Low-Intensity Vibration as a Therapy for the Treatment of Traumatic Muscle Injury

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THESIS

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This thesis is dedicated to my wife, Nicole, without whom, none of this would have been possible.

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CONTRIBUTION OF AUTHORS

Chapter 1 is an introduction and overview of the dissertation that explains the significance and aims of the research question. Chapter 2 is a review of literature that gives a broad overview of relevant concepts pertaining to the research question. Chapter 3 is a published manuscript (citation) for which I was the first author. I performed the experiments involving the 90 Hz protocol, created the figures, and wrote the manuscript. My research advisor, Dr. Timothy Koh, devised the research plan and assisted in the writing of the manuscript. Dr. Stefan Judex contributed to the research plan. Dr. Eileen Weinheimer-Haus performed the experiments involving the 45 Hz protocol and trained me on the injury model. Chapter 4 is a series of my own unpublished experiments seeking to determine the mechanisms by which LIV-induced improvements in muscle healing occur. I anticipate that this work will be published as a co-authored manuscript with my research mentor, Dr. Timothy Koh, as the corresponding author. Chapter 5 represents my synthesis of the research including a summary of findings for each specific aim, conclusions that can be made, and future directions.

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LIST OF ABBREVIATIONS

LIV	Low-Intensity Vibration
WBV	Whole-Body Vibration
IGF-1	Insulin-Like Growth Factor 1
Akt	Protein Kinase B
mTOR	Mammalian Target of Rapamycin
FOXO	Forkhead Box
MuRF1	Muscle Ring Finger 1
VML	Volumetric Muscle Loss
FFMT	Functional Free Muscle Transplantation/Transfer
ECM	Extracellular Matrix

SUMMARY

Traumatic muscle injuries are a devastating consequence of motor vehicle accidents, limb salvage surgeries for the removal of tumors and military combat. These injuries are characterized by a significant loss of tissue and inadequate healing leading to impaired muscle function, joint stiffness, and loss of mobility. Low-intensity vibration (LIV) is a promising candidate for physical therapy that provides mechanical stimulation without requiring active muscle contraction. The goal of this study was to determine if LIV improves the healing of injured muscle and if these effects are mediated by IGF-1 and associated downstream pathways using a mouse model of laceration injury and a cell culture model of myoblast differentiation. We found that whole-body LIV (WBV) at 45 Hz and 0.4g increased myofiber area, while WBV at 90 Hz and 0.2g increased both myofiber area and diameter at day 14 in a mouse model of laceration injury. Additionally, we showed that local LIV at 90 Hz and 0.2g increased both myofiber area and diameter in injured muscle at day 14 post-injury while also increasing percent area of peripherally-nucleated myofibers (associated with mature muscle) and decreasing percent damaged area. These LIV-induced effects on muscle were not associated with an increase in IGF-1 protein or mRNA expression. Furthermore, LIV also increased differentiation and growth of cultured myotubes at day 6 post-differentiation. There was an increase in total Akt in muscle at the day 7 post-injury and in cultured myotubes at day 3 post-differentiation while, phosphorylated to total Akt ratio increased at day 14 post-injury in muscle and at day 6 post-differentiation in cultured myotubes. Taken together, LIV enhances growth of myofibers following laceration injury, muscle cells are capable of directly transducing LIV signals into enhanced differentiation and growth, and Akt may be involved in this process. Therefore, LIV could be a promising therapeutic option for the healing of traumatic muscle injury.

I. INTRODUCTION

1. Background and Rationale:

Traumatic Muscle Injury

Traumatic muscle injuries are a devastating consequence of motor vehicle accidents, limb salvage surgeries for the removal of tumors[1] and military combat[2]. These injuries are characterized by a significant loss of tissue and inadequate healing leading to impaired muscle function, joint stiffness, and loss of mobility[3–6]. The regenerative capacity of muscle is inversely related to the severity of the injury with less severe injuries able to regenerate fully and more severe injuries resulting in permanent functional deficits due in part to denervation and scar formation. Recent reports indicate that volumetric muscle loss injury, a type of traumatic muscle injury, contributes to permanent disability rating in 65% of patients among a type III open tibia fracture cohort and lifetime disability costs for such patients are estimated between 340,000 and 440,000 USD[7]. In addition to these healthcare costs, patients also experience loss of time for work and reduced quality of life

Current Treatments for Traumatic Muscle Injuries

Currently, the most common treatment for traumatic muscle injury is free functional muscle transfer. This procedure has shown mild success in small muscle groups such as the forearm and facial muscles[8–10]. Treating larger muscles of the lower extremity has resulted in inconsistent results with some patients only partially recovering functionality and others still requiring amputation[11]. Researchers are exploring alternative options with the goal of

regenerating functional muscle tissue with variable results. Such experimental treatments include minced muscle grafts, decellularized extracellular matrix scaffolds, and cell therapy[12–20].

Standard of care for patients with less severe muscle injuries, such as sports-related strains, involves early mobilization of the muscle[21,22]. Physical therapy treatment for traumatic muscle injuries such as VML have shown minimal improvements in function with no ability to regenerate lost tissue[23]. However, when combined with other treatments such therapy can enhance regenerative outcomes[24,25]. A major limitation to physical therapy in traumatic injury is immobility and pain[26]. Thus, alternative forms of mechanical stimulation that do not require active muscle contraction could be useful.

Low-Intensity Vibration as Physical Therapy for Traumatic Muscle Injury

LIV is a promising candidate for mechanical stimulation that does not require active muscle contraction. Physical therapists have turned to low-intensity vibration (LIV) for the treatment of neurological disorders by inducing positive effects on motor control[27–30] or as an adjunct to exercise with applications of warming up muscle, improving power, preventing falls in elderly, or reducing lower back pain[31]. LIV is defined as vibration with a magnitude less than 1g acceleration. To our knowledge, LIV is not yet being used clinically as a treatment for tissue injury; however, some researchers have begun to investigate the regenerative capabilities of vibration. LIV has been shown to ameliorate bone loss, enhance bone regeneration, reduce damage from pressure ulcers in aged mice, and improve healing in diabetic mouse skin following injury. [32–36]. However, the effects of LIV on regenerating muscle have yet to be studied.

The Effects of LIV on Muscle Mass

Although little is known about the effects of LIV in muscle injury, previous studies have tested the effects of vibration on muscle size in humans and mice as well as cultured muscle cells. Three months of whole-body vibration in older human adults with sarcopenia increased skeletal muscle mass index[37], however six months of local vibration in postmenopausal women had no effect on muscle mass in the quadriceps[38]. Lastly, two studies using cultured muscle cells showed that vibration induced increased myotube differentiation, one of which was associated with increased MyoD and myogenin expression, the other with increased M-cadherin expression. The differences in the outcomes and mechanisms implicated in these studies may be due to the differences in study design and vibration parameters. Despite these differences, it appears that that there is a range of vibration signals that can induce myogenesis or growth of muscle fibers. However, whether LIV is beneficial for muscle repair following injury remains to be determined.

The regulation of muscle mass is a delicate balance of hypertrophic and atrophic signaling pathways. Akt plays a central role in the regulation of both hypertrophy and atrophy in skeletal muscle[39]. Akt and its downstream target, mammalian target of rapamycin (mTOR) were upregulated in models of muscle hypertrophy and downregulated in models of muscle atrophy in both mice and humans[40,41]. In a model of young developing mice, Akt activated by phosphorylation at Ser473, was significantly increased in vibrated mice at days 5, 10, 15, and 20 as compared to control mice[42]. Additionally, inhibition of PI3K, an activator of Akt, suppressed the vibration-induced increase in myotube size, number and fusion[43]. Taken together, Akt is a possible mediator of vibration enhanced muscle growth; however, little is known about whether Akt mediates the effects of LIV on muscle repair following injury.

