Non-thermal ultrasound (US) is commonly used in an attempt to improve tissue repair and regeneration, although the efficacy of this practice is not firmly established. US is normally described in terms of duty cycle and spatial averaged intensity (SAI; W/cm²). The combination of these two, spatial averaged temporal averaged intensity (SATA), provides an indication of total energy delivered per unit of time. There is an untested suggestion that using non-thermal US treatments with an equivalent SATA, but that use different combination of duty cycle and SAI might produce different results. Following skeletal muscle injury there is a relatively defined series of events that take place during repair1-3. Previous research has investigated only a few dependent variables at discreet points in time. To better identify the influence of ultrasound on skeletal muscle regeneration, it is vital to examine multiple protocols as well as multiple markers of regeneration at various time points. Therefore, the purposes of these studies were to examine the influence of non-thermal ultrasound markers on skeletal muscle regeneration and to compare 4 different combinations of duty cycle and SAI representing two common SATA intensities. Design and Setting: Multifactorial designs comparing treatment, duty cycle, SATA, and time were used. A bilateral contusion injury to the gastrocnemius via a
drop mass technique was performed. US administration commenced 24-hr post-injury and was delivered 5 minutes daily on 4 consecutive days. Rats received the US treatment on their left hindlimb, and the contralateral right hindlimb served as a non-US control.

**Subjects:** Male Wistar rats were used in this study and the protocol was ILACUC approved. **Measurements:** Dependent variables included muscle mass, fiber cross-sectional area, centrally localized nuclei, embryonic myosin heavy chain, MGF (muscle specific IGF-1), M-cadherin, and MyoD. Data were analyzed using a factorial MANOVA. **Results:** US increased muscle mass more than no treatment ($P < 0.0001$) and increases in muscle mass were found with the continuous US treatment compared to the pulsed duty cycle treatment at the same SATA ($P = 0.019$). The interaction of treatment and SATA on fiber cross-sectional area ($P = 0.021$) and centrally located nuclei ($P = 0.016$) is statistically significant. In the second experiment, ultrasound treatments also statistically significantly affected MGF levels compared to non-treated hind limb ($P = 0.029$). Ultrasound had no effect on muscle mass ($P = 0.251$). There was an effect observed for the day post-injury for MGF ($P = 0.014$) but not for mass ($P = 0.290$). Post-hoc testing revealed that the MGF value on 1 day post-injury was statistically greater than the values observed for days 3 and 4 after injury. **Conclusions:** The specific non-thermal ultrasound treatments we studied had beneficial effects on skeletal muscle regeneration following blunt trauma. When comparing US treatments providing identical energy delivery, a continuous duty cycle has a greater influence on skeletal muscle regeneration following blunt trauma than does a pulsed duty cycle.
ACKNOWLEDGMENTS

I would like to thank the National Athletic Trainer’s Research and Education Foundation and Proctor and Gamble for funding a portion of this dissertation project.

I would like to thank my advisor, Steve Devor, Sport and Exercise Science; committee members, Mark Merrick, Athletic Training, and Mark Failla, Human Nutrition; for all their help and guidance during this process.

I also wish to thank the following people at The Ohio State University, for all their help with data collection and analysis:

Dr. Jaimy Lekan, Sport and Exercise Science

Dr. Lawrence J. Druhan, Dorothy M Davis, Heart and Lung Institute

Dr. Macdonald Wick and Dr. John Mark Reddish, Animal Science

Finally I wish to thank family and friends for their love and support, especially my husband Matthew, without whom none of this would have been possible.
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2. Livecchi NM, Merrick MA, Ingersoll CD, Stemmans CL. Pre-athletic training students perform better on written tests with teacher-centered instruction. *Journal of Allied Health.* 2004; 33:212-216


FIELD OF STUDY

Major Field: Education
Specialization: Muscle Physiology, Therapeutic Modalities
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CHAPTER 1

INTRODUCTION

Satellite cells (SC) originate during embryonic development from myogenic cells that are closely attached to the myotubes (formed from the myoblasts). Once the basal lamina of a developing cell encloses these clusters of cells they are known as satellite cells. These cells then “satellite” the muscle fiber between the sarcolemma and the basal lamina. When an injury to skeletal muscle has been sustained, a defined series of events occur. Injury stimulates the activation of satellite cells and also the release of MGF (muscle specific insulin-like growth factor), as well as other associated chemical mediators that will assist in the inflammation process. These secreted products attract monocytes and macrophages to the injured area during the inflammatory stage of the degeneration process. Initiation of the regeneration process is associated with the satellite cells transitioning from the G0 phase of the cell cycle to G1. SCs will then begin dividing, and once division is completed some will diffuse across the membrane and enter the muscle fiber. After crossing the membrane, SCs differentiate into myoblasts (muscle precursor cells), recognize and align with other myoblasts and fuse to form the myotube. These cells, in conjunction with the fibers already present, will then begin to
develop from primary myotubes (embryonic myosin) into secondary myotubes, and ultimately be expressed as adult isoforms of the myosin heavy chain protein\textsuperscript{1,2}.

**Ultrasound**

Therapeutic ultrasound (US) is commonly utilized by allied health professionals in rehabilitative settings. The majority of the recent research has examined how the thermal effects of US influence pain reduction\textsuperscript{4-6}, increase perfusion\textsuperscript{7-9} and collagen extensibility\textsuperscript{10-13}, decrease muscle spasm\textsuperscript{5,14} and joint stiffness\textsuperscript{15-18}, and general tissue healing\textsuperscript{18-20}. However, there has been little research focused on the markers of skeletal muscle regeneration in the non-thermal US literature\textsuperscript{20-24}. Most studies have examined whole muscle outcomes post-injury\textsuperscript{22,23,25} (i.e., mass changes, area size, and force generation), while very few have examined mechanistic pathways or even cellular activity\textsuperscript{26,27} associated with the changes. Although available results represent a fundamental starting point, more detailed inquiry is needed.

Dyson’s \textsuperscript{20,28} early work examined gross variables such as mass, rather than more complex variables at the cellular level. Her work revealed that US can increase post-injury tissue mass and mean area, but more importantly she examined the possibility that different protocols produce different results. Both pulsed and continuous duty cycles were demonstrated to significantly affect outcomes following injury. Rantanen \textit{et al.} \textsuperscript{27} examined the effect of US on a few specific markers of muscle regeneration, including satellite cell proliferation. Rats received US following a contusion injury to gastrocnemius muscle. There was a dramatic increase in SC proliferation, but no change in differentiation. Based on these outcomes he suggested that the overall benefit of US is
minimal. This conclusion may be premature. It is possible that the varied results may be due to protocol of the US selected, but more importantly the timing of the modality.

Satellite cells are a crucial component of skeletal muscle development and regeneration. It is clear from the work of Rantanen et al.\textsuperscript{27} that satellite cell proliferation (number) alone is not an adequate indicator of regeneration. Instead, regeneration can only be adequately described when both proliferation and differentiation into myotubes are examined. Although these processes are tightly regulated, there is still need for much research examining the role of the environment, and the influence of US. Questions remain regarding the role of different US protocols on SCs at a mechanistic level.

\textbf{Therefore, the purpose of these studies was to examine the biological effects of therapeutic ultrasound following a blunt trauma on skeletal muscle regeneration including satellite cells, myogenic regulatory factors and muscle specific insulin like growth factor.}

In order to more effectively address this research question separate experiments have been conducted. Each experiment is based on the one previous and serves to work through methodological techniques as well as more definitive set of dependent variables.
**Specific Aims for Experiment 1**

The purpose of the first experiment was to examine the effects of four different ultrasound protocols that vary mode and dose on markers of skeletal muscle regeneration. The following four ultrasound protocols were selected: a.) 0.5w/cm² at 20% duty cycle; b) 1.5w/cm² at 20% duty cycle; c.) 0.1w/cm² continuous duty cycle; and d.) 0.3w/cm² continuous duty cycle. To achieve the purpose, the following hypotheses were tested.

1. Compared with contusion injured non-ultrasound treated GTN muscles, contusion injured ultrasound treated GTN muscles will exhibit greater values for:
   a) Muscle mass;     b) Fiber cross-sectional area;
   c) Satellite cell number

2. Compared with contusion injured 20% duty cycle-ultrasound treated GTN muscles, contusion injured 100% duty cycle-ultrasound treated GTN muscles will exhibit greater values for:
   a) Muscle mass;     b) Fiber cross-sectional area;
   c) Satellite cell number

3. Compared with contusion injured 0.1w/cm² cont duty cycle-ultrasound treated GTN muscles, contusion injured 0.3w/cm² cont duty cycle-ultrasound treated GTN muscles will exhibit greater values for:
   a) Muscle mass;     b) Fiber cross-sectional area;
   c) Satellite cell number
Specific Aims for Experiment 2

The purpose of the second experiment was to examine the effects of different drop mass weights on the level of embryonic myosin heavy chain (EmbMHC). This experiment was performed in order to obtain a more adequate injury from the contusion apparatus. The results we observed from Experiment 1 suggest that 171g was not heavy enough to induce a meaningful injury resulting in a measurable amount of regeneration. Therefore, to better assess the effects of ultrasound post-injury, we examined the following four different masses 171g, 200g, 220g, 240g. The following hypothesis was tested. As the weight of the drop mass increases the level of skeletal muscle damage will increase proportionally. Since EmbMHC is produced during regeneration, the levels of EmbMHC will also increase proportionally as the drop mass weight increases.
Specific Aims for Experiment 3

The previous experiments have examined a small subset of indicators of regeneration at a single point in time. The specific aim of this experiment was to examine the influence of ultrasound (US) treatments at several times following contusion injury on a larger set of biological markers of skeletal muscle regeneration. In addition to examining the activation of satellite cells (SCs), we plan to address the issue of differentiation of the SCs. The following hypotheses were tested:

1. Compared with contusion injured non-ultrasound treated gastrocnemius (GTN) muscles, contusion injured ultrasound treated GTN muscles will exhibit greater SC activation as assessed by:
   a) greater quantity of M-cadherin protein; and
   b) greater expression (quantity) of MGF mRNA

2. Compared with contusion injured non-ultrasound treated GTN muscles, contusion injured ultrasound treated GTN muscles will exhibit greater amount of SC differentiation into myoblasts as determined by a greater quantity of MyoD protein

3. Compared with contusion injured non-ultrasound treated GTN muscles, contusion injured ultrasound treated GTN muscles will exhibit greater myofiber regeneration exhibited by a greater quantity of EmbMHC protein.
CHAPTER 2

LITERATURE REVIEW

Introduction

Review of the inflammatory response, satellite cells, muscle regeneration, myogenic regulation and the influence of ultrasound on skeletal muscle regeneration follows.

Inflammation and Regeneration

Acute inflammation is a component of the body’s response to injury. Although some inflammation is required for proper repair or regeneration of tissues, excess inflammation will delay proper healing. Understanding the process of inflammation will aid the clinician’s ability to better treat an acute injury. Majno and Joris have defined inflammation as follows, “Inflammation is a response to injury of vascularized tissues. Its purpose is to deliver defensive materials (blood cells and fluid) to the site of injury. It is not a state but a process.” This section will focus on the inflammation process, and the difference between primary and secondary injury.
Vascular and Cellular Events of Inflammation

Inflammation is characterized by vascular and cellular events that are required for proper healing to take place. Table 2.1 outlines the vascular and cellular events that occur following injury.²⁹,³⁰

<table>
<thead>
<tr>
<th>Vascular</th>
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<tr>
<td>Vasoconstriction (transient)</td>
<td>Neutrophils-Leukocyte</td>
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<td>Vasodilation</td>
<td>Chemotaxis</td>
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<td>Increased permeability</td>
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<td></td>
<td>Margination</td>
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<td>Diapedesis</td>
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<td>Recognize and Attack</td>
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<td></td>
<td>Macrophages</td>
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Table 2.1. Events that occur during the inflammation process.

Vascular changes function to promote cellular and fluid accumulation at the injured area. Initially, there is transient vasoconstriction which is then followed by vasodilatation. Vasodilatation decreases the rate of blood flow to the injured area thereby, facilitating leukocytes to adhere to and emigrate through the vessel. Finally, there is an increase in vascular permeability that is typically observed as edema or joint effusion.³⁰ This altered permeability also allows for the excretion of transudate and potentially exudate into the interstitial space.²⁹

Cellular activities during this review will focus on the neutrophils and macrophages. Neutrophils, the most abundant members of the leukocyte family are found in the blood and bone marrow. Their lifetime after entering circulation is relatively short i.e. 12-20 hours. They are characterized by a spherical shape and contain little endoplasmic reticulum, few mitochondria and a subdivided nuclei.²⁹ Most neutrophils are not activated during their lifespan unless an injury has occurred; whereby injured cells secret chemotactic signals to recruit them. Neutrophils detect chemotactic signals via
spatial awareness; monitor chemical gradients from one side of their cell surface to the
other. They are able to detect very minute differences in their surroundings. Upon
recognition of the signal, neutrophils initiate their activation process adhering to the
vessel wall (margination). The protein selectin is needed to begin the rolling and sticking
process. They continue to roll along the vessel wall decreasing their rate of speed until
two similar integins attach and adhere to the vascular endothelial wall. Neutrophils then
migrate between adjacent endothelial cells to enter the interstitium (diapedesis). Once
within the interstitial space, cellular space, neutrophils often recognize target areas via the
process of opsonization. There are three types of opsonins: IgG antibody, C3b fragment
of complement, and nonspecific opsonins. Neutrophils can also “attack” in an
opsonization independent manner e.g. killing bacteria in the lungs.

