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The Effect of Radiation on Myofiber Properties in Mouse Skeletal Muscle

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THE EFFECT OF RADIATION ON MYOFIBER PROPERTIES IN MOUSE SKELETAL MUSCLE

by

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ABSTRACT

Radiotherapy has been proven as an effective and necessary treatment for cancer. The dose given is dependent on cancer type and location. It has been previously established that skeletal muscle is the most radiation tolerant tissue in the body. With that being said there is a current gap in the literature that is missing the effect of radiation doses on the histological properties of skeletal muscle. The overall purpose of this study is to determine the effect that a single unfractionated dose of 16Gy radiation has on histological properties of myofibers when compared to 4 fractionated doses of 4Gy. 24 C57/BL6 female mice under went hind limb irradiation procedures at UNC Chapel Hill under Dr. Ted Bateman and were sacrificed two weeks later. Tissues were then shipped to USC where serial sectioning was done on a cryostat, MHC and SDH staining procedures were all conducted. Upon analysis the fiber type specific myosin heavy chain isoforms IIA and IIB CSA both decreased significantly (p=.04, p=.002). The SDH activity of each treatment group showed a significant decrease in glycolytic fibers and an upward trend in small oxidative fibers in regards to mean CSA but in terms of distribution showed an increase of smaller oxidative fibers (p=.03) and a loss of larger glycolytic fibers (p=.02) in the un-fractionated 16Gy group (n=7). The findings of this study show that unfractionated radiation dosing has atrophic effects on individual myofiber histological properties when compared with 4 fractionated 4Gy treatments.
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CHAPTER 1

Introduction

Cancer is currently the number two cause of death in the United States of America [1]. The CDC projects that in the year 2013 about 1,660,290 new cases of cancer will be diagnosed with a projected 580,350 cancer deaths [1]. The total number of deaths alone will translate to about 1600 deaths per day just from cancer [1, 2]. Radiotherapy is a treatment that is given to over half of cancer patients [2]. According to the National Cancer Institute approximately 60% of people who have cancer will receive radiotherapy [3]. It uses high-energy particles or waves such as X-rays or even gamma rays in some instances to damage or control the rapid uncontrolled cell growth that is cancer [3]. There is a lack of literature and studies on myofibers and how varying radiation doses affects them differently in terms of overall distribution and cross sectional area.

The treatment can be administered in 3 different ways [4]. External radiation is a method of treatment that involves a radiotherapy machine called a linear accelerator to direct beams of radiation into the desired area of tissue where the cancer or tumor may be located, and is also the most common type of radiotherapy [5]. The standard unit for the radiation is the Grey (Gy) [5]. The dose given as well as the course of treatment is often almost dependent on the type of cancer and location of the disease in the body [6]. Curative doses are around 60-80 Gy and most lymphomas are treated with 20-40 Gy doses [5]. Doses of 8-20Gy are used typically as boost doses for breast cancer as well as treatments for skin melanomas and larynx cancer [7]. The total dose being used in this
masters thesis is of 16Gy is a common dosage used for pelvic irradiation due to cervical cancer [8]. The next type of radiation treatment is Internal radiation and often some type of radioactive source from a wire, seed, pellet or balloon that is “implanted” within the individual in the body near the tumor or cancerous cell growth [9]. This form of therapy is not very common however, it does in fact minimize damage to surrounding tissues seeing as how it is implanted directly near the target and does not have to pass through multiple layers of tissue [7]. The third and final form of radiotherapy treatment is called systemic. This type of radiotherapy uses drugs that are known as radiopharmaceuticals [9] These drugs are administered either intravenously or in pill form and often require a short stay in the hospital.

In terms of dosimetry, radiotherapy is Radiation used locally can minimize the side effects often seen with chemotherapy [10]. Lung cancer radiotherapy is the standard procedure for treatment in 70 to 90 % of all individuals diagnosed with small cell lung cancer and those with non-small cell lung cancer are given radiation about 17 % of the time or in combination with chemotherapy 35% of the time, and this is in both male and female populations [1]. Prostate cancer in men is another common cancer that is one of the main killers and with this illness around 42% of all patients diagnosed will undergo some type of radiotherapy treatment [11]. The prescribing physician of the radiotherapy must also take into consideration the overall health of the patient as well as the location and size of the cancer [9]. Most radiotherapy is given locally and this can serve as a benefit as opposed to chemotherapy, which exposes the entire body to harmful cellular damaging chemicals that do not distinguish between good and bad cells systemically throughout the body [12]. Radiotherapy has been proven as an effective and often
necessary treatment for cancer. There are some serious side effects of the therapy that can afflict the patient’s quality of life both during and post treatment time periods. Out of all patients who receive radiotherapy treatment around 80% of them report the number one side effect of fatigue [2].

Skeletal muscle itself is the largest organ in the body [13]. Skeletal muscle fibers all have varying degrees of oxidative capacity. Oxidative capacity is defined as a measure of the maximal capacity of a tissue (usually muscle) to use oxygen; expressed as microliters of oxygen consumed per gram of tissue per hour [14]. This is directly related to the amount of mitochondria in the skeletal muscle fibers themselves [15]. The mitochondria interacts with the oxygen supply in the body in order to make cellular energy ATP[16] [15]. The amount of mitochondria in skeletal muscle is directly heavily dependent on the type of muscle fiber as well as the actual energy and metabolic demands for any given sub-cellular region within that myofiber [17]. When the electron transport chain is stressed and the amount of redox reactions becomes too excessive to meet the energy demands then things like superoxides form and make free radicals [18]. Free radicals are dangerous superoxides that cause damage on a cellular level both systemically and locally and recent publications are showing they may have a direct hand in the aging process [19]. Skeletal muscle fibers are generally categorized into two groups Type I and Type II, with Type I being the slower more oxidative fiber [20]. Within Type II there is a sub-group known as Type IIa, also known as oxidative fast twitch fibers higher oxidative capacity [20]. These fibers generate ATP through the glycolytic cycle but also have a very high mitochondrial count which still allows them to obtain ATP through oxidative metabolism [21]. While Type IIa may fatigue quicker than
Type I they do possess some capability to resist fatigue [21]. The second sub-group is known as Type IIb or glycolytic fast twitch. They have an almost white appearance due to low myoglobin levels and contain a very low amount of mitochondria that gives them an extremely low oxidative capacity. That low oxidative capacity allows Type IIb to fatigue very quickly [20]. Type IIx is actually a subject of much research and current debate in the scientific community. This fiber is thought to be a transitional switch fiber, which means that in order for a Type IIa to switch to IIb and vice versa it must first become a IIx [20]. This means that the fiber switches its MHC isoform expression depending on the stimulus it undergoes [20]. The vast majority of studies have shown how the radiation damages satellite cells via interrupting the satellite cells mitosis process by causing “breaks” in the cells DNA [4].

The current gap in the literature of irradiation studies shows that skeletal muscle satellite cells and myoblast activity are hindered greatly with radiotherapy doses however, little to no research has been conducted on myofibers and radiation therapy. Previous studies do show evidence of oxidative stress, which would follow along with a potential change in myofiber distribution in a mouse model [22]. The overall purpose of the proposed study is to determine the changes in myosin heavy chain expression, size and fiber distribution as well as changes in oxidative myofiber expression, size and fiber distribution with varying doses of radiation. This is a study that is necessary to gain a grasp on the concept of the potentially dangerous side effects in the skeletal muscle post treatments and overall what radiation does to the fibers of the muscle itself. Based on previous literature and evidence our central hypothesis is that one single dose of 16Gy
will have a greater alteration on MHC and oxidative myofiber expression, size and fiber distribution and when compared with 4 treatments of 4Gy.

Specific Aims

Using previously collected skeletal muscle from 24 female C57/BL6 mice immuno-histochemical staining methods will be employed to test the central hypothesis with these two specific aims.