The Role of IGF-1 on the Regulation of Skeletal Muscle Mass

Insulin-like growth factor 1 (IGF-1) is a well-known regulator of skeletal muscle mass in part by upregulating Akt/mTOR signaling and downregulating myostatin signaling[39]. IGF-1 expression is increased in models of stretch-induced hypertrophy[44], overload-induced hypertrophy[45], and compensatory hypertrophy[46] in rodents. Furthermore, transgenic mice overexpressing muscle-specific IGF-1 showed an increase in muscle hypertrophy[47]. Two isoforms, IGF-1Ea, a muscle-specific isoform, and mechanogrowth factor (MGF) are upregulated in muscle after mechanical stimulation. Both isoforms increased within 4 days of stretch in rabbit EDL muscles[48] and in older humans on a prolonged resistance exercise program[49]. Thus, the expression of local IGF-1 depends on mechanical signals received by the muscle and likely is a positive contributor to muscle mass.

Whether vibration stimuli induce IGF-1 has not been clearly demonstrated. Vibration applied to young men or women with fibromyalgia resulted in no changes in serum IGF-1 levels[50–52]. Alternatively, vibration in combination with isometric squatting in aged men and women did increase serum IGF-1 levels[53]. In addition, LIV increased local IGF-1 levels in mouse skin wounds[35]. Although IGF-1 is involved in many hypertrophic and atrophic pathways in muscle, its role as a mediator of LIV induced myofiber growth after injury has yet to be elucidated.

2. Purpose

Patients who suffer from traumatic muscle injury typically experience immobility and are in need of treatment that does not require active muscle contraction. LIV is a promising treatment option due to its non-invasive application of mechanical stimulation that does not require active

muscle contraction. Muscle responds to mechanical stimulation which can increase muscle size, however, the effects of vibration on injured muscle have yet to be elucidated. The goal of this study is to determine if LIV improves the healing of injured muscle and if these effects are mediated by IGF-1 and associated downstream pathways.

3. Central Hypothesis

The central hypothesis of this proposal is that LIV can improve healing of traumatic muscle injuries by increasing local IGF-1 levels and PI3K/Akt/mTOR signaling, while decreasing expression of genes related to atrophy via the ubiquitin-proteasome system.

Therefore, the specific aims of the study are as follows:

4. Specific Aims

Specific Aim 1: To determine the effect of LIV on muscle repair in a mouse model of traumatic muscle injury.

Aim 1.1. Determine whether altering amplitude, frequency and/or mode of application alters efficacy in the treatment of traumatic muscle injury.

Hypothesis 1.1a: LIV applied at both 90 Hz frequency and 0.2g acceleration and 45 Hz and 0.4g will be capable of improving muscle healing since both have been shown to have positive effects in biological tissues such as bone and skin wounds[35,54,55].

Hypothesis 1.1b: LIV applied locally to the lower leg will improve muscle healing more than whole-body LIV because muscle responds to mechanical loading which is associated with the release of growth factors that could positively effect regeneration in a paracrine fashion[44–46,48]. While WBV may also stimulate muscle to a certain extent, the

signal received in this manner may be different or dampened since it will first have to travel through the skeleton and tendons, and then to the muscle, possibly eliciting a systemic response.

Aim 1.2. Determine the effect of LIV on expression of total IGF-1, local IGF-1 isoforms, and related hypertrophic/atrophic downstream targets in lacerated gastrocnemius muscle.

Hypothesis 1.2: LIV will increase the mRNA and protein expression total IGF-1, local IGF-1 isoforms, and Akt after 7 and 14 days of treatment in injured muscle because other forms of mechanical stimulation, such as stretch, have been associated with increased levels of IGF-1 in muscle[44–46,48] and Akt is a major downstream target from IGF-1 that regulates muscle mass[39–41].

<u>Specific Aim 2</u>. Determine whether muscle cells directly sense LIV signals and transduce them into increased growth.

Aim 2.1. Determine the effect of LIV at 90 Hz and 0.2g on of myotube differentiation and size in C2C12 cells.

Hypothesis 2.1: LIV will increase differentiation and size after 6 days of treatment because previous studies have also shown increased differentiation and size in myotubes albeit with different and/or more intense protocols[42,43].

Aim 2.2. Determine the effect of LIV on expression and/or accumulation of total IGF-1, local IGF-1 isoforms, and related hypertrophic/atrophic downstream targets in C2C12 cells.

Hypothesis 2.2: LIV will increase the expression and accumulation of total IGF-1, local IGF-1 isoforms, and Akt after 3 and 6 days of treatment in cultured myotubes because other forms of mechanical stimulation, such as stretch, have been associated with increased levels of IGF-1 in muscle[44–46,48] and Akt is a major downstream target from IGF-1 that regulates muscle mass[39–41].

5. Summary of Findings

In our first study described in Chapter 3, we found that whole-body LIV (WBV) at 45 Hz and 0.4g increased myofiber area, while WBV at 90 Hz and 0.2g increased both myofiber area and diameter at day 14 post-muscle injury. Neither protocol, however, changed the percent of damaged area in the injury. We therefore concluded that WBV improves healing following muscle injury by enhancing myofiber growth. Although we do not have sufficient evidence to conclude which vibration protocol is optimal for muscle healing, we plan to proceed with the 90 Hz and 0.2g protocol for future experiments since there was an increase in both area and diameter whereas the 45 Hz and 0.4g protocol only showed an increase in area, not diameter.

Our second study described in Chapter 4 sought to determine if local LIV was as effective as WBV in healing injured muscle when applied at the same frequency and acceleration as was established in the previous study. We showed that local LIV at 90 Hz and 0.2g increased both myofiber area and diameter in injured muscle at day 14 post-injury. There was also a significant increase in percent area of peripherally-nucleated myofibers (associated with mature muscle) and a decrease in percent damaged area suggesting that local LIV improves muscle

healing at least as much as WBV. LIV also increased differentiation and growth of cultured myotubes at day 6 post-differentiation. Contrary to our hypothesis, these LIV-induced effects on muscle were not associated with an increase in IGF-1 protein or mRNA expression. However, there was an increase in total Akt in muscle at the day 7 post-injury and in cultured myotubes at day 3 post-differentiation. Furthermore, phosphorylated to total Akt ratio increased at day 14 post-injury in muscle and at day 6 post-differentiation in cultured myotubes. Taken together, muscle cells are capable of directly transducing LIV signals into enhanced differentiation and growth, and Akt may be involved in this process.

II. LITERATURE REVIEW

1. Muscle Injury and Repair

1.1 Early Events following Injury

Skeletal muscle repair is a complex process governed by a diverse population of myogenic, inflammatory, vascular, and fibrotic cells that contribute to the repair response in a tightly orchestrated fashion[56]. Immediately following damage to the sarcolemma, an influx of calcium leads to increased proteolytic and hydrolytic activity which further damages the muscle tissue. This leads to activation and accumulation of inflammatory cells in the injury that release cytokines and growth factors capable of regulating many aspects of the repair process[57,58]. Injury also causes a release of hepatocyte growth factor (HGF) from the basal lamina which activates satellite cells, the muscle-specific stem cell that is located beneath the basal lamina of the muscle. Once activated, satellite cells migrate towards the injury site and differentiate into committed myoblasts that further proliferate within the injury[59]. These myoblasts begin to fuse with each other, and then with the host myofibers forming nascent myotubes.

1.2 Restoring Muscle Mass following Injury

Over the next several weeks, these nascent myotubes further mature by reforming contractile proteins and undergoing hypertrophy followed by connective tissue deposition in areas where myofiber regeneration is insufficient[60]. Additionally, blood vessels and nerves must be reformed to restore perfusion and innervation to ensure new muscle is fully functional. Deficiencies in any of these processes could impair restoration of muscle function. Overall, muscle mass is determined by the balance of protein synthesis and protein degradation at any given time. Hypertrophy occurs when there is an enhanced rate of protein synthesis, and atrophy

occurs when there is an enhanced rate of protein degradation. Hypertrophy is an increase in muscle mass and can occur in response to mechanical overload via strength training, ablation of synergistic muscles, or reloading after unloading as well as by anabolic signaling from hormones such as testosterone[39]. Thus, enhancing hypertrophy has potential to improve recovery following muscle injury.

