial swelling in the brain and liver tissue of rats. The induction of MPT was observed using a spectrophotometer, and the rapid reduction of light absorbance indicated a rise in mitochondrial swelling. The mitochondria from both brain and liver tissues, treated with 100 μM Ca^{2+}, caused a large decrease in light absorbance. This Ca^{2+}-induced mitochondrial swelling led to an increase in the release of cyt *c* from mitochondria, detected by western blot assay (Kobayashi et al., 2003). These studies suggested that the release of cyt *c* can be caused by DNA damage, ROS-induced oxidative stress, lipid peroxidation, or Ca^{2+}-induced mitochondrial swelling leading to cell death.

Caspase-9 (Casp-9)

Caspase-9 (casp-9) is a family of cysteine-aspartic protease (caspase) involved in apoptosis and found in the mitochondria, cytosol, and nucleus. Casp-9 is an initiator caspase of the caspase cascade and plays a critical role for the apoptotic pathway in many

tissues. Procaspase-9 is the form expressed in an inactive proenzyme, and cleaved caspase-9 is the active form of proenzyme caused by apoptotic signals (Kuida, 2000). Caspase-9 has three domains: N-terminal pro-domain, large subunit, and a small subunit. The N-terminal pro-domain contains the caspase activation domain (CARD) where it binds to CARD of Apaf-1 (Li et al., 2017). The release of cyt *c* and activated Apaf-1 by apoptotic signals cause dimerization of procaspase-9 resulting in formation of cleaved casp-9. Once it is activated, the caspase cascade initiates with cleaved casp-9 cleaving another procaspase-9 for its dimerization or activating the executioner caspases including caspase-3, -6, and -7, leading to apoptosis (Druskovic, Suput, & Milisav, 2006; Zhivotovsky, Samali, Gahm, & Orrenius, 1999).

Cleaved casp-9 is an essential enzyme to initiate apoptosis via caspase cascade in mitochondria. In the HeLa cell with immunodepletion of casp-9, it failed to activate the executioner caspase, caspase-3. According to Li et al., (1997), the protease cascade leading to apoptosis was initiated as soon as cleaved casp-9 rapidly activated caspase-3 in normal healthy HeLa cell. In another experiment, ROS such as H_2O_2 induced p53-dependent apoptosis. The loss of mitochondrial membrane permeability due to an injection of H_2O_2 (25 and 50 μM) in HeLa cells resulted in the release of cytochrome *c* leading to a significant increase in the cleavage of procaspase-9 compared to the control without the H_2O_2 treatment ($p<0.01$). The 25 μM H_2O_2-induced activation of procasp-9 significantly increased the activation of the caspase-3-mediated apoptosis ($p<0.001$) (Pallepati & Averill-Bates, 2011). In Wang et al. study (1999), flavonoid-induced ROS activated the procaspase-9 in human promyelocytic leukemia HL-60 cells treated with 60 μM of flavonoid, a strong antioxidant treatment. The time-dependent cleavage of

procaspase-9 was increased after 12 hours of flavonoid treatment by western blot assay, and it was consistent with DNA fragmentation and the activity of caspase-3. The study suggested that flavonoid metabolic process induced the ROS production due to its structure which led to mitochondrial dysfunction, and resulted in the activation of casp-9 and caspase-3, leading to apoptosis. In a cancer cachexia mouse model, increased caspase-dependent proteolytic activity was observed due to weight loss with the depletion of skeletal muscle and adipose tissue. Among mice with weight loss (from 2% to 25%) after MAC 16 or MAC 13 tumor implantation, the activity of caspase-9 and caspase-3 increased by 177% and 98%, respectively, in gastrocnemius muscles (Belizário, Lorite, & Tisdale, 2001). This suggested that the weight loss was caused by caspase-dependent apoptosis in skeletal muscle cells. From these previous studies, cleaved caspase-9 plays a critical apoptotic initiator enzyme which induces the activation of caspase-3, leading to apoptosis in cells.

Doxorubicin-Induced Apoptosis

Lipid Peroxidation

Lipid peroxidation is the oxidative degradation of fatty acids, especially polyunsaturated fatty acids due to their multiple double bonds in between methylene bridges that possess primarily reactive hydrogen atoms. The lipid peroxidation is initiated by ROS such as ·OH and H_2O_2 taking electrons from lipids in the cell membrane producing the fatty acid radicals. The fatty acid radical is not a stable molecule by losing an electron, which leads to reacting with oxygen, forming peroxyl-fatty acids. The final products of lipid oxidation are malondialdehyde (MDA) and 4-hydroxynonenal (HNE), and they are important biomarkers of oxidative stress (Mylonas & Kouretas, 1999). MDA

and HNE are considered to be mutagenic and causal agents for many diseases such as diabetes, chronic inflammation, neurodegenerative disease, osteoarthritis, and different types of cancer (Tiku, Narla, Jain, & Yalamanchili, 2007; Zarkovic, 2003).

According to Myers et al. (1977), DOX-induced lipid peroxidation results from increased ROS production by DOX metabolism with NAD(P)H and xanthine oxidase. Their study showed that MDA was detected in normal mouse cardiac tissues two days after the injection of DOX (15 mg/kg) in the peritoneum while the control cardiac tissues without the DOX injection had no MDA detected. After four days of the injection, MDA reached a peak concentration of 53 ± 10 μM. (Myers et al., 1977). Another study found that the formation of conjugate diene-containing lipids, resulted from peroxidation of unsaturated lipids, increased in cultured cardiomyocytes of Wistar rats exposed to 1 μM DOX. The DOX-treated cardiomyocyte had a significant increase of 1.6 fold in comparison to the cells not exposed to DOX in the first three hours after drug exposure ($p<0.01$). Also, there was a linear correlation between the conjugate diene-lipid level and the time of exposure in the first three hours ($r=0.996$) (Hrelia et al., 2002). The increase in intracellular ceramide, a biomarker of lipid peroxidation, was observed in rat cardiac myocyte with one hour treatment of DOX (0.5 μM). The DOX-induced lipid peroxidation led to apoptosis after 7-day of the DOX treatment in cardiomyocyte. Lipid peroxidation-induced apoptosis was prevented in cardiac myocyte with the treatment of L-carnitine (200 μg/ml), a compound with protective effect on cardiac metabolism (Andrieu-Abaddie et al., 1999).

Deoxyribonucleic Acid (DNA) Damage

Several mechanisms have been proposed for DOX-mediated deoxyribonucleic acid (DNA) damage, including topoisomerase inhibition, DNA adduct formation, and oxidative stress by ROS (Yang, Teves, Kemp, & Henikoff, 2014). The DOX-induced topoisomerase II poisoning has been clinically used to increase double-strand DNA breaks, resulting in DNA damage and cell death. Topoisomerase II is an ATP-dependent enzyme that binds DNA supercoils and entangled DNA and releases torsional stress formed during DNA replication and transcription. Additionally, topoisomerase II is important for separating two topologically-linked daughter chromosomes during mitosis, and topoisomerase II inhibition prevents healthy cells from dividing into two daughter cells resulting in apoptosis (Carpenter & Porter, 2004; Pommier, Leo, Zhang, & Marchand, 2010). Doxorubicin-induced topoisomerase II inhibition has a similar mechanism as etoposide which traps a topoisomerase II at breakage sites, stabilizes the cleavage complex, and impedes DNA resealing (Nitiss, 2009; Wu et al., 2011). In primary mouse embryonic fibroblasts (MEF) with DNA topoisomerase II β knockout (*top2*$\beta^{-/-}$), the level of DOX-induced γ-H2AX, a key DNA damage signal induced by double-strand DNA breaks, reduced in comparison with MEFs with DNA topoisomerase IIβ (T*OP2*$\beta^{+/+}$). This study suggests that the DOX mediates DNA damage signal γ-H2AX with topoisomerase IIβ (Lyu et al., 2007).

Additionally, topoisomerase IIβ was mainly involved in DOX-induced heart failure, which is a side effect of damaging non-dividing cells. It has been shown that T*OP2*$\beta^{+/+}$ cardiomyocytes of mice, injected with 25 mg/kg DOX and harvested 16 hours later had an increase of p53-inducible gene (Trp53inp1) that regulates apoptosis by 200-

fold. The genes involved in the apoptotic pathway, such as Apaf1, Bax, and Fas, showed an upregulation of their transcriptions. The upregulations of pro-apoptotic genes were only expressed in DOX-treated T*OP2*$\beta^{+/+}$ mice but not in *top2*$\beta^{-/-}$ mice with the same DOX treatment (Zhang et al., 2012). These findings suggest that DOX treatment can force cardiomyocytes into apoptosis by double-strand DNA breaks utilizing topoisomerase IIβ.

Doxorubicin is also able to cause DNA adduct formation as a DNA intercalator and activate DNA damage responses leading to topoisomerase II-independent cell death (Chaires, Herrera, & Waring, 1990; Chen, Gresh, & Pullman, 1986; Swift, Rephaeli, Nudelman, Phillips, & Cutts, 2006). Doxorubicin is more likely to intercalate at the adjacent GC base pairs due to specific hydrogen bond formation between DOX and guanine. A covalent bond mediated by formaldehyde, generated by free radical reactions from lipids and spermine, plays a primary role to stabilize DOX and DNA interaction. This interaction involves DOX forming a covalent bond with guanine on one strand of DNA mediated by formaldehyde and hydrogen bonds with guanine on the opposing strand (Taatjes, Gaudiano, Resing, & Koch, 1996). In cultured breast cancer cells (MCF-7), the elevated level of formaldehyde was observed with the highest concentration of DOX treatment (50 μM). The DOX sensitive MCF-7 cells had a higher formaldehyde count by 16.7 ± 4.3 compared to the DOX-resistant MCF-7 cells (Kato, Burke, Fenick, Taatjes, & Bierbaum, 2000). Another DOX-induced DNA adduct experiment was performed in the promyelocytic leukemia cell line HL-60 and the mitoxantrone resistant variant HL-60/MX2, which has a decreased expression of topoisomerase IIα and no detectable levels of topoisomerase IIβ. Doxorubicin concentration-dependent DNA

adduct formation in the presence of formaldehyde increased from ~2 adducts per 10 kb DNA for one µmol/L of DOX to ~8.5 adducts per 10 kb DNA for four µmol/L of DOX. The cells were also treated with a combination of DOX (1, 2, and 4 µmol/L) and pivaloyloxymethyl butyrate (AN-9, 50 µmol/L), a compound that releases formaldehyde upon hydrolysis. The adduct formation with the combination treatment increased DOX concentration-dependent apoptosis in both HL-60 and HL-60/MX2 cells, 30-50%, and 20-50% respectively. The activity level of Caspase-3/7, the executioners in apoptosis, was also measured, and the pattern of the activity was matched with the pattern of induction of apoptosis by sub-G_1 DNA content analysis and by DNA fragmentation. They showed that DOX-induced DNA adducts in the formation of formaldehyde could lead to a DOX concentration-dependent apoptosis in leukemia cells (Swift et al., 2006).

Although evidence suggests that DNA damage-induced apoptosis in cancer cells and cardiomyocytes by topoisomerase II and DNA adducts with DOX treatments, they are unlikely to be the important mechanisms of doxorubicin action. According to Pang et al. (2013), human melanoma cells were treated with a standard clinical dosage, 9 µM of DOX with inhibition of topoisomerase II and it strongly induced apoptosis which was led by histone eviction. Additionally, a clinical dose for DOX (typically >10 µM) causes only 4.4 ± 1.0 adduts/10^7 base pair DNA, and it is a small fraction of total DOX (Coldwell, Cutts, Ognibene, Henderson, & Phillips, 2008).

Further studies were investigated for more mechanisms of DOX-induced DNA damage besides the inhibition of topoisomerase II and DNA adduction. A different mechanism was found that DOX can also be oxidized into doxorubicin-semiquinone radicals through redox cycling by oxidoreductases including NAD(P)H and xanthine

synthase (Doroshow, Synold, Somlo, Akman, & Gajewski, 2001). Due to the instability of semiquinone radicals, they immediately react with oxygen and produce ROS including $^{\bullet}O^{-}_{2}$ and H_2O_2 leading to DNA damage (Berlin & Haseltine, 1981; Minotti et al., 2004). According to Myers (1998), DOX can bind to metal molecules and form a DOX-iron complex. This bond catalyzes H_2O_2 to ·OH which is a highly reactive molecule.