Neutrophils role in inflammation is to find the infection, kill it and then die in the
act; due to the method of killing sometimes healthy tissues fall victim in the process. This
is accomplished typically via two different methods. One is through oxygen (O2)
dependent killing. O2 dependent killing is more common method and is accomplished
through an increase in oxygen consumption so that reactive oxygen species (ROS) can be
released i.e. H2O2 and O2-. The other method is oxygen independent killing, this is
observed when neutrophils are called to areas that have little to no oxygen available. And
in these cases bacterialcides, lysosomes, and proteases are released.

Sometime after injury, though not clearly defined, monocytes will leave the blood
and enter tissue becoming macrophages. They are the second line of defense in acute
inflammation. Macrophages are phagocytes and kill foreign agents. One main difference
is a longer half-life (months) compared to neutrophils (<24hrs) as well as the type of
killing. Macrophages have more selective kills, therefore it is not the widespread killing seen with neutrophils. Macrophages, like neutrophils, respond to chemical mediators and will use lysosomes and bactericides to induce phagocytosis.

**Primary vs. Secondary Injury**

Inflammation occurs in all types of tissue with magnitude determined mainly by the extent of the injury that has occurred. Injury can occur via two distinct mechanisms, primary injury and secondary injury. The goal of this section is to distinguish between the two types of injury and give a clinical perspective.

Ken Knight \(^{31}\) first coined the terms “primary and secondary injury” (Figure 2.1). He explained primary injury as the actual trauma that took place: the sprain, the contusion trauma etc. The important distinction is that clinicians cannot prevent primary injury. Primary injury may affect any tissue. The extent of the injury will determine the degree of inflammation.
Secondary injury occurs in tissues that were otherwise healthy and not involved in the initial trauma. The predominant type of secondary injury was first referred to as secondary hypoxic injury but was more recently renamed secondary ischemic injury. Ischemia refers to a decrease in blood flow to a certain area, whereas hypoxia represents a decrease in oxygen. Thus the two are related, but different. Figure 2.1 shows that there is a decrease in blood flow following injury due to clotting factors. This decrease in blood flow can lead to ischemia in healthy tissue. The problem with ischemia, in addition to the decrease in oxygen, is a decrease in both substrate delivery and waste removal. The decrease in oxygen and substrate availability can impair mitochondrial function. One response to decreased supply of molecular oxygen is a transition to anaerobic metabolism. Anaerobic metabolism produces acidic by-products that decrease the pH.
level in the cell as a result of impaired waste removal. The combination of decreased oxygen tension and increased acidity can cause additional problems including DNA fragmentation and membrane disruption. Alteration in membrane proteins, especially the sodium-potassium pump, alters cellular homeostasis. For example, disruption to the sodium-potassium pump can lead to an influx of sodium and calcium and efflux of potassium. This imbalance causes the cellular swelling and eventual disruption of the membrane causing its contents to spill (including lysosomes) into the extracellular space.

**Regeneration in Muscle**

Injury directly affects proteins in the muscle including their structure, quantities and function. Following injury, a defined series of events will occur to stimulate the degeneration-regeneration process to restore the muscle cell. (Figure 2.2) Injury stimulates the activation of satellite cells (SC) and also the release of muscle-specific insulin-like growth factor (MGF) and associated chemical mediators that will assist in the inflammation process. Activated SCs, in combination with other mediators, attract neutrophils and macrophages to the injured area during the inflammatory stage of the degeneration process. The degeneration process destroys the necrotic material. Once the debris has been removed, protein synthesis begins to regenerate the muscle fiber. The required time completing this process is largely dependent on the extent and type of injury.
Once the regeneration process begins, satellite cells become mitotically active and leave the $G_0$ phase of the cell cycle and enter $G_1$. The muscle then secretes MGF and via an autocrine fashion increases the proliferative potential of the SCs. This stimulation has been observed to peak within 24hrs post injury suggesting the important role of this growth factor. SC will then begin dividing and once completed some diffuse across the membrane and enter the muscle fiber. Other cells will remain located between the sarcolemma and the basal lamina waiting for chemical signals that may induce differentiation to other needed tissues (i.e. blood vessel, connective tissue, etc.) or they will re-enter the quiescent state until another injury occurs. Those that cross the membrane, differentiated into myoblasts (muscle precursor cells), recognize other myoblasts in the area, line up together and finally fuse into a myotube. These cells in conjunction with the fibers already present will then begin to develop from a primary
myotube (expressing embryonic myosin) to a secondary myotube (expressing neonatal myosin) followed by the expression of adult myosin heavy chain.\textsuperscript{1,2}

Research suggests that the proliferative potential of satellite cells may be limited by senescent changes associated with aging and the number of times that the degeneration-regeneration cycle occurs.\textsuperscript{1,35-37} Schultz et al\textsuperscript{35} examined the effects of multiple degeneration-regeneration cycles on proliferate capacity of satellite cells. Multiple injuries were induced via the injection of marcaine to the rat \textit{extensor digitorum longus} (EDL). They observed that the mean colony size of satellite cells was significantly following 2 or more regenerative processes reduced compared to control. They observed, “…a direct relationship between the number of mitotic divisions…and the number of divisions that remain,” and that they are inversely proportional.\textsuperscript{35} Thus, in addition to the senescence associated with aging, multiple injuries over time may account for longer recuperation time following injury in the older populations.\textsuperscript{36}

The type of injury sustained may play a role in the regeneration process.\textsuperscript{1} It has been observed that short-term denervation stimulates satellite cells to begin dividing where as longer-term denervation tended to deplete the overall pool of satellite cells.\textsuperscript{1,36, 38} One study\textsuperscript{38} examined satellite cells behavior on both normal and denervated \textit{flexor digitorum brevis} muscles in rats. Resistance to recruitment into the proliferation cycles following 16 and 32 weeks of denervation was noted. This resistance may contribute to the overall inability to repair muscle fibers completely following a traumatic nerve injury. In contrast, shorter duration of denervation did not affect the proliferate potential of satellite cells; a similar response was recently demonstrated in older animals.\textsuperscript{37} Those
findings suggest that if the nerve injury can be repaired, the muscle has strong potential to regenerate regardless of age.

**Aging and injury**

The senescent changes associated with aging effects the cellular environment that the muscle resides in as oppose the the muscle fibers themselves. Similarly to young animals and people, the success of regeneration depends largely on two important factors: activation of the satellite cells and an adequate environment to complete the process. Muscle grafts have been performed on rats to demonstrate that it is the environment in which the muscle regenerates is more critical than the age of the muscle cell. Grafts were repeatedly taken from older animals and placed into young animals with successful regeneration after surgery. This important finding suggests that the muscle will regenerate if the proper mediators are present to ensure the completion of the process. The limiting factor for muscle regeneration and healing may be the decrease hormones and growth factors that occur in aged organisms.

**Location of Satellite Cells**

Skeletal muscle satellite cells are non-differentiated cells that play an important role in the development and regeneration of muscle tissue. These cells are normally dormant but once activated will proliferate and differentiate into myotubes. Satellite cells (SC) originate during embryo development from myogenic cells that are closely attached to the myotubes (formed from the myoblast). Once enclosed by the basal lamina, these clusters of cells they now become known as satellite cells. These cells then “satellite” the
muscle fiber between the sarcolemma and the basal lamina. One definitive characteristic of satellite cells is that the basal lamina completely surrounds it. In addition to the basal lamina, characteristics of these cells include: high nuclear to cytoplasmic ratio, few organelles, and increased in heterochromatin relative to the myonuclei. The large quantity of heterochromatin explains the typical quiescent state.

**Satellite Cell Number in response to aging**

SC number changes both with age and the physical demands placed upon the muscle fiber. This dynamic population is influenced by many factors. First, the senescent changes associated with the aging process seem to be the most influential factors. The greatest quantity of SC is found during early development and then decreases in two main stages. During the first stage there is a sharp postnatal decline that occurs over the course of about 2 months in mice and 9 years in humans. In mice, approximately 32% of the muscle nuclei are SCs and this percentage will drastically decrease to range of 1-5% at the 2 month of age Similar changes have been noted to occur in humans. The rapid decline is associated with the marked increase in muscle growth that occurs from birth to adolescence. The second decline occurs at a much slower rate during aging. Humans typically decrease about 1% per decade after age 40. Although this has been clearly demonstrated in animal models, there are small discrepancies in humans. Some of the discrepancies in human data may be due to the muscle that was examined. Roth et al. examined the vastus lateralis and found no change in the number of satellite cells between 20-30 yr old relative to 65-75 yr olds. The lack of difference may be associated with the muscle selected for examination as well as the population of the older individuals. Many of the senescent changes associated
with aging occur after the age of 65. The mean age of the older group was 65, although a few subjects were much younger. Kadi and colleagues suggest that using the \textit{tibialis anterior} may be more representative of changes associated with aging. The \textit{tibialis anterior} is one of the prime muscle associated with walking, an everyday activity, whereas the recruitment of the \textit{vastus lateralis} is activity dependent. A significant decrease of 25-37\% was observed for satellite cell number in both males and females. The decrease in satellite cell number associated with aging may be a contributing factor for the slower recovery and regeneration after injury in elderly people.

\textbf{Changes with fiber type}

In addition to age, muscle fiber type has much influence on the number of satellite cells seen at various ages. Many animal studies have demonstrated that SCs account for approximately 1-4\% of the myonuclei in a Type II muscle fiber and almost 3-4x as many in a Type I fiber. The mechanism responsible for this process is not well understood, but is believed to occur during embryo development since myosin heavy chain is set prior to birth (six months in utero). An early study observed that an increase in SC density is associated with proximity to capillaries, myonuclei and motorneuron junctions.\textsuperscript{2,43} Gibson et al\textsuperscript{40} examined the soleus and \textit{extensor digitorum longus} (EDL) in rats and observed both a decline in satellite cell number with aging and with the fiber type. (Table 2.2)
<table>
<thead>
<tr>
<th>Muscle</th>
<th>Age</th>
<th>Percent of SC</th>
<th>Number of SC (x10^5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soleus</td>
<td>1 month</td>
<td>9.6</td>
<td>5.2</td>
</tr>
<tr>
<td></td>
<td>12 months</td>
<td>6.6</td>
<td>7.3</td>
</tr>
<tr>
<td></td>
<td>24 months</td>
<td>4.7</td>
<td>5.4</td>
</tr>
<tr>
<td>EDL</td>
<td>1 month</td>
<td>7.0</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>12 months</td>
<td>2.9</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>24 months</td>
<td>1.9</td>
<td>1.3</td>
</tr>
</tbody>
</table>

Table 2.2. Absolute number of satellite cells ⁴⁰

The differences in numbers may be due to recruitment and load of type I versus type II adult myosin fibers. Since type I fibers are typically postural or anti-gravity muscles, these muscles are in constant use therefore subjected to daily recruitment and force production. This leads to repeated degeneration-regeneration cycles and thus an increased utilization of SCs. Therefore more SCs are needed to accommodate this demand. ³⁵

**Cell Cycle Characteristics**

Satellite cells function to provide additional myonuclei during growth and regeneration after trauma. The number of myonuclei generated is largely dependent on the cell cycle as well as the number of completing divisions.⁴⁴ An important distinction is that satellite cells are heterogeneous.⁴⁵,⁴⁶ The combination of cell cycle time and lack of homogeneity provide regulate muscle development and regeneration. Schultz ⁴⁴ used Sprague-Dawley rats to determine the actual satellite cell cycle time. The following times were observed for each part of the cycle (Table 2.3).
Most SCs are in G0, or a senescent state, until stimulated by an external factor. Only about 80% of the cells complete the cycle in 32 hr. The remaining 20% respond much more slowly with some never leaving the G0 phase.44, 47 This control mechanism ensures that 1) there will always be a future supply of SC and 2) the amount of regeneration or repair that occurs is proportional to the extent of tissue damage. This also suggests that there are many chemical signals and growth factors that are needed for proper muscle regeneration to take place.

**Regulation of Satellite Cells during Regeneration**

Myogenic regulatory factors (MRFs) are critical components for skeletal muscle regeneration. MRFs interact with other proteins to either promote or inhibit the proliferation of satellite cells and their differentiation into myotubes. Each MRF expression is dependent on their function as well as their location in the cell.

**Myo-D and M-Cadherin (M-Cad)**

Myo-D is one of the most prominent regulatory factors for muscle regeneration, as well as the one of the first to be expressed in response to injury.48 Myo-D promotes proliferation and differentiation of satellite cells. It is expressed by both proliferating satellite cells and differentiated cells, but not on quiescent satellite cells. This
regulatory factor only becomes active if injury or a repeated load has been inflicted on
the muscle. Myo-D was also recently suggested to have silencing HADC (hydroxal
adenylatecyclase).49 Once bound to this protein Myo-D has been observed to block cell
proliferation through the inhibition of myogenin. This mechanism illustrates the tight
regulation associated with satellite cell regeneration and how exact placement of proteins
directly influences the timing and quantity of cell replication that takes place.

Another MRF that promotes the regeneration and skeletal muscle is m-cadherin or
m-cad. M-cad is associated with the development of myotubes. M-cad expression is
transient and tends to peak and fall rapidly.34 It appears within a few hours and then
peaks by approximately 48 hours. The main function of m-cad is to align active myoblast
so that fusion into a myotube can take place.50, 51 Absence of this protein has been
speculated to result in improper fusion and therefore, poor regeneration. However,
Hollenagel 52 observed that in m-cadherin knockout mice proper fusion still occurs due to
the increase in n-cadherin, an analogous protein that is unregulated following nerve
trauma. She suggested that there is redundancy in the regenerative process, especially
with the cadherin proteins so that proper regeneration can take place under numerous
types of injury.