1.2.1 The Role of IGF-1/Akt/mTOR on the Regulation of Skeletal Muscle Mass

Insulin-like growth factor 1 (IGF-1) is a well-known regulator of skeletal muscle mass. IGF-1 expression is increased in models of stretch-induced hypertrophy[44], overload-induced hypertrophy[45], and compensatory hypertrophy[46] in rodents and transgenic mice overexpressing muscle-specific IGF-1 showed an increase in muscle hypertrophy[47]. IGF-1-induced hypertrophy is associated with the upregulation of protein kinase B (Akt) – mammalian target of rapamycin (mTOR) signaling[39]. Genetic induction of constitutively active Akt resulted in significant increase in muscle fiber size whereas genetic inhibition of Akt blocked hypertrophy[61]. Akt is an upstream activator of mTOR, which in turn mediates muscle growth by its activation of p70S6K[39]. Inhibition of mTOR with rapamycin prevents muscle growth during muscle regeneration following myotoxin-induced injury[62]. Furthermore, genetic deletion of p70S6K causes muscle atrophy[63]. Therefore, IGF-1 plays an important role in promoting muscle growth through the Akt-mTOR-p70S6K pathway and is likely an important mediator of healing after injury.

Atrophy can be considered the opposite of hypertrophy and typically occurs with aging, caloric/nutrient restriction, inactivity, loss of neural input, or catabolic signaling from hormones such as cortisol. The ubiquitin-proteasome system is a major pathway for protein degradation in

muscle. Briefly, single or chains of ubiquitin molecules attach to proteins, labeling them as targets for degradation by a large enzyme called the proteasome[64]. Atrogin-1 and Muscle Ring Finger 1 (MuRF1) are two muscle-specific transcription factors that control this process[65]. Atrogin-1 and MuRF1 knockout mice are lose significantly less muscle mass following denervation compared to wild type mice[66]. MuRF1 has been shown to ubiquitinate large sarcomeric proteins such as titin[67]. Atrogin-1 targets MyoD for degradation, a transcription factor associated with muscle proliferation and differentiation during development and regeneration[68]. Atrogin-1 and MuRF1 are regulated by the FOXO family of transcription factors[69]. FOXO-Atrogin-1/MuRF1 activity is upregulated by myostatin, a protein in the TGFβ family known to induce muscle atrophy[70]. Interestingly, IGF-1 is also a mediator of these pathways. IGF-1 can inhibit atrophy by downregulating myostatin signaling[71]. Furthermore, when activated, Akt phosphorylates FOXO transcription factors, sequestering them in the cytoplasm and inhibiting atrophy-related transcription[41]. Thus, preventing atrophic signaling during muscle repair could also benefit recovery.

1.3 Models of Muscle Injury

Muscle injury is studied using a variety of injury models in animals. Each injury model has distinct repair responses and outcomes. There are several injury models that represent less severe muscle injuries experienced by a large portion of the population throughout daily life. Exercise-induced injuries are among the most relevant to the general public due to the wide-spread participation in recreational sports. These injuries can be categorized as contusions, strains or tears. Animal models that study these injuries are typically less severe in nature and employ stimuli such as downhill running or lengthening contractions via electrical stimulation to

cause a muscle overload injury that recovers full functionality within 2 weeks[72–74]. Freeze injury induced by applying a probe cooled on dry ice directly to the muscle is a more severe physical method for creating injury which is unique for its ability to also destroy mononuclear cells in the zone of injury making it a good model to study cellular infiltration and migration following injury. These injuries fully recover within 1 to 3 months depending on the number and duration of freeze application[75]. Ischaemia-reperfusion injury can be created by temporarily cutting off the blood supply via arterial occlusion. This models injury experienced in hospital settings due to prolonged tourniquet application. Muscle damaged in this way is capable of full functional recovery within 2 weeks.[76,77]. Myotoxin injection with chemicals such as cardiotoxin, notexin, or bupivacaine is commonly used to induce complete myofiber necrosis[78–80]. Muscle injured using the aforementioned methods display impressive regenerative capacity, completely healing within 2 to 3 weeks, likely due to the blood supply and neuromuscular junctions remaining intact. While these may be efficient and reproducible models, they may not be representative of regeneration following more severe injuries in humans.

Conversely, traumatic muscle injury seen in military combat, automobile accidents, or surgical events such as tumor removal pose unique complications for affected patients as these injuries typically involve a disruption of the blood supply and denervation leading to impaired muscle regeneration, excessive fibrosis, and subsequent functional deficits[7]. Models of traumatic muscle injury have been used in the form of crush, laceration[5,81], or volumetric muscle loss (VML) by blunt resection[82]. Crush injuries are caused by blunt force directly to the muscle and healing responses can vary depending on the amount of force applied. Laceration involves making a thin cut through the belly of the muscle, which in turn also cuts the blood and nerve supplies but does not remove any tissue. VML is a severe form of laceration where the tissue is

lacerated and a portion of tissue that is at least 20% of the muscle mass is removed. These models more closely represent what occurs following severe injury because of excessive fibrosis and lack of innervation resulting in persistent functional deficits and disability. To address these unique challenges of traumatic muscle injury, researchers have begun to explore possible treatments to improve regeneration of functional muscle tissue.

1.4 Existing Treatment for Traumatic Muscle Injury

Currently, the most common treatment option for VML is functioning free muscle transplantation (FFMT) in the form of vascularized muscle flaps. FFMT involves the transfer of a normal, vascularized muscle to an injured, destroyed, or denervated muscle site. FFMT is technically challenging, specifically in terms of nerve repair and the ensuring optimal muscle tension, and also requires a significant post-operative therapy[83]. This procedure has shown some clinical success when using small muscle groups[8,9,84], but treating larger muscles has shown inconsistent results with limited functional recovery in the most severe injuries[11]. This limited recovery is usually due to prolonged ischemia in the grafting site or donor site morbidity[85]. This has led researchers to explore alternative approaches to restoring functional muscle tissue following traumatic muscle injury.

1.5 Experimental Treatments for Traumatic Muscle Injury

1.5.1 Minced muscle grafts

Another form of autograft has been explored which involves mincing muscle prior to implanting into the injury site. A recent study performed with a porcine VML model found 32% increase in strength at 12 weeks when treated with minced muscle. These results were associated with a reduction in fibrosis although the amount of scar tissue was still extensive[12]. In a mouse TA VML model using GFP transgenic mice, donor cells contributed to muscle fiber regeneration, albeit without any functional improvements[86]. Furthermore, Hurtgen et al applied minced muscle grafts to a rat TA VML injury with concomitant tibial osteotomy and found that animals had improved fracture healing and muscle function when treated with minced muscle grafts which was associated with a reduction in CD45+CD11b+ monocyte/macrophage and CD3+ T lymphocyte response suggesting a possible role in modulating the immune response[87]. Earlier studies performed by Corona, Ward and colleagues explored this treatment in the previously mentioned rat and porcine models of VML[13,88]. These studies also found sparse pockets of muscle regeneration in the defect, usually near the border of the injury, along with partial improvements in functional measurements. Thus, minced muscle grafts show the ability to only partially improve healing from traumatic muscle injury.

1.4.2 Decellularized Extracellular Matrix Scaffolds

Researchers have turned to tissue engineering techniques, exploring the possibilities of implanting decellularized scaffolds made of various connective tissues or extracellular matrix (ECM) components. These can be made from natural polymers such as collagen[89] or fibrin[90] or from the decellularization of a variety tissues[91]. The purpose of decellularized scaffolds is to

provide a regenerative environment that releases chemotactic and/or immunomodulatory signals to promote a healing response[92] while simultaneously reducing host rejection. A commonly used scaffold has come from porcine small intestine submucosa (SIS-ECM). In a study using a VML model of injury in the quadricep of mice, SIS-ECM increased vascularity and innervation at the border of the defect, although extensive scar tissue developed throughout the rest of the defect by day 56 post-injury[14]. Another study using the same model found a similar fibrotic response alongside some modulation of the macrophage (MP) response. iNOS+ cells, representative of the pro-inflammatory MP population, were decreased at day 3, while Fizz1+ cells, representative of the anti-inflammatory MP population, were increased at all time points. There was also evidence of perivascular stem cell migration into the defect at days 7 and 14, and increased innervation at day 56, however functional outcomes were not assessed[16]. Although mild changes were seen in a mouse model of VML, SIS-ECM was not sufficient for muscle regeneration in a dog VML model[18]. Although some mild improvements were observed with the SIS-ECM scaffold, it does not appear to provide regeneration of an appreciable amount of functional muscle.