Aim1: To determine if skeletal muscle fiber type IIa/IIb fiber distribution and size are differentially affected by radiation dose.

Rationale:

With the presented study having two different dose treatments it is logical to examine if the radiation doses are in fact causing a change in myosin heavy chain IIa/IIb expression as well as distribution and mean cross sectional area.

Aim2: To determine if the distribution and size of oxidative and glycolytic myofibers are differentially affected by radiation dose.

Rationale:

Specific aim2 will establish if the myofiber metabolic capacity related to size and phenotype oxidative metabolism differentially alters the response to radiation dose.
CHAPTER 2

Review of Literature

Cancer is currently a major health problem in the United States as well as worldwide [1]. Approximately one in every 4 deaths in the United States are due to cancer [1]. While there are many different kinds of treatments and plans currently available for cancer, one is much more prevalent and common amongst patients. Radiotherapy will be given to approximately 60% of all people who are diagnosed with cancer [9]. This is an astounding amount of people that will have some part of their body exposed to radiation. While radiation is believed and widely accepted to be a life saving and often necessary treatment, that does not mean that it is a completely safe system of treatment. Radiation can be a dangerous and harmful thing especially depending on treatment location as well as the dose that is being administered [23]. A great deal of previously published literature has established that radiation does in-fact kill healthy cells as well as stopping the “tumor” or cancerous cells from metastasizing (spreading) to another part of the body [4]. According to the National Cancer institute, when dosing for radiation therapy it is often taken into consideration which dose will be high enough to effectively kill targeted cells yet low enough to limit damage to healthy cells [4]. In
terms of research much of this area is relatively untouched when it comes to skeletal muscle myofibers with only a few studies looking at actual morphological properties. Most radiation research has been done with varying doses of all sorts but the main source of interest with these previously published studies are macrophage infiltration as well as damage to satellite cells. A large amount of studies have been conducted in order to establish the fact that radiation damages DNA on a molecular level and therefore interrupts cellular mitosis which in turn stops rapid and uncontrolled cell division (cancer) (, but at the same time can do extensive damage to cardiomyocytes and other very important cells necessary for physiological function. With that being said the specific effects of myofiber morphology has not been looked at in great detail with one of the major studies published coming from a joint collaboration between the University of Wisconsin and the USSR that flew rats into space and analyzed fiber expression and regeneration [24]. This study was primarily focused on regeneration and not fiber type expression or any type of alteration in oxidative capacity. It is logical to hypothesize that if there is going to be a change in myosin heavy chain expression their will most likely be a change in oxidative capacity (metabolism) of those myofibers.

The literature review for this Master’s Thesis is going to be divided into three sections: (1) Radiation and its known effects on cells and skeletal muscle (2) Skeletal muscle morphological structure and fiber types (3) Oxidative capacity and skeletal muscle, this literature review is meant to give a general overview of radiation and what has been established in terms of how it affects cells and what is known about its effects on skeletal muscle. This review will also encompass skeletal muscle structure (myofibers) and general background and established knowledge on skeletal muscle as
well as detailed information on myosin heavy chain expression seeing as how it is one of the aims of the study. The final section will feed off of the second and detail what oxidative capacity is in skeletal muscle fibers and what are known to cause alterations to such properties.

2.1 Radiation

While there are many different treatments now for cancer radiation is the most common with chemotherapy coming in second place just behind it. Radiation is an essential part of treatment for cancer and with modern technology it has become safer and more efficient than ever. With that being said that does not mean that radiation is a cure for cancer. It must be understood that there is no perfect treatment for this disease and given the age and technological advances radiation is a treatment that has withstood the test of time and has been refined tremendously since its implementation. This section is geared toward clearly defining what radiation is and elaborating on the dosimetry, types of radiation and the effects that it is known to have on healthy and cancerous cells as well as known effects on skeletal muscle.

Radiation and Skeletal Muscle

Radiation is a word that stemmed from 1550s, from Latin radiationem which means “to beam or shine” [25]. Radiation in terms of a practical definition is high energy that is emitted in waves or particles (ions) [25]. These high-energy beams or particles are classified into two main types of radiation. Ionizing radiation such as X rays and gamma rays, is radiation that can ionize atoms in the material it interacts with and Non-Ionizing radiation such as micro and radio waves that can not ionize atoms in the material it
interacts with [8]. In terms of our study we will be focusing on Ionizing radiation particularly X rays. Radiotherapy is often given using X rays and it is called external beam radiotherapy [26]. The patient must sit or lie extremely still in order for this procedure to be conducted and often the patient receiving treatment is restrained in some type of specially designed cast or apparatus [26]. This is by far the most common type of radiotherapy and will usually use either superficial (kilovoltage) X rays or deep (megavoltage) X rays depending on the type of cancer being treated [27]. The type of rays used will always be determined by the oncologist and radiologist in order to plan the best treatment possible while ensuring the comfort and highest possible rate of survival for the patient [23]. Now in terms of dosing the radiation this is a process that is completely unique to each patients situation. The health, age weight, height and most importantly the location and the type of cancer will all come into play when establishing how to effectively dose the radiotherapy treatments for the patient. The standard unit in radiation dosing that is used in almost all of the published literature is the Gray (Gy) [28]. The Gray is a way of saying the dose that has been absorbed by the tissue. It is not uncommon to see radiation reported in rads as well and just for reference purposes 1Gy = 100 rads [29]. Typically all of the units will vary depending on the type of radiation being administered as well as the type of ionizing particle being used. In terms of actually dosing a patient it is often common to spread out the radiotherapy treatments in order to allow the good cells that may have been damaged time to heal before the next treatment [30]. However, with that being said some radiotherapy treatments require one single “big blast” of radiation and this is sometimes also employed in order to keep cancer from reoccurring such as in the case of breast cancer [23]. The patients all had
similar cases of breast cancer and were given their initial fractionated 50Gy radiation treatment. Following treatment some of the patients were then assigned to receive a “boost dose” of a single shot of 16Gy radiation in order to prevent tumor recurrence [23].

The previous report from the EORTC data center found that the trial established that for patients 40 years old or less, an additional radiation boost (16Gy to the tumor bed) reduced the 5-year local recurrence rate from 20% to 10% [23]. This establishes that a single dose of moderate to low intensity can be extremely important in terms of prevention or recurrence of the disease. As mentioned before it is important to note that all radiation dosing and treatment comes with consequences and possible side effects [31].

SIDE EFFECTS AND CONSEQUENCES OF RADIATION

Radiation as mentioned briefly before does not distinguish between healthy and cancerous cells. This is important because there are side effects that patients undergoing treatment must be ready to face. The radiation itself proceeds to enter the body or localized region in most cases [32]. It stops the cell growth by damaging the DNA of each cell, which will hinder or stop mitosis all together [29]. We consider chromosomes to be the most important of the cell seeing as how they carry all of the genetic material necessary for cellular division and replication [33]. In addition to damaging the chromosomes there are also repair cells that can repair damage and if these are also damaged by radiation then repair of the damaged chromosomes impossible [29]. Thus, finding an effective dose of radiation to stop division and repair but not completely kill the cells all together is the problem that many oncologist face. Typically four things will happen as an effect of radiation. The first is that the cells are undamaged by the dose and
will continue to function normally as if nothing had ever happened. The second is that
the cells are damaged, the repair cells repair the problem and the cell continues once
again to operate on a normal level and this can include repairing of chromosomes [34].
The third biological effect that can happen is that the cells are in fact damaged, repaired
but will no longer operate normally. If the damaged cell must perform a task before it
has had time to repair itself it will either not perform correctly or the repair will not be
completed correctly, which results in improper function [34]. These types of cells can be
dangerous due to the fact that they may still be able to reproduce but being damaged will
cause them to reproduce at a rapid uncontrolled rate (cancer). The fourth and final
biological effect is complete cellular death. If a cell is severely damaged by the radiation
then it is beyond repair and will die [12]. This depends on the sensitivity of the cells that
are exposed to radiation. It is often said that skeletal muscle is the most radiation
resistant tissue in the body. The tissue or system that has been deemed the most
sensitive is that of the blood or the hemopoietic system [4]. Doses typically given as
acute radiation leave the body more susceptible to things such as internal or damage to
the nervous system. Most of the time when exposed to an acute dose death does not
occur from the radiation exposure but rather damaged to the GI tract and things such as
internal bleeding [5]. Many radiotherapy patients report having trouble eating or keeping
food down and this is the reason why. The dose has damaged the cells in the GI tract
thus making nutrient absorption that much harder. In terms of skeletal muscle there is not
a great deal of radiation studies.