**Myostatin**

In addition to promoting satellite cell and myoblast activity, a portion of MRFs
function to inhibit or slow down this process. Myostatin is a MRF that inhibits the
proliferation of satellite cells during regeneration. However, myostatin does not enhance
protein degradation or muscle atrophy. Myostatin inhibits the progression of the G1 and
G2 (RNA and protein synthesis) by up-regulating p21. P21 inhibits cdk which is
required for phosphorlyation of pRb.\textsuperscript{53} It has been speculated that myostatin may have beneficial effects in postnatal growth by regulating the amount of satellite cells that enter the cell cycle. Researchers \textsuperscript{53} examined genetic knockout mice that lacked the gene for myostatin and showed a higher percentage of satellite cells that were activated compared with wild type mice. Myostatin also appears to affect adipocytes. Li \textsuperscript{54} observed that the relationship between lean body mass and adipose tissue was significantly different in myostatin knockout mice (MKO) compared to wild type. The MKO had higher lean body mass and lower levels of leptin and adipose tissue than the wild type mice counterparts. Li and colleagues \textsuperscript{54} suggest that in addition to the role that myostatin has with inhibiting muscle fiber regeneration it may also up-regulate leptin expression which induces the adipocytes to proliferate, thereby increasing the amount of adipose tissue relative to the amount of muscle or lean body mass.

**Growth Factors**

Growth factors have been found to influence satellite cells by increasing proliferation following injury. One growth factor that has been demonstrated to have a positive role in regards to activation of satellite cells is hepatocyte growth factor (HGF) in conjunction with the c-met protein receptor.\textsuperscript{55-57} HGF is a ligand for the c-met receptor, which is expressed on both quiescent and active satellite cells. One of the unique properties of HGF is that is produced both in regenerating tissue and in normal skeletal muscle. It functions to increase migration of the satellite cells into the muscle \textit{in vitro}.\textsuperscript{55,56} Miller et al \textsuperscript{55} observed that exogenous HGF increased satellite cell number, but that high doses of HGF actually inhibited muscle differentiation.
Another growth factor found in skeletal muscle is vascular endothelial growth factor (VEGF). VEGF is found predominantly in the vasculature and is up-regulated following trauma, and especially ischemic injury. The main function is to aid in angiogenesis so that oxygenated blood can be transported to specific areas. Germaini et al. ⁵⁸ observed that this growth factor is up-regulated during ischemic injury and promotes proper regeneration under this condition. VEGF stimulates angiogenesis and proliferation of satellite cells. Germaini et al. noted that the types of growth factors that are up regulated might be dependent on the type of injury as well as the severity of the trauma induced.

**Insulin-like growth factor (IGF)/ Muscle specific insulin-like growth factor (MGF)**

Insulin-like growth factors, IGF-I in particular, are structurally similar to proinsulin and also stimulate cell replication and modulate metabolism. IGF-I has been associated specifically with increasing proliferation and differentiation in skeletal muscle. It recently has been shown to enhance absorption of various nutrients and to aid in inhibiting protein or muscle breakdown. ⁵⁹ In addition to muscle development, IGF-I plays a role in physiological adaptations of muscle to overload and stress as seen in hypertrophy. Such adaptations in response to resistance training may be due to increases in the number and activation of satellite cells. ⁶⁰ Proper functioning of IGF-I is compromised in subjects with muscular dystrophy which adds to the decrease in regeneration and heightened degeneration. ⁶¹

During development and growth IGF-I mRNA levels are transiently higher due to the need for increases in myofibers. ⁵⁹ IGF-I protein levels are also present in higher
levels post-injury and more recently increased level of mRNA has been detected after single and repeated episodes of resistance training.

**Structure**

Insulin-like growth factor I (IGF-I) is a single-chain polypeptide with 70 amino acid and three intra-chain disulphide bridges. IGF-I has structural similarities to the B and A chains of proinsulin. The majority of the folding seen in the tertiary structure is due to its hydrophobic core (Figure 2.4).

![Schematic diagrams showing tertiary structure of proteins in the insulin family.](image)

IGF-I is produced both in the liver and locally in skeletal muscle, and there are many isoforms of IGF-I. IGF-Ia is a systemic isoform produced in the liver whereas mechano-growth factor (MGF) is produced locally within the muscle. The one main structural difference between MGF and other forms of IGF-I is the lack of glycosylation. MGF is therefore susceptible to degradation and has a shorter half-life. Local forms of IGF that are produced in the muscle have been shown to have both paracrine and autocrine function.
In addition to the structural similarities with proinsulin, IGF-I also interacts with a specific receptor causing an intracellular ligand-activated tyrosine-specific protein kinase activity.\textsuperscript{64} The receptor is a membrane glycoprotein containing 2 alpha and 2 beta subunits. Disulfide bonds stabilize this quaternary structure.\textsuperscript{64} The receptor has high affinity binding for IGF-I and relatively low affinity for insulin and IGF-II.

**Splice Variants**

Two distinct isoforms are expressed in animal skeletal muscle. The isoform IGF-IE\textsubscript{a}, similar to IGF-1 is secreted by the liver. This isoform seems to have anabolic effects and may play a role in the aging process. The second isoform is expressed during mechanical changes in the environment. Since its expression is regulated by mechanical activity, it has been renamed from IGF-IE\textsubscript{c} (IGF-IE\textsubscript{b} in rats) to MGF—mechano-growth factor. This isoform is generated by alternative splicing of the primary RNA transcript, of IGF-1 transcript. The IGF-1 gene has two 5’ promoter regions. MGF is derived from splicing beginning at the promoter 1 region (Figure 2.5).\textsuperscript{63} Promoter 1 is stimulated by mechanical stresses, although it may be also influenced by growth hormone.
Differences in IGF-1 and MGF

IGF-1 was first identified by Blundell in 1980. It is synthesized and stored in the liver until needed. Recently, Yang and Goldspink reported splice variants in IGF-1. They showed increased MGF protein following stretch and stimulation but not stimulation alone, suggesting a mechanical stimulus is needed to produce this protein. Hill and Goldspink also observed different responses from these two hormones. Following stretch and stimulation MGF mRNA peaked within 24hrs and then rapidly declined. In contrast, IGF-1 mRNA was still rising up to day 7. Bupivicaine injection attenuated the response of MGF mRNA reached peak levels at day 4 when the levels of IGF-1 were much higher and response more rapidly. The rapid response of the MGF mRNA expression suggest a local production and storage, as well as the autocrine response in contrast to the endocrine response mediated by IGF-1
Alterations in Concentration

Concentrations of specific proteins may vary. One of the most profound changes that influences IGF-1 and MGF is stretch and resistance exercise. Increases are observed when stretch is applied. The bimodal response likely reflects changes in MGF and IGF-1 separately since MGF was not well established at the time of the report. Hameed et al. examined a hypertrophy model and a regression model. Levels of IGF-1 were increased with the hypertrophy model and the levels stayed elevated related to control even after the muscle went through a regression or no activity period. This finding suggested that IGF-1 is not influenced by atrophy or lack of activity. McKoy et al. examined the influences of resistance exercise and aging. The amount of IGF-1 produced was similar for both young and elder men but MGF was absent in the elder group whereas an increase was demonstrated in the young. This suggested that MGF production was attenuated with aging and may explain the delayed healing response in older individuals. Psilander et al. also examined hypertrophy as an environmental effect but due to the type of analysis used to measure IGF they suggested a decrease in IGF with exercise. This interpretation may be due to the fact that they did not analyze splice variants separately; as illustrated when the analysis was performed again with IGF-1Ea was removed the decrease in IGF-1 was not observed. It is clear that IGF-1 and MGF concentrations increased with stretch and resistance exercise.

Effects on Skeletal Muscle

IGF-1 and MGF have beneficial effects on skeletal muscle including promoting satellite cell activation and protein synthesis. Adams and Haddad observed that DNA
content and protein quantity are strongly related. Using an overload model they were able to increase the protein and DNA of IGF-1. They attributed the increase in hypertrophy to protein synthesis. Charvarthy \(^6^7\) examined the influence of IGF-1 infusion following repeated bouts of casting (immobilization). Animals were casted for 10 days and then returned to mobile states for 3 weeks. After the third period, half of the group was infused with IGF-1 and the other half with saline. The IGF group was able to recover 46% of the lost muscle mass due to immobilization. They suggest that over time and even through aging that IGF would elicit positive effects and help to recover decrease mass associated with senescent changes or immobility. The same grouped also examined the role of IGF-1 on cell cycle and satellite cell proliferation\(^6^8\). They observed that IGF-1 influenced the pRb (retinoblastoma protein) by down regulating p27kip1. P27kip1 inhibits phosphorylations of pRB needed for satellite cells to enter the S phase of the cell cycle and replicate (Figure 2.6).\(^6^8\)

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**Figure 2.5. IGF influence on SC activation**

![IGF influence on SC activation diagram](image-url)
Whereas IGF-1 influences p27kip1 Hill and Goldspink examined the spice variant of MGF and observed that in addition to increasing proliferation of satellite cells, MGF silencing the genes that induced differentiation while the proliferation is taking place. This observation suggests that MGF has its own specific receptor independent of IGF-1 receptor. They also note that since MGF has more transient activity that the role of MGF is more with activation of satellite cells and the early phases of skeletal muscle regeneration where as IGF-1 is more with later stages of regeneration including myofibrillar protein synthesis and the increase in the hormone have long lasting effects. Finally, Rabinosky observed an increase in proliferation and differentiation of myoblast, but he also demonstrated that IGF-1 can have positive effects on neural injuries illustrated via nerve transection model. IGF-1 promotes the healing associated with nervous injury and may aid in returning a proper neural muscular junction during regeneration.

**Other Regulatory Factors**

P27Kip1 is a cell cycle regulator of satellite cells. Higher levels of expression are seen in adult SC as oppose to young or adolescent SC. This has been consistent with various conditions that either prevent or may attenuate typical activation of SC following injury or trauma. When p27Kip1 is up regulated there is inhibition of the protein cdk2. Once inhibited, this kinase cannot phosphorlyate pRb (retinoblastoma protein) therefore leaving it in the inactive form, which in turns inhibits the S-phase entry of the cell cycle. The implication then is that the satellite cells are unable to synthesize DNA and therefore replicate.
STAT3 is a membrane protein that has transcriptional activity. STAT3 main
targets include cyclin-D and Bcl-2, which are anti-apoptosis regulatory protein. STAT3
will then increase transcription thereby increasing the proliferation and development.
Wozinak\textsuperscript{56} examined c-met which is a cell membrane protein that is the receptor for
HGF. It was observed that c-met is present in both quiescent and active satellite cells but
not in fully differentiated cells. The function of this cell receptor is to bind HGF and
induce proliferation. C-met is also a prominent marker for satellite cells since it is found
in both the active and inactive states.

Finally, the membrane receptor protein IP3 is critical for the proper formation of
the neuromuscular junction (NMJ).\textsuperscript{71} IP3 allow for the proper aggregation of Ach
receptors along the NMJ so that proper connection between the muscle fiber and the
nerve can take place.

**Ultrasound**

The ultrasound (US) literature regarding skeletal muscle regeneration is quite
limited. Most of the studies examine whole muscle outcomes\textsuperscript{20,23,72} (i.e. mass changes,
area size, force output) and very few examine mechanistic pathways or even cellular
activity.\textsuperscript{26,27} The majority of studies that examine whole muscle functions or changes
post-injury take make large assumption on the role of US. They are a fundamental
starting point but at the same leave even more questions to answers. Dyson’s\textsuperscript{20,23} early
work and her measurements of whole muscle are probably due more so to laboratory
techniques especially since the US machine used in the studies is not comparable to any
used clinically today. Her work does show that US can increase tissue mass and mean
area post-injury, but more importantly she examined the idea of different protocols as
independent variables. Both pulsed and continuous settings were demonstrated to have significant effects following injury. A more recent study\textsuperscript{72} examined a different mode of US delivery—underwater on skeletal muscle contraction induced injury. They found that after 7 days of continuous US isometric force (Po) had increase and this value was used as an indication of function. This particular protocol was shorter in time frame and higher intensity than the previous work by Dyson, however these studies are unable to determine the mechanistic role of US.

At the other end of the spectrum, researchers have examined the \textit{in vitro} effect of US.\textsuperscript{26} Although difficult to extrapolate clinically, it was a closer means to try to answer what is going on at the cellular level. The US settings used in this study were altered to suit an \textit{in vitro} environment; the main problem is that the US induced cellular damage before increasing mitotic activity. Cellular damage is normally not elicited during \textit{in vivo} studies unless the intensity is set really high.\textsuperscript{23} In majority of cases where biopsy or whole muscle tissues are removed the damage noted is from the injury. The fact that damage was induced via the US may be due to the settings chosen especially the intensity may be too high. Rantanen\textsuperscript{27} went one step further and examined the effect on specific marker of muscle regeneration, satellite cell proliferation. Rats received US following a contusion injury to gastrocnemius muscle. He observed a dramatic increase in SC proliferation but was unable to show a change regarding differentiation. It is then suggested that the overall significance of US it very little. This deduction may be premature. It is possible that the varying results may be due to protocol of the US selected but more importantly the timing of the modality. The treatments were given in spurts and often added a day of rest or no treatment. The day of “no treatment” may have
been enough to deter any positive effect that was being created. As with US timing seems
to be of the essence.

Ultrasound is the new hot topic area for research and has been under scrutiny
since the late 1990s. One of the reasons for this controversy was a review paper written
by Robertson and Baker\textsuperscript{73} on the efficacy of ultrasound. In this review they conclude that
ultrasound is an ineffective modality based on the 10 papers they reviewed.