Different types of bioscaffolds have also been explored. A urinary bladder matrix (UBM) scaffold was explored alongside an autograft, in a rat tibialis anterior VML model. At week 2 and 8 post-injury, there was significantly more myosin heavy chain in the defect in the autograft group. Although this seems obvious since muscle tissue was implanted into the defect, it is important to note that there was little necrosis or atrophy associated with this treatment. Alternatively, the UBM healed with more extensive fibrosis which was associated with sustained TGF-β out to 8 weeks. UBM also had an increased pro-inflammatory MP response compared to autograft with no differences in anti-inflammatory MP[15]. Lastly, a decellularized skeletal muscle

scaffold supplemented with minced muscle in a rat TA VML model resulted in improved force production and reduced the fibrotic response after 12 weeks[93]. Taken together, these data suggest that ECM alone cannot regenerate functional muscle tissue, resulting in the formation of non-functional scar tissue instead. Any functional improvements seen in these therapies are thought to be due to improved force transmission through a "functional fibrosis" within the defect[94]. Thus, there is a need for therapies that can minimize scar formation to allow for muscle fiber regeneration.

1.5.3 Growth Factor Therapy

Growth factors play in an important role in regulating the endogenous phases of healing following injury and therefore have been explored as a therapy to treat muscle injury. Hepatocyte growth factor (HGF) delivered via a bioengineered fibrin scaffold enhanced myoblast differentiation at day 14 and angiogenesis at days 14 and 60 in a model of VML injury in a mouse TA[90]. Vascular endothelial growth factor (VEGF) applied to an ischemic injury in mice via hydrogel injection improved angiogenesis and neuronal regeneration[95]. VEGF combined with insulin-like growth factor 1 (IGF-1) improved muscle regeneration of mouse ischemic injury by improving vascularization, myoblast proliferation, and fibrosis[76]. It is important to note that ischemic injury response is different from the response in traumatic muscle injury due to its ability to recover full functionality without additional treatment while traumatic muscle injury results in persistent functional deficits. Nonetheless, growth factors have the potential to improve muscle regeneration.

1.5.4 Cellular Therapy

Although growth factors play a supporting role, various cell populations are critical to regenerating damaged tissue. Capitalizing on these cellular therapies has been a goal of research over the recent years. The most obvious candidate to improve muscle regeneration is the satellite cell. These cells remain in a quiescent state beneath the basal lamina until activated. Upon injury, satellite cells are activated, asymmetrically proliferate, differentiate into myoblasts which further proliferate and differentiate, eventually fusing with existing myofibers to repair the injury. Satellite cells delivered via hydrogels have been shown to improve regeneration following injury from myotoxin[96,97] and partial ablation[98]. It is difficult to isolate and maintain these cells in vitro, however. CD133+ cells are muscle-derived stem cells closely associated with blood vessels in skeletal muscle. These cells are capable of ex vivo expansion and have been shown to repopulate the stem cell niche and improve regeneration following cryoinjury[99].

Finally, mesenchymal stem cells have shown promising regenerative potential by acting in a paracrine/endocrine fashion that benefits many aspects of repair including cell recruitment, migration, proliferation, and differentiation as well as angiogenic and immunomodulatory effects[100]. Moreover, they are easily harvestable from bone marrow and adipose. Muscle regeneration has improved in VML models with the addition of MSCs from various sources[101–103]. Although the mechanism of action has not been identified, cellular therapies could play a beneficial role as part of a multifaceted treatment program for someone recovering from traumatic muscle injury.

1.5.5 Physical Therapy and Mechanical Stimulation

A defining characteristic of functional skeletal muscle tissue is the ability to respond to changes in mechanical loading. Physical therapy is widely used as an adjunct treatment for muscle injury. Physical therapists use a variety of mechanical stimulations to treat patients such as strengthening exercises, stretching, massage, electrical stimulation, or vibration. Mechanical stimulation has potential to provide to improve muscle repair. In 1975, Jarvinen applied crush injury to 242 rat gastrocnemius muscles and treated them with either motorized treadmill running, immobilization, or a combination of both and performed histological analysis[21]. In general, exercised muscles showed a greater inflammatory response and hematoma with less necrosis early after injury compared to immobilized muscles. Exercise also resulted in both increased myofiber regeneration and fibrosis with regenerated fibers being oriented parallel to the direction of the original muscle. On the other hand, the regenerated fibers penetrated further into the scar tissue following immobilization[21]. A more recent study found that voluntary wheel running alone starting at 7 days post-VML injury in rats resulted in 17% increased force generation with no additional improvements related to other regenerative outcomes[23]. Thus, physical activity may impact many aspects of the repair process in including inflammation, myofiber regeneration, and scar formation.

To address the issue of traumatic muscle injury, recent studies have combined physical therapy with other experimental therapies. Voluntary wheel running starting at day 7 post-injury found a reduction in scar formation compared to starting immediately following VML injury in mice. This study combined the exercise treatment with with a bioscaffold and cell therapy and found accelerated myogenesis, improved force production, and reduced fibrosis which was associated with increased neuromuscular junctions and angiogenesis compared to

non-exercised controls[24]. Additionally, mechanical stimulation via compressive pulses applied through a magnetized ferrogel in a mouse model of notexin and ischemia induced injury improved regeneration and contractile force at day 14 by reducing fibrosis and inflammation[104]. Similarly, voluntary wheel running combined with an aligned collagen scaffold increased vascular density and neuromuscular junctions, albeit without an increase in myofiber density or size[89]. Finally, one group performed a GFP+ bone marrow transplantation into irradiated WT mice that were injured with notexin in one leg and left uninjured in the other. These mice underwent either forced exercise via motorized treadmill or overload via synergist ablation. GFP+ bone marrow-derived cells were found in the repairing muscles and this accumulation was increased in response to both forms of exercise suggesting that muscle activity may also be capable of systemic immunomodulation[105]. Taken together, mechanical stimulation may have a synergistic effect on muscle repair when applied with other regenerative therapies and optimizing mode of application, intensity, timing, and other variables is of great importance to improve healing following traumatic muscle injury.

2. Vibration Therapy

Vibration as a means of exercise or therapy has been a topic of curiosity for decades. Over the past century, countless vibration machines have been marketed claiming everything from fat loss to skin tightening. However, chronic exposure to vibrations, as is often experienced with the use of power tools, is detrimental to the nervous system leading to disorders such as Raynaud's syndrome and vibrating white finger[106]. More recent reports, however, claim that vibration has positive effects on muscle size and differentiation[42,43,81], bone health[54], circulation[107–109], and neurological disorders[27–30] among others. In the clinical setting, vibration

therapy is very appealing for its non-invasive application and low risk of side effects when vibrations are low intensity. Patients suffering from traumatic muscle injury often experience pain or immobility preventing them from engaging in physical therapy requiring active muscle contraction. Therefore, vibration therapy is a promising alternative for treatment of traumatic muscle injury.

Vibration therapy can be defined as periodic oscillations that transmit a force from a device (the actuator) to the human body (the resonator)[31]. Signals transmitted to the body depend on a number of factors including amplitude, displacement, frequency, acceleration, and mode of transmission. Amplitude is defined as half of the peak to peak displacement with values ranging from fractions of a millimeter to several centimeters. Frequency is defined by the number of oscillations occur per unit time typically measured in cycles per second, or Hertz (Hz)[31]. Vibration intensity is typically measured by the acceleration resulting from the combined amplitude and frequency and is usually represented in units of acceleration due to gravity (g)[31]. **Low-intensity vibration is of particular interest because of its lack of reported side effects and is defined as vibration that is less than 1g acceleration.**

In addition to vibration parameters, other factors can also affect the transmission of the signal including mode of transmission. The vibration can be transmitted to the body while standing on a platform (whole body vibration, WBV) or locally to a certain part of the body using various devices. Additionally, tissue properties such as the amount of fat, muscle volume, and muscle activation can also affect the signal due to differences in tissue properties. Mechanical properties of tissues can have amplifying effects due to resonant frequencies or damping effects due to elasticity, thereby altering the way the signal travels throughout the body. Researchers have explored the effects of a wide range of vibration intensities ranging from 0.2

g[81] to 17 g[110] acceleration in the skin and muscle tissue, and skeletal, cardiovascular and nervous systems of healthy, young, athletically-trained, aged, post-menopausal, and neurologically impaired humans. Studies have also been performed in similar tissues in rodent and porcine animal models. These varying vibration parameters and experimental conditions lead to many challenges when comparing studies and making conclusions from the current literature. None-the-less, with more researchers investigating the effects of vibration, trends can be observed in the literature.