Doxorubicin-induced ROS can lead to oxidative stress and result in DNA damage and apoptosis (Thorn et al., 2011). In previous studies, researchers suggested that the redox cycling of DOX by NADH dehydrogenase induced oxidative stress and led to DNA base modifications. Oxidative DNA bases such as thymine glycol and 8-hydroxyguanine are mutagenic and are produced by ·OH characteristic chemical species in human chromatins (Basu, Loechler, Leadon, & Essigmann, 1989; Shibutani, Takeshita, & Grollman, 1991). Another study showed that erythrocytes from breast cancer patients had a significant increase in DNA base oxidation by DOX-induced ROS after 72 to 96 hours of intravenous infusion of 1 μM of DOX ($p<0.02$) (Doroshow et al., 2001). In H9C2 rat cardiomyocytes, the oxidized DNA bases, including 8-hydroxyguanine and pyrimidines, were observed and induced the activity of p53 (L'Ecuyer et al., 2006). The relationship between oxidative stress and DNA damage were examined in cultured cardiomyocyte of rats. With the 1 μM of DOX treatment for four and eight hours, DNA damage increased as oxidative stress increased. Also, the accumulation of p53 and cleaved caspase-3 expression were detected by western blot assay ($p<0.05$). The apoptotic cell death was also observed after eight hours of DOX treatment. DOX-induced oxidative stress and p53 expression were reduced by a free radical scavenger, N-Acetyl-Cystine, indicating that DOX-induced ROS resulting in p53-dependent apoptosis in

cardiomyocyte. In the p53 mice model with 6 mg/kg of DOX treatment for four weeks, an increased in impaired contractile function and apoptotic cardiomyocytes in p53 wild type mice model were detected compared to p53 heterozygous knockout mice ($p<0.05$). It was suggested that p53 accumulation and apoptotic cardiomyocyte were caused by DOX-induced ROS, resulting in DNA damage (Yoshida et al., 2009).

In another study, a significantly elevated level of cytosolic cyt *c* was observed in mitochondria of DOX (20 mg/kg) treated cardiomyocytes of rats compared to the control group ($p=0.033$). Also, the positive correlation of cytosolic cyt *c* and caspase-3 activity was found in the DOX-treated group ($r=0.88$, $p=0.003$). These findings suggested that the *in vivo* levels of cyt *c* in the cytoplasm of cardiomyocytes directly affect caspase-3 activity, which results in apoptosis. The significantly elevated level of 8-iso-$PGF_{2\alpha}$, a marker of oxidative stress to lipids, was detected in the cytosol of rats treated with DOX ($p=0.036$). This result suggests that DOX induces oxidative stress *in vivo* and leads to an elevated cyt *c* release resulting in apoptosis of cardiomyocyte of rats (Childs, Phaneuf, Dirks, Phillips, & Leeuwenburgh, 2002). These DNA base modifications and oxidative stress by ROS can promote mutations and impair the functions of replication and transcription, resulting in cell death (Jena, 2012).

Doxorubicin and Skeletal Muscle

The DOX chemotherapy has been associated with myotoxicity in many tissues, including cardiac and skeletal muscle. Dorxorubicin-induced skeletal muscle toxicity has resulted in an increase in muscle dysfunction, fatigue, and atrophy depending on time and injection dosage (Brehdal, Pfannenstiel, Quinn, Hayward, & Hydock, 2016; Hydock et al., 2011; Smuder, Kavazis, Min, & Powers, 2011; Van Norren et al., 2009). This

deleterious effect of DOX is a serious problem because it directly affects activities of daily living (ADL) requiring the utilization of skeletal muscle in cancer patients undergoing DOX chemotherapy (Schwartz, 2000).

The contractile dysfunction of extensor digitorum longus (EDL) of the mouse was observed with DOX treatment (50 – 175 μM) for 0 – 2 hours in *ex vivo* and *in vitro* assays. Due to the significantly longer relaxation time at 90 – 100% of the maximal force in all of the different dosages of DOX ($p<0.05$), maximal force, contraction and relaxation velocity significantly decreased in a time- and dose-dependent manners ($p<0.05$). The slower relaxation velocity is associated with impaired relaxation, which is directly related to the rate of calcium (Ca^{2+}) clearance from the cytoplasm. They further investigated a calcium influx with oxidative stress, which was applied to C2C12 myotubes and resulted in producing ROS due to a mitochondrial malfunction. Even though the increase in ROS led to cell death in C2C12 cells, the increase in Ca^{2+} influx was not observed (MacLennan, 2000; Van Norren et al., 2009). However, Abramson, Buck, Salama, Casida, and Pessah (1988) found evidence that DOX (< 10 μM) induced Ca^{2+} release by 50-60% in rabbit skeletal muscle sarcoplasmic reticulum (SR), which stores Ca^{2+} and regulates contraction and relaxation in skeletal muscle. This study suggested that DOX-induced Ca^{2+} release from SR has a direct dose-dependent interaction with the ryanodine receptor (RYR) complex, a calcium release channel, by increasing the sensitivity of calcium activator site of RYR.

The increase in intracellular Ca^{2+} release can trigger the caspase cascade and calpain, a Ca^{2+}-dependent protease (on caspase protease) involved in apoptosis (Momeni, 2011). A piece of evidence showed that 20 mg/kg of DOX injection in soleus (SOL)

muscle of rats 24 hours before sacrifice activated protease, including calpain and caspase-3. The ratio of calpain-to-calpastatin (calpain inhibitor) was significantly higher compared to the control group without DOX ($p<0.05$). Also, the expression of cleaved caspase-3, often activated by casp-9 in the caspase cascade, was significantly higher than the control group in a western blot assay ($p<0.05$). These results showed that the DOX treatment triggered apoptosis via caspase protease and on caspase protease in SOL muscle.

Additionally, one of the contractile proteins of skeletal muscle, actin, was observed as a marker of muscle proteolysis. The increase in activity of calpain and caspase-3 significantly lowered the actin level in SOL with DOX treatment compared to the control group without DOX and the endurance exercise training group without DOX treatment ($p<0.05$). The percentage of myofilament release showed a significant increase ($p<0.05$) in SOL with 20 mg/kg of DOX injection due to the loss of actin leading to reduction of skeletal muscle cross-sectional which can cause the contractile dysfunction (Smuder et al., 2011). Another study showed that Ca^{2+}-mediated calpain activation significantly increased in the diaphragm, plantaris, and soleus of rat model with the clinical dosage (20 mg/kg) of DOX injection 48 hours prior to sacrifice. The active calpain can cause the release of cyt *c* and AIF by breaking the bond of cardiolipin and inner mitochondrial membrane (Min et al., 2015). Then, the release of cyt *c* and AIF triggers DNA fragmentation and initiates apoptosis in cells.

Doxorubicin-induced contractile dysfunction and atrophy can be triggered by a large amount of DOX-induced ROS production, leading to oxidative stress in skeletal muscle (Min et al., 2015; Montaigne et al., 2011). In Min et al. study (2015), the

significant increase in mitochondrial ROS emission was detected in the diaphragm, plantaris, and soleus muscles with 20 mg/kg of DOX treatment in rats ($p<0.05$). Besides, the mitochondrial-targeted antioxidant, SS31, was utilized with DOX treatment in all three tissues, and the DOX-mediated mitochondrial ROS emission was prevented. They also observed oxidative damage in muscle proteins triggered by the DOX-mediated mitochondrial ROS production. The level of 4-HNE, a biomarker of lipid peroxidation, was significantly increased with the DOX treatment (20 mg/kg) compared to the control group without the DOX injection ($p<0.05$). Important to note, the tissues with SS31 treatment prevented the increase in 4-HNE, indicating that the DOX-mediated mitochondrial ROS emission resulted in oxidative stress in the skeletal muscle, causing lipid peroxidation. The increase in mitochondrial ROS emission by the DOX treatment in diaphragm led to a significant reduction in diaphragmatic specific force production compared to the control group. Additionally, there was a significant decrease in myofiber cross-section area (CSA) in all three skeletal muscles including the diaphragm, plantaris and soleus muscles ($p<0.05$), however, the reduction in myofiber CSA was protected with the antioxidant treatment of SS31 in these tissues. This indicated that DOX-induced mitochondrial ROS caused contractile dysfunction and skeletal muscle atrophy.

Interestingly, cardiolipin, the anchorage protein for AIF and inner mitochondria membrane, can also be readily peroxidized in ROS-induced oxidative environment and promote the opening of MPT leading to release of AIF including cyt *c* besides the mechanism that the Ca^{2+}-mediated calpain activation dephosphorylates cardiolipin (Montaigne et al., 2011). Therefore, DOX treatment induces mitochondrial ROS production leading to oxidative damage in skeletal muscle and promotes the opening of

MPT pores by ROS-mediated cardiolipin dephosphorylation causing the release of AIF from the mitochondria.

Doxorubicin-induced mitochondrial ROS production can also upregulate the muscle regulatory proteins, causing muscle degradation in skeletal muscle. For example, E3 ubiquitin ligases, including atrogin-1/MAFbx and MuRF1, are mainly involved in muscle protein degradation. The upregulation of these proteins can be found in many catabolic situations such as inflammation and starvation (Dogra et al., 2007; Jagoe, Lecker, Gomes, & Goldberg, 2002). Evidence showed that the exposure of 0.2 μM DOX to C2C12 myotube led to a significantly increased level of atrogin-1/MAFbx mRNA, along with a rise in protein 24 hours after the exposure ($p<0.05$). However, DOX treatment in C2C12 myotubes altered neither of the levels of MuRF1 mRNA or MuRF1 protein level. To ensure the increase in atrogin-1/MAFbx mRNA and protein levels was affected by the DOX-induced mitochondrial ROS emission, C2C12 was treated with the mitochondrial-targeted antioxidant, SS31. The level of atrogin-1/MAFbx mRNA was reduced with the SS31 treatment and the decrease in myotube width and the loss of contractile proteins including myosin and actin, caused by DOX treatment, were prevented as well (Gilliam et al., 2012).

These previous studies showed that the DOX treatment induced apoptosis via activating Ca^{2+}-mediated calpain leading to the increase in the opening of MPT pores in mitochondria which released AIF including cyt *c* to the cytoplasm. In addition, DOX-induced mitochondrial ROS production could increase the level of MPT pores by lipid peroxidation of mitochondrial membrane and the mRNA level of E3 ubiquitin ligases

involved in muscle protein degradation such as atrogin-1/MAFbx. The DOX-mediated apoptosis in skeletal muscle leads to contractile dysfunction and muscle atrophy.

Creatine

Creatine (Cr) is an organic compound that is endogenously synthesized with two amino acids, arginine, and glycine, that are combined by the enzyme arginine:glycine amidinotransferase (AGAT). The combination of arginine and glycine forms guanidinoacetate which is methylated with S-adenosyl methionine by guanidinoacetate N-methyltransferase, leading to the formation of Cr. The synthesis of Cr primarily occurs in the liver and is transported to other organs that store and utilize Cr through the blood. By utilizing its concentration gradient, Cr is diffused and transported through sodium and chloride dependent transporter into tissues from the blood. Most of Cr (95% of total Cr) becomes stored in skeletal muscle while the remaining 5% of the Cr can be found in the blood, brain, liver, and kidney. In addition, Cr can also be obtained by dietary intake, and the average Cr uptake from the diet is ~1g/day in young adults (Walker, 1979).

Creatine kinase (CK) can convert Cr and ATP to phosphocreatine (PCr) and adenosine diphosphate (ADP). PCr can be seen as an energy buffer in the PCr energy system because it can form ATP and Cr through the reversible reaction of CK. This reversible reaction of PCr plays an essential role in rapid ATP production during high-intensity, short duration (~10 seconds) exercises such as sprints and repeated bouts of maximum weight lifting. The formation of ATP and Cr from PCr becomes favored due to the low pH level caused by the rapid accumulation of lactic acid produced by high-intensity anaerobic exercise (Demant & Rhodes, 1999; Walker, 1979; Yquel, Arsac, Thiaudiere, Canioni, & Manier, 2002). The accumulation of intracellular Cr

concentration from 19 to 32 mM in rat SOL and extensor digitorum longus (EDL) muscles increased the maximum Ca^{2+}-activated force by 104 ± 1 and 105 ± 1%, respectively, by increasing Ca^{2+} sensitivity in skeletal muscle. This study suggested that an increase in Cr concentration resulted in increased maximum force in skeletal muscle (Murphy, Stephenson, & Lamb, 2004).