**Research Methodologies and Their Conclusions**

Robertson and Baker performed a thorough MEDLINE (and other search engines)
review of current literature using ultrasound. They came up with 36 papers to subject to
the following criteria:

1. Adequate control group, placebo group
3. Measurable outcomes
4. Detailed methodologies
5. Adequate sample size especially in case where results were not significant.
6. Appropriate statistics.

After re-examining the 36 papers with these criteria they ended with 10 papers that were
included in this particular review. The papers spanned 1986-1999 and the areas of
treatment were from all different areas of the body. Areas of treatment included: molar
extraction (2), epicondylitis of the elbow, peritoneal injury, breast engorgement,
osteoarthritis of the knee, ulcers, shoulder pain, carpal tunnel, and calcification of tendon
in shoulder. Eight of the ten papers observed no significant difference in the treatment of
ultrasound compared to control for the outcomes measured. The last two observed a
significant effect of using ultrasound compared with control. From these results and
based on their criteria Robertson and Baker concluded that ultrasound is an ineffective clinical modality.

**Draper Critique**

Following the publication of this article a critique of the paper was asked of Dave Draper PhD ATC, a researcher with emphasis on modalities including ultrasound. His criticism focused on few distinct areas. He emphasized the size of the treatment area and time of the treatment. He stated that many of these experiments in review had people treating areas anywhere from 10-100x the size of the ultrasound head; when effective treatment area size is 2x. He also addressed distinct properties of ultrasound including frequency stating that the depth of the sound wave would directly impact whether or not a treatment effect would be observed. He disagreed with Robertson and Baker’s conclusions based on poor methodologies of the papers reviewed.

**Another Look**

There are some short-comings to this review paper. First, the type of injury of the subjects was so vast that any generalization from the effects of ultrasound would be difficult at best to draw. Ultrasound (US) is one modality that can provide multiple effects from temperature increase to micro massage, etc. Selecting projects with vast area and very different measurable outcomes increases the difficulty. The main concern of Robertson and Baker was adequate control and blinding. In modality research blinding is very difficult to do since the majority of the treatments have some sensation associated with it. Even US in many cases will elicit a warming sensation when administered
properly. Today, many researchers avoid this bias by using an animal model. The papers used in this review used blinding procedures and a placebo group (US is used but the machine is not giving off energy.)

Another criterion used was measurable outcomes. A thorough description of the measurable outcomes for each paper is not given but after personal review many of these papers lack commonality, similarly to site of injury treated. A common variable measured was pain via an analogue scale. Whereas in many research practices this is an appropriate way to measure pain, however pain itself is highly variable. Similarly, “tissue healing” was sometimes used. This alone is a vague variable and unlikely to be an objective measurement. One study used ulcer size to indicate tissue healing, trying to quantify and provide an actual pre to post measurement.

The fourth criterion was related to methodologies. To meet this criterion each study had to have listed the parameters used for the ultrasound how it was administered in such detail that the study could be replicated. Only one study listed the US ERA (effective radiating area), none listed BNR (beam nonuniformity ratio), few listed the actual size of the application in most cases it was estimated by Robertson and Baker as well as the majority of the treatment areas were estimated. Time was provided in most of the cases or a range was given, as well as the frequency used and intensity was noted. In my opinion if you have criteria that states methodologies need to be specific so that the study can be replicated then estimating important factors like treatment area is a gross error. Estimating applicator size and treatment size are two important factors that determine the time of the treatment, which directly relates to efficacy of the treatment. It was stated in their discussion that not all the criteria were held to the same standard.
In the eight papers that failed to demonstrate an effect, many of them had to do with the parameters selected. In the molar extractions papers an area 3X an estimated sound head was treated for 5 minutes. Similarly, peritoneal injury, breast engorgement, osteoarthritis of the knee, ulcers, shoulder pain all treated areas at least 3x great and some up to 100x greater than the sound head. Draper states that it would be impossible to generate any appreciable heating effect to these tissues due to great size difference and time not being adjusted. The average time for a treatment was 5 minutes. Draper’s statement is correct and based on his classic US paper in order to reach appreciable temperatures an area 2x the sound head (or better the ERA) should be given for 10 minutes. ERA is the effective rating area and will always be smaller than the actual sound head due to the size of the crystal generating the sound wave. The only problem with using Draper’s method is that we now assume that a heating effect is required for proper healing and that may not be the case as suggested from a classic non-thermal ultrasound paper by Dyson in 1968. No matter if it is thermal or non-thermal the area plays a critical role in the ability to generate any therapeutic effect.

One study used epicondylitis of the elbow and failed to demonstrate an effect. This is probably due to the frequency chosen in the parameter. A frequency of 1MHZ was chosen which is optimal at depths between 2.5-5cm. The epicondyle is so superficial that the sound wave most likely passed right through the tissue. A frequency of 3MHZ would have been the proper choice. It may be that when this paper was done the only frequency the US unit could produce was 1MHZ, which was common at that time. However, a conclusion drawn of ineffectiveness would be inappropriate since incorrect parameters were used.
The last two studies did show a therapeutic effect. Both studies came out of the same lab within a year of each other: carpal tunnel, and calcification of a shoulder tendon. In both cases the main difference was the duration of the treatment. The treatment area sizes were 3X that of the sound head but each treatment was administered for 15 minutes. There is a strong possibility that the reason for the effect was due to the time as opposed to the area size. This may be cause for future research examining time as the key component instead of intensity.

**Conclusions**

The Robertson and Baker paper had good merit in that they tried to use specific criteria to select papers to review. However, the selection criteria were lost when it did not remove papers that had improper treatment protocol. The criteria focus on research design but failed to incorporate proper design using therapeutic ultrasound. The conclusions they have drawn, ultrasound is an ineffective modality, are inconclusive due to improper parameters and lack of agreement between subject populations and dependent variables. New data is also coming to light about ultrasound units and that differences are present between units and within units themselves. An updated review needs to be done that groups ultrasound studies by usage i.e. temperature studies, tendon elasticity, skeletal muscle regeneration. It may be possible that US is effective more or less effective in different injuries.

**Ultrasound Influence**

Ultrasound works on the basis that electrical energy passes to a transducer that then vibrates a crystal to generate sound waves. A transducer is, “a device that converts energy from one form to another...electrical energy to ultrasound.” The sound wave
then requires a medium for which to travel through, water, gel, second skin, etc to be used therapeutically. As the sound waves travel through medium the amplitude and (intensity) SAI will attenuate. As it reaches difference densities associated with the medium the wave will reflect, refract and some will be absorbed. The intensity applied initially will decrease exponentially with the distanced traveled. This is where the depth of the target tissue and US frequency become important variables. Attenuation is often described in terms of half-value thickness, or the dept that will decrease the original intensity to half. Typically an application using a frequency of 3MHz has a half-value at approximately 0.8 cm, where as 1MHz is approximately 2.3 cm. Therefore choosing the appropriate frequency will often times determine whether or not your treatment will be effective.

**Cavitation and Acoustical Streaming**

The majority of the literature regarding non-thermal ultrasound efficacy was attributed to two main rationales, cavitation and acoustical streaming, although recent studies lack a possible explanation for the beneficial or absence effect of ultrasound and another speculates what truly may be happening at the cellular level.

Cavitation is defined by, “any observable activity involving a bubble or population of bubbles stimulated into motion by an acoustical field.” Basically gas bubbles within a fluid medium will begin to oscillate, expand and contract, in a regular fashion while the sound wave is being generated. As long as the oscillation remains regular in nature then stable cavitation will develop over the course of the repeated cycles. If improper US settings are chosen, i.e. intensity is too high, the gas bubbles can
rapidly expand and collapse and in some cases explode, this is termed unstable cavitation. Stable cavitation results in “…secondary motions, high shear stress in or near the tissue, microstreaming and implosion…” Acoustical streaming then is the unidirectional movement of fluid in an ultrasound field. Acoustical streaming will set up boundaries of cells that will form velocity gradients and generate different stresses on cells and their membranes. As the cells and ions are exposed to these stresses they will be displaced and as a result changes in membrane permeability, changes in ion flux etc may occur. The main theory is that the stresses alter the cell’s membrane permeability and therefore alter the calcium channels. This lead to an increase of calcium into the cell which may act as secondary messengers and aid in protein synthesis. At the time of these speculations laboratory technique were not as advanced as today however, to date there is little scientific research that continuous to examine this rationale. Majority of the literature has used either a cell culture model or created an acoustical model used to mimic the one generated by an ultrasound unit but none to date have demonstrated these effects in vivo modeled after clinical practice.

**Frequency Resonance Hypothesis**

A review in 2002 by Lennart Johns hypothesized another possibility for the cellular effects induced by ultrasound. He speculated that instead of cavitation from gas bubbles and acoustical streaming that was originally hypothesized, that it was the individual proteins absorbing the mechanical energy from the sound wave and that was altering their structure or function. This phenomenon is known as the frequency resonance hypothesis and it is threefold in nature. This suggests that
“…energy provided to the enzyme by the ultrasound wave may induce transient conformational shifts in certain enzymatic proteins, altering the enzyme’s activity and overall function...or it may result in dissociation of functional multimolecular complexes...or it may release a sequestered molecule by dislodging an inhibitor molecule from the complex...”

The first part of this hypothesis suggests that ultrasound can cause a transient conformational change in a protein that would allow the active site to become exposed and possibly through the addition of a phosphate become an active enzyme. Another possibility is that the shearing forces generated may dislodge a molecule from its complex and turn the protein inactive. Finally the third portion of this hypothesis suggests that the shearing forces could also dislodge an inhibitor protein from the active site thereby allowing access to this site and the protein to become functional. This hypothesis is different from the previously proposed in two ways. First, it suggests that the sound waves are directly being absorbed by the individual proteins and not influenced by expanding and contracting gas bubbles or movement of cells in a fluid. Secondly, this hypothesis broadens the idea of the influence of ultrasound on cell and tissue structures. We tend to focus on the therapeutic effects of ultrasound and only consider ultrasound hindering regeneration if the protocol selected is inappropriate. This hypothesis suggests then that not only can ultrasound help cells (proteins) to become active (illustrated by Rantanen’s work) it can also cause proteins to become less active. The main shortcoming of this paper, however, is that it is pure speculation from a molecular biology point of view and there is no data as of today that begins to address this hypothesis.
**Ultrasound devices**

Another point that should be mentioned is that not every ultrasound device is the same nor is all the applicator heads within the same device. Recent research\textsuperscript{75-77} has suggested that there is between variability among ultrasound manufacturers as well as within variability for each device. These findings help to explain why there are so many discrepancies in the ultrasound literature. We are now finding out that just because we have purchased a machine by the same manufacturer and used the same protocol as a previous study we may not get the same results. This has previously been accounted for operator differences when in fact it may actually be the machine itself as oppose to the person using it.

**Research Injury Models**

Many times in order to better understand a pathologic state or influences on injury an animal model is used. There are numerous models of injury that can be chosen to answer a specific research question. The following sections will illustrate three common injury methods used as well as their generalizability to clinical practice.

**Contusion Model**

Blunt trauma is very common in athletic competition and many of the current modalities are used to treat this type of injury. Contusions were first introduced as a crush injury, an *in situ* technique using forceps. This generated skeletal muscle trauma and induced inflammation but the problem was that it was difficult to generalize to an injury that would be sustained in sport and you have use careful technique so as to make the extent of injury reproducible. Crisco and colleagues\textsuperscript{89,90} devised a reproducible drop
mass technique that would cause focal injury and since a constant mass was used the
ingury would be reproducible. This technique is also performed *in vivo* so it leaves less
chance for confounding results due to inducing the injury. This technique has been used
for many years now to study acute inflammation as well as the influence of therapeutic
modalities. A known mass slides through a plastic tube and hits an impactor that directly
hits one focal area on the animal. Most commonly it has been used to induce trauma to
the gastrocnemius muscle, but has also be used with hamstring muscle group. See Table
2.4 for the characteristics of all the models being discussed.

**Contraction Induced Damage (CID)**

CID is an *in situ* method whereby an electrode is place on a specific muscle and it
is forced to contract against a lengthen state. CID is used as a model for injuries
associated with eccentric loading or strains to the muscle. CID is dependent mainly on
the velocity of the contraction,\(^9\), but can be reproduced using the same parameters. In
humans the model most commonly used to capture this effect was to induce delayed
onset muscle soreness via eccentric contraction. The limitation with this particular model
was that the effect of DOMS is not the same as acute inflammation and very few
modalities have a positive effect on it. Using CID acute inflammation can be studied as
well as functional outcomes like force output.