2.1 Vibration and Muscle

To date, the majority of studies exploring the effects of vibration on muscle focus on neuromuscular outcomes related to strength and power in exercise. A recent, comprehensive review of these studies shows some conflicting evidence with many studies reporting that vibration increases in isometric strength of various muscle groups while others reporting no change in these outcome measures[111]. Notably, these studies apply signals that are intended to mimic or induce an exercise stimulus and are thus not categorized as LIV due to accelerations ranging from 5 g to 32 g, much greater than the 1g that defines LIV. While improving neuromuscular performance could have some relevance to this thesis, these studies were performed in healthy, intact muscle. Therefore, the effects of LIV on damaged muscle is an area that is yet to be explored.

A number of studies have tested the effects of vibration on cultured muscle cells, and uninjured muscle in humans and mice. Wang et al applied LIV at 5, 8, and 10 Hz with an amplitude of 0.4 mm (0.04 g to 0.16 g acceleration) to confluent C2C12 myoblasts in growth

medium. There was an increase in myotube number, size, and fusion when vibrated for 10 minutes per day for 3 and 6 days, and allowed to culture for another 3 days after each treatment[43]. These measurements corresponded with an increase in gene expression of MyoD and myogenin; both markers of myogenic differentiation. Ceccarelli et al applied vibration in vivo to developing mice and in vitro to primary myoblasts[42]. Although this was a model of development and not regeneration, some important observations were made. Vibration at 30 Hz and 11 mm amplitude (40 g acceleration) was applied directly to cultured myoblasts for 1 hour per day over a period of 7 days, resulting in increased cell fusion[42]. This study also found a significant increase in expression of M-cadherin, an indicator of satellite cell activation, in mouse TA muscles, primary myoblasts, C2C12 myoblasts, and L6C11 myoblasts when treated with vibration. Thus, vibration signals of varying intensities are capable of inducing fusion and growth of cultured myoblasts.

Further studies have investigated the effects of LIV on muscle in aging individuals in humans and mice. Three months of whole-body vibration at 12 Hz and 3 mm displacement (1.74 g) in older adults with sarcopenia increased skeletal muscle mass index[37], however six months of local vibration of 30 or 45 Hz and 1.7 or 3.6 g acceleration in postmenopausal women had no effect on muscle mass in the quadriceps[38]. In an exercise study performed in young men, occlusion combined with vibration at 30 Hz and 2.56mm displacement (1.5 g) increased satellite cell activation and myogenin as assessed histologically[112]. Notably, none of the vibration protocols in these studies would be categorized as LIV. The differences in the outcomes and mechanisms implicated in these studies is likely due to the differences in study design and vibration parameters. Nevertheless, it appears that that there is a range of vibration signals that

can induce myogenesis or growth of muscle fibers. However, whether LIV is beneficial for muscle repair following injury remains to be determined.

The regulation of muscle mass is a delicate balance of hypertrophic and atrophic signaling pathways. Akt plays a central role in the regulation of both hypertrophy and atrophy in skeletal muscle[39] and preliminary evidence indicates that this pathway may mediate at least some of the positive effects of vibration in muscle. In the study by Ceccarelli et al using the developmental mouse model, Akt activated by phosphorylation at Ser473, was significantly increased in vibrated mice at days 5, 10, 15, and 20 as compared to control mice[42]. In addition, Wang et al found that inhibition of PI3K, an activator of Akt, suppressed the vibration-induced increase in myotube size, number and fusion[43]. Taken together, Akt is a possible mediator of vibration enhanced muscle growth, however the effects of LIV on muscle repair have yet to be studied.

2.2 Vibration and IGF-1

The effect of vibration on production of IGF-1 is not fully understood. Vibration applied to young men at 30 Hz/1.5 mm displacement, 30 Hz/3 mm displacement, 22 Hz/4mm displacement and women with fibromyalgia at 30 Hz/2mm displacement simultaneously with resistance exercises resulted in no changes in serum IGF-1 levels[50–52]. Vibration of 30 Hz/4 mm displacement in combination with isometric squatting in aged men and women did increase serum IGF-1 levels, however[53]. Another study applying vibration training in combination with isometric squatting increased leg extension force production as well as IGF-1Ec (MGF) and peroxisome proliferator-activated receptor **γ** coactivator 1α (PGC1α) expression in the muscle[113].

Furthermore, local IGF-1 levels increased with LIV application at 45 Hz and 0.4g for 30 min/day and 5 days/week in mouse skin wounds[35]. Due to its involvement with many different hypertrophic and atrophic pathways in muscle, IGF-1 may be a mediator of LIV induced myofiber growth after injury. However, the effect of LIV on IGF-1 levels in muscle remains to be elucidated.

2.3 Vibration, Hormones and other Growth Factors

In addition to IGF-1, muscle repair is mediated by a complex network of other hormones and growth factors that work in both endocrine and/or paracrine fashion. A study looked at the effects of hypoxia and high intensity cycling with and without vibration on angiogenesis by the production of matrix metalloproteinases 2 and 9 (MMP), and VEGF. Degradation of the ECM by MMP-2 and MMP-9 clear space for VEGF to promote new blood vessel formation within the injury. Vibration led to a significant increase in VEGF for both normal and hypoxic oxygen levels compared to the non-vibration group immediately following and up to 4 hours following treatment. MMP-2 and MMP-9 increased for all groups[114], suggesting that cycling alone increased these factors regardless of oxygen levels or the presence of vibration. An additional trend within the test subjects was the effect of training level. Highly trained individuals showed much greater increases in VEGF than those who were less trained. This is a variable that should be controlled for in future studies. Rittweger et al. found opposing results in regard to VEGF. In a vibration plus squat protocol, there was no significant difference in VEGF levels between vibration and control immediately following and up to one hour after treatment[115]. These data suggest that the effect of vibration on angiogenic signaling is complex and future research must

take into account oxygen levels, exercise intensity, activity level, and the current state of inflammation when determining how vibration may play a role.

Growth hormone and testosterone have many functions related to metabolism, growth, and proliferation. A few studies have looked at the effect of vibration on the level of these hormones. Most evidence shows no effect of vibration on testosterone levels[50,53,116,117] with the exception of one study that showed an increase when a very high intensity signal was applied(26 Hz, 4mm displacement, 17g acceleration.)[110] The evidence regarding GH and vibration is more conflicted. Two studies show an increase in GH[110,117], while another showed no change[53]. More studies need to be performed to determine if these anabolic hormones are affected by a vibration stimulus.

Cortisol is a hormone typically released as a stress response in the body. Many researchers have looked at the change in cortisol levels when vibration was applied. This may give insight into the level of stress an individual's response to this treatment. Human and animal studies have shown that vibration increases cortisol[53,118], whereas other human studies have shown decreases[110,117], and another showed no change[116]. This result cannot be explained by variations in intensity as one of the studies showing an increase used an acceleration of 15 g[53] while the other used 0.3 g[118] whereas one of the studies that showed no change in cortisol used 17 g acceleration[110]. The application of exercise, intensity of vibration, duration of vibration, age and sex of the subjects all vary between these studies which likely induce variability in the measured factors.

Vibration may have an effect on hormones and growth factors although findings are still not clear. Repair mechanisms relevant to muscle healing may be IGF-1 and factors effecting

angiogenesis. The effects of vibration on growth factors involved in the repair of traumatic muscle injury remains to be elucidated.

2.4 Vibration and Blood Flow

Adequate circulation is vital to proper healing. Traumatic muscle injury is typically associated with a disruption in blood supply, so restoring muscle perfusion would be beneficial to healing. Several studies have shown that vibration increases blood flow velocity through various arteries of the periphery in humans and pigs[119–123]. Other studies performed in the humans and found increases in microcirculatory blood flow with the application of vibration[108,124–127]. Taken together, the evidence suggests that different intensities of vibration are able to increase blood flow in peripheral circulation and microcirculation This is valuable to the field of traumatic muscle injury because the restoration of blood flow to the injury as well as the capillaries within the damaged muscle is necessary for functional recovery. The effects of vibration on blood flow in muscle has not yet been explored.