THE POTENTIAL FOR RADIATION INDUCED REACTIVE OXYGEN SPECIES

The cellular damage done above can cause significant stress to the cells and in return this
can either be repaired or cause significant damage beyond repair which leads to improper function and cellular death [35]. Reactive oxygen species or ROS is defined as molecules that are formed by the incomplete one-electron reduction of oxygen [10]. These reactive oxygen intermediates encompass singlet oxygen; superoxides; peroxides; hydroxyl radical; and hypochlorous acid [19]. They contribute to the microbicidal activity of phagocytes, regulation of signal transduction and gene expression, and the oxidative damage to nucleic acids; proteins; and lipids [19]. ROS is a frequent marker of damage in terms of skeletal muscle and biological tissues in the body [22]. It occurs when the mitochondria encounters some type of problem in the electron transport chain, typically this is “problem” is due to some type of stimulus or simply an overload [15]. An overload in the electron transport chain typically leads to uncoupling which is a defense mechanism for the body to help limit the production of ROS [36]. Alone these superoxides are not extremely reactive but they can travel through the body disrupting cellular processes as well as deactivating enzymes and initiating lipid peroxidation [36]. With ROS being a marker for oxidative stress it is important to know that ROS itself cannot be directly measured but rather certain proteins that are elevated are markers of high levels of ROS [37]. Radiation has been found shown to cause oxidative stress at a low dose such as in Tongsheng et. al. where they irradiated mice with varying doses of 2 and 4Gy and actually saw that within two hours of irradiation Bax which is a marker protein for ROS was elevated [38]. Thus concluding that with irradiation there was a long playing production of ROS [38]. While this is an important finding is it necessary to point out that his was done in A549 lung cancer cells not in actual skeletal muscle, none the less the proves the concept that radiation can induce production of reactive oxygen
species. The same results were corroborated by Yamamori at Hokkaido University in Japan where they took the same A549 cells and ran a similar experiment although they used a slightly larger dose of 10Gy [22]. In a study conducted by Fedorva et al. they found that proteins scavenge about 50-75% of free radicals [39]. This is significant in the fact that x irradiation has been shown to induce the production of free radicals.

Furthermore skeletal muscle in mammals is where about 25% of total protein turnover occurs [39]. Fedorva used an acute dose of x-irradiation at 5Gy to induce oxidative stress. Through this small dose they saw an immediate and high generation of ROS via various markers and all at different time points [39]. Fast twitch muscles are more prone to oxidative damage and show a more rapid decline during aging than slow twitch myofibers such as Type 1 or even Type IIa. A major find of this particular study was that there was irreversible carbonyl modifications at all time points, particularly modifications to creatine kinase and actin [39].

**Radiation and its Effects on Skeletal Muscle**

The effects of radiation on skeletal muscle is very limited, it is often deemed the most radiation resistant tissue but with that being said it has not been studied in great detail [40]. There are a few studies that have examined the effects of radiation and skeletal muscle. According to Mihaela Jurdana, radiation inhibits satellite cells, by damaging the DNA beyond repair by the polymerases [34]. He notes that lower levels of radiation can disable mitotically active satellite cells but not the post mitotic myonuclei, preventing compensatory hypertrophy of the skeletal muscle. Muscle satellite cells are undifferentiated mononuclear myogenic clels that can be found in skeletal muscle of various animals [33]. After irradiation Jurdana found that there were a low amount of
satellite cells to fuse to form new fibers or to fuse to overloaded cells to allow hypertrophy to continue during overload. Olive et al. noted that a single small dose of radiation at 2Gy affected single skeletal muscle cells (satellite and myoblast) in the midst of development and induced apoptosis [35].

Figure 2.1. Jurdana et al. demonstrates his working model to show how low doses of radiation affected the neuro-muscular junction NMJ and blocked satellite cell proliferation.

Bandstra et. al. studied mice exposed to 1 and 2 Gy doses to simulate space flight and noted that muscle fibers cut from the triceps brachi had fewer fibers with smaller diameters [41]. This is important when one thinks about it because this could mean a possible loss of IIa fibers, which would fall in line with AIM 1 of the proposed thesis study. It is important to note as well that Bandstra et. al. also found an increase in centralized nuclei which is a known marker of muscle regeneration and remodeling (34). The term regeneration is typically used to describe the renewal and or repair of damaged or destroyed cells or tissues with the potential to restore functional activity of those tissues or cells [42]. There are two phases to regeneration and muscle remodeling [43]. The first is the degenerative phase which involves necrosis and cleaning of the damaged tissue [42]. The regenerative phase involves cellular proliferation which is critical in order for regeneration to be achieved. The expansion of myogenic cells offers a plentiful source of myonuclei for the repair [44]. Upon proliferation the myogenic cells then
differentiate and fuse which leads to new myofiber formation. Histological analysis when analyzing regeneration skeletal muscle typically shows centralized nuclei [42]. When looking at the study by Bandstra they also noted centralized nuclei had a significant increase when radiation was used thus meaning that damage had occurred to the skeletal muscle [41]. Radiation is commonly used as a way to knock down overload response as well as protein synthesis [45]. As previously mentioned it has a hindering effect on myogenic satellite cells [46]. These satellite cells are undifferentiated. In a paper by Adams et al. they ran a study that took rats out to 90 days in order to hopefully disprove the fact that previously conducted studies were not allowing enough time for a possible recruitment of extra-muscular stem cells that could perhaps infiltrate the damaged muscle and differentiate into satellite cells and allow for hypertrophy to resume as normal [47]. What they actually found was that after the single dose of 25Gy gamma radiation even at 90 days appeared to attenuate the overload response and did not induce an increase in CSA or myofibrilllar protein content [47]. They did see an increase in DNA concentration and mRNA however this could be due to the infiltration of immune cells while repairing the induced damage to the skeletal muscle. So it appears that the radiation had disabled satellite cells and even though there was growth in terms of body weight and muscle weight of the rats, this is most likely attributed to the natural growth of the rats over the course of a 90 period seeing as how they were not on a restricted diet or special caloric intake regulation they did indeed grow but the radiation did prevent the normal hypertrophy overload response that was seen in the non irradiated overload mice [47]. It is important to note again that this study along with most of the literature on skeletal muscle and radiation is using only one single dose, and the proposed masters
thesis is looking at fractionated and un-fractionated radiation in order to see if the fractionated has less negative affects when compared with un-fractionated and control.