**Bupivicaine Injection**

Another commonly used method to induce injury is via bupivicaine injection.
Bupivicaine or marcaine is injected into 3 distinct areas of the muscles. This will
produce injury along the entire fiber as oppose to focal point seen with contusion models.
This model has been used alone to study force output and regeneration of skeletal muscle\textsuperscript{92} and as a means to attenuate the effects of CID.\textsuperscript{93}

<table>
<thead>
<tr>
<th>Damage</th>
<th>Contusion</th>
<th>CID</th>
<th>Bupivicaine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injury</td>
<td>Focal</td>
<td>Diffuse and random through out a fiber</td>
<td>Whole muscle damage</td>
</tr>
<tr>
<td>Severity</td>
<td>Mimic blunt trauma, \textit{in vivo}</td>
<td>Mimic eccentric loading, strain, \textit{in situ}</td>
<td>\textit{In vivo}, neurotoxin</td>
</tr>
<tr>
<td>Reproducible</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Histology</td>
<td>Easy to quantify</td>
<td>Difficult to quantify</td>
<td>Easy to quantify</td>
</tr>
<tr>
<td>Regeneration Potential</td>
<td>Good</td>
<td>Good</td>
<td>Full within 60 days force output is 90% of control</td>
</tr>
</tbody>
</table>

Table 2.4. Characteristics of specific models of injury

\textbf{Generalizability}

When designing an experiment, the model chosen should be based on the specific experimental question asked. For example, if the research question is to explore whole muscle damage then bupivicaine injection would be a proper choice. The main difficulty with this model comes when generalizing to clinical applications of modalities. The likelihood of a clinician treating patients experiencing an injury that is similar to a bupivicaine injection is minimal at best. Therefore the injection model although excellent at producing a uniform maximal injury, may not be the most appropriate choice for examining questions related to clinical practice. CID or the contusion injury model may be a better choice but having proper technique and practice is required to sustain the appropriate amount of damage in order to see a treatment effect. Velocity and mass seem to be the biggest player for contraction induce injury model. The disadvantage of CID is
the random diffuse injured tissue. It involves the whole fiber, typically seen with a clinical evaluation of a grade 2 (partial tear of the muscle) or 3 strain (complete tear), but since it is so diffuse it is difficult to quantify the amount of damage from a biochemistry or histology perspective. Functional outcomes, i.e. force output are easily measurable; however, it is very difficult to ascertain mechanistic questions relative to the treatment. The contusion model is influenced directly by mass of the animal and the weight dropped. If the ratio of drop mass to animal size favors the animal (the animal is too large) than a superficial injury will be generated and limit the amount of results or effect seen. If the ratio of drop mass to animal size favors the drop mass (mass is too heavy) then damage may occur to structures other than the muscle in question i.e. bone. The placement of the animal’s hind limb is critical to sustain an appropriate injury that is consistent on all the animals.

Conclusions

All models of injury have some limitations. The selection of a model should be based on the specific research question, as well as the outcome variables of interest. The difficulty in selecting a model is creating a balance between internal validity and generalizability. If a basic science/physiology question about skeletal muscle regeneration is being asked, then bupivicain injection may provide the best model of injury. If clinical application or treatment protocols are in question a model like contusion or even in some cases the CID would generalize more easily to a specific population.
CHAPTER 3

METHODS AND MATERIALS

Experiment 1: Ultrasound delivery method and energy transfer study

A 2x2x2 (treatment x SATA x duty cycle) factorial design was chosen to examine the effects of therapeutic ultrasound on skeletal muscle regeneration process following a contusion injury in a rat model. Seventy-eight Forty (40) 8-month old male Wistar rats were studied. The rats were procured from Harlan-Sprague Dawley, Incorporated (Indianapolis, Indiana), and the animals are descended from animals of the Wistar Institute (Philadelphia, Pennsylvania). The Ohio State University Laboratory Animal Care and Use Committee approved all experimental procedures. Animals were housed two per cage, were fed commercial rat chow food and tap water ad libitum, and were on a 12h:12h light:dark cycle.

The independent variables of this experiment were treatment (US, injury control), spatial averaged temporal average intensity [SATA] (0.1 or 0.3 W/cm²) and duty
cycle (20% or 100%). Note that equivalent amounts of US energy are produced by protocols $A$ and $C$, and also by $B$ and $D$. Duty cycle and the intensity are varied.

<table>
<thead>
<tr>
<th>Duty Cycle</th>
<th>SATA</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A$</td>
<td>$B$</td>
</tr>
<tr>
<td>0.5w/cm²</td>
<td>1.5w/cm²</td>
</tr>
<tr>
<td>20%</td>
<td>20%</td>
</tr>
<tr>
<td>$C$</td>
<td>$D$</td>
</tr>
<tr>
<td>0.1w/cm²</td>
<td>0.3w/cm²</td>
</tr>
<tr>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>

The dependent variables for this study included the following as measured in GTN of both hind limbs:

1.) muscle mass;
2.) fiber cross-sectional area; and,
3.) satellite cell proliferation, indicated by number

The 40 animals received a bilateral contusion injury to the right and left GTN muscles. Animals then served as their own controls with the left GTN receiving therapeutic US treatment and the right GTN receiving no therapeutic US treatment. To provide a uniform muscle contusion injury, a reproducible drop mass technique was used (Appendix A). Following administration of inhalant anesthetic, animals were weighed and hind limbs were shaved and cleaned with alcohol. The mid-belly region of the GTN was determined through palpation, and marked with permanent marker. Each animal was positioned in the injury device prone with the hind limb fully extended and positioned to ensure an impact directly over the mid-belly region of the GTN muscle (Appendix B). The muscle contusion injury was produced by a mass falling through a clear Lucite guide tube. The mass (171 grams) was dropped from 110cm height onto the top of an impactor
(6.4 millimeter radius). Following the bilateral injury, a five day time-release tablet containing bromodeoxyuridine (BrDU) (Sigma; St. Louis, MO.) was inserted subcutaneously into the scruff of neck of the animal. After recovering from anesthesia, animals were returned to their cages and allowed to move about freely. Animals were observed for normal activity.

Animals then were randomly assigned to one of four different US treatment groups. For each treatment group, unilateral (left hind limb) US administration began 24-hrs post contusion injury, and was administered in a parallel fashion for 5 minutes daily on four consecutive days. The size of the chosen US treatment area is approximately 2.0 cm², which is based upon the size of a Wistar rat GTN muscle. Our US applicator is 1 cm², which yields an effective radiating area (ERA) of 1 centimeter squared ± 20% according to the Mettler Electronics Corporation Sonicator 730 instruction manual. The appropriate size of the area to be treated using US is the conventional standard of twice the ERA of the crystal in the applicator. A commercially available ultrasound gel was used as a coupling medium. All treatments were performed in the morning between the 0800 and 1000 hours (Appendix C).

On the fifth day, post-injury, animals were anesthetized by an intraperitoneal injection of sodium pentobarbital. The right and left GTN muscles were excised, trimmed of excess fat, connective tissue and weighed (Mettler-Toledo). The tissue was then mounted on cork board with Tissue Tek, and flash frozen in isopentane cooled in liquid nitrogen for
subsequent analyses. Animals were euthanized via cervical dislocation following dissections. Tissue was stored at -80°C until further analysis.

Ten (10) micron serial cross-sections were prepared with a cryostat and sections were mounted on a slide. One set of slides were incubated with Anti-BrdU kit containing polyclonal anti-rat laminin antiserum and monoclonal anti-BrdU antibody (Sigma; St. Louis, MO.). The reaction with the monoclonal anti-BrdU antibody labels the proliferating cells in the S-phase of mitosis. BrdU labels all cells, in S-phase. The other set of slides were stained with hematoxalin and eosine to determine fiber cross-sectional area. Slides were examined microscopically and digital photographs were prepared and analyzed using Image J software (National Institute of Health).

Centrally located nuclei (CLN) were also counted to address the nature of satellite cell proliferation. CLN provide a gross marker for cell regeneration. The digital photographs prepared from the H&E slides were used to determine a count of centrally located nuclei. (Figure 3.1) One hundred (100) cells per hind limb were counted for both determining CLN and cross-sectional area (CSA) – equate to 1000 cells per treatment group (n = 10 per group).
Figure 3.1 Centrally located nuclei. The cell on the left has nuclei that have moved toward the center of the cell in contrast to the cell on the right where nuclei are located around the periphery.

**Statistical Analysis:** A repeated measure 2x2x2 factor MANOVA was conducted (SPSS version 12); within factor was treatment and between factors were duty cycle and SATA. Statistical significance was established *a priori* at $p < 0.05$.

**Experiment 2: Increasing drop mass weight study**

The second experiment utilized multiple drop masses and was conducted to determine a better mass from which to elicit a more profound and more easily quantified injury to the gastrocnemius muscle (GTN). Ten 4-month old male Wistar rats were studied. The Ohio State University Institutional Laboratory Animal Care and Use Committee (ILACUC) approved all experimental procedures outlined in this section. Animals were housed two per cage, received food and water *ad libitum*, and were on a 12h:12h light:dark cycle.
The **independent variable** was mass with; 170g, 200g, 220g, 240g masses used in this study. Two animals were used for each mass group and both hind limbs were injured (n=4).

The **dependent variable** for this study was quantity of **embryonic myosin heavy chain** protein and was measured in GTN of both hind limbs.

The eight rats received a bilateral contusion injury to the right and left GTN muscles as previously described in experiment 1. Two of the animals served as true control, only being administered anesthetic without injury. On the fifth day, under sodium pentobarbital anesthesia the right and left GTN muscles of the animals were excised, trimmed of excess fat and connective tissue and weighed on an analytic balance (Mettler-Toledo) as in experiment 1. The tissue was then flash frozen in liquid nitrogen for subsequent analyses. Animals were euthanized via cervical dislocation. Tissue was stored at -80°C for until further analysis.

Tissue was pulverized under liquid nitrogen into a powder and homogenized in a Newcastle buffer solution (50mls: 7.5mls of 1M Tris, 9.5mls of 20% SDS, 25mls 8M Urea, 8mls of dH₂O). Once the sample was a powder, 500ul of buffer solution was added. Samples were then placed on ice until centrifugation at 5000 x g for 10min at 4°C. If little to no supernatant was evident, samples were centrifuged for an additional 10min. Supernatant was collected and the pellet was discarded. Supernatant samples were stored at -80°C until the following morning. Protein concentration was determined using a BCA protein analysis kit (Pierce; Rockford IL) as directed by the manufacturer using a microplate reader.
Samples were also used in conjunction with the Newcastle buffer to determine a more accurate measurement of buffer to sample ratio. Tissue (0.5g ± 0.02g) was transferred to three separate weigh dishes and pulverized under liquid nitrogen to a powder. Test volumes of Newcastle buffer added to each sample was 500ul, 750ul, 1000ul respectively. Samples were placed on ice until centrifuging at 10,000g for 10min at 4°C. A second set of tissue was pulverized to a powder under liquid nitrogen. A 1:5 and 1:10 ratio of sample to buffer was performed; then boiled for 5min and spun at 21,000g for 10min. Due to the nature of the samples/supernatant and the inability to of the supernatant to remain in a liquid form, the BCA protein analysis kit was not performed at this time.

**Experiment 3: Low energy SATA on MGF, M-cad, MyoD**

This study utilized a 2 X 4 factorial multivariate analysis of variance (MANOVA) design. This design also employed a skeletal muscle contusion injury model in the rat as previously described. Thirty-six (36) 4-month male Wistar rats were studied. Animals were housed two per cage, and treated the same as in experiment 1 and 2.

The independent variables of this study were ultrasound and day. Ultrasound has two levels: treatment (0.3 w/cm² continuous duty cycle for 5minutes at a frequency of 3mHz.) or no treatment and day of tissue collection from animal post-injury (day 1, 2, 3, or 4).

The dependent variables included

1) Embryonic myosin;
2) Muscle specific insulin-like growth factor (MGF);
3) M-Cadherin; and,
4) Myo-D
Four (4) of the 36 animals were not subjected to the contusion injury, to differentiate between the response of the dependent variables that occur from natural maturation and those from the contusion injury and modality intervention. The remaining (32) animals were randomly assigned to treatment groups based on weight to ensure homogeneity of the groups. On Day 0 animals received a bilateral contusion injury as described in experiment 1. One difference noted is that the drop mass was increased to 240g. This was based on the change in behavior of the animals (showing signs of injury) during experiment 2.

Therapeutic ultrasound treatment was initiated 24 hours post contusion injury on the left (L) GTN only. A Mettler Electronics Corporation (Anaheim, California) Sonicator 730 with a 1 centimeter squared applicator was used for all ultrasound treatments. The right (R) GTN of each animal served as a non-ultrasound treated control muscle. Ultrasound treatment (continuous, frequency of 3.3 MHz, intensity 0.3 W/cm²) was administered once daily for 5 minutes per day and for four consecutive days post contusion injury, except for the animals that will be sacrificed at earlier time points for specific tissue analysis. All ultrasound treatment occurred between the 0800 and 1000 hours. Then in afternoon (1300-1500 hours), the right and left GTN muscles of eight of the animals were excised under sodium pentobarbital anesthetic (administered via intraperitoneal injection) and the animals were euthanized via cervical dislocation. The muscles was trimmed of excess fat and connective tissue, weighed on an analytic balance (Mettler-Toledo) to establish muscle mass. A portion (approximately 30mg) from middle GTN was placed in
an RNAse-free microcentrifuge tube containing 630ul of RNAlater (Ambion Inc.). The sample was stored at 4°C overnight and placed in a -80°C freezer. The remaining middle 1/3 of the GTN was flash frozen in liquid nitrogen and stored at -80ºC until analysis.

Muscle samples in the RNAse-free microcentrifuge tubes were taken to the Micro-array Genetics Core Laboratory of the Davis Heart and Lung Research Institute at OSU College of Medicine for RNA extraction and real time RT-PCR analysis.

**RNA purification**

The muscle tissue was removed from the RNAlater and transferred to an Eppendorf tube containing 1 mL Trizol reagent (Invitrogen). A stainless steel pellet was added and the tissue was homogenized in a TissueLyser (Qiagen) for 6 minutes with a frequency of 30 per sec. Chloroform (200uL) was added to the sample, shake vigorously, and centrifuge at 12000g for 15 min at 4°C. The aqueous (upper) phase was transferred to a new tube and an equal volume of 70% Ethanol was added. The resultant mixture was used for total RNA purification using the RNeasy Mini Kit (Qiagen). Total RNA samples were quantified by absorption at 260 nm. The ratio of 260:280 was in the range of 1.8-2.0. RNA free plastics and reagents were used throughout.

**Reverse Transcription**

0.5 ug total RNA from each sample was used for reverse transcription in a reaction volume of 25 uL using the TaqMan Reverse Transcription kit (Applied Biosystems). The reverse transcription was primed using random hexamers.