Creatine monohydrate supplementation is well known to facilitate in increasing total Cr concentration in muscles. The ingestion of 20g of creatine monohydrate supplement for five days increased the muscle total Cr concentration and PCr concentration by 18% and 10%, respectively (Casey, Constantin-Teodosiu, Howell, Hultman, & Greenhaff, 1996). Another study showed a similar result with the intake of 20 to 30g of creatine monohydrate per day for two days significantly increasing total Cr concentration by 20 to 40% in the quadriceps femoris muscles of 17 healthy participants. This increase in total muscle Cr concentration can enhance performance and decrease fatigue during anaerobic exercise (Harris, Söderlund, & Hultman, 1992). In Hultman, Söderlund, Timmons, Cederblad, and Greenhaff study (1996), the effects of creatine loading treatment was also investigated in vastus lateralis muscle of health men. The group with 20 g/day Cr supplements for six days followed by 2 g/day for the next 28 days had a rapid increase in total Cr concentration by ~16 mmol/kg dry mass after 6-day of the 20 g/day Cr consumption. The Cr loading group maintained the elevated total Cr concentration in vastus lateralis muscle after 28-day when the group without the Cr loading protocol declined its total Cr concentration ($p<0.05$).

Many researchers have shown that the effect of the creatine monohydrate supplementation is positively associated with exercise performance. The Cr loading (9g

per day for six days) significantly increased the volume of the 1-rep max (RM) deadlift compared to the placebo group without the Cr supplementation ($p=0.010$) (F. Rossouw, Kruger, & J. Rossouw, 2000). Nine weeks of Cr supplementation (20g per day for five days followed by 5g per day for the rest of days) with a strength training program showed an improvement in exercise performance in collegiate football players (M. Bemben et al., 2001). The Cr supplement group showed significant increases in body weight and lean body mass post Cr supplement consumption ($p<0.05$). Also, anaerobic strength improved in all three lifts, including bench press, power clean, and squat by 5.2%, 3.8%, and 8.7%, respectively. In addition, the Wingate bicycle ergometer test was performed to measure anaerobic power and capacity, and the Cr group improved in both anaerobic power and anaerobic capacity by 19.6% and 18.4%, respectively (M. Bemben et al., 2001). The improvement in exercise performance with Cr supplementation also was shown in sprint performance. The intake of 5g Cr supplement for five days significantly increased body weight in professional male sprinters compared to pre-Cr supplement consumption ($p<0.05$). The participants in the Cr group showed a significant decrease in 100 m sprint time from 11.68 seconds to 11.59 seconds ($p<0.02$) (Skare et al., 2001).

Creatine monohydrate supplementation also showed the effect in myofibrillar protein and myosin heavy chain (MHC) protein with resistance training for six months. Total body mass and strength significantly increased in the Cr supplementation group compared to the control group and the placebo group only with the resistance training ($p<0.05$). Additionally, the myofibrillar protein expression and the MHC protein expression increased by 57.92% and 17.49%, respectively, in the Cr supplement group with the training (Willoughby & Rosene, 2001). The effect of Cr supplementation during

exercise training enhances the power, strength, and speed by delaying fatigue and increasing the muscle protein expression.

Although many studies have focused on the improvement of exercise performance with the Cr supplement, recent investigations have proposed the antioxidant effect of Cr in the oxidative environment. Lawler et al. (2002) investigated the association between antioxidant scavenging capacity (SAC) and creatine. In a dose-dependent manner, a significant relationship between SAC and Cr concentration was found (r=0.88), which indicated the direct antioxidant capacity of Cr. The antioxidant effect of Cr only affected on $^{\bullet}O^{-}_{2}$ and peroxynitrite ($OONO^{-}$) in this study. The treatment with 40 mM Cr resulted in a significant increase in quenching $^{\bullet}O^{-}_{2}$ compared to the control group. The SOD equivalent units of $^{\bullet}O^{-}_{2}$ quenching in the control group and Cr group were 4 ± 9 and 68.0 ± 32.5, respectively. A similar result was shown in H_2O_2 quenching with 40 mM Cr; however, the significant difference was not observed between the control and Cr groups. The $OONO^{-}$ quenching also significantly increased in a dose-dependent manner, and the most effective Cr concentration of the removal of $OONO^{-}$ was found to be at 60 mM Cr. These results indicate that Cr possesses the direct antioxidant capacity in the removal of $^{\bullet}O^{-}_{2}$ and $OONO^{-}$. The formation of Cr with arginine can explain the ability of Cr to scavenge free radicals. Arginine has an antioxidant capacity by quenching $^{\bullet}O^{-}_{2}$, generated by xanthine oxidase or released from endothelial cells. Since Cr is synthesized with arginine and glycine, the study suggests that the antioxidant effect of arginine may be possessed in Cr as well (Lawler et al., 2002).

In Guimarães-Ferreira et al. (2012) study, H_2O_2 production significantly decreased in Cr-treated SOL and EDL muscles of rat ($p<0.01$ and $p<0.05$, respectively).

The SOL and EDL were treated with 5 g/kg creatine for six days. The percent change of H_2O_2 compared to the control group without Cr treatment in SOL and EDL muscles were 59 % and 76.3%, respectively. Creatine supplementation in SOL and EDL did not affect the level of antioxidant enzyme mRNA including the primers for SOD, mitochondrial manganese SOD, and catalase indicating the direct antioxidant capacity of Cr.

Another study performed investigated the antioxidant capacity of Cr with acute exercise training in rats. The acute exercise training group with a 2% creatine monohydrate diet for 28 days had a significant increase in total muscle Cr concentration ($p<0.05$) compared to the control group that did not receive the Cr diet. Plasma thiobarbituric acid reactive species (TBARS), a byproduct of lipid peroxidation, and total lipid hydroperoxide were observed as markers for oxidative stress-induced ROS in skeletal muscle. In SOL and gastrocnemius muscles with the Cr diet, the level of TBARS decreased by 20% after six hours and total lipid hydroperoxide level also decreased by 20% after six hours. These results indicated that the 2% Cr monohydrate diet directly facilitated the antioxidant activity in increased oxidative stress markers caused by acute exercise (Deminice & Jordao, 2012).

An excessive ROS production in skeletal muscle is one of many mechanisms that trigger apoptosis via mitochondrial dysfunction in skeletal muscle. According to Ceddia and Sweeney (2004), 5’ adenosine monophosphate-activated protein kinase (AMPK) signaling plays a key role in ROS-induced mitochondrial dysfunction/impairment, muscle atrophy, and sarcopenia, a loss of muscle mass. It has been suggested that Cr supplement increased in the activity of AMPK via glucose oxidation. The production of $^{14}CO_2$, radioactive carbon dioxide, was observed as a marker of glucose oxidation. In L6 rat

myoblasts treated with 0.5 mM Cr, the basal production of $^{14}CO_2$ significantly increased by 1.4-fold compared to the control group without the Cr treatment ($p<0.05$). AMP kinase activity was measured, and the significant increase in AMPK activity was observed with a ~2.2-fold increase ($p<0.05$). This study suggested that the increased activation of AMPK activity with Cr treatment might prevent mitochondrial dysfunction. However, it was unknown if AMPK activity also increased in oxidative damaged skeletal muscle.

Even though the exact mechanism of Cr supplementation preventing ROS-induced apoptosis is not known, the Cr supplement has shown to prevent apoptosis in ischemic cells via activating the anti-apoptotic pathway. The H9c2 cardiomyocytes treated with 0.5 μM of DOX, significantly decreased the cell viability by 35% ($p<0.001$). The Cr treatments (2.5 mM Cr, 5 mM Cr, and 10 mM Cr) significantly increased compared to the only DOX treated cell in a dose-dependent manner ($p<0.05$). The 50.4% decreased cell viability was also observed in ischemic cardiomyocytes, restricted with blood supply, compared to the control group ($p<0.001$). Unlike the DOX treated cells, the 2.5 mM Cr treatment did not increase the cell viability; however, the combination of 5 mM ribose and 2.5 mM Cr significantly improved cell viability by 30% ($p<0.01$). The apoptotic enzymes, cleaved caspase-3 and poly ADP-ribose polymerase (PARP), were observed in control, Cr and, ribose treated ischemic H9c2 cells. The Cr and ribose treatment significantly reduced the protein expressions of cleaved caspase-3 and PARP compared to the untreated ischemic cell ($p<0.5$). The increased activation of Akt pathway, known as an anti-apoptotic pathway, was observed in Cr and ribose treated ischemic cardiomyocytes ($p<0.05$). The study suggested that the Cr and ribose

supplementation could activate the anti-apoptotic pathway and prevent apoptosis in DOX treated and ischemic cardiomyocytes. However, the antioxidant effects of Cr supplement treatment were not found to prevent DOX-induced apoptosis in cardiomyocytes (Caretti et al., 2010).

Conclusion

DOX is a highly effective chemotherapeutic drug used in many types of cancer. Due to its structure, DOX often goes into redox cycling with NAD(P)H and induces the release of ROS, leading to lipid peroxidation, DNA damage, and oxidative stress in cells (Doroshow, 1986; Gewirtz, 1999). The usage of DOX in the clinic has been limited because the excessive release of ROS results in toxicities and triggers apoptosis in many tissues, including heart and skeletal muscle (Minotti et al., 2004). The contractile dysfunction, including maximal force, contraction, and relaxation velocity, is induced by DOX treatment, which increases mitochondrial ROS production. Doxorubicin-induced apoptosis in skeletal muscle is initiated via the opening of MPT pores which often is induced by Ca^{2+}-mediated mitochondrial swelling, the excessive release of the mitochondrial ROS production, and activation of calpain which releases AIF including cyt *c* into cytoplasm (Min et al., 2015; Smuder et al., 2011; Van Norren et al., 2009).

The release of cyt *c* from mitochondria is a biomarker for the initiation of apoptosis. The mitochondrial ROS production causes DNA damage and lipid peroxidation, which damages the inner mitochondrial membrane. The damage of the membrane in mitochondria breaks the bond between cardiolipin and cyt *c*, leading to the release of cyt *c*. Additionally, ROS production induces the proapoptotic Bcl-2 family proteins including Bax/Bak and release cyt *c* through the pore formation of Bax/Bak into

the cytoplasm from mitochondria. The formation of apoptosome with Apaf-1 and cyt *c* cleaves procaspase-9 and initiates the apoptotic caspase-cascade, resulting in apoptosis (Cory & Adams, 2002; Saelens et al., 2004). The regulation of apoptosis is critical in skeletal muscles because the decreased in muscle contractile proteins results in contractile dysfunction and muscle atrophy (Gilliam et al., 2012; Min et al., 2015).

Creatine is an organic compound, primarily synthesized in the liver and obtained by the diet. The ergogenic capacity of Cr via the PCr energy system has shown with the reversible reaction of CK. The rapid production of ATP from PCr and ADP occurs during high-intensity, and short duration exercises that lowers the pH and favors the formation of Cr and ATP. Creatine supplementation increases total muscle Cr concentration in skeletal muscle and improves exercise performance by delaying fatigue and increasing contractile muscle proteins with exercise training (Cory & Adams, 2002; Saelens et al., 2004).

Besides the positive effect of Cr on exercise performance, some researchers suggest a direct antioxidant capacity of Cr. The ability to quench ROS, $^{\bullet}O^{-}_{2}$, and $OONO^{-}$, has shown (Lawler et al., 2002). Additionally, decreased levels of H_2O_2, TBARS, and lipid hydroperoxide has been observed with Cr supplementation and exercise training in skeletal muscles (Deminice & Jordao, 2012; Guimarães-Ferreira et al., 2012). Creatine supplementation possibly prevents ROS-induced mitochondrial dysfunction and apoptosis via activation of the AMPK pathway and reduces apoptotic enzyme activities by activating the anti-apoptotic Akt pathway (Ceddia & Sweeney, 2004; Caretti et al., 2010).

In summary, DOX is a highly effective therapeutic drug for many types of cancers; however, its clinical usage is limited due to producing an excessive amount of ROS in heart and skeletal muscles during the process of its metabolism. The DOX-induced ROS release in mitochondria leads to lipid peroxidation, DNA damage, and oxidative stress triggering apoptosis in cells. The apoptosis is initiated by initiator apoptotic enzymes, cyt *c* and casp-9. Doxorubicin-induced mitochondrial ROS production releases cyt *c* into cytoplasm and activates casp-9 leading to apoptosis. Doxorubicin-induced apoptosis is critical in skeletal muscles because it results in contractile dysfunction and muscle atrophy. Recent findings have suggested that Cr supplement reduces mitochondrial ROS production and potentially prevents initiation of apoptosis in skeletal muscles with its direct antioxidant effect; however, its exact mechanism is not yet fully understood.

This study demonstrates the potential intervention for preventing the initiation of apoptosis caused by DOX-induced ROS production in mitochondria with creatine supplementation. As previous literatures mentioned, the antioxidant capacity of creatine should reduce apoptosis, which is initiated by DOX-induced ROS production and oxidative stress in skeletal muscles.