Radiation Specific Damage to Satellite Cells

So it has been well documented and established that radiation can afflict satellite cell function as well as population number. Such studies conducted by Rosenblatt et al. are classic examples of the damage that radiation can do to satellite cell activity. They ran a study that looked at compensatory overload in regards to satellite cells in the EDL muscle of mice [46]. After a four-week period they sacrificed the mice and analyzed the mass, fiber type percentage and the overall size of the EDL were measured. What they found was the percent of smaller fibers as well as their size decreased with irradiation as well irradiation and overload [46]. What I would like to point out is that this study did in fact use ionizing radiation however, they used gamma radiation as opposed to x-ray irradiation, which is used in the presented masters thesis. The gamma radiation is known for being more aggressive and deeper penetrating. An interesting thing to note is that the overloaded muscle whether it was irradiated or not had a significant increase in type IIx myofibers and a decrease in the amount of IIb fibers [48]. This means that satellite cells are required for muscle hypertrophy induced by synergist incapacitation but they do not appear to be necessary for maintenance of or change in myosin heavy chain phenotype expression. So with that being said what we know about radiation and skeletal muscle is largely limited to satellite cells and muscular remodeling through induced damage from the radiation. There are very few if any studies that solely analyze radiation and its effects on skeletal muscle [49]. Radiation in most studies appears to simply a way to knock down satellite cell activity and a way to block the compensatory hypertrophy
response. The dose and type of ionizing radiation does indeed play a role in the amount of damage that is being seen in these studies. Obviously, the higher the dose of radiation the more of an effect there is going to be. Where our study is novel is the fact that we are using x-irradiation which is common for breast and skin cancer [26]. It is also novel in the fact that our dose is lower than what is typically used as a total treatment. However, we are able to detect a change which means that if someone receiving a single dose at the 16Gy intensity could see some effects it is also important to note that even though our main effect is seen in single un-fractionated doses a cancer patient could in fact receive fractionated doses at 16Gy which could have an acute effect possibly in between treatments.

2.2 Skeletal Muscle Morphology

Skeletal muscle as previously mentioned is the largest organ in the body. It is often sectioned and analyzed in order to view its morphology. Morphology of skeletal muscle is defined as the actually composition and structure of the muscle. It deals with total fiber size and shape as well as fiber type specificity and the distribution of the fibers throughout the muscle [50]. Changes in morphology can occur via many different stimuli which include overload, aging, exercise, detraining, cancer and even radiation [21]. This section is geared towards addressing what skeletal muscle morphology is made up of and what will be analyzed in terms of the proposed thesis study and how radiation has been shown in previously published literature to induce changes in muscle morphology.

Skeletal Muscle Phenotypes.

There are 3 main fiber types that make up skeletal muscle, Type 1, Type IIa, and
Type IIb [51]. These fibers and the amount of them present in the specific muscle can vary greatly depending on such things as stimulus or something such as disease like cancer or muscular dystrophy [51]. These fibers are also present in varying amounts due to the fact that each fiber type has a specific metabolic capacity and metabolism that it is responsible for carrying out in order to feed the muscles. Type I muscles are known as slow oxidative myofibers and use the process of oxidative phosphorylation in order to synthesize ATP for energy needs [14]. Type IIa is known as fast oxidative they can vary between the oxidative and glycolytic pathway depending on energy needs of the muscle [52]. Type IIb are known as fast twitch glycolytic myofibers. The difference of energy use between fast and slow twitch muscle has been mostly studied in small mammals such as mice and rats by indirect approaches such as oxygen consumption [53]. Typically the Type 1 fiber is known for being slow to contract due to its low level of ATPase activity [52]. The IIa fiber less mitochondria than a Type I but has more than a IIb which as the lowest mitochondria count of them all. This is important because the amount of mitochondria in these fibers is what dictates the metabolic pathway that they use in order to produce energy. A lack of mitochondria means that oxidative phosphorylation is typically not going to be the way that energy is produced [54]. Hence a IIb fiber is known as a “glycolytic” fiber due to the fact they have little to no mitochondrial capacity which forces them to very prevalent in muscles that are typically non load bearing such as the EDL which was studied in the previously mentioned Rosenblatt paper [46]. Type IIa fibers are known for being a fiber that is mildly oxidative and can switch back and forth between pathways depending on needs. These fibers are best suited for mild to intermediate intensity exercises such as mid distance running or jogging [50]. They have
a high concentration of myoglobin and are a deep red in color. In terms of contraction velocity they have a faster one than a Type I fiber. The Type IIb fiber is purely a fast twitch fiber. Typically they are very large in size with the average CSA being around 3000 um² [55]. These fibers have the fastest contraction velocity out of all of the skeletal muscle fiber types [55]. The IIb fiber is the most powerful however, typically consumes the most energy (ATP) due to the intensity of its shortening velocity and its high power output (43ZHENG-HEHE). Now as mentioned previously there can indeed be myosin heavy chain isoform shifts that occur under certain stimulus. The most common of these stimuli being weight bearing or (overload activities) that can induce a glycolytic IIb fiber to transition into a IIa oxidative fiber in order to meet the demands of constant continued overload stimuli [56]. Something that is still of rather common debate is the role of the IIx fiber. While these fibers were not specifically analyzed in the proposed thesis study they are indeed important to talk about. The common theory of IIx fibers is that they are actually a transitional fiber that is only present when a myosin heavy chain isoform shift is occurring seeing as how it has a intermediate contractile speed that falls directly between IIa/IIb [57]. Initially IIx was thought to be the same as IIb but recent research and data on contractile velocity and power output has found that it is in fact its own distinct fiber type [57].

**Myosin Heavy Chain Shifts**

Radiation has been shown to dull the overload response in terms of compensatory hypertrophy but not myosin heavy chain shifts [55]. In the study by Adams et al. they noted that while the radiation hindered satellite cell proliferation there appeared to be no damage in terms of the immune response and interestingly the radiation did not appear to
have an affect on myosin heavy chain isoform shifts with the overload. The muscles measured on a myosin heavy chain gel showed that the overloaded mice did in fact undergo an isoform shift from IIb $\rightarrow$ IIa [58, 59]. Thus showing that the radiation does not necessarily always affect the myofiber isoform expression. In another study that was conducted by Phenlan and Gonyea in 1997, which used a similar gamma radiation procedure that was just like Rosenblatt et al, they noted that the myofibers that were irradiated and overloaded stained positively for embryonic myosin heavy chain which is a special form of the isoform only present in new regenerating muscle fibers [60]. This study was particularly interesting due to the fact they used a higher dose and found that the radiation did not actually hinder the amount of fibers in the soleus that were producing embryonic myosin heavy chain isoform. Thus, meaning that the radiation may indeed knock down satellite cells and prevent hypertrophy through overload even when IGF-1 appeared to be elevated [60]. This means that although the muscle did not hypertrophy with irradiation there was an increase in embryonic myosin heavy chain expression meaning that the muscle is regenerating and still repairing itself but not growing any larger with the overload stimuli due to the irradiation [60]. This is important to note since it is evidence that irradiation does not prevent a shift in myosin heavy chain. The shift in phenotypes occurs typically when skeletal muscle is either being overworked constantly such as in an overload model or underworked such as in an unloading model. In a study done by Talmadge et al. they found that rats that were flown into space for 6 days and experience zero gravity conditions (unloading) showed an increase in the expression of IIx/IIb fibers in the gastrocnemius muscle due to the non weight bearing nature of the space flight. The normally heavy oxidative myofibers in the soleus had
switched to a more glycolytic type II fiber [61]. In conjunction with the space flight they also ran a hindlimb suspension group in order to show a similar effect and after 14 days noted a significant increase in the amount of IIx MHC isoform [61]. In regards to fiber shifts one of the key mechanisms that has been studied widely is PGC-1a or peroxisome proliferator activated receptor-y coactivator, and it is a transcriptional activator that plays key roles in mitochondrial biogenesis and in our case myosin heavy chain isoform expression and shifts. Mortensen et al. found that overexpression of PGC-1a in skeletal muscle increased citrate synthase activity by nearly twofold at 14 days. This leads to a more oxidative shift in myosin heavy chain isoforms which means more oxidative type I and type IIa fibers [62]. When PGC-1a is able to be overexpressed it is what essentially drives the fast to slow oxidative fiber type switch. Based on previous studies listed above that take note of the myosin heavy chain isoform shifts that occur under radiation stimuli one could say that radiation has no effect or hindering effect on the expression of PGC-1a which may be why we do not see much of a muscle mass change in the proposed thesis but rather myosin heavy chain shifts [62]. Now some radiation studies such as Rosenblatt et al. did note a decrease in muscle mass size but that could be attributed to the higher dose and different type of ionizing radiation that they had used [48]. As previously mentioned in the above study most of the irradiation literature that is published is about it being used as a model to decrease satellite cell response to overload and regeneration. The other main purpose that radiation is found in literature for is for the purpose of spaceflight investigation and unloading in order to further analyze the radiations atrophic effects when combined with anti-gravity conditions [24].
2.3 Oxidative Capacity of Skeletal Muscle