**Real Time PCR**
For real time PCR, 2 μL of a 1:4 dilution of each cDNA was added to 23 μL of reaction mix. The reaction mix contained 12.5 μL 2X Taqman Universal PCR Master Mix (Applied Biosystems), 1.25 μL 20X Pre-Developed TaqMan primer/probe mix (Applied Biosystems), and 9.25 μL H2O. Two Pre-Developed TaqMan primer/probes were used: Eukaryotic 18S rRNA as the endogenous control, and rat specific MGF primer/probe set (Applied Biosystems cat. No. Rn01503688_m1) as the target. The reactions were carried out in an ABI7700 Real Time Thermocycler, and analyzed using SDS version 1.9.1 (Applied Biosystems). Reactions for quantifying 18S and MGF were performed in duplicate and triplicate, respectively.

Protein extraction for SDS Page

The rest of the samples were homogenized in a Urea-Thiourea buffer at a ratio of 1mg of sample to 30μl of buffer. Tissue was pulverized into powder under liquid nitrogen and 45mg of sample (+ 0.002mg) was mixed with 1350μl of buffer. Samples were placed in a 65°C water bath for 2 min and then placed on ice for 5 min. Samples were then centrifuged at 10,000 x g for 10min and placed in a -20 freezer for subsequent electrophoretic analysis. All non-injured control samples were combined into one tube to eliminate the variability between animals.

Beta galactosidase (lot number 59J3153N, 8.89 mg/ml; Worthington Biochemical Corp., New Jersey) was used to control for loading error. For each sample 5μl of beta
galactosidase was added to 84ul of soluble sample this allowed for a known concentration of 0.5ug/ul for all samples.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was used to separate the proteins of differing molecular weight with a 10% resolving gel and a 3% stacking gel. (See Appendix D for actual quantities used.) Mini-Protean gel II unit (Bio-Rad Systems) was used to run gels. A 15-well comb was used to analyze samples from 6 animals per gel and 6 total gels per dependent variable. After completion proteins were transferred to nitrocellulose membrane and subsequently probed for embryonic myosin chain, m-cadherin, and myo-d with appropriate antibodies. Manufacturer recommendations for antibody dilutions were used as a starting level.

Multiple bands were detected on pictures therefore; dilutions of primary antibodies were performed. The antibody for embryonic myosin was diluted at 1:1000, 1:10K, 1:100K. Beta galactosidase antibody was dilutions at 1:3000 (manufacture recommendation), 1:10K, 1:100K, and 1:1M. M-cadherin antibody was diluted at 1:500. The chemiluminescence kit (ECL) was also tested for cross reactivity. Three (3) gels were transferred to nitrocellulose membranes and all were blocked for 30min at 37ºC in BLOTTO. All were washed in PBST as previously described. Membrane 1 was then immediately tested with the ECL kit, membrane 2 was first incubated with primary antibody, washed in PBST then tested with the ECL kit; and membrane 3 was first incubated in secondary antibody, washed, and tested with the ECL kit. Images were captured and analyzed digitally. Samples were re-run using a new source of positive
control, beta galactosidase (Sigma #G-8511, concentration of 0.5mg; gift from Dr. Wick). Beta gal was reconstituted in 200μl of dH₂O and 3μl was added to each diluted (1:10) sample. Loading volume for gels was 10μl of pre-stained standard and 30μl of sample.

**Statistical Analysis**

Means ± standard error of the mean were calculated and reported. Data was analyzed repeated measure multivariate analysis of variance (MANOVA). Differences detected by the MANOVA, were compared by Tukey test. Student’s t-test was used to determine if there were differences between initial and final weights of the animal. Statistical significance was established *a priori* at \( p < 0.05 \).

**Power Analysis and Power Calculation:** To make an *a priori* estimate of N, we used mean and variance data from both Rantanen *et al.*\(^27\) who examined ultrasound and satellite cell proliferation and from Hill and colleagues\(^34, 66\) who examined muscle injury and regeneration. Using a proposed power at 0.8 and an effect size of 0.7, the estimated sample size was calculated to be 8 per group utilizing the effect size curves for alpha set at 0.05 for a two-tailed test.
CHAPTER 4

RESULTS

Experiment 1: Ultrasound delivery method and energy transfer study

During pilot work with the Anti-BrdU kit, satellite cells were not detected. Active satellite cells were expected to stain brown or black. Although small changes were noticed around the edges of the muscle section it was not clear that these represented satellite cells. Further testing lead to the conclusion that cross-sectional cuts were not appropriate to determine the location of satellite cells. Because the nature of the local injury it precluded obtaining transverse sections. Therefore we were forced to discard satellite cells as a dependent variable and centrally located nuclei are the only marker for satellite cell regeneration examined.

A multivariate treatment ($F_{3,33} = 9.05, P<0.0001, 1-\beta = .991, \eta = .451$) and treatment x SATA interaction ($F_{3,33} = 3.92, P = 0.017, 1-\beta = .781, \eta = .263$) was observed for the linear combination of all skeletal muscle regeneration variables. US increased muscle mass more than no treatment ($F_{1,35} = 25.895, P < 0.0001, 1-\beta = .999, \eta = .425$) and increases in muscle mass was found with the continuous US treatment compared to
the pulsed duty cycle treatment at the same SATA (F_{1,35} = 6.045, P = 0.019, 1-\beta = .667, \eta = .147). An increase in the number of centrally located nuclei was found compared to the non-treated leg (F_{1,35} = 3.275, P = 0.079, 1-\beta = .421, \eta = .086) as well. The interaction of treatment and SATA on fiber cross sectional area (F_{1,35} = 5.863, P = 0.021, 1-\beta = .653, \eta = .143) and centrally located nuclei (F_{1,35} = 6.460, P = 0.016, 1-\beta = .696, \eta = .156) is significant; suggesting that if treated, SATA makes a difference on both CSA and centrally located nuclei.

<table>
<thead>
<tr>
<th>SATA</th>
<th>Duty Cycle</th>
<th>Muscle Mass (g)*§</th>
<th>CSA</th>
<th>CLN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Untreated</td>
<td>Treated</td>
<td>Untreated</td>
</tr>
<tr>
<td>.10</td>
<td>20%</td>
<td>3.13 ± 0.44</td>
<td>3.30 ± 0.34</td>
<td>888.78 ± 270.15</td>
</tr>
<tr>
<td>.10</td>
<td>100%</td>
<td>3.24 ± 0.16</td>
<td>3.40 ± 0.20</td>
<td>1015.30 ± 101.02</td>
</tr>
<tr>
<td>.30</td>
<td>20%</td>
<td>3.02 ± 0.23</td>
<td>3.09 ± 0.30</td>
<td>1116.50 ± 123.50</td>
</tr>
<tr>
<td>.30</td>
<td>100%</td>
<td>3.35 ± 0.27</td>
<td>3.51 ± 0.44</td>
<td>1090.50 ± 265.05</td>
</tr>
</tbody>
</table>

Table 4.1. Means and standard deviations for dependent variables (CSA = cross-sectional area of muscle, CLN = centrally located nuclei). * indicates that treated muscle has greater mass (p < .05); § indicates muscle treated with 100% duty cycle is greater than when treated with 20% (p < .05).
Figure 4.1 Interaction of treatment and SATA on fiber cross-sectional area. \((P = .021)\)

Figure 4.2 Interaction of treatment and SATA on centrally located nuclei \((P = .016)\).
Figure 4.3 Ultrasound treatments increase muscle mass more than no treatment ($P < .0001$). This is independent of the duty cycle and SATA used for the ultrasound treatment.

Figure 4.4 Increases in muscle mass was observed with the continuous US treatment compared to the pulsed duty cycle treatment at the same SATA ($P = .019$).
Figure 4.5 The number of centrally located nuclei observed compared to the non-treated leg ($P = .079$) ($0 =$ no treatment; $1 =$ treatment)
**Experiment 2: Increasing drop mass weight study**

Analysis Difficulties:

After initial procedure samples were refrozen until needed, then defrosted in ice but the supernatant never returned to liquid form. It remained as a gel-like substance and was not usable. Rapid warm in 65°C water bath did not alter sample state. Supernatant was then re-pulverized under liquid nitrogen and another 300ul of buffer was added but due to the large pellet that was remaining it was determined that little to no myosin was extracted. Therefore SDS-PAGE was not performed.

We then piloted different ratios of samples to buffer to determine if 1) not enough buffer was used in the beginning or 2) if the speed at which the samples were spun too slow to create a small pellet. In all cases even when a 1:1000 sample to buffer ratio was used and samples spun at 10,000 x g the supernatant became gelatinous at room temperature. It was then determine that this buffer would not be appropriate for this type of muscle tissue and buffer was changed to urea-thiourea buffer at a ratio of 1mg sample: 30ul of buffer. Therefore, no data could be generated due to insolubility of the protein into the Newcastle buffer.
Experiment 3: Low energy SATA on MGF, M-cad, MyoD, EmbMHC

A multivariate effect of treatment \((F_{2,27} = 5.605, P = 0.009, 1-\beta = .816, \eta = .293)\) and days post-injury \((F_{6,56} = 3.260, P = 0.008, 1-\beta = .902, \eta = .259)\) was observed for the linear combination of all skeletal muscle regeneration variables. Ultrasound treatments significantly decreased MGF mRNA levels compared to non-treated hind limb \((F_{1,28} = 6.605, P = 0.016, 1-\beta = .699, \eta = .191)\). Ultrasound had no effect on muscle mass \((F_{1,28} = 2.723, P = 0.110, 1-\beta = .357, \eta = .089)\). There was an effect observed for the day post-injury for MGF \((F_{3,28} = 7.303, P = 0.001, 1-\beta = .968, \eta = .439)\) but not for mass \((F_{3,28} = 0.870, P = 0.468, 1-\beta = .215, \eta = .085)\). Post-hoc testing revealed that the MGF value on 1 day post-injury was statistically greater than the values observed for days 3 and 4 after injury. Student’s dependent t-test revealed no difference between the initial and final weights of the animals \((P = 0.742)\).

Table 4.2. Means and standard deviations for dependent variables (MGF = muscle specific insulin-like growth factor). * indicates that cycle threshold value (ddCt) is greater on day 1 post-injury than day 3 or 4 \((p < .05)\); § indicates muscle treated ultrasound has lower values for MGF compared to those not treated.

<table>
<thead>
<tr>
<th>Day(s) post-injury</th>
<th>Muscle Mass (g)</th>
<th>MGF (ddCt)§</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td>Treated</td>
</tr>
<tr>
<td>1</td>
<td>1.75 ± 0.12</td>
<td>1.75 ± 0.15</td>
</tr>
<tr>
<td>2</td>
<td>1.60 ± 0.19</td>
<td>1.65 ± 0.24</td>
</tr>
<tr>
<td>3</td>
<td>1.72 ± 0.14</td>
<td>1.72 ± 0.15</td>
</tr>
<tr>
<td>4</td>
<td>1.69 ± 0.14</td>
<td>1.72 ± 0.16</td>
</tr>
</tbody>
</table>
Figure 4.6 MGF mRNA Amplification Curve. The threshold (the value that determines Ct) was set at 0.01. dCt is the difference between Ct 18S – Ct MGF. ddCt is the dCt – Ct for non-injured controls.
Figure 4.7 MGF mRNA response following blunt contusion injury. Day 1 is statistically different from Days 3 and 4 ($p < 0.05$).

Figure 4.8 MGF mRNA response following blunt contusion injury. Day 1 is statistically different from Days 3 and 4 ($p < 0.05$)
Figure 4.9 Comparison of ultrasound treated vs. non-treated muscle mass by days post-injury. (p > 0.05)
Figure 4.10. First trial at western analysis for embryonic myosin heavy chain (223 kDa) and beta galactosidase (116 kDa). Lane 1 is control uninjured tissue. The next lanes are hind limb pairs in the order of treated then non-treated. The molecular weight marker did not appear on western analysis. There is too much streaking and cross reactivity. There should be two distinct bands, one at 223KDa and the other at 116KDa. It is difficult to determine the actual molecular weight of the beta gal.
Figure 4.11. Titration of Beta galactosidase (beta gal) antibody at 1:3000 dilution. Three distinct bands appear. Lanes are loaded from left to right with a concentration of beta gal. Lane 1 = 0.2mg/ul, lane 2 = 0.3mg/ul, lane 3 = 0.4mg/ul, lane 4 = 0.5mg/ul, lane 5 = 0.6mg/ul
Figure 4.12. Second titration of Beta galactosidase (beta gal) antibody at 1:10,000 dilution. Three distinct bands appear. Lanes are loaded from left to right with a concentration of beta gal. Lane 1 = 0.2mg/ul, lane 2 = 0.3mg/ul, lane 3 = 0.4mg/ul, lane 4 = 0.5mg/ul, lane 5 = 0.6mg/ul
Figure 4.13. Third titration of Beta galactosidase (beta gal) antibody at 1:1M dilution. Three distinct bands appear. Lanes are loaded from left to right with a concentration of beta gal. Lane 1 = 0.5mg/ul, lane 2 = 0.4mg/ul, lane 3 = 0.3mg/ul, lane 4 = 0.2mg/ul..
Figure 4.14. Second trial at western analysis for embryonic myosin heavy chain with new beta galactosidase and sample at a 1:10 dilution. The first lane is control uninjured tissue. The next lanes are hind limb pairs in the order of treated then non-treated. There is less streaking but it lacks specificity still and possibly cross reactivity. We assume that the monoclonal antibody is reacting with the embryonic myosin at 223kDa. We are unable to detect the beta gal.
Figure 4.15. Western Analysis for m-cadherin with new beta galactosidase and a sample 1:10 dilution. The first lane is control uninjured tissue. The next lane is a blank and then follows are hind limb pairs in the order of treated then non-treated. There is less streaking, better separation, but it lacks specificity still and possibly cross reactivity. We assume that the monoclonal antibody is reacting with the embryonic myosin at 223kDa. We are unable to detect the beta gal.
Figure 4.16. Gel stained with Coomassie blue. There is good separation and purity. This gel was run to ensure proper separation and proper run time for electrophoresis. The gel is a little overloaded which may have contributed to poor separation of bands especially on standard) but still clean lines. Therefore the problem lies with the antibodies.
Figure 4.17. Third at western analysis for embryonic myosin heavy chain (sample 1:10 dilution). This time no primary antibody for beta gal was used. It was omitted to see if it was the beta gal antibody that was cross reacting with the myosin antibody. The first lane is control uninjured tissue. The next lanes are hind limb pairs in the order of treated then non-treated. There is streaking and it lacks specificity still cross reactivity. This illustrates a potential blocking issue. We assume that the monoclonal antibody is reacting with the embryonic myosin at 223kDa.
Figure 4.18. Western Analysis for myo-d (sample 1:10 dilution). This time no primary antibody for beta gal was used. This was done to see if it was the beta gal antibody that was cross reacting with the antibody. Lane 1 is control uninjured tissue. Lane 2 is a blank. Lanes 3-10 are hind limb pairs in the order of treated then non-treated. There is streaking and it still shows cross reactivity. This illustrates a potential blocking issue. We assume that the monoclonal antibody is reacting with the myo-d at 45kDa.
Experiment 1: Ultrasound delivery method and energy transfer study