2.5 Vibration and Inflammation

A common characteristic of damaged tissues that do not heal well is a persistent inflammatory response. This sustained release of pro-inflammatory cytokines prevents the injury from transitioning into the reparative stages of healing[128]. Few studies have looked at the impact of vibration on inflammation, but understanding this effect is necessary for the treatment of traumatic muscle injury with vibration. A study of male recreational runners looked at the effect of vibration on the progression of delayed onset muscle soreness (DOMS). DOMS is associated with increases in IL-6. The subjects ran downhill for 40 minutes to establish muscle damage and

then went through three 1-minute bouts of vibration passively to the calf and quadricep separated by 45 seconds rest. IL-6 was significantly decreased at 24 hours and 5 days post exercise and neutrophils were significantly reduced at 24 hours post compared to controls[129]. Weinheimer et al. found a trend towards a decrease in inflammatory markers and phenotype in mice treated with low intensity vibration. These mice also showed a decrease in neutrophils at day 7[35]. This evidence suggests a possible reduction in inflammation with an acute bout of vibration to a variety of populations. Conversely, one study performed a vibration plus exercise protocol on older adults over 9 weeks showing no changes in inflammatory markers, including IL-6[130]. This suggests that vibration may only have an acute effect on inflammation rather than long term. Nonetheless, one could speculate that a vibration protocol applied regularly following muscle injury could prove to be beneficial due to this acute anti-inflammatory effect.

2.6 Vibration and Oxygenation

Following tissue damage, metabolism is increased in order to perform the cellular functions required for repair. A few studies have explored the effects of vibrations on local metabolism and tissue oxygenation. Games and Sefton studied how WBV effects tissue oxygenation in the lower leg. They found that WBV applied to healthy subjects at 50 Hz and 2mm amplitude led to an increase in total hemoglobin, but a decrease in oxygenated hemoglobin (O2Hb) compared to baseline values as measured by near-infrared spectroscopy(NIRS)[109]. They claimed this was possibly due to an increase in muscle perfusion and oxygen utilization although this hypothesis would have to be tested further. One study by Zange et al. looked at the effect of vibration on oxygenation of the unloaded lower leg. This study is valuable because it looks at the effect of vibration alone while many other studies only look at vibration when combined with

exercise. Male subjects were exposed to vibration at 15 and 25 Hz with 5 mm displacement. Unlike the study by Games and Sefton, this study found that there was an increase in O2Hb and a decrease in total Hb by near-infrared spectroscopy(NIRS)[131]. The authors claim that the tissue preferentially extruded deO2Hb from the tissue, changing the ratio of Hb in the muscle. With seemingly opposite results, it is interesting to see that these two groups still suggested similar conclusions, that tissue oxygenation was improved.

The evidence regarding oxygenation with vibration and exercise together is as conflicting as the studies without exercise. Coza, Nigg, and Dunn found an increase in oxygen utilization, total hemoglobin, EMG activity, and oxygen recovery of the gastrocnemius medialis (GM) among males performing dynamic calf raises[132]. This study also occluded the artery while applying the vibration. This was an important technique, because without the occlusion, new blood flows in constantly. The new blood brings new oxygen. By cutting off this oxygen, it makes it more feasible to compare the vibration group to the control group without the confounder of blood flow. Rittweger et al. found an increase in tissue oxygenation index (TOI) and O2Hb following vibration compared to the dynamic shallow squat group using NIRS[115]. Yamada et al. studied the effect of WBV on oxygenation of the vastis lateralis (VL.) A squat group was compared to a group that received WBV while they squatted. There was no group that was treated with only WBV. The squat plus WBV group showed a decrease in O2Hb and an increase in total Hb[133]. This study merely suggested that vibration can serve as an efficient training stimulus because of the apparent increased consumption of O2 by the tissue. It seems, however, that to make these claims, one would have to control for more factors. Lastly, Cardinale, Ferrari, and Quaresima found no change in GM or VL oxygenation with squatting exercise and different frequencies of vibration[134].

Zange et al. performed another study that indirectly looked at ATP production rather than tissue oxygenation as a measure of metabolism. This study also occluded the arteries in the lower leg. By measuring phosphocreatine in the muscle of the occluded leg with and without vibration, they found that vibration led to a 60% increase in ATP consumption over the control[135]. This protocol also included 3 minutes of plantar flexion at 40% of the maximal voluntary contraction during the treatment.

The literature is unclear about whether vibration actually increases metabolism. The variety of protocols and outcome measures makes comparison difficult. It is also difficult to know if an increase in metabolism would be affected by an injured environment. In times of severe hypoxia like the rat DTI model by Sari et al., improving oxygenation seemed to improve healing of the injury[136]. Mild hypoxia, however, is sometimes a trigger for angiogenesis through HIF-1α and vascular endothelial growth factor (VEGF) so some amount of hypoxia may be necessary for triggering the proper signaling pathways during the proliferative and remodeling stages of healing. Thus, altering metabolism in the injury is another possible mechanism by which LIV may influence muscle repair.

2.7 Vibration and Skin

Sporadic research has targeted chronic wound healing as a possible area of application for vibration therapy. Mechanical stimulation of the wound via vibration may alter circulation, local metabolism, hormones, growth factors, and inflammation in a non-invasive way. Understanding how vibration benefits skin will prove to be beneficial when exploring LIV-induced mechanisms of muscle repair.

One study explored the effects of vibration on patients with Stage I pressure ulcers (PU). If left untreated, the severity of pressure ulcers worsens and damages the underlying muscle leading to deep tissue injury. Patients were treated with vibration therapy 3 times per day for 15 minutes per treatment at 47 Hz for 7 days or until the PU healed. Significantly more patients healed in 7 days with vibration than the control group. Authors speculate that this was due to improvements in circulation. A similar study from explored the effects of vibration on deep tissue injury in rats. They found improved oxygenation in the injury area indicated by reduced hypoxia inducible factor-1α (HIF-1α.). Additionally, vibration prevented necrosis and the subsequent deterioration of the injury deeper into the muscle.

Two studies performed in skin also found beneficial effects of vibration. Weinheimer et al. conducted a skin wound healing and vibration study on db/db mice that looked at a number of outcomes. The mice that were treated with LIV showed an improvement in wound closure at 15 days post-injury, an increase in granulation tissue thickness and angiogenesis at 7 days post. These improvements coincided with increases in MCP-1, VEGF, and IGF-1[35], all thought to be involved in angiogenesis. This study shows that vibration may have an effect on the healing environment through the production of growth factors. Furthermore, vibration applied locally to the lower leg found that after twelve weeks of treatment, 62% of patients with venous ulcers completely healed compared to 40% in the control group. These studies demonstrate the positive effects of vibration on injuries in skin but the effects of LIV on muscle injury have yet to be explored

3. Conclusion

Traumatic muscle injury significantly decreases quality of life, often leading to permanent disability. Vibration therapy is a promising intervention for the healing of traumatic muscle injury. The non-invasive nature of vibration therapy could be valuable for non-ambulatory patients. This review has presented evidence on how vibration therapy may improve healing factors related to muscle repair. The evidence to date supports LIV-induced increase in myofiber growth and improvement of circulation at the macro and micro levels, although the specific mechanisms that mediate these processes are yet to be elucidated.