A muscle's oxidative capacity is defined as its maximal capacity to use oxygen in microlitres of oxygen consumed per gram of muscle per hour [63]. Factors, which affect the oxidative capacity of muscles, include the activity of oxidative enzymes (e.g. succinic dehydrogenase) fibre-type composition and availability of oxygen [50]. With that being said, if there is going to be some type of change in myosin heavy chain isoform expression then this most likely will alter oxidative capacity of the muscle, which falls in line with Aim 2 of the proposed masters thesis. As mentioned above fiber type specific changes can occur in skeletal muscle ultimately altering the amount of mitochondria present in the particular fiber that is currently being expressed. An alteration in oxidative capacity alters the type of metabolism being used. A slower Type I fiber has the most mitochondria and is therefore the most oxidative of them all and has the highest oxidative capacity [64]. Typically producing ATP thorough oxidative phosphorylation and fueling the contracting muscle that is undergoing slow and intermediate exercise [50].

Stimuli That Can Alter Oxidative Capacity.

Changes in oxidative capacity can be altered by numerous stimuli. These stimuli can range from aging or even mechanical. In a particular study done by Conley et al. they conducted a human trial that looked at the difference between adult humans and elderly individuals on the parameters of mitochondrial content in skeletal muscle as well as oxidative capacity [65]. Upon analysis of the results they found that as human beings age the ability of the muscle to effectively utilize oxygen. The mitochondria content drops significantly in the elderly population, which leads to a decreased aerobic capacity as indicated by VO2 maximal data [65]. In another study that was published, the
investigators looked at body composition in relation to oxidative metabolism and make up of skeletal muscle in women. As mentioned previously there are numerous ways that can alter the oxidative capacity of skeletal muscle which in turn essentially alters the muscles substrate use and ultimately the total metabolism. The researchers took body composition from obese women and found that they were becoming insulin insensitive when they had high levels of adipose tissue. The insulin insensitivity was then altering the and high adipose tissue was pusing the muscle toward an increased capacity for anaerobic re-synthesis of ATP as well as increased glycolytic capacity [65]. Essentially they found that when there was excess adipose tissue the muscle tended to favor that as a substrate which resulted in the decreased oxidative capacity and citrate synthase activity which is a key enzyme that is involved in the aerobic generation of cellular energy [65]. When citrate synthase activity decreases then you are typically going to see a shift toward a more glycolytic metabolism. In White et al. they looked at alterations of mouse skeletal muscle oxidative capacity in relation to the min (cancer) mouse [63]. What they saw was that cancer cachexia (wasting) caused a drop in mitochondrial DNA, and with this there were no alterations in myosin heavy chain expression and PGC-1a appeared to be suppressed. Now it has also been documented oxidative capacity is directly related to skeletal muscle mitochondria content and quality [62]. If these mitochondria are stressed via something like radiation or cancer then there can be oxidative stress (ROS), which, is mentioned, previously in the above studies in the introduction. White managed to demonstrate that cancer cachexia does not appear to show elevated levels of oxidative stress however there is suppressed PGC-1a signaling which is both responsible for myosin heavy chain shifts as well as mitochondrial biogenesis [56]. Since this was
repressed then it is apparent that the lack of biogenesis accounts for some of the decrease in oxidative capacity as well as there being no shift in myosin heavy chain fiber type expression in both red and white muscle [63]. The alterations in oxidative capacity have been very well documented in regards to cancer and other stimuli such as exercise or unloading. In a study conducted by Riley et al. rats were flown into space and orbited earth for 12.5 days [24]. When they analyzed the tissue of the rats for SDH activity there were not many changes mitochondrially but they did not significant decreases in oxidative activity in the subsarcolemmal region of the skeletal muscle of the rats. This shows that with non weight-bearing activity muscle can induce a change in order to adapt the environment. The muscles were in an anti-gravity environment so therefore they decreased oxidative capacity and switched to a more glycolytic metabolism [66]. The oxidative properties of muscle are relatively untouched in terms of x irradiation. There a few studies that truly examine whether or not the damage induced by ionizing radiation differentially affect the oxidative myofibers. It is well established though that IIb myofibers are much more susceptible to damage via ROS or other stimuli than IIa myofibers are [67]. With that being said it would appear that through radiation activation of ROS as mentioned previously in this literature review that the IIb fibers would most likely be the myofibers damaged and thus more prone to remodeling and regeneration [68]. Thus this would lead to an overall decrease in size of these fibers such as seen by Rosenblatt in his numerous studies that were conducted with radiation treatments to the EDL and soleus muscles. The effect that this could have on oxidative capacity would mean that the myofiber oxidative metabolism may be pushed toward a more oxidative type IIa fiber if the IIb fiber is more susceptible to the type of damage induced by
ionizing radiation such as x or gamma rays [69]. With that being said it appears that radiation does indeed have an effect indirectly via various mechanisms on oxidative capacity as well as metabolism of skeletal muscle.
CHAPTER 3

THE EFFECT OF RADIATION ON MYOFIBER PROPERTIES

ABSTRACT

Radiotherapy has been proven as an effective and necessary treatment for cancer. The dose given is dependent on cancer type and location. It has been previously established that skeletal muscle is the most radiation tolerant tissue in the body. With that being said there is a current gap in the literature that is missing the effect of radiation doses on the histological properties of skeletal muscle. The overall purpose of this study is to determine the effect that a single unfractionated dose of 16Gy radiation has on histological properties of myofibers when compared to 4 fractionated doses of 4Gy. 24 C57/BL6 female mice underwent hind limb irradiation procedures at UNC Chapel under Dr. Ted Bateman and were sacrificed two weeks later. Tissues were then shipped to USC where serial sectioning was done on cryostat, IIA/IIB and SDH staining procedures were all conducted. Upon analysis the fiber type specific myosin heavy chain isoforms IIA and IIB CSA both decreased significantly (p=.04, p=.002). The SDH activity of each treatment group showed a significant decrease in glycolytic fibers and an upward trend in small oxidative fibers in regards to mean CSA but in terms of distribution showed an increase of smaller oxidative fibers (p=.03) and a loss of larger glycolytic fibers (p=.02) in the un-fractionated 16Gy group (n=7). The findings of this study show that
unfractionated radiation dosing has atrophic effects on individual myofiber histological properties when compared with 4 fractionated 4Gy treatment.

**INTRODUCTION**

Cancer is currently the number two cause of death in the United States of America [4]. The CDC projects that in the year 2013 about 1,660,290 new cases of cancer will be diagnosed with a projected 580,350 cancer deaths [4]. The total number of deaths alone will translate to about 1600 deaths per day just from cancer [4]. Radiotherapy is a treatment that is given to over half of cancer patients [9]. According to the National Cancer Institute approximately 60% of people who have cancer will receive radiotherapy. It uses high-energy particles or waves such as X-rays or even gamma rays in some instances to damage or control the rapid uncontrolled cell growth that is cancer (8,22). There is a lack of literature and studies on myofibers and how varying radiation doses affects them differently in terms of overall distribution and cross sectional area.