CHAPTER III

METHODS

Experimental Design

All the procedures were approved by University of Northern Colorado Institutional Animal Use and Care Committee and followed the Animal Welfare Act. Ten-week-old male Sprague Dawley rats (Envigo, Indianapolis, IN, N=120) were individually housed and maintained on a 12:12 hour light-dark cycle at normal room temperature and humidity. Rats were provided with rodent chow and water *ad libitum*. They were randomly divided into three groups: normal chow (CON, n=40), 2% creatine chow (2% Cr, n=40), and the creatine loading diet consisting of one week of 4% creatine chow followed by three weeks of 2% creatine chow (4% Cr, n=40). Each group was then, again, randomly assigned into two groups which included 1-day injection prior to sacrifice (1, n=20) and 3-day injection prior to sacrifice (3, n=20). Lastly, each group was randomly divided into two groups: doxorubicin (DOX, n=10) and saline (SAL, n=10). The treatments for the groups are shown in figure 1. The 2% Cr groups were provided with chow that contained 2% creatine (Sigma-Aldrich, St.Louis, MO) for four weeks, while the 4% Cr groups were provided with 4% creatine chow (Sigma-Aldrich, St.Louis, MO) for one week followed by 2% creatine chow for the remaining three weeks. Body

mass (g) for all animals were measured once a week during the four-week nutritional intervention and prior to sacrifice.

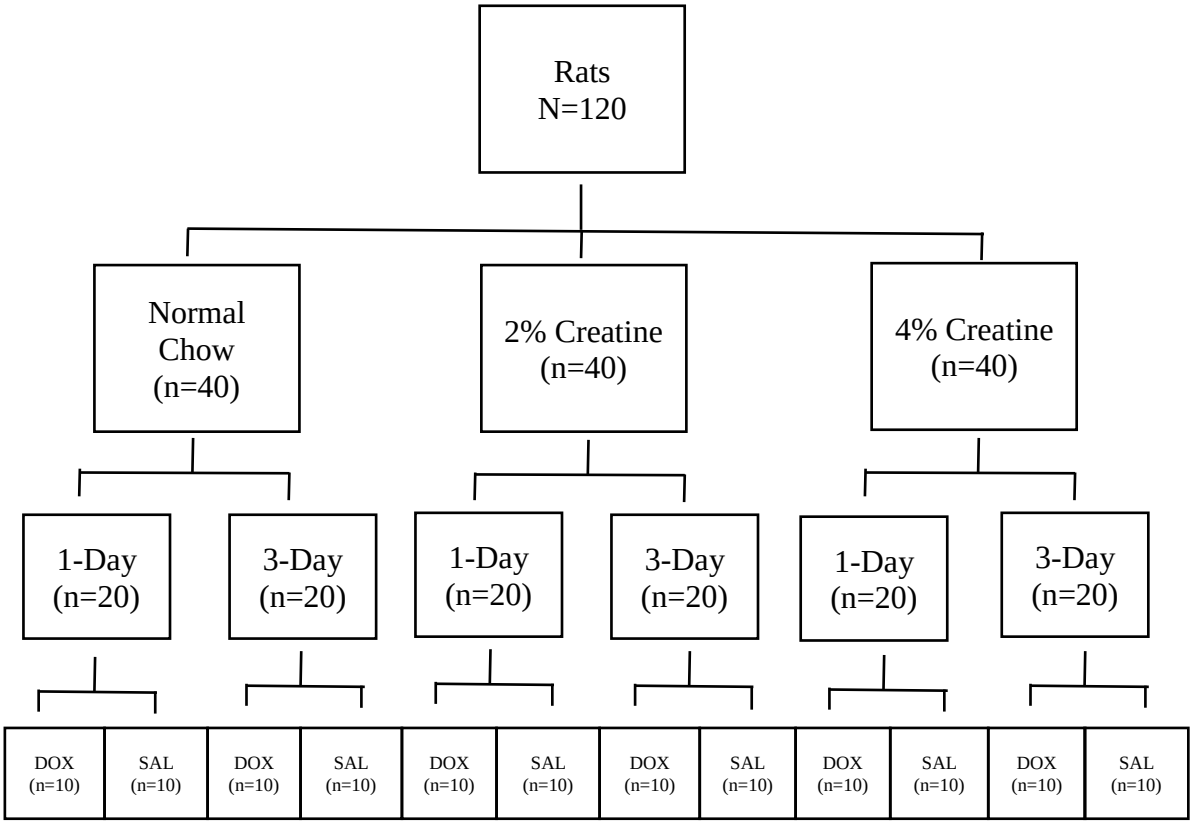

Figure 1. Experimental Design of Diet Treatments for Groups.

Drug Treatment

DOX-1 and SAL-1 were injected with 15 mg/kg body weight of DOX hydrochloride (Bedford Labs, Bedford, OH) and 3 mL of SAL, respectively, one day prior to sacrifice. DOX-3 and SAL-3 were also injected with DOX 15 mg/kg body weight and 3mL of SAL, respectively, three days prior to sacrifice.

Biochemical Analysis

Extensor Digitorum Longus and Soleus Preparation

At the determined point, each animal was injected with sodium pentobarbitol (50 mg/kg) in the lower left abdominal region prior to sacrifice. The tail was pinched to ensure the absence of a tail-pinch reflex indicating no pain or discomfort. The left extensor digitorum longus (EDL) and left soleus (SOL) were removed, weighed, and flash frozen in liquid nitrogen. All tissues were stored in a laboratory freezer at -80°C until biochemical analysis.

Homogenate Preparation

Frozen portions of the EDL and SOL between 0.07 g and 0.1 g were used for homogenization. Muscle samples were manually homogenized with radioimmunoprecipitation assay (RIPA, SigmaAldrich: St. Louis, MO) buffer (1:10 tissue mass:volume) and 10 µL of protease enzyme inhibitor (SigmaAldrich: St. Louis, MO) in a glass tissue homogenizer. Cell membranes were further disrupted with a sonicator (Sonic disembrator model 100, Fisher Scientific, Hampton, NH) with ten, one second pulses. Samples were then transferred to centrifuge tubes and centrifuged for ten minutes at 10,000*g*. The supernatant was collected, and total protein concentration analyzed using the Bradford (Bradford, 1976). Protein concentration was standardized with RIPA buffer, and an equal volume of Lammeli buffer was added to each sample.

Western Blotting

Electrophoresis was conducted to separate proteins by molecular weight (kDa) using an Xcell II blot module (Invitrogen, LifeTechnologies) on 4-20% gradient Tris-Glycine precast gels (LifeTechnologies, Carlsbad, CA). Before loading onto gels, samples were heated in boiling water for two minutes and transferred to an ice bucket for ten minutes. Gels were placed and locked in the Xcell II blot module and the middle section was filled with running buffer (100 mL Tris-Glycine Running Buffer (10X): 900 mL deionized (DI) water). Each 15 µL of sample was loaded on to gels, and 10 µL of SeeBlue®Plus2 protein ladder (Novex, LifeTechnologies) and 10 µL of MagicMark™ XP standard ladder (Novex, Lifetechnologies) were also loaded on the gels as standards. The outside of the chamber was also filled with Tris-glycine running buffer. Gels were run at 125 V and 0.04 A until proteins migrated to the bottom of gels.

Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Novex, LifeTechnologies) with two filter papers soaked in transfer buffer (40 mL Tris-Glycine transfer buffer, 200 mL methanol, and 760 mL DI water). The soaked membranes, filter papers, and pads were stacked in the following order: 1) two soaked pads on the bottom 2) one filter paper 3) gel-1 4) membrane 5) one filter paper 6) one soaked pad 7) filter paper 8) gel-2 9) membrane 10) filter paper 11) two soaked pads. The soaked membranes were rolled with glass pipette to remove air bubbles between the gels and the transfer buffer was poured into the middle of chamber, and DI water filled the outside of the chamber. The protein transfer was run for 90 minutes at 25 mV and 0.10 A. Once proteins were transferred onto the membranes, the membranes were washed with 20 mL of DI water for five minutes, four times, and then blocked with 10 mL of SuperBlock

blocking buffer (Thermoscientific, Rockford, IL) for thirty minutes using a laboratory rocker. PVDF membranes were rinsed again with 20 mL of DI water for five minutes, four times.

The membranes were incubated for 15 – 18 hours with 10 mL of mouse monoclonal primary antibodies for cytochrome *c* (1:200, Santa Cruz Biotechnology: Dallas, TX) or cleaved caspase-9 (1:200, Santa Cruz Biotechnology: Dallas, TX). The mouse monoclonal antibody GAPDH (1:500, Santa Cruz: Dallas, TX) was used as the loading control. PVDF membranes were washed with 20 mL of TBST for five minutes and repeated four times and rinsed with 20 mL of DI water for five minutes, twice. Membranes were incubated in 10 mL of anti-mouse secondary antibody (1:1000, Santa Cruz: Dallas, TX) for one hour. Membranes were again washed with 20 mL of TBST for five minutes, four times and then were rinsed with 20 mL of DI water for five minutes, twice.

Protein imaging was completed using enhanced chemoluminescence (ECL) (C-Digit, Li-Cor, Lincoln, NE), and protein expression was quantified by ImageJ software (NIH, Bethesda, MD). 750 mL of luminol and enhancer (C-Digit, Li-Cor: Lincoln, NE) were added to the membrane and allowed to incubate for five minutes. Standard sensitivity was used at first then if the image was not detected clear, high sensitivity was used.

Statistical Analysis

Data were analyzed using GraphPad and presented as mean±standard error of the mean (SEM). Outliers above or below two standard deviations (SD) from the mean were removed before data analysis. A two-way ANOVA was performed to determine main

effects of Cr (diet) and DOX (drug) along with diet and drug interactions in cytochrome *c* and cleaved caspase-9 protein expression. Tukey's *post hoc* testing was performed if there were significant main effects of interactions to identify where differences existed. Significance was set at $\alpha=0.05$.

CHAPTER IV

RESULTS

The purpose of this study was to exam in the effects of the two different treatments of Cr supplementation on cytochrome *c* and caspase-9 protein expressions in SOL and EDL from rats administered DOX injections at two different time points. Prior to sacrifice, five rats did not survive (two from 4% Cr-DOX-1, one from 4% Cr-SAL-3, and two from 4% Cr-DOX-3).

Biochemical Analysis

The 1-SOL, 1-EDL, 3-SOL, and 3-EDL were analyzed to determine the effects of two different Cr treatments (2% Cr for four weeks and the Cr loading consisting of 4% Cr for one week followed by 2% Cr for three-week) and DOX administration at 1-day and 3-days prior to sacrifice on the protein expressions of cyt *c* and casp-9. Protein expression was expressed relative to the expression of mouse monoclonal antibody GAPDH as the loading control. Cytochrome *c* expression is shown in Table 1 (1-SOL and 3-SOL) and Table 2 (1-EDL and 3-EDL), and casp-9 expression is shown in Table 3 (1-SOL and 3-SOL) and Table 4 (1-EDL and 3-EDL).

Table 1
Cyt c Western Blot expressions

Muscle	CON-SAL	CON-DOX	2% Cr-SAL	2% Cr-DOX	4% Cr-SAL	4% Cr- DOX
1-SOL	0.31 ± 0.03	0.37 ± 0.06	0.34 ± 0.03	0.39 ± 0.03	0.49 ± 0.05	0.40 ± 0.03
3-SOL	0.30 ± 0.03	0.48 ± 0.11	0.49 ± 0.04	0.60 ± 0.09	0.34 ± 0.06	0.44 ± 0.10

Note. Cyt *c*= cytochrome *c*, 1-SOL= one-day soleus, 3-SOL = three-day soleus, CON-SAL = control diet saline, *n=10*, *n=8*, CON-DOX = control diet doxorubicin, *n=9*, *n*=10, 2% Cr-SAL = 2% creatine diet saline, *n=8*, *n=8*, 2% Cr-DOX = 2% creatine diet doxorubicin, *n=8*, *n=10*, 4% Cr-SAL = creatine loading saline, *n=10*, *n=8*, 4% Cr-DOX = creatine loading doxorubicin, *n=8*, *n=9*. Values are means ± SEM.

Table 2
Cyt c Western Blot expressions

Muscle	CON-SAL	CON-DOX	2% Cr-SAL	2% Cr-DOX	4% Cr-SAL	4% Cr- DOX
1-EDL	0.24 ± 0.10	0.41 ± 0.15	0.41 ± 0.14	0.35 ± 0.11	0.27 ± 0.11	0.39 ± 0.15
3-EDL	0.56 ± 0.13	0.63 ± 0.14	0.69 ± 0.16	0.54 ± 0.13	0.60 ± 0.15	0.61 ± 0.05

Note. Cyt *c*= cytochrome *c*, 1-EDL= one-day extensor digitorum longus, 3-EDL = three-day extensor digitorum longus, CON-SAL = control diet saline, *n=10*, *n=10*, CON-DOX = control diet doxorubicin, *n=10*, *n*=10, 2% Cr-SAL = 2% creatine diet saline, *n=10*, *n=10*, 2% Cr-DOX = 2% creatine diet doxorubicin, *n=10*, *n=9*, 4% Cr-SAL = creatine loading saline, *n=10*, *n=10*, 4% Cr-DOX = creatine loading doxorubicin, *n=8*, *n=7*. Values are means ± SEM.