The purpose of this experiment was to examine the effects of four different therapeutic ultrasound protocols that vary in mode and dose on markers of skeletal muscle regeneration. We specifically set out to examine muscle mass, fiber cross-sectional area and satellite cell number. Due to the transverse rather than longitudinal orientation of the muscle in the cryostat satellite cell number has been replaced with centrally located nuclei, another marker of regeneration. We hypothesized that ultrasound will enhance regeneration and that a continuous duty cycle of 0.3W/cm² would be the most effective protocol to enhance regeneration.

An overall multivariate effect for treatment and an interaction of treatment and (spatial averaged temporal average) SATA was observed for skeletal muscle regeneration. This means that ultrasound treatments and the combination of using US and the SATA chosen influences skeletal muscle regeneration. The interaction terms suggested that if treated, SATA makes a difference on both fiber cross-sectional area (CSA) and centrally located nuclei (CLN). (Figure 4.1 and 4.2) These figures illustrate
that using an SATA of 0.1W/cm² may be more appropriate than using 0.3W/cm². In the animals that were treated with 0.3W/cm² the limb that was not treated demonstrated a higher CSA and higher percentage of CLN. One rationale for this conflicting result is the variability in the data as expressed by the large error bars on the graphs. Large error bars indicated high variability in the data; therefore it is difficult to make definite conclusion based on this result and know that it was the treatment that caused these changes and not some confounder. The large variability, especially seen with centrally located nuclei (Figure 4.2, 4.5), may be due to the localized injury as well as the amount of the drop mass used.

**Centrally located nuclei**

Centrally located nuclei (CLN) were determined from taking cross-sections from the middle of the *gastrocnemius* muscle in order to maximize the chance of getting the portion of muscle with the greatest injury. Using a standard H&E stain one can visualize the muscle cell nucleus under a light microscope and determine which ones have moved toward the center of the cell versus remaining in the periphery.\(^8^4,8^5\) Nuclei in the center of the center indicate the cell is undergoing regeneration as oppose to the nuclei that remained in the periphery. Two possible reasons for the large variability seen with this data fall within the injury model used and the amount of weight used to induce the injury. The injury model selected for this research creates a focal injury this is much different than the injury that is seen when contraction induced injury \(^8^0,8^7\) or injury induced by marcaine injection.\(^8^1,8^2\). The injury can be as severe but it affects only a certain diameter or the muscle instead of a more of the fiber length or in some cases the entire fiber. A
contusion model injury is more applicable to the clinical setting. Athletic Trainers deal with contusion type injuries often and in order to more accurately address questions regarding treatment protocols it is important to make the injury more similar to what will be actually treated in a real-life setting. The drop mass weight used in this study was based on the work of Crisco and colleagues at 171g; however centrally located nuclei were not used as a marker of regeneration in his paper. He used a vimentin staining to examine myoblast activity but no numbers were provided with the “dramatic increase” seen post-injury. It is difficult as a reader to determine amount of an injury was actually present. They measured the size of the hematoma generated but the saphenous vein is very superficial in this region and it is possible that its location could have produced a noticeable hematoma unrelated to muscle damage and this would cause over estimation of the amount of injury that was actually induced to the skeletal muscle beneath it. There was also a size difference in the animals used, Crisco’s animals average about 330g where ours were slightly larger averaging closer to 450-500g. He does note having 27% fracture rate of the tibia and we had none. The amount of injury generated would then be confounded by the overall size of the animal, i.e. a smaller animal thereby sustaining a larger injury. The injury sustained by this weight as illustrated by CLN (Figure 4.5), shows that at most approximately 10% of the muscle fibers undergoing regeneration with the average being between 5-6%. This low injury percentage reinforces the argument that little injury was sustained. Therefore it would be difficult to see any therapeutic effect of the ultrasound, if present, since the injury effect is so small. CLN has been used as measurements of regeneration in diaphragm muscle and to our knowledge this is one of the first studies to apply this method to a blunt contusion model in skeletal muscle.
Another potential confounder was the technique of using the cryostat has a learning curve and therefore some inconsistencies. Although portion were cut form the center of the muscle, it is difficult assess whether the same exact area was selected on every sample. Also due to biological variability within each animal it is difficult to state that using an same measurement would be in the area of greatest injury on every animal. Therefore it is more likely the combination of these factors may have been enough to affect the results. This variable may be very useful in future studies to better determine the exact middle of the injury and from there a portion can be removed for further biochemical analysis.

**Wet Muscle Mass**

We did observe an increase in treated muscle mass versus injured control and those treated with a continuous ultrasound exhibited a greater mass than those receiving a pulsed ultrasound treatment (Figure 4.3, 4.4). When examining the results related to mass there is a statistically significant difference but when comparing the numbers from Table 4.1 more closely it becomes more apparent that these numbers are probably not biologically significant. The effect sizes for this variable range from 0.26-0.90 demonstrating a medium effect size. Therefore mass is something that should be included with experiments, however, not as a main dependent variable. This increase in mass supports the previous work of Dyson and colleagues\(^2\), who also demonstrated an increase in tissue mass following repeated treatments. Dyson equated an increase in mass to be increase in tissue regeneration. Dyson’s argument with increased mass = increase tissue regeneration is more a factor of when the experiment was conducted.
rather than to specific methodologies. Today, mass is more appropriately used as a
descriptor, similar to body composition or VO2max, than has an indicator of muscle
regeneration. The lack of specificity is the main difficulty with using mass as a dependent
variable rather than a descriptive variable. Mass is a generic measurement, meaning that
the number obtained from the scale represents the whole piece of muscle and not just the
amount of protein due to regeneration. Since we did not obtain any biological markers of
inflammation or take into account edema formation it is nearly impossible to ascertain
what that number truly represents. In addition, muscle mass is associated with many
confounding variables especially the skill level of the person performing dissections.
Therefore, it is difficult to decide how much was affected by the actual dissection due to
the learning curve associated with this technique.

Experiment 2: Increasing drop mass weight study

The purpose of the second experiment was to examine the effects of different
drop mass weights on the level of embryonic myosin heavy chain expression. This study
was performed in order to obtain a more substantial injury from the contusion apparatus
and to select a dependent variable with less technician variability. We hypothesized that
as the drop mass weight increased so would the levels of embryonic myosin heavy chain.

The data collection portion of this experiment went smoothly. Animal behavior
did not change until 220g was used as the drop mass and the behavior continued at a drop
mass of 240g. Animals were very guarded, protecting their hind limbs and did not
resume normal cage activity for approximately 20min. It was noticed there was no
fracture to the tibia. During the biochemistry analysis the supernatant was unusable and
more than likely little to none myosin extraction occurred. It is difficult to ascertain why
the Newcastle buffer was not an appropriate buffer since the combination of 8M urea,
20% SDS, and 1.5M Tris should be able to solubilize most tissues, including skeletal
muscle. This buffer has been used in other cases with injured or damaged tissue,
however, majority of those experiments were performed on mice and we were using in on
rat tissue. One would suspect that altering the proportion of buffer to sample would have
minimized the issue with larger tissue quantity but that was not the case. This buffer has
been used successfully in muscular dystrophy (mdx) mice (unpublished results from our
lab), possibly due to the low proportion of muscle relative to connective tissue compared
to the localized injury model we were using.

Different studies were piloted and based on these results the supernatant was still
unable to be used. The one positive result from this study was the determination of a
more appropriate buffer that would increase myosin and protein extraction—Urea-
thiourea buffer would then be used for the next study.

Since no data could be gathered from this study the weight used in the next study
was based solely on anecdotal evidence based on animal behavior and since behavior has
changed at both 220 and 240g, the 240g weight was used to ensure that an appropriate
injury would be generated.

**Experiment 3: Low energy SATA on MGF, M-cad, MyoD**

The purpose of this experiment was to examine the influence of ultrasound (US)
treatments at several times following contusion injury on a larger set of biological
markers of skeletal muscle regeneration. In addition to examining the activation of
satellite cells (SCs), which is one of the initial steps during skeletal muscle regeneration, we plan to address to the issue of differentiation of the SCs. We hypothesized that the hind limb treated with ultrasound would exhibit greater quantities of m-cadherin, MyoD and embryonic myosin heavy chain proteins as well as a greater expression of MGF mRNA.

**Muscle-specific IGF-I**

There was a statistical significance decline in the MGF mRNA levels treated with ultrasound compared to the injured control but this did not affect the biological decline. Values for mRNA peaked at Day 1 post-injury and rapidly declined to normal values by day 4 (Figure 4.6 and 4.7). Our decline follows Hill et al. who also demonstrated similar response following mechanical-induced damage. MGF is a splice variant of insulin-like growth factor 1 (IGF-1) and is predominantly up-regulated by mechanical stresses placed on the muscle. MGF functions in an autocrine fashion and exhibits a relatively short half-life. It has been suggested that MGF is responsible for the initial activation of satellite cells and later the influence would come from IGF-1 as one of the predominant hormones needed continue the up-regulation of protein synthesis and complete the repair process. We hypothesized that the ultrasound would increase the mRNA levels of MGF based on the idea that this growth factor is up-regulated by mechanical stresses. We speculated that the mechanical stress associated with the sound waves would further enhance this up-regulation; however, based on the results observed from this experiment this may not be the case. To our knowledge this is the first experiment to examine MGF mRNA using a contusion injury model and therapeutic ultrasound. It is possible then that the ultrasound caused a biological shift to the left and
the MGF mRNA peaked earlier and the level that we encounter represents the decay of the hormone instead of the rise. In order to examine this phenomenon we would have had to take samples at time points closer to the ultrasound treatment. Our first samples were taken roughly 3-5hrs post-treatment, if we had taken them at 1 and 2hr post-treatment we may have been able to observe greater levels of mRNA. There is some variability with using a localized injury method as illustrated in Figure 4.7. Once the gastrocnemius muscle was harvested and weight a portion of approximately 30mg was retained from the center for RT PCR analysis. It is possible that to the nature of the injury some of the muscle from the 30mg may have been uninjured tissue and therefore is partially responsible for the variability seen in the samples taken for day 1 post-injury. As the days progressed the amount of variability decreases and this may be due to better technique for sample removal. Previous research has demonstrated an increase in other growth factors including basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) following ultrasound treatments. These two growth factors function during angiogenesis and are thought to aid in overall tissue healing. Ultrasound has been thought to aid in the delivery of these factors so as to induce blood vessel growth following injury. It has also been suggested that VEGF may function in an autocrine manner to aid skeletal muscle repair following an ischemic injury. Some of the differences may lie in method of ultrasound delivery. The work of Reher and colleagues used a cell culture model to examine the effects of US and also used a difference US machine and settings. Their settings were used at both a 1MHz and 45MHz frequency—the lower the frequency the deeper depth of penetration. It is often difficult to compare results demonstrated in vitro versus in vivo; especially when talking about
energy delivery. As US travels through the body and encounters different mediums there is a loss of energy and attenuation that takes place. Therefore recommendations for treatments that are based solely on cell culture studies may need to be altered when introducing them into an animal model or a person.

**Wet Muscle Mass**

During this experiment there was no differences observed between treated and untreated and across days (Figure 4.8). This is in contrary to our previous study (Exp 1) where there was a statistical significance was observed for muscle mass. The lack of difference may have been due to better dissection techniques therefore less chance of taking muscles others than the gastrocnemius. It may also be due to cross-sectional design of this study where separate groups of animals were used for each day and thereby decreased the chance of seeing any change. We had anticipated an increase in muscle mass since a greater drop mass was used (an increase of approximately 70g) to induce the injury as well as smaller animals were used in this study. An increase in mass was expected and mostly likely attributed to edema and inflammation from the injury. Therefore, it was surprising not to observe any noticeable change even across days. We are confident that the lack of difference is not due to a change in eating habits or weight loss since there was no difference between initial and final body weights of the animals.