The most common type of radiation treatment is known as external radiation [4]. External radiation is a method of treatment that involves a radiotherapy machine called a linear accelerator to direct beams of radiation into the desired area of tissue where the cancer or tumor may be located, and is also the most common type of radiotherapy [5]. The standard unit for the radiation is the Grey (Gy) [5]. The prescribing physician of the radiotherapy must also take into consideration the overall health of the patient as well as the location and size of the cancer [9]. Most radiotherapy is given locally and is either fractionated which means the treatments are spread out over time or un-fractionated which means the total prescribed dose of radiation is given all at once[12]. Radiotherapy
has been proven as an effective and often necessary treatment for cancer. There are some serious side effects of the therapy that can afflict the patient’s quality of life both during and post treatment time periods. Out of all patients who receive radiotherapy treatment around 80% of them report the number one side effect of fatigue [2].

Skeletal muscle itself is the largest organ in the body [13]. Skeletal muscle fibers all have varying degrees of oxidative capacity. Oxidative capacity is defined as a measure of the maximal capacity of a tissue (usually muscle) to use oxygen; expressed as microliters of oxygen consumed per gram of tissue per hour [14]. This is directly related to the amount of mitochondria in the skeletal muscle fibers themselves [15]. The mitochondria interacts with the oxygen supply in the body in order to make cellular energy ATP[16][15]. The amount of mitochondria in skeletal muscle is directly heavily dependent on the type of muscle fiber as well as the actual energy and metabolic demands for any given sub-cellular region within that myofiber [17]. When the electron transport chain is stressed and the amount of redox reactions becomes too excessive to meet the energy demands then things like superoxides form and make free radicals [18]. Free radicals are dangerous superoxides that cause damage on a cellular level both systemically and locally and recent publications are showing they may have a direct hand in the aging process [19].

Skeletal muscle fibers are generally categorized into two groups Type I and Type II, with Type I being the slower more oxidative fiber [20]. Within Type II there is a sub-group known as Type IIa, also known as oxidative fast twitch fibers higher oxidative capacity [20]. These fibers generate ATP through the glycolytic cycle but also have a very high mitochondrial count which still allows them to obtain ATP through oxidative
metabolism [21]. While Type IIa may fatigue quicker than Type I they do possess some capability to resist fatigue [21]. The second sub-group is known as Type IIb or glycolytic fast twitch. They have an almost white appearance due to low myoglobin levels and contain a very low amount of mitochondria that gives them an extremely low oxidative capacity. That low oxidative capacity allows Type IIb to fatigue very quickly [20]. This means that the fiber switches its MHC isoform expression depending on the stimulus it undergoes [20]. The vast majority of studies have shown how the radiation damages satellite cells via interrupting the satellite cells mitosis process by causing “breaks” in the cells DNA [4]. We are aiming to determine if the radiation dosing is important in regards to altering Myosin heavy chain expression and in accordance with that myofiber metabolic properties related specifically to oxidative capacity.

The current gap in the literature of irradiation studies shows that skeletal muscle satellite cells and myoblast activity are hindered greatly with radiotherapy doses however, little to no research has been conducted on myofibers and radiation therapy. Previous studies do show evidence of oxidative stress, which would follow along with a potential change in myofiber distribution in a mouse model. The overall purpose of the proposed study is to determine the changes in myosin heavy chain expression and metabolism for morphological changes by differing doses of radiation. This is a study that is necessary to gain a grasp on the concept of the potentially dangerous side effects in the skeletal muscle post treatments and overall what radiation does to the fibers of the muscle itself. Based on previous literature and evidence our central hypothesis we believe that 16Gy will have a greater alteration on MHC phenotype and oxidative metabolism when compared to 4 separate treatments of 4Gy.
**METHODS**

*Animals.* Twenty four C57/BL6 female mice were housed at UNC Chapel Hill and underwent irradiation procedures under Dr. Ted Bateman. All mice were treated in accordance to proper IACUC protocols and procedures.

*Irradiation.* Mice were separated into three separate treatment groups and all irradiation procedures began on the same day. 24 C57BL/6 mice underwent hind-limb irradiation procedures at the University of North Carolina Chapel Hill under the direction of Dr. Bateman. The procedure for irradiation was similar to their previously published paper [41]. The mice were anesthetized with 4 % isoflurane and immobilized using a custom cast made to stabilize the hind-limbs for the procedure. Sedation was maintained throughout the procedure using 2.5 % isoflurane. The irradiation began on day 1 for both treatment groups. The 16Gy received only one treatment while the 4x4Gy mice received their first 4Gy treatment the same day that the 16Gy received their single treatment. Control mice were treated with the exact same sedation and restraint protocol but were not exposed to radiation. The mice were then allowed two weeks from the first treatment for recovery from irradiation procedure and to resume normal dietary eating and activity. At two weeks end, all 24 mice were sacrificed humanely in accordance with UNC Chapel Hills IACUC committee and all tissues were immediately snap frozen in liquid nitrogen and stored at -80°C for further analysis at a later time point.

*Immunohistochemistry Procedures.* Myofiber cross sectional area (CSA) was determined via Image J analysis software (NIH, Bethesda, Maryland, USA). All muscles were serial sectioned on Leica Cryostat at 10 um thickness from the midbelly of the Tibialis Anterior
The slides were then stained using Succinate Dehydrogenase (SDH) solution and IIa/Iib myosin heavy chain isoform expression kit (Vector Labs, Burlingame, CA). Primary IIA and IIB antibody were obtained via (Hybridoma Bank, Iowa City, Iowa). The detection of myosin heavy chain isoforms IIA and IIB will be conducted using the Vectastain kit for each respective fiber type. This is a proven and validated method of immunohistochemistry. The samples are mounted on a positively charged slide and then fixed in cold acetone for 1 minute. The slides are then washed in a solution of PBS-BSA and milk then put into a .6% solution of H2O2 in order to make the membrane more permeable. Slides are then taken and the cuts are circled with a hydrophobic pen in order to keep antibody from mixing. The slides are then blocked with respective blocking solutions depending on fiber type and incubated for 1 hour at room temperature. The slides are then put into primary antibody SC-71 (IIA) and BFF3 (IIB) and left overnight in cold room for incubation. Slides are then removed the next day washed in PBS-BSA and milk then put into secondary antibody, covered and then put into incubator at 37 degrees Celsius for one hour. Slides are removed after 1 hour, washed in plain PBS then have ABC reagent added to them for 30 minutes at room temperature. The slides are then washed in PBS again and given DAB reagent for 6 minutes, which is responsible for color changing of the individual fibers. Slides are then washed in dH2O, dried and mounted with Permount.

**Statistical Analysis.** All data collected will be initially logged into Microsoft excel in order to organize and obtain average values. The data will then be transferred into Prism 6 software for statistical analysis. A one way ANOVA will be ran on both light and dark stain groups and figures generated respectively for each one as well as a side by side
comparison of the dark and lights. Analysis from the ANOVA will determine if there is a difference between control and radiation treatment groups in terms of percentage of dark and light stained fibers. Chi square analysis were run for frequency distributions of means in Sigma Stat. Statistical significance was set at P<0.05.

RESULTS

Experimental Design

Figure 1 shows the experimental design for the study. 24 C57/BL6 mice were randomized into 3 treatment groups Control, 4x4Gy, and 16Gy. The irradiation procedures were conducted by Dr. Ted Batemen at UNC Chapel Hill and were similar to previously conducted study by Bandstra et al. The control group received no irradiation and on day one of the study the 4 separate 4Gy (4x4Gy) received their first dose of 4Gy x irradiation to the hindlimbs and the single un-fractionated 16Gy group received their only radiation treatment to the hindlimbs. Only the 4x4Gy group continued to receive radiation throughout the week at days 3, 5 and 7. At day 14 of the study functional data was taken and the mice were sacrificed with blood and muscle tissue being collected for analyzation.