Table 3
Casp-9 Western Blot expressions

Muscle	CON-SAL	CON-DOX	2% Cr-SAL	2% Cr-DOX	4% Cr-SAL	4% Cr- DOX
1-SOL	0.69 ± 0.05	0.69 ± 0.06	0.66 ± 0.07	0.79 ± 0.09	0.91 ± 0.11	0.89 ±0.10
3-SOL	0.65 ± 0.15	0.82 ± 0.14	0.81 ± 0.10	0.98 ± 0.14	1.00 ± 0.16	1.03 ± 0.23

Note. Casp-9= caspase-9, 1-SOL= one-day soleus, 3-SOL = three-day soleus, CON-SAL = control diet saline, *n=9*, *n=10*, CON-DOX = control diet doxorubicin, *n=9*, *n*=10, 2% Cr-SAL = 2% creatine diet saline, *n=8*, *n=9*, 2% Cr-DOX = 2% creatine diet doxorubicin, *n=10*, *n=10*, 4% Cr-SAL = creatine loading saline, *n=10*, *n=9*, 4% Cr-DOX = creatine loading doxorubicin, *n=10*, *n=9*. Values are means ± SEM.

Table 4
Casp-9 Western Blot expressions

Muscle	CON-SAL	CON-DOX	2% Cr-SAL	2% Cr-DOX	4% Cr-SAL	4% Cr- DOX
1-EDL	0.05 ± 0.01	0.06 ± 0.01	0.07 ± 0.02	0.09 ± 0.02	0.09 ± 0.02	0.06 ± 0.01
3-EDL	0.06 ± 0.01	0.07 ± 0.01	0.07 ± 0.02	0.06 ± 0.02	0.08 ± 0.02	0.10 ± 0.02

Note. Casp-9= caspase-9, 1-EDL= one-day extensor digitorum longus, 3-EDL = three-day extensor digitorum longus, CON-SAL = control diet saline, *n=10, n=7*, CON-DOX = control diet doxorubicin, *n=10, n*=9, 2% Cr-SAL = 2% creatine diet saline, *n=8, n=9*, 2% Cr-DOX = 2% creatine diet doxorubicin, *n=10, n=8*, 4% Cr-SAL = creatine loading saline, *n=10, n=8*, 4% Cr-DOX = creatine loading doxorubicin, *n=6, n=6*. Values are means ± SEM.

Cytochrome *c*

The protein expression of cyt *c* was measured to see if there was an initiation of apoptosis with the effects of Cr supplementation and DOX administration. In the 1-SOL, no significant effects of the Cr supplementation (p=0.08) and the DOX injection (p=0.85) were observed. The interaction between the Cr supplementation and the DOX injection was not significant (p=0.17). The cyt *c* expression, however, in the CON-DOX increased by 19.4% compared to the CON-SAL group. 2% Cr-DOX showed an increase of 14.7% from the 2% Cr-SAL group while there was a 18.4% decrease of cyt *c* expression in the 4% Cr-DOX group compared to 4%-Cr SAL (Figure 2). There were no significant effects of the Cr diet treatments (p=0.10) and the DOX administration (p=0.09) in the 3-SOL. No significant interaction between the Cr diet treatments and the DOX administration was observed (p=0.87). In the CON groups, DOX increased the expression of cyt *c* by 60%. There was a 22.4% increase in 2% Cr-DOX compared to 2% Cr-SAL. DOX also increased the protein expression by 29.4% in 4% Cr group (Figure 3).

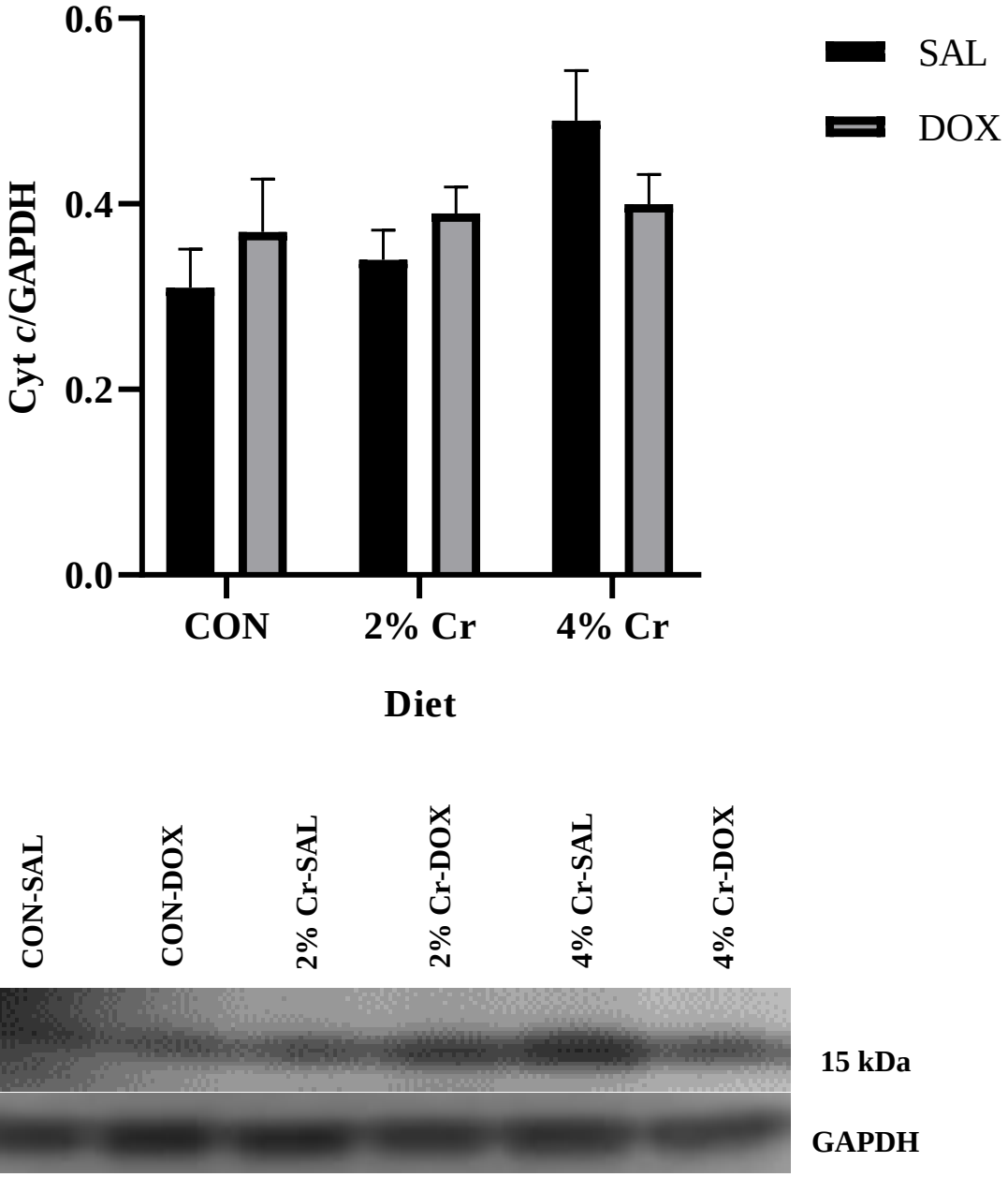

Figure 2. Cyt *c* expression in 1-SOL.
Cyt *c*= cytochrome *c* (15 kDa), GAPDH= loading control, 1-SOL= one-day soleus, SAL= saline, DOX= doxorubicin, CON= control diet, 2% Cr= 2% creatine diet, 4%= creatine loading; control diet saline, *n=10*, control diet doxorubicin, *n=9*, 2% creatine diet saline, *n=8*, 2% creatine diet doxorubicin, *n=8*, creatine loading saline, *n=10*, creatine loading doxorubicin, *n=8*. Values are mean ± SEM.

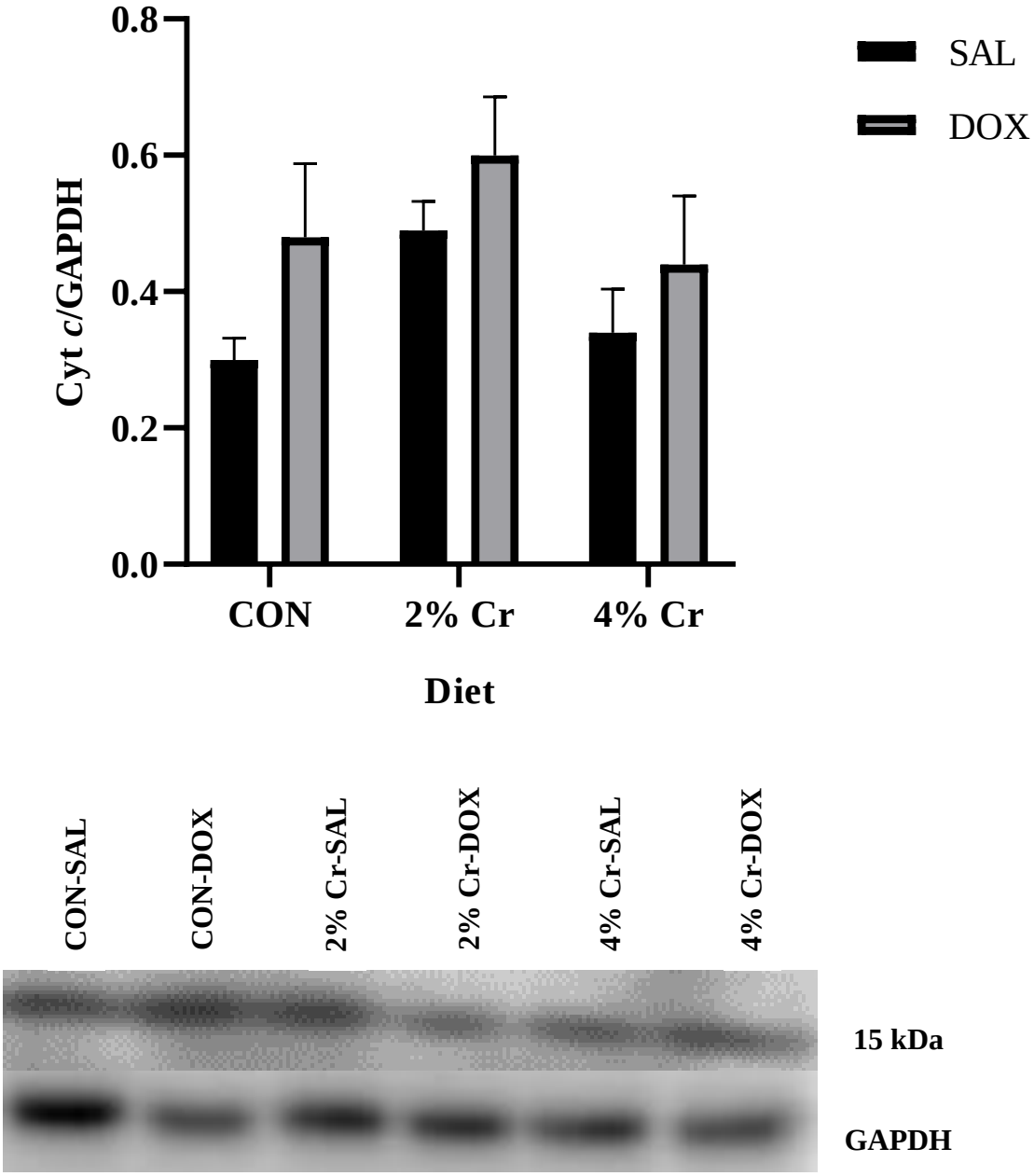

Figure 3. Cyt *c* expression in 3-SOL.
Cyt *c*= cytochrome *c* (15 kDa), GAPDH= loading control, 3-SOL= three-day soleus, SAL= saline, DOX= doxorubicin, CON= control diet, 2% Cr= 2% creatine diet, 4%= creatine loading; control diet saline, *n=8*, control diet doxorubicin, *n=10*, 2% creatine diet saline, *n=8*, 2% creatine diet doxorubicin, *n=10*, creatine loading saline, *n=8*, creatine loading doxorubicin, *n=9*. Values are mean ± SEM.