**Embryonic Myosin, MyoD and M-cadherin**

These variables were selected to better answer questions relative to muscle regeneration. Embryonic myosin heavy chain would help to address the total amount of
regeneration that took place. The other 2 variables were chosen to help address the questions of proliferation and differentiation. Myo-D promotes proliferation and differentiation of satellite cells. It can be found on proliferating cells, differentiated cells but the key determining factors is that Myo-D is not on quiescent satellite cells. M-cad is associated with the development of myotubes. M-cad is very transient and tends to peak and fall at a rapid rate. Western Blot Analysis was the chosen method to analyze these variables. Whereas this was probably the most appropriate choice for analysis there are many difficulties that were encounter during this process (see Figures 4.9-4.17).

The main difficulties seem to be with cross-reactivity of the antibodies and the antigens and the selection of the positive control. From the all the different variations with dilutions that were done a couple “pearls” have emerged. First, multiple antibodies require more than one positive control. In fact one should be selected for each primary antibody separately. One of the difficulties with using beta galactosidase is finding one that has high purity. It took us three times to find a pure product that would create a band at 116KDa. It is also important to use a more appropriate pre-stained standard and to load the antigen with the standard. One limitation with the Western Analysis was the lack of pure antigen to better titrate the ratio of antigen:antibody needed to get better results. Financial constraints may this virtually impossible but this would definitely be a strong recommendation for follow-up studies. It seems that the difficulty results more from determining the appropriate ratio prior to sample testing than actually performing the analysis.
The influence of Ultrasound

Based on what we have observed from these few experiments it is difficult to suggest which hypothesis, if either, is really applicable. The first problem with these hypotheses is that neither one of them have done testing in an in vivo model. In vitro models are great starting points; however, it is difficult to extrapolate the results from a cell culture model to either an animal model or a person. Every theory has a beginning and from there pilot work performed to see if in fact the theory works but somewhere it must be applied to what is done in clinical practice. The cavitation theory has been tested and there are some studies that demonstrated that both cavitation and acoustical streaming occur however none of the experiments have been modeled after a therapeutic protocol and majority have not even used a standard ultrasound unit to induce the effects. The majority of the research done on cavitation and streaming dates to the early 1970s to the middle of the 1980s,87,88 a couple more studies as of late were conducted the most recent being 2004.84 None of these studies can be generalized to fit clinical practice, which still leaves this particular theory up to scrutiny. When examining the resonance hypothesis, this opens the possibilities to other cellular mechanisms and takes a viewpoint from a molecular biology angle rather than a physical science one. This hypothesis intuitively makes sense. When an ultrasound head is place in water and turned on one can see ripples produces from the sound waves generated. It is plausible then to think that the same could occur within the body; sound waves bumping into proteins causing conformational changes that lead to either an up or down regulation of the protein. The problem is still the same however—there is no current data to support or refute this theory.
When examining our data, it is too soon to say which side is more plausible. We were able to demonstrate both positive and negative effects through the application of ultrasound treatments. From our first study we observed an increase in mass, centrally located nuclei and in some cases increases in fiber cross-sectional area. The latter study demonstrated a decrease in MGF mRNA following treatment when ultrasound was applied and no change in muscle mass. The ability of ultrasound to enhance and inhibit proteins or cause biological shifts in protein response should be addressed. This area of skeletal muscle regeneration is vast and there are so many different proteins that interact with each other. We have attempted to examine only a handful of these protein based on previous literature as important factors in the regeneration process. Little though is understood on what influence ultrasound will produce following blunt trauma. For example does ultrasound influence proteins directly as suggested by the resonance hypothesis or does it work more in a cascading effect or positive feedback. Some limitations of these experiments are the timing of tissue harvest, techniques associated with tissue analysis as well as the learning curve involved. Although there was some difficulties experienced we are able to suggest that initiation of ultrasound modality post-injury is important. All protocols for these studies commenced 24hrs post-injury. We were hoping to enhance the factors that would up-regulate satellite cell proliferation but unfortunately, due to constraints, were only able to show it negatively influencing a growth factor that induces satellite cell proliferation. What we cannot say for certain is what caused this down-regulation, was it is the intensities or the timing of the overall treatment regime. These are questions for future research.
Epilogue

The difficulty with doing this type of research falls mainly with the audience that it is addressed, either the basic scientist or the clinician. Being part of a young profession can be exciting especially when your role is to help find answers to questions that seem easy at least in theory. There appears to be, however, a division between the clinical world and the world of basic science and when one tries to bridge this gap more problems arise. Clinicians will be the first to admit that they want to read research that will help advance their practice and improve the current treatment regimes. The difficulty with generating evidence-based practice research is that it must marry with basic science and most clinicians do not have the background to fully understand the distinction. On the other hand basic scientist love the challenge of answering a question but amidst all the biochemistry the underlying clinical application and the “so what” gets lost.

If given 6 more months and unlimited funds there are a few areas that I would like to continue to explore. First, I would purchase pure antigens and a new set of antibodies. A titration should be performed using both the antigen and antibody to better determine a dilution factor that will elicit a more profound effect. Many of the problems we encountered, especially with the final project, were due to poor reaction or cross reactivity of the antibodies. I would also like to determine positive controls for each antibody. As we have discovered using only one causes almost as many problems as not having one. Both the antigens and the positive controls need to loading in the standard or loaded in a lane all by themselves—this needs to occur to confirm that was the ECL kit is lighting up is indeed the protein of interest. Finally one large “pearl” that was learned during this
process was that manufacturer recommendations are seldom effective and more pilot work needs to be done with the blocking steps involved with Western Analysis. Different times and temperatures were performed with the milk blot following the transfer but additional pilot work needs to be completed with washing in PBST and try different percentages of Tween-20. It may be necessary to use a higher concentration in order to obtain clear blots.

A limitation of these studies is the amount of sample available post-injury. The contusion model elicits a focal injury and this may propose difficulties when analyzing tissues. Because of the local injury it is inevitable that non-injured tissue will become part of the sample. The mixing of injured and non-injured muscle leads to increased variability in the results and also can dilute the injury effect that one is trying to determine. Future research with this model needs to be the following. First, an updated injury device where force outputs can be calculated needs to be created. Having force outputs will allow for better quantification of injury and can then be used with muscles other than the gastrocnemius. Once force is known a regression model can be built based on animal mass, since we know that this can be a confounder with this type of injury. Based on this model, histology (quantify satellite cells) and biochemistry analysis such as ELISA or RT PCR can be used to better quantify embryonic myosin and therefore the extent of injury can be better predicted. Using the cryostat and staining techniques to better see the area of injury will help to minimize the amount of non-injured tissue that would be combined with the overall sample. The addition of force to biological markers will allow for a more effective repeatable injury and it will be based on the extent of injury rather than animal behavior. This model could also be used to examine
confounding variables associated with bone fractures and other inflammatory markers associated with injury.

Regarding ultrasound, additional well-designed projects need to be performed to help build the knowledge base. There are many confounding factors relative to devices and energy output which makes it difficult to reproduce previous work. This however should not be a reason to assume that the modality does not work—it just means it will take longer to get repeatable results. The poor quality of existing research makes it difficult to establish the efficacy or lack thereof of therapeutic ultrasound. Therefore, once we can achieve reproducible results, a dose-dependent relationship curve needs to be established. Currently we are basing research protocols off of clinical practice but we are not confident that those parameters are even effective. We may need to treat longer or more times per week. A dose-dependent curve would help to address these issues and provide the data necessary to demonstrate to clinicians and scientists the biological influences of this modality. Ideally, we want to base clinical practice on science. However, it will be useful to begin our dose-response investigations with current clinical protocols before we branch out to more obscure protocols.

**Conclusions**

The methods associated with ultrasound intervention are still in their infancy and it will take many more studies to begin to answer these clinical questions. Our work has shown some beneficial effects of ultrasound with regard to skeletal muscle regeneration. Points to consider are the role of duty cycle in determining ultrasound protocols and the desired energy output or SATA in order to aid the regeneration process.
BIBLIOGRAPHY


APPENDIX A

THE CONTUSION DEVICE
The contusion apparatus. (Photograph courtesy of B.M. Eder, 2000).
APPENDIX B

ANIMAL POSITIONING
The animal was positioned prone with the hind limb stretched. Impactor was placed on the mid-belly of the gastrocnemius.
APPENDIX C

ULTRASOUND APPLICATION
The animal was positioned prone and hind limb was secured. Ultrasound treatments were administered for 5 min and the sound head moved in a parallel fashion.
APPENDIX D

BIOCHEMISTRY PROCEDURES
**Procedure for Electrophoresis (adapted from Dr. Wick’s lab class ANS/FST 868)**

1. Glass plates, spacers, combs were cleaned with 70% EtOH.

2. Plates were assembled and testing with dH₂O for leaks, apparatus was left to set for approximately 10min to ensure that no leaks were present. Then inside was dried with paper towel.

3. 6.5mls of the resolving mixture was poured and overlaid with isopropanol alcohol. A potion was retained in pipette to check for polymerization. Once the gel sets (~20min) pour alcohol off.

4. Place comb and slowly pipette the stacking gel being careful not to allow air bubbles to form. Retain left over portion in pipette to check for polymerization.

5. When set slowly remove comb and rinse with 1x PAGE running buffer.

6. Gently load thawed samples: 10ul of pre-stained standard (Pierce, Rockford IL) and 10ul of sample

7. Remove from casting apparatus and place in electrophoresis apparatus add running buffer—use a long funnel to fill in between the cassettes.

8. Secure electrodes run for ~1hr at 200V.

The gel was then transferred to a nitrocellulose membrane.

**Procedure for membrane transfer (adapted from Dr. Wick’s lab class ANS/FST 868)**

1. Place the tray on a relatively level surface.

2. Place two plastic bubble screens in the bottom of the tray.

3. Place the cathode in the bottom of the tray.

4. Place a plastic bubble screen, ribbed side down, on the cathode.
5. Place a SCOTCH-BRITE pad in the tray and fill tray with transfer buffer to a level even with the SCOTCH-BRITE pad. Squeeze the pad to expel all bubbles.

6. Place a sheet of filter paper (Pierce) then place nitrocellulose membrane on top of the filter paper, cut to the size of the SCOTCH-BRITE pad. Be sure not to trap bubbles under the blotting paper.

7. Position the gel on the membrane, two (2) gels can be placed on the membrane at one time.

8. Place another transfer membrane on the gel. Make sure there is no air bubbles.

9. Place a piece of filter paper over the membrane being careful not to trap any bubbles.

10. Place SCOTCH-BRITE pads over the filter paper, add buffer until tray is almost full. Additional blots can be put between these pads.

11. Place a plastic bubble screen, ribbed side up, on the pad and place the anode (banana connector should be in the upper right corner) on it.

12. Position the two-holed plastic anode cover over the anode.

13. The sandwich should need to be compressed about 2 mm. to slide into the tray holder. If not, the gel may slide during the electro blotting and another SCOTCH-BRITE pad should be added to the sandwich.

14. Keeping the tray level, slide the tray into the tray holder. Be careful not to pinch the electrode cover between the tray and the tray holder as the tray will jam and be difficult to remove.

15. Slowly tip the GENIE to its vertical running position. Add buffer if it appears the final buffer level will not cover the blotting area.
16. Connect the GENIE to the battery charger and begin blotting. Left-hand connection is (-), cathode; Right-hand connection is (+), anode.

19. Run for 45min at 1Amp (~20V) when finished place membrane in BLOTTO and store in 4°C overnight to block, (can also block for 30min at 37°C)

**Western Immunoblot Analysis**
1. Membrane was removed from walk-in and washed in PBST 3 times for 5min on a plate shaker (999ml 1xPBS + 1ml of Tween 20)

2. Incubated with primary antibody for 30min at 37°C
   a. Embryonic Myosin 1:1000 dilution
   b. M-cadherin 1:250 dilution
   c. Myo-D 1:500 dilution

3. Washed 3x in PBST for 5min on plate shaker

4. Incubate with secondary antibody for 30min at 37°C

5. Washed 3x in PBST for 5min on plate shaker

6. Prepare ChemiLucent Working Solution (Chemicon International; US)
   a. 1:1000 dilution of peroxide solution in peroxide buffer. Mix equal parts of luminal/enhancer solution and peroxide solution.
   b. This can be stored at RT for several hours

7. Transfer membrane to a Ziploc bag and add 4mls of chemilucent solution and incubate for 3min at room temperature. Remove membrane from baggie and place on plastic wrap

8. Multi-Fluro-s Imager with a CCD camera was used to take pictures (Alpha Innotech Corp, San Leandro, CA)
   a. FluroChem Imaging System software v.2.0
b. Sensitivity/Resolution—medium/medium

c. Exposure times—25, 45, 60, 120 seconds

d. Black color was set to 700 and white tab was moved almost to top ~1000

e. Save images to hard drive

Solutions

**4X Lower TRIS pH 8.8**

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**4X Upper TRIS pH 6.8**

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**Running Buffer (10X)**

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<td>10% APS (µL)</td>
<td>60 µL</td>
<td>80 µL</td>
<td>80 µL</td>
<td>75 µL</td>
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3% STACKING GEL

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<td>dH₂O (mL)</td>
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<td>4X Upper TRIS (mL)</td>
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<td>10% APS (µL)</td>
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40 mM TRIS                      4.84 gm
240 mM glycine                  18 gm
Methanol                        20 mL
0.1 \% SDS                      10 mL of 10\% SDS
q.s. with dH₂O                  1000 mL

10 X PBS

32 gm NaCl
8 gm KCl
57.6 gm Na₂HPO₄
9.6 gm KH₂PO₄
q.s. to 1000 mL dH₂O
pH 7.2

BLOTTO

50 mL 10 X PBS
450 mL dH₂O
15 mL Tween 20
25 gm Non-fat dry milk
APPENDIX E

RAW DATA
## Raw data table—experiment 3.

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<th>Muscle Mass Right</th>
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