Body weight and TA muscle mass

At 8 weeks of age the C57/BL6 mice were assigned to one of three treatment groups and monitored for 14 days. This time point was chosen in order to allow some recovery time from the irradiation procedure. Radiation to the hind limb appears to have no overall effect on body weight. In addition to no change in body weight there was also no change reported in hind limb muscle mass or grip strength. In table 1 it appears that at
this particular time point there is not altered muscle mass, body mass, or function when the hind limbs are directly exposed to irradiation.

*Myosin Heavy Chain IIA/IIB representative images*

Figure 2A. shows representative images for both myosin heavy chain IIA and IIB. Control group is on the top left with 4x4Gy in the middle and 16Gy on the right. The first row shows myosin isoform IIA. The arrows are pointing to the dark positively stained fibers for IIA and clearly represent the decrease in fiber size across the treatment groups. The second row shows once again shows arrows pointing to dark positively stained fibers for IIB and the change in size of the fibers is visually distinguishable.

*Myosin Heavy Chain IIA/IIB fiber percent distributions.*

Figures 2 B and C. are myosin heavy chain IIA and IIB percent distribution. No change was found with both 4x4Gy and 16Gy treatments in either the IIA or IIB isoforms.

*Myosin Heavy Chain IIA/IIB Cross Sectional Area*

Figure 3A shows IIA and IIB cross sectional area for all 3 treatment groups. When analyzed with a one-way ANOVA the IIA fibers saw a significant decrease in fiber size only with the 16Gy radiation treatment (p=.04). This finding does agree with some previously published literature that shows higher doses of radiation can in fact cause a loss in myofiber size such as seen by Rosenblatt et al. A one way ANOVA revealed that IIB fibers showed a significant decrease with both 4x4Gy and 16Gy radiation treatments (p=.002). This falls in line with literature that states glycolytic fibers are more susceptible to damage induced by radiation and potential ROS (reactive oxygen species)
[54]. These findings point to some type of remodeling or atrophy occurring in the actual myofibers themselves.

Upon looking at the fiber type distribution of mean CSA for IIA in figure 3C it is apparent that the 4x4Gy shows no significant shift in fiber size distribution. However, the 16Gy shows a significant shift towards a smaller fiber type with significance at <1000 p=.02, > 1500 p=.032. This means that the radiation is inducing a shift toward a overall smaller fiber size which explains the decrease in total CSA as previously depicted in figure 1. Figure 3D shows the IIB fiber type distribution of mean CSA. There was significance for both 4x4Gy and 16Gy at <2000 p=.002 and >3000 p=.03. This again points to a shift toward a smaller IIB fiber, which coincides with the overall CSA data and again reinforces that aspect that IIB glycolytic fibers are more susceptible to radiation induced damage and potential oxidative stress.

_Succinate Dehydrogenase Representative Images_

Figure 4A shows representative images for succinate dehydrogenase activity from left to right are Control, 4x4Gy and 16Gy. The arrows point to dark (high activity) and light (low activity) fibers.

_Succinate Dehydrogenase Fiber percent distributions and CSA_

Figure 4B shows SDH dark stain fiber percentage distribution. There was no significant change across all 3 treatment groups although the amount of SDH dark stain fibers appeared to be trending upward. Figure 4C shows SDH light fiber percentage distribution across all 3 treatment groups. A significant decrease in light stain (low activity) was found with both 4x4Gy p=.04 and 16Gy p=.002. The decrease in light
fibers points to radiation causing a potential change toward a more oxidative fiber. Figure 4D shows total mean CSA for both dark and light stained SDH fibers. There was no significance found with a one-way ANOVA across all treatment groups for both light and dark. The light fibers appeared to be trending toward a loss of CSA but were unable to achieve significance. Figure 4C shows the frequency distributions of the means for SDH dark stained fibers for all treatment groups. Significance was found at <750 p=.03 and trending with > 1750 p=.07 with only the 16Gy group being significant compared to control. Figure 4D was shows the frequency distribution of the means for light stained fibers and once again for all 3 treatment groups. Chi square analysis was done between various two group combinations with significance being found for both 16Gy and 4x4Gy compared to Control at <2000 p=.02, p=.01, >3000 p=.01 respectively.

**DISCUSSION**

This study was not geared toward looking at molecular mechanisms so much as it was geared toward looking at actual changes that the radiation induced in the myofibers themselves. Our data shows that while expression of MHC IIA/IIB was not differentially altered with radiation, size and frequency distribution of the means was indeed affected differentially and supports in part that 16Gy has a more powerful and damaging effect on myofiber properties in mouse skeletal muscle.

Previous studies have shown that Reactive Oxygen Species or (ROS) are what can sometimes induce damage due to them scavenging for the missing electron and activating lipid peroxidation [35]. Radiation has been found shown to cause oxidative stress at a
Figure 3.5 Radiation ROS Model.

low dose such as in a particular Japanese university study where they irradiated mice with
varying doses of 2 and 4Gy and actually saw that within two hours of irradiation Bax
which is a marker protein for ROS and apoptosis was elevated [18, 38]. One of our
biggest findings in this study was the decrease in both IIA as well as IIB cross sectional
area. Our IIA fibers only saw a decrease with the single un-fractionated dose of 16Gy.
The IIB fibers saw significant decreases with both the 4x4Gy and 16Gy radiation
treatments. The possibility that the IIA fibers being more oxidative appears to attenuate
or protect from some of the damage from the fractionated radiation dose where as the
single un-fractionated dose of 16Gy appears to induce more damage with both the IIA as
well as the IIB in terms of overall cross sectional area. 4x4Gy was unable to alter the
size and frequency distribution of the IIA fibers. This could be due to the fact that the
IIA fiber has more mitochondria and can filter and handle excess ROS produced by the radiation treatment better if the dose is fractionated into smaller treatments. It has been shown that oxidative muscle has a much more robust expression of antioxidant enzymes when compared to glycolytic muscle such as in Figure 3.5 [70]. The single larger blast of 16Gy appears to have an “overload” effect and not even the high level of antioxidant enzyme activity in IIA can save it from atrophy. The IIB fibers obviously having a lower expression level of these antioxidants are more susceptible to the induction of potential atrogens leading to atrophy [70]. The ROS is most likely up-regulating the expression of atrogens as well as the ubiquitin proteosome pathway and thus leading to fiber atrophy and shifts within the frequency of the means.

The distribution of means with IIA fibers saw a shift toward a smaller fiber type but only with the 16Gy compared to control was significance achieved. This helps support our mean loss in cross sectional area. Although no necrosis of fibers was seen in the sections like other radiation studies with higher doses fibrosis, the shift toward a smaller fiber type without changes in distribution points to some form of remodeling in response to damage induced by the un-fractionated radiation dose [71]. These superoxides are probably scavenging throughout the body trying to find an electron to stabilize in disrupting oxidative metabolism and protein synthesis leading to atrophy of the IIB and in some cases smaller IIA fibers that are more easily affected by the stress [61, 70]. However, even though there was no change in IIB percentage we did indeed have a loss of light stain SDH fibers. This is only slightly surprising as there can be changes in oxidative capacity without changes in MHC phenotype and vice versa. Our MHC phenotype data is similar to two irradiation studies done by Rosenblatt et al. where
they detected that while there was no change in MHC percentage there was a decrease in CSA as well as an increased incidence of smaller fibers in the distribution of means [46, 48]. SDH staining is semi quantitative, which means it may pick up oxidative stain for some fibers that would normally be pure glycolytic. While we did not pursue a IIX stain it was initially thought that IIX was the same as IIB but recent research and data on contractile velocity and power output has found that it is in fact its own distinct fiber type [57]. It is in fact possible that the we may have had some IIX stain positive for IIB and that may explain the lack of loss of IIB fibers when compared to SDH light fibers[72].