In the 1-EDL, the effects of the Cr diet treatments and the DOX administration were not significant (p=0.89 and p=0.46, respectively). There was no significant interaction with the Cr supplementation and the DOX injection (p=0.63). Doxorubicin administration increased cyt *c* expression by 70.8% in the CON group. A 14.6% decrease was observed in the 2% Cr-DOX compared to the 2% Cr-SAL group while the expression of cyt *c* increased by 30.8% in the 4% Cr-DOX group compared to the 4% Cr-SAL group (Figure 4). In the 3-EDL, there was no significant effects of the Cr supplementation observed (p=0.99). The DOX injection showed no significant effect either (p=0.84). The interaction between the Cr diet treatments and the DOX injection was not significant (p=0.71). Cytochrome *c* expression had a 12.5% increase in the CON-DOX group compared to the CON-SAL group. The DOX administration decreased the protein expression by 21.7% in the 2% Cr group. There was a 1.7% increased expression of cyt *c* observed in the 4% Cr-DOX group compared to the 4% Cr-SAL group (Figure 5).

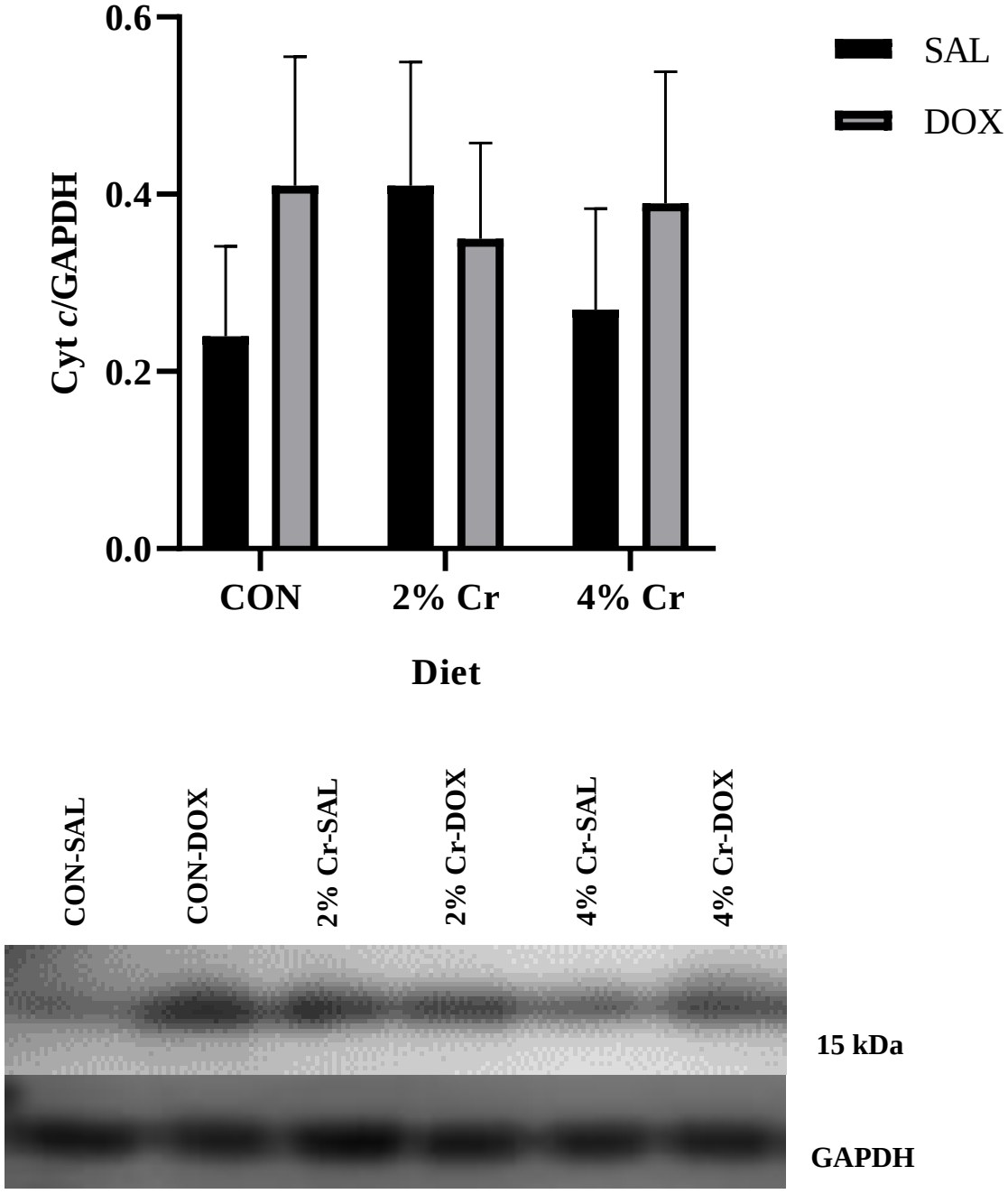

Figure 4. Cyt *c* expression in 1-EDL.
Cyt *c*= cytochrome *c* (15 kDa), 3-EDL= one-day extensor digitorum longus, SAL= saline, DOX= doxorubicin, CON= control diet, 2% Cr= 2% creatine diet, 4%= creatine loading; control diet saline, *n=10*, control diet doxorubicin, *n=10*, 2% creatine diet saline, *n=10*, 2% creatine diet doxorubicin, *n=10*, creatine loading saline, *n=10*, creatine loading doxorubicin, *n=8*. Values are mean ± SEM.

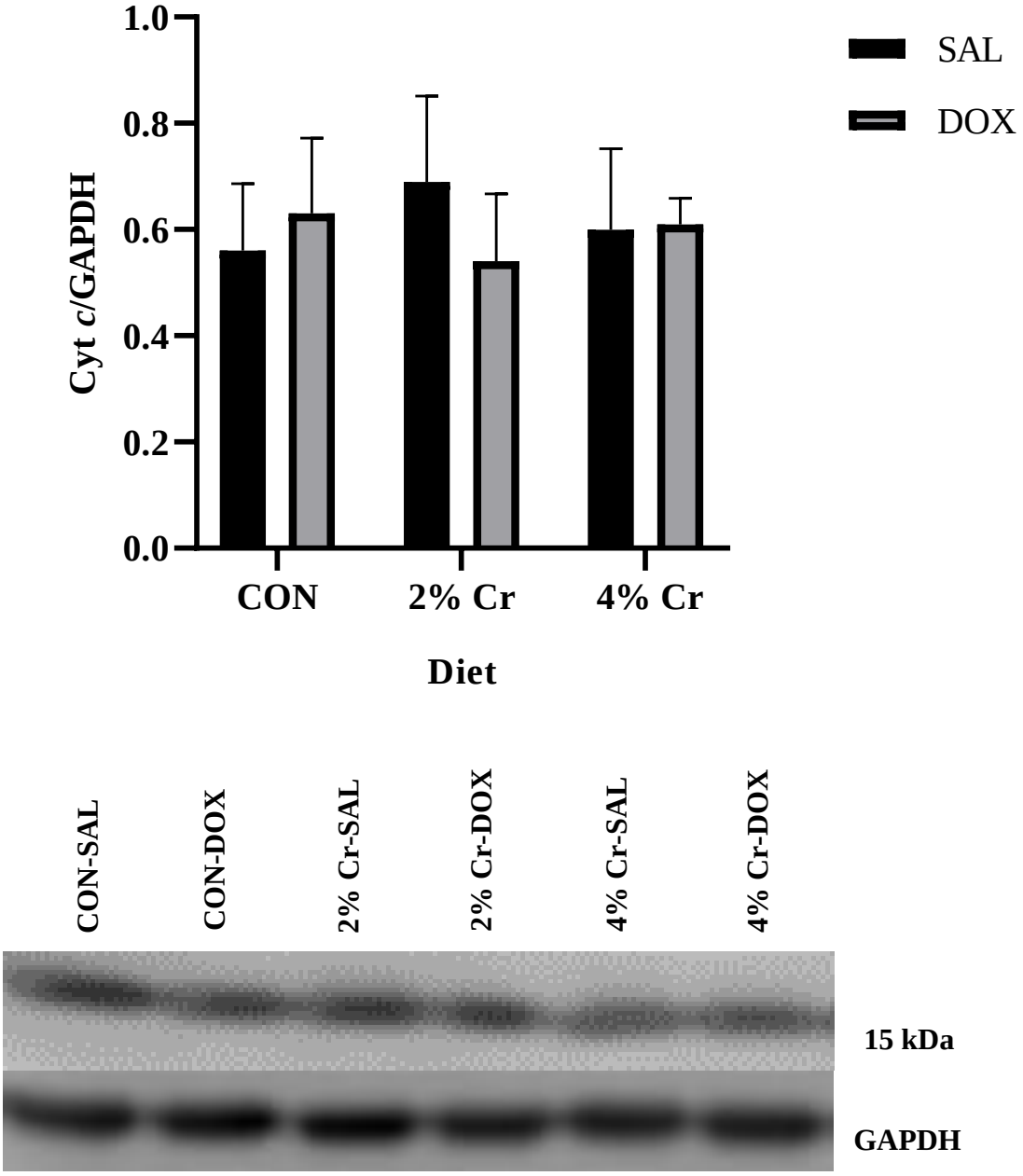

Figure 5. Cyt *c* expression in 3-EDL.
Cyt *c*= cytochrome *c* (15 kDa), GAPDH= loading control, 3-EDL= three-day extensor digitorum longus, SAL= saline, DOX= doxorubicin, CON= control diet, 2% Cr= 2% creatine diet, 4%= creatine loading; control diet saline, *n=10*, control diet doxorubicin, *n=10*, 2% creatine diet saline, *n=10*, 2% creatine diet doxorubicin, *n=9*, creatine loading saline, *n=10*, creatine loading doxorubicin, *n=7*. Values are mean ± SEM.

Caspase-9

The casp-9 expression was examined to see the initiation of caspase cascade-mediated apoptosis. There were no significant effects of the Cr supplementation and the DOX administration ($p=0.63$ and $p=0.09$, respectively). The interaction between the Cr supplementation and the DOX administration was not significant ($p=0.68$). In the 1-SOL, the expression of casp-9 of the CON group remained the same with the DOX injection. The 2% Cr-DOX had a 19.7% increase compared to the 2% Cr-DOX group. The DOX injection decreased the protein expression of casp-9 by 2.2% in 4% Cr-DOX group compared to the 4% Cr-SAL (Figure 6). No significant effects of the Cr diet treatment and the DOX injection were observed in the 3-SOL ($p=0.21$ and $p=0.34$, respectively). No significant interaction was observed with the Cr diet treatment and the DOX administration ($p=0.88$). The DOX administration in all three Cr diet groups (CON, 2% Cr, and 4% Cr) increased the casp-9 expression by 26.2%, 21.0%, and 3%, respectively, compared to the SAL group (Figure 7).