III. MANUSCRIPT 1

Low-intensity Vibration Improves Muscle Healing in a Mouse Model of Laceration Injury

(Published in the Journal of Functional Morphology and Kinesiology, 2018, 3, 1; doi:10.3390/jfmk3010001)

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1. Abstract

Recovery from traumatic muscle injuries is typically prolonged and incomplete leading to impaired muscle and joint function. We sought to determine whether mechanical stimulation via whole-body low-intensity vibration (LIV) could 1) improve muscle regeneration and 2) reduce muscle fibrosis following traumatic injury. C57BL/6J mice were subjected to laceration of the gastrocnemius muscle and were treated with LIV (0.2 *g* at 90 Hz or 0.4 *g* at 45 Hz for 30 min/d) or non-LIV sham treatment (controls) for 7 or 14 days. Muscle regeneration and fibrosis were assessed in hematoxylin and eosin or Masson's trichrome stained muscle cryosections, respectively. Compared to non-LIV control mice, myofiber cross-sectional area was larger in mice treated with each LIV protocol after 14 days of treatment. Minimum fiber diameter was also larger in mice treated with LIV of 90 Hz/0.2 *g* after 14 days of treatment. There was also a trend toward a reduction in collagen deposition after 14 days of treatment with 45 Hz/0.4 *g* ($p = 0.059$). These findings suggest that LIV may improve muscle healing by enhancing myofiber growth and reducing fibrosis. The LIV-induced improvements in muscle healing suggest that LIV may represent a novel therapeutic approach for improving the healing of traumatic muscle injuries.

Key words: skeletal muscle injury; laceration; low-intensity vibration; muscle regeneration; fibrosis

2. Introduction

Traumatic muscle injuries are among the most common injuries experienced during military combat. Approximately 70% of combat injuries involve the musculoskeletal system, many of which in recent conflicts have been caused by improvised explosive devices that cause devastating soft tissue injury[2]. Recovery is typically prolonged and incomplete and the inadequate healing response is associated with impaired muscle function, joint stiffness and loss of mobility[3–6]. The impaired healing of traumatic muscle injuries is likely due, in part, to a disruption in blood supply and subsequent ischemia and development of fibrosis. Current therapies include anti-inflammatory strategies and physical therapy. However, we and others have demonstrated that blocking components of the inflammatory response can lead to impaired muscle healing and reduced muscle growth[5,137–142]. In addition, physical rehabilitation in the form of voluntary wheel running has resulted in modest functional improvements and increases in muscle mass associated with upregulation of markers of fibrosis, but no hypertrophy or hyperplasia of muscle fibers after 8 weeks[23]. This indicates that the improvements in function may be due to "functional fibrosis". The lack of significant improvements in muscle function resulting from existing therapeutic approaches indicate that additional therapies are needed.

Skeletal muscle is remarkably sensitive to changes in mechanical loading. Resistance exercise and other forms of mechanical loading increase muscle mass, while reduced loading by immobilization or microgravity lead to muscle atrophy[143–145]. Mechanical stimulation via low-intensity vibration (LIV), defined as vibration with a magnitude less than 1g acceleration, can be considered a physical rehabilitation modality. Whole body mechanical stimulation via LIV has been shown to increase bone and muscle mass in growing mice and to attenuate the loss of bone and muscle during reduced loading situations[32–34]. Furthermore, LIV has been shown to accelerate bone regeneration in a cranial defect in rats[55]. With respect to tissue repair, mechanical stimulation via negative pressure therapy is commonly used to improve skin wound healing, including combat-related blast injuries [146], and we have recently shown that LIV improves

the delayed healing of skin wounds in diabetic mice [35]. However, little is known about the influence of any type of mechanical stimulation on healing of damaged muscle.

We therefore sought to determine whether mechanical stimulation via LIV could improve muscle healing following traumatic injury. We hypothesized that LIV would 1) improve muscle regeneration and 2) reduce muscle fibrosis following traumatic injury in mice. In this manuscript, we present our initial results bearing on these hypotheses.

3. Materials and Methods

Animals

C57BL/6J mice were obtained from Jackson Laboratories and housed individually in a pathogen-free, barrier facility with a 12 hour light/dark cycle at a constant temperature and humidity. Experiments were performed on male mice 11-13 weeks old. Following traumatic injury of the gastrocnemius muscles, mice were randomly assigned to one of three LIV treatment groups (90 Hz, 14 days treatment; n=14 mice, 45 Hz, 14 days treatment; n =18 mice, 90 Hz 7 days treatment; n=6 mice,) or non-LIV control (n=16, n=18, and n=6 mice, respectively) treatment, starting on the day of wounding. Uninjured control mice were also subjected to the LIV protocol (n=3-5 mice). All procedures involving animals were approved by the Animal Care Committee at the University of Illinois at Chicago.

Muscle Injury

Bilateral laceration of the gastrocnemius muscles was used as a model of traumatic injury and was performed as previously described[147]. Briefly, mice were anesthetized and a longitudinal incision was made through the skin on the posterior hindlimb. A scalpel was used to lacerate the lateral gastrocnemius transversely at its widest point from the central neurovascular complex (taking care to

preserve its integrity) to the lateral edge of the muscle which is approximately 4 mm. The laceration goes through the entire thickness of the mid-belly of the muscle which is approximately 2-3 mm thick. The skin was closed, and the procedure was repeated on the contralateral leg. Muscles from injured and non-injured control mice were harvested at the indicated time points.

Whole Body Low-Intensity Vibration

For LIV, mice were placed in an empty cage directly on the vibrating plate and LIV was applied vertically at either 90 Hz with peak acceleration of 0.2 *g* or 45 Hz with peak acceleration of 0.4 *g* for 30 min per day for either 7 or 14 days (Figure 1)[35]. Non-vibrated controls were placed in a separate empty cage but were not subjected to LIV. The mechanical signals were calibrated using an accelerometer attached to the inside surface of the bottom of the cage, so that the signals produced were indeed those transmitted to the feet of the mice. In addition, the amplitude of the vibrations (< 100 μm) are small enough that the cage does not move relative to the plate and the vibrations of the plate and the cage are in sync. The protocols used for this study were chosen based on their ability to induce positive biological effects in animals. The 90 Hz/0.2 g protocol has been used to ameliorate bone loss in rodents[54]. The 45 Hz/0.4 g protocol has been used to accelerate bone regeneration in a cranial defect and improve wound healing in rodents[35,55].

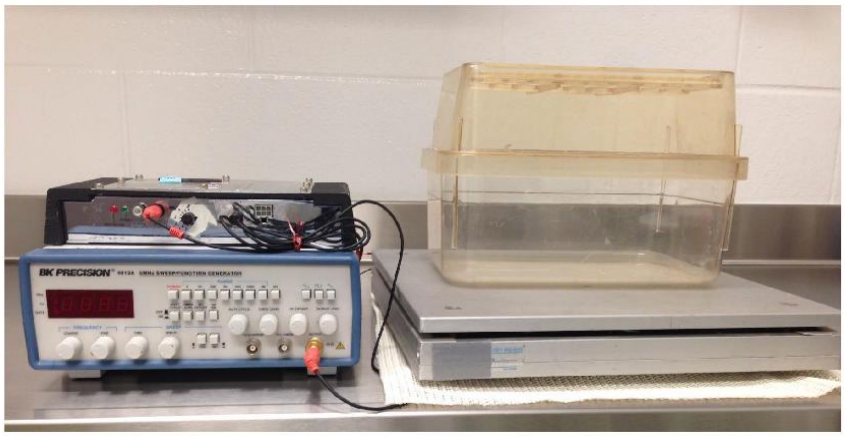

Figure 3.1. *Equipment used to deliver whole-body low-intensity vibration (LIV) to mice.* Mice were placed in an empty cage directly on the vibrating plate, and LIV was applied vertically at either 45 Hz or 90 Hz with peak acceleration of either 0.4 *g* or 0.2 *g* for 30 min/day. The non-vibrated controls were similarly placed in a separate empty cage but were not subjected to LIV.

Histology

Muscle regeneration and fibrosis were assessed by histological analysis as previously described[147]. Gastrocnemius muscles were harvested, embedded in freezing medium and flash frozen in 2-methylbutane cooled on dry ice. Serial transverse 10μm-thick cryosections were taken throughout the entire injured portion of the muscle. Sections with the greatest percentage of damaged, non-regenerated area were then selected for further analysis by staining with hematoxylin and eosin and Masson's trichrome, and immunohistochemistry.