Phenlan and Gonyea in 1997, used a similar gamma radiation procedure that was just like Rosenblatt et al, they noted that the myofibers that were irradiated and overloaded stained positively for embryonic myosin heavy chain which is a special form of the isoform only present in new regenerating muscle fibers [46, 60]. This embryonic myosin heavy chain may also play a role in the remodeling of skeletal muscle via radiation induced damage and should be pursued with further investigation using x irradiation.

Now in our study the loss of CSA in IIB fibers as well as loss of SDH light activity fiber percentage points to radiation oxidative stress and the glycolytic fibers being highly susceptible to the superoxides [70]. The IIB fibers would most likely be the myofibers damaged and thus more prone to remodeling and regeneration [42]. With that being said the IIB fibers appear to be susceptible to radiation as a whole seeing as how the fractionated as well as 4x4Gy un-fractionated dose caused a loss of cross sectional area in IIB glycolytic fibers as well as significant shifts in frequency distribution toward a smaller fiber size. Where as the 4x4Gy did not induce any kind of change in the
oxidative/IIA fibers which as mentioned before is most likely due to high anti-oxidant expression.

The SDH (dark) high activity of our myofibers showed significant shifts toward a much smaller fiber type with 16Gy upon analysis of SDH fiber CSA distributions, which coincides with our IIA oxidative fiber data that also had a significant shift toward a smaller fiber. The SDH (light) stain or low activity also had a significant shift toward a smaller fiber CSA but it was with both 4x4 as well as 16Gy. This once again confirms that the low oxidative activity (glycolytic) fibers are more susceptible to radiation induced damage possible via ROS [73]. Now while the IIA fiber did not have any significant percentage change we did indeed see shifts toward a smaller CSA, which coincides with Rosenblatt et al [46]. In our study we effectively showed that a single un-fractionated dose appears to alter fiber size by forcing the muscle toward a smaller myofiber (size especially for IIB) for both oxidative as well as glycolytic and that 4 fractionated doses of 4Gy appears to have little to no affect on oxidative myofibers. While we have found that radiation decreases myofiber size the exact mechanism and signaling process is unknown and requires further investigation to determine exactly why these particular changes are occurring.
# Tables

**Table 3.1. Body/muscle weight and grip strength.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Body weight (g)</th>
<th>Grip strength (N)</th>
<th>Tibialis anterior (mg)</th>
<th>TA:BW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>21.9 ± 0.3</td>
<td>0.70 ± 0.03</td>
<td>37 ± 1.4</td>
<td>1.7 ± 0.06</td>
</tr>
<tr>
<td>4x4 Gy</td>
<td>8</td>
<td>22.0 ± 0.3</td>
<td>0.67 ± 0.03</td>
<td>37 ± 1.0</td>
<td>1.6 ± 0.04</td>
</tr>
<tr>
<td>16 Gy</td>
<td>8</td>
<td>22.2 ± 0.2</td>
<td>0.67 ± 0.03</td>
<td>35 ± 0.6</td>
<td>1.6 ± 0.04</td>
</tr>
</tbody>
</table>

**Table 3.2. Summary of Findings**

<table>
<thead>
<tr>
<th>Fiber Type</th>
<th>4x4Gy</th>
<th>16Gy</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA</td>
<td>Size (um2)</td>
<td>Frequency (%)</td>
</tr>
<tr>
<td>IA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SDH</td>
<td>High</td>
<td>Low</td>
</tr>
</tbody>
</table>

- Arrows indicate the direction of change between treatments.
Figure 3.1. Experimental Design. The above figure shows the experimental design for radiation procedures as conducted by Dr. Ted Bateman and his staff at UNC Chapel Hill. 24 female C57/BL6 mice were randomized into one of 3 treatment groups. Control being no radiation, 4x4Gy meaning 4 separate treatments of 4Gy and 16Gy being one single dose of radiation. All radiation mice began treatments on day 1 of the study and only the 4x4Gy group continued to receive treatment throughout the first week. At day 14 grip strength was taken and the mice were sacrificed according to UNC Chapel Hills IACUC committee guidelines with blood and tissue being collected.

Figure 3.2: A. Myosin Heavy Chain Isoforms Depicted above are representative images for Myosin Heavy Chain Isoforms IIA and IIB. Top row are IIA fibers bottom row are IIB fibers. Left to right are Control, 4x4Gy, and 16Gy. B Myosin Heavy Chain IIA Percentages is % of fibers stained positive for myosin heavy chain IIA across all 3 treatment groups. C. Myosin Heavy Chain IIB Percentages is % of fibers stained positive for myosin heavy chain IIB across all 3 treatment groups. All values are means expressed as percentages and are plotted with SEM. Analysis conducted with one way ANOVA.

Figure 3.3. Myosin Heavy Chain IIA/IIB CSA and Distributions. A. Cross Sectional Area (CSA) for dark stained myosin heavy chain IIA all values reported are means ± SEM. A. Cross Sectional Area (CSA) in um² dark stained myosin heavy chain IIA and IIB fibers * denotes significance. B. Myosin heavy chain IIA frequency distribution of means for all treatment groups all values reported are means expressed as percentages. Black bars denotes significant range *denotes significance control to 16Gy. C. Myosin heavy chain IIB frequency distribution of means for all treatment groups, all values reported as means expressed as percentages. Black bars denotes significant range, * denotes significance control 16Gy, *** denotes significance control to 4x4Gy and 16Gy.

Figure 3.4. Succinate Dehydrogenase Representative Images. Depicted above are representative images for Succinate Dehydrogenase Stain (SDH). Left to right are Control, 4x4Gy, and 16Gy. Arrows denote the dark and light stained fibers SDH Percent Distributions and CSA B. Shows the fiber percentage of dark and light stained SDH myofibers across all treatment groups. All values are means expressed as percentage, * denotes significance C. SDH CSA for all 3 treatment groups. All values are expressed as means in um² ± SEM. D. SDH dark stain myofiber distribution for all 3 treatment groupsBlack bars denote significant region, *denotes significance of control to 16Gy. E. SDH light stain distribution for all 3 treatment groups. Values are means ± SEM. Statistical significance was set at p < 0.05. Black bars denote significant region, * denotes significance of control to 16Gy, *** denotes significance of control to both 4x4Gy and 16Gy Black bars denote significant region, *denotes significance of control to
16Gy. E. SDH light stain distribution for all 3 treatment groups. Values are means ± SEM. Statistical significance was set at p < 0.05. Black bars denote significant region, * denotes significance of control to 16Gy, denotes significance of control to both 4x4Gy and 16Gy Black bars denote significant region, * denotes significance of control to 16Gy. E. SDH light stain distribution for all 3 treatment groups. Values are means ± SEM. Statistical significance was set at p < 0.05. Black bars denote significant region, * denotes significance of control to 16Gy,  * denotes significance of control to both 4x4Gy and 16Gy.
Figure 3.1. Shows the research design of the study with irradiated and non-irradiated groups.

Figure 3.1. The above figure depicts representative images for MHC IIA/IIB staining with varying radiation treatments.
Figure 3.2 Shows Myosin Heavy Chain IIA/IIB phenotype expression.

Figure 3.3 The above figure shows MHC IIA/IIB cross sectional area (CSA)
Figure 3.3 Shows Myosin Heavy Chain CSA and distribution of means
Figure 3.4 The above figure shows representative images for SDH staining with varying radiation treatments.
Figure 3.4 The above figure shows SDH cross sectional area (CSA)
Figure 3.4 Shows SDH representative images and total morphology
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