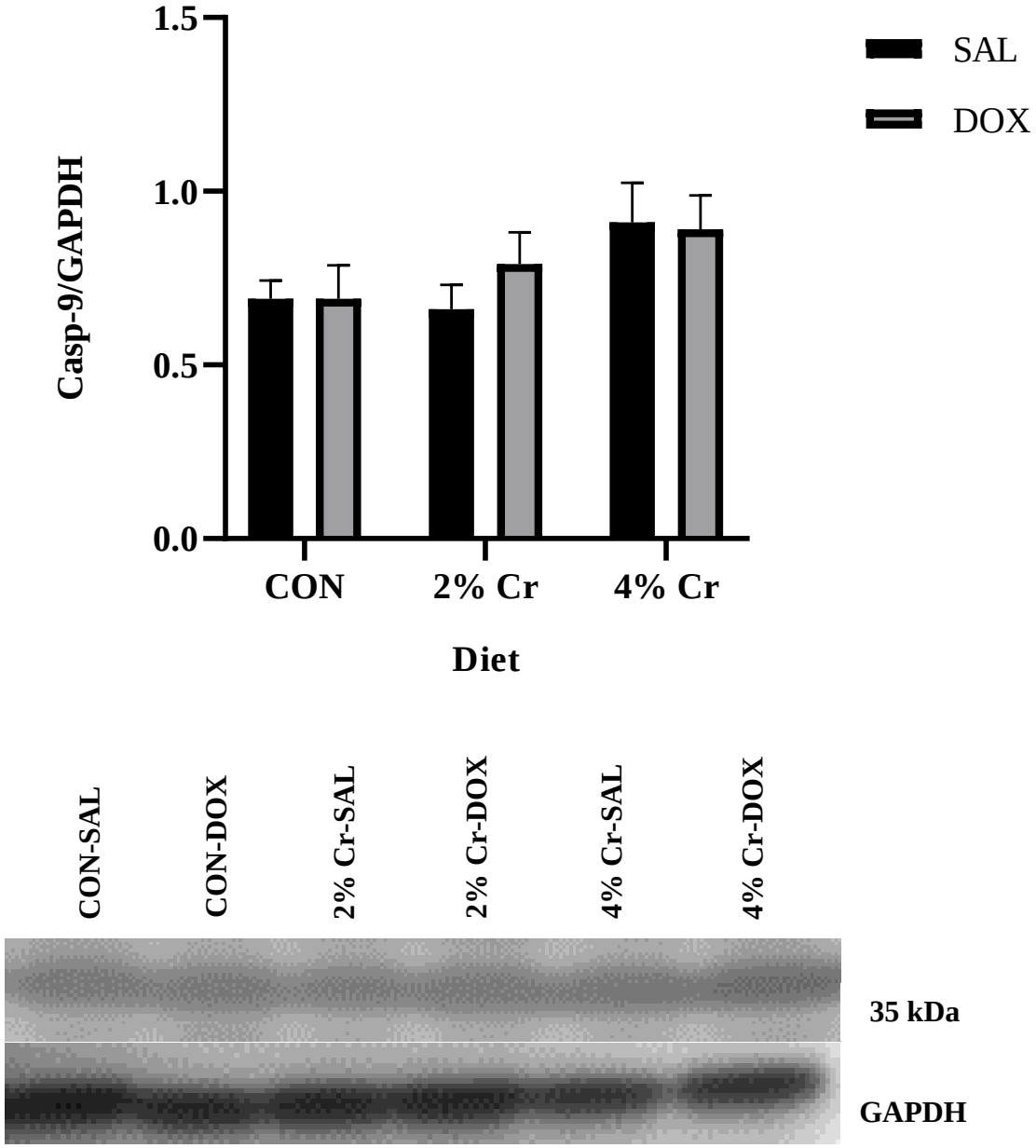

Figure 6. Casp-9 expression in 1-SOL.
Casp-9= caspase-9 (35 kDa), GAPDH= loading control, 1-SOL= one-day soleus, SAL= saline, DOX= doxorubicin, CON= control diet, 2% Cr= 2% creatine diet, 4%= creatine loading; control diet saline, *n=9*, control diet doxorubicin, *n=9*, 2% creatine diet saline, *n=8*, 2% creatine diet doxorubicin, *n=10*, creatine loading saline, *n=10*, creatine loading doxorubicin, *n=10*. Values are mean ± SEM.

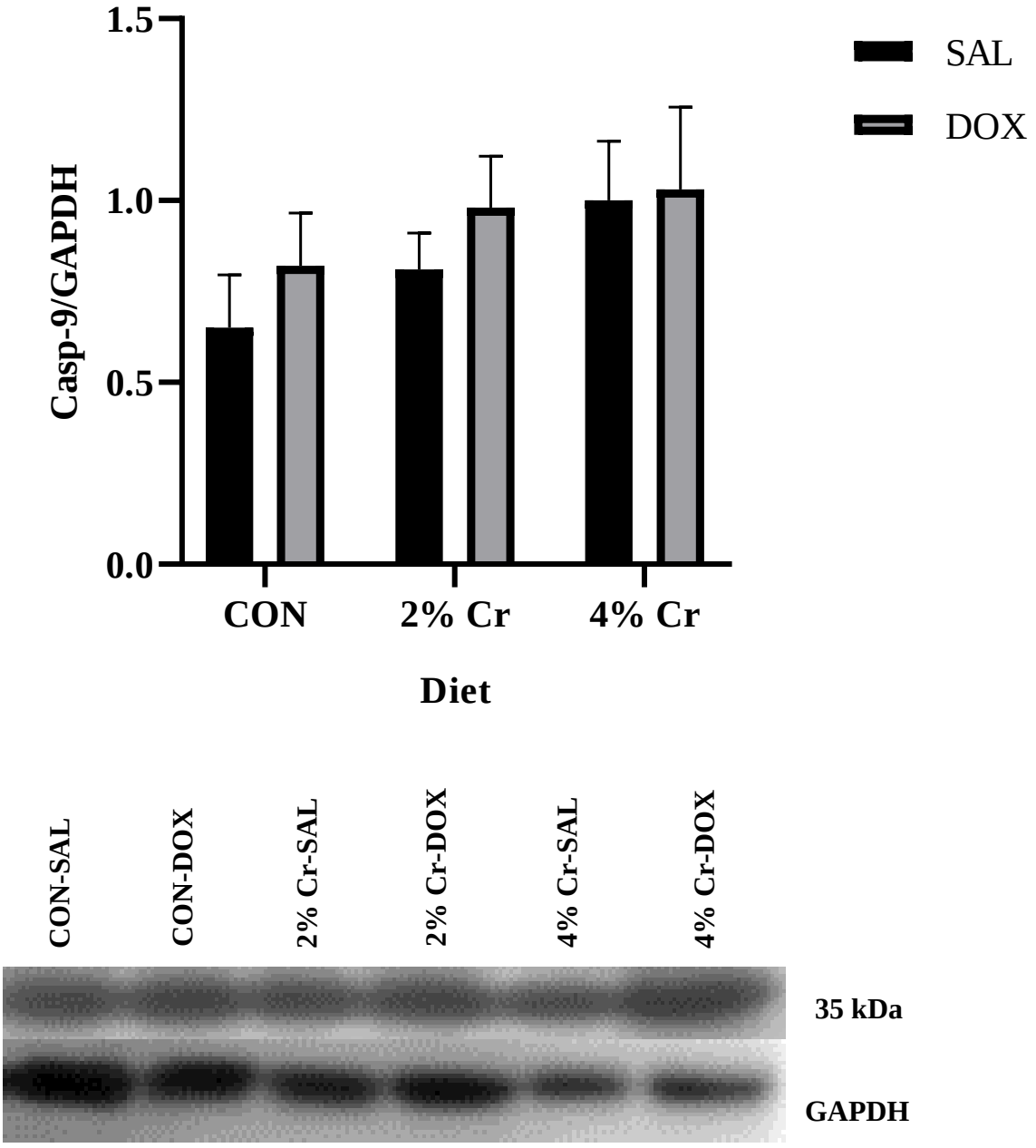

Figure 7. Casp-9 expression in 3-SOL.
Casp-9= caspase-9 (35 kDa), GAPDH= loading control, 3-SOL= three-day soleus, SAL= saline, DOX= doxorubicin, CON= control diet, 2% Cr= 2% creatine diet, 4%= creatine loading; control diet saline, *n=10*, control diet doxorubicin, *n=10*, 2% creatine diet saline, *n=9*, 2% creatine diet doxorubicin, *n=10*, creatine loading saline, *n=9*, creatine loading doxorubicin, *n=9*. Values are mean ± SEM.

In the 1-EDL, no significant effects were observed in the Cr diet treatments ($p=0.13$) and the DOX administration ($p=0.21$). The interaction with the Cr supplementation and the DOX injection was also not significant ($p=0.27$). The DOX injection increased the protein expression of casp-9 in the CON and 2% Cr groups by 20.0% and 28.6%, respectively. However, the 4% Cr-DOX group had a 33.3% decrease compared to the 4% Cr-SAL (Figure 8). In the 3-EDL, there were no significant effects in the Cr supplementation ($p=0.10$) and the DOX administration($p=0.54$). The interaction between the Cr diet treatment and the DOX injection was not significant ($p=0.51$). A 16% increase was measured in the CON-DOX group compared to the CON-SAL. The DOX administration in the 2% Cr group decreased the casp-9 expression by 14.3% while the 4% Cr-DOX increased the expression by 25.0% compared to the 4% Cr-SAL (Figure 9).

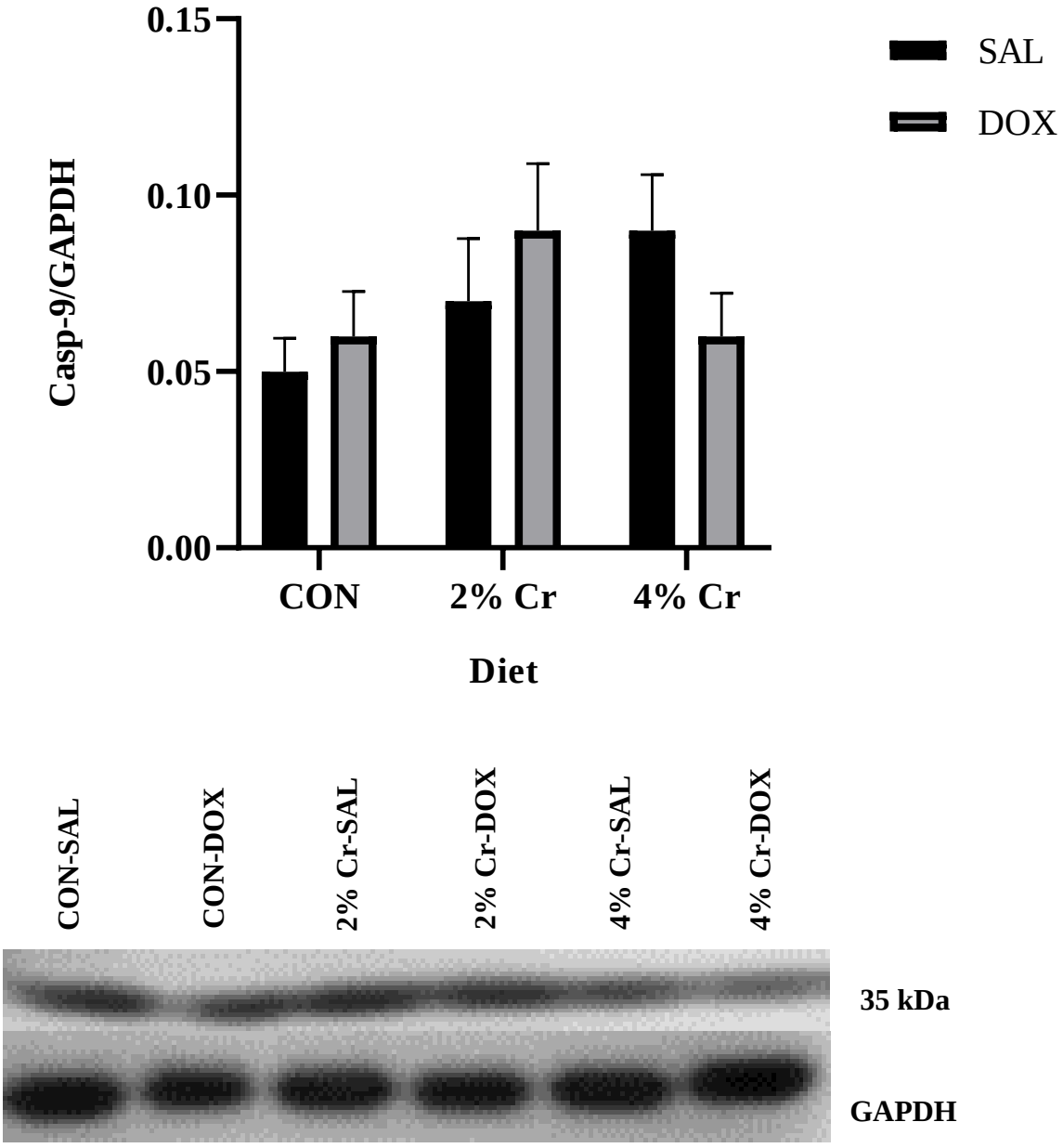

Figure 8. Casp-9 expression in 1-EDL.
Casp-9= caspase-9 (35 kDa), GAPDH= loading control, 1-EDL= one-day extensor digitorum longus, SAL= saline, DOX= doxorubicin, CON= control diet, 2% Cr= 2% creatine diet, 4%= creatine loading; control diet saline, *n=10*, control diet doxorubicin, *n=10*, 2% creatine diet saline, *n=8*, 2% creatine diet doxorubicin, *n=10*, creatine loading saline, *n=10*, creatine loading doxorubicin, *n=6*. Values are mean ± SEM.

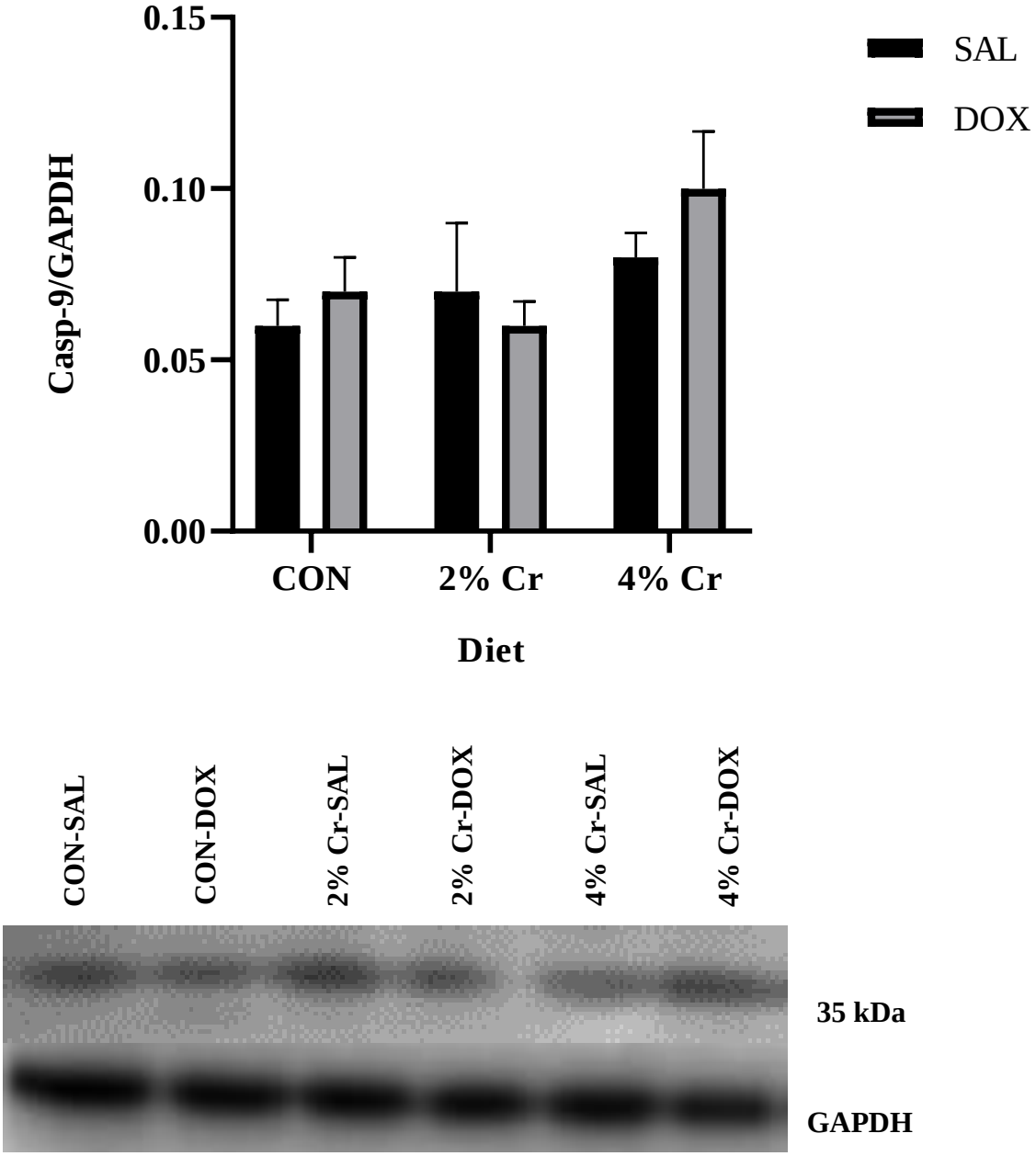

Figure 9. Casp-9 expression in 3-EDL.
Casp-9= caspase-9 (35 kDa), GAPDH= loading control, 3-EDL= one-day extensor digitorum longus, SAL= saline, DOX= doxorubicin, CON= control diet, 2% Cr= 2% creatine diet, 4%= creatine loading; control diet saline, *n=7*, control diet doxorubicin, *n=9*, 2% creatine diet saline, *n=9*, 2% creatine diet doxorubicin, *n=8*, creatine loading saline, *n=8*, creatine loading doxorubicin, *n=6*. Values are mean ± SEM.

CHAPTER V

DISCUSSION

The main purpose of this study was to determine changes of the initiator apoptotic enzyme expressions cyt *c* and casp-9 in type I and type II skeletal muscles treated with two different Cr supplement treatments and two different time points of DOX administrations. In addition, the interaction between the Cr supplementation and the DOX administration was examined in this study. The results showed no significant effects of the Cr supplementations and the DOX injections on cyt *c* and casp-9 in both the SOL and EDL. No significant interaction was observed between the Cr diet treatment and the DOX administration.

Even though the results did not indicate significant effects of Cr supplementation and DOX injections, a potential trend toward Cr supplementation providing protection with DOX administration in 1-EDL and 3-SOL were observed. The expression of cyt *c* of the 3-SOL was attenuated in 4% Cr-SAL and 4% Cr-DOX by 30.6% and 26.7%, respectively, compared to the 2% Cr-SAL and 2% Cr-DOX groups, respectively. Also, the attenuated casp-9 expression was observed in the 1-EDL, treated with the Cr loading diet treatment compared to the 2% Cr-DOX group. According to Hultman et al. (1996), the Cr loading treatment increases total Cr concentration in skeletal muscle compared to a steady amount of Cr supplemented. This finding may explain that the Cr loading treatment group possibly had a higher total Cr concentration in the SOL and EDL

compared to the 2% Cr treatment and thus may have attributed to prevent the initiation of apoptosis in this study.

The gradual increase in the casp-9 expression, although not significant ($p>0.05$), was observed in 1-SOL and 3-SOL with both the SAL and DOX injection. This finding can be explained by the dimerization of cleaved casp-9. When procaspase-9 becomes activated by Apaf-1, the cleaved casp-9 can lead to the activation of another procaspase-9 that was next to for its dimerization. The overexpression of cleaved casp-9 led to a 48% increase in cleaved casp-9 expression in HeLa cells (Druskovic et al., 2006). Additionally, the SOL muscles have a higher percentage of type I muscle fibers, also known as slow-twitch fiber, compared to the EDL muscles (type IIb fiber/fast-twitch fiber) in Sprague-Dawley rats (Armstrong & Phelps, 1984). The type 1 muscle fibers, often utilized for aerobic activities, contain a higher oxidative capacity due to the higher concentration of mitochondria and capillaries while the type IIb fibers has a low mitochondrial density and less capillaries (Powers & Howley, 1995). Doxorubicin treatment often triggers ROS production from mitochondria via DNA damage and lipid peroxidation, which can lead to apoptosis (Min et al., 2015; Montaigne et al., 2011). Since SOL muscles have a higher mitochondrial density than the EDL muscles, DOX treatment could have been more effective with mitochondrial-ROS production and led to the trend toward higher expression cleaved casp-9 in the SOL muscles.

Previous studies have proposed the prevention of apoptosis with the Cr supplementation via increasing the activity of anti-apoptotic pathway (Caretti et al., 2010), mitochondrial dysfunction signaling pathway (Ceddia & Sweeney, 2004) and reducing ROS level in an oxidative environment (Lawler et al., 2002). Besides the

defense mechanism of Cr, protein synthesis can be stimulated by Cr supplementation. In Ingwall, Weiner, Morales, Davis, and Stockdale study (1974), 5 mM of Cr supplement in cultured embryonic skeletal muscle with radioactive environment increased the synthesis rate of two major contractile protein, actin and myosin heavy chains but did not affect muscle degradation rate. Creatine supplement treatments did not significantly attenuate the initiator apoptotic enzyme expressions in either type I or type II skeletal muscles in the current study. With previous findings, Cr supplements may have affected protein synthesis more rather than preventing the apoptosis in oxidative environment.

Multiple studies report that the combination of Cr supplementation and exercise training increases body mass, anaerobic strength and power (M. Bemben et al., 2001), improves in speed (Skare et al., 2001), and increases in myofibrillar protein and MHC protein expressions (Willoughby & Rosene, 2001). Besides the improvement of exercise performance, Cr also possesses the antioxidant capacity for quenching ROS, specifically $^{\bullet}O^{-}_{2}$ and $OONO^{-}$ according to previous research (Lawler et al., 2002). This antioxidant capacity of Cr was observed in oxidative skeletal muscles that was induced by acute exercise (Deminice & Jordao, 2012). However, Cr supplementation alone may not improve skeletal muscle function or increase in contractile muscle protein expression. According to Norman et al. (2006), a similar Cr supplement study without exercise was done in colorectal cancer patients for eight weeks, and the results showed that grip strength, knee extension strength, and hip flexion strength had no significant difference between the Cr intervention group, consisting of 20 g/day for one-week followed by 5 g/day for 7 weeks, and the control group. The body cell mass (BCM) of the Cr intervention group was also not significantly different from the control group. These

results suggest that Cr supplementation alone cannot improve skeletal muscle functions and increase protein synthesis. In this study, exercise was also not performed along with the Cr supplement treatments. This may explain the increase in cyt *c* and casp-9 expressions in the skeletal muscles because the Cr supplementation alone cannot induce its antioxidant effects without exercise.

Although the effects of Cr supplementation were not significant on the initiator apoptotic enzymes with the DOX treatment in the current study, many researchers have suggested Cr supplementation as a therapeutic agent in many disorders. According to Kley, Tarnopolsky, and Vorgerd (2013), Cr supplementation significantly increased in maximum voluntary contraction by 8.47% in patients suffering from muscle dystrophies. Also, the significantly higher number of patients answered "feeling better" with the Cr supplementation in the survey ($p<0.05$). Another study examined Cr's effects in Parkinson's disease, neurodegenerative disorder that affects motor systems in skeletal muscle. Patients with Cr supplementation combined with resistance training had significantly increased muscular strength in biceps curl, chest press, and leg extension ($p<0.05$). Additionally, muscle endurance for chest press and leg extension significantly increased by 38% and 95%, respectively, in the Cr supplementation group with resistance training (Hass, Collins, & Juncos, 2007). In Huntington disease involving mitochondrial dysfunction and oxidative injuries, Cr supplementation reduced the oxidative stress level in patients. Serum 8-hydroxy-2'-deoxyguanosine (8OH2'dG) was observed as a biomarker of DNA oxidative injury and its level significantly reduced by 9.11 ± 13.93 pg/mL after 16 weeks of the Cr supplementation (5 g/day) ($p<0.0042$) (Hersch et al., 2006). These promising therapeutic effects of Cr supplementation from previous studies

should be investigated for future prevention and therapeutic mechanism for a variety of disorder even though the current study did not find significant effects of Cr supplementation in DOX treated skeletal muscles.

The limitation in this study was the uncertain and unequal amount of Cr consumed by rats. The DOX group especially could have had less amount of the Cr supplement compared to the SAL due to the loss of appetite, a common side effect of DOX (Coates et al., 1983). Another limitation of this study was the unequal sample sizes in each group due to the death of rats prior to tissue collections and removal of outliers from the data set.

Further studies should investigate the effects of Cr supplementation and DOX administration on the initiation of apoptosis in skeletal muscles. The rate of protein synthesis and degradation should also be explored as previous studies discussed this along with the apoptotic enzyme expressions. In addition, DOX-induced ROS production should be measured to indicate the oxidative stress level in skeletal muscles. The consumption of the Cr supplement should be measured to standardize the amount of Cr stored in skeletal muscles as well. Exercise training should be also considered with Cr supplementation to enhance the effects of Cr.

Conclusion

The present study examined the effects of Cr supplementation and DOX administration on cyt *c* and casp-9 expressions in type I and type II skeletal muscles. In addition, the interaction between the Cr supplementation and DOX administration was examined in this study. There were no significant main effects of the Cr diet treatment or DOX injection observed. The interaction between the Cr supplementation and the DOX

injection was also not significant. However, a potential trend toward Cr providing protection was observed in 1-EDL and 3-SOL by attenuating the increased expressions of cyt *c* and casp-9 with the Cr loading treatment. Future research should be conducted with the new interventions previously suggested to better understand the mechanisms of Cr supplementation and the DOX administration in skeletal muscles.

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APPENDIX A

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL

UNIVERSITY OF NORTHERN COLORADO

IACUC Memorandum

To: David Hydock

From: Laura Martin, Director of Compliance and Operations

CC: IACUC Files

Date: December 28, 2017

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

The UNC IACUC has completed a final review of your protocol "Nutrition and Exercise in Cancer Treatment-Induced Muscle Dysfunction".

The protocol review was based on the requirements of Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training; the Public Health Policy on Humane Care and Use of Laboratory Animals; and the USDA Animal Welfare Act and Regulations. Based on the review, the IACUC has determined that all review criteria have been adequately addressed. The PI/PD is approved to perform the experiments or procedures as described in the identified protocol as submitted to the Committee. This protocol has been assigned the following number 1711CE-DH-R-20.

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

Sincerely,

Laura Martin, Director of Compliance and Operations