Regeneration was quantified in hematoxylin and eosin-stained sections by morphological analysis on five representative images of each muscle section obtained using a Nikon Instruments Eclipse 80i microscope with a 40x objective, a DS-Fi1 digital camera, and NIS Elements software (Nikon, Melville, NY). Images were taken within the muscle belly and care was taken to avoid extramuscular connective tissue. Fibers were identified as either centrally-nucleated or peripherally-nucleated with no evidence of damage. Centrally-nucleated fibers likely represent both fibers that have undergone denervation and those

in the process of regeneration[148]. Percent of total fibers that were classified as centrally- or peripherally-nucleated were then quantified using ImageJ (NIH). Damaged area was quantified by subtracting the area of all fibers from the total area within the field of view.

Collagen accumulation was quantified using Masson's trichrome staining. Three to six 20x images were taken of the injured site in each muscle using a 20x objective on an Eclipse 80i microscope with DS-Fi1 camera and NIS-Elements BR software. Masson's trichrome stains muscle fibers red, nuclei black, and collagen blue. Collagen accumulation was quantified as the percent of the total image area stained blue.

Platelet endothelial cell adhesion molecule-1 (PECAM-1), a marker for angiogenesis, was identified using anti-mouse CD31 antibody (clone 390; 1: 100 in PBS; BioLegend, Inc., San Diego, CA, USA); whereas macrophage accumulation was assessed using an anti-mouse F4/80 antibody (clone BM8; 1: 100 in PBS; eBioscience, Inc., San Diego, CA, USA). Slides serving as negative controls received PBS instead of primary antibody. Briefly, sections were air-dried, fixed in cold acetone, washed with PBS, quenched with 0.3% hydrogen peroxide, and washed with PBS. Sections were blocked with buffer containing 3% bovine serum albumin and then incubated with primary antibody for 1 hr at room temperature and then overnight at 4°C. Sections were washed in PBS, incubated with biotinylated mouse adsorbed anti-rat IgG (1: 200 in PBS; Vector Laboratories Inc., Burlingame, CA, USA) followed by avidin D horseradish peroxidase (1:1000 in PBS). Sections were then developed with 3, 3'-diaminobenzidine (ImmPACT DAB, Cat. No. SK-4105; Vector). Three to six 20x images were taken of the injured site in each muscle using a 20x objective on an Eclipse 80i microscope with DS-Fi1 camera and NIS-Elements BR software. Angiogenesis and macrophage accumulation were quantified as the percent of the total image area stained using ImageJ (NIH).

Statistics

Values are reported as means ± standard error. Data were tested for homoscedasticity and those passed were compared using two-sided t tests and those that did not pass were compared using the nonparametric Mann-Whitney test. Differences between groups were considered significant if $P \leq 0.05$. Graphpad Prism Version 7.00 was used to generate all figures.

4. Results

LIV Protocol using 90 Hz and 0.2g for 14 day treatment period

Body mass (27.3 ± 1.3 vs. 27.1 ± 1.1 g; $p>0.05$) was not different between LIV and non-LIV groups, suggesting that mice tolerated the LIV protocol well. Consistent with our hypothesis, LIV treatment at 90 Hz/0.2 g improved healing of lacerated gastrocnemius muscle at day 14 post-injury **(Figure 2 A, B)**. Compared to non-LIV but injured control mice, both the minimum fiber diameter and the cross-sectional area of individual myofibers were significantly larger in mice treated with LIV at 14 days post-injury (**Figure 2 C, D**). When centrally-nucleated and peripherally-nucleated myofibers were assessed separately, minimum fiber diameter of both were significantly larger in LIV treated mice (**Figure 2 E**), whereas cross-sectional area showed only a trend in this direction **(Figure 2 F)**. In contrast to the increase in muscle fiber size, the percent area occupied by centrally-nucleated myofibers and peripherally-nucleated myofibers was not different between LIV-treated and non-LIV control mice, however there may be a trend towards a decrease in damaged area with LIV ($p = 0.198$) (**Figure 2 G**).

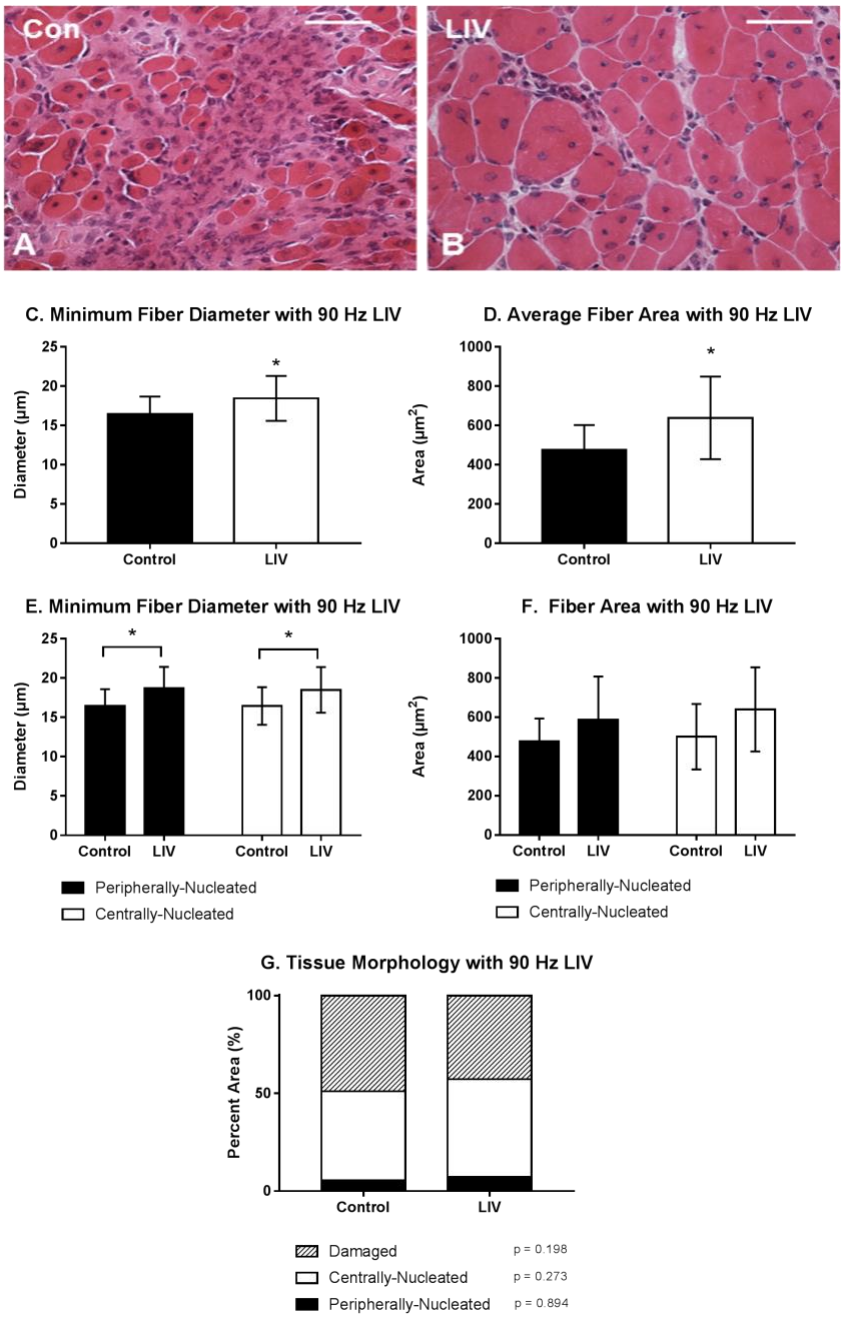

Figure 3.2. ***Low-intensity vibration (LIV) at 90 Hz and 0.2 g enhances myofiber growth at day 14 post-injury following laceration muscle injury in mice***. Gastrocnemius muscles were lacerated and collected for histological analysis at day 14 post-injury. (A, B) Representative images of hematoxylin and eosin-stained sections (scale bar = 50 μm, 40x magnification). (C, E) Average minimum myofiber diameter, (D, F) average cross-sectional area of individual myofibers, and (G) percent area of injury that consists of peripherally-nucleated fibers, centrally-nucleated fibers, or damaged tissue was quantified in five 40x fields per muscle in hematoxylin and eosin-stained sections. (C and D) All fiber types averaged together. (E and F) Myofibers grouped by type. Data are presented as mean ± SE. $*P \leq 0.05$.

LIV Protocol using 45 Hz and 0.4g for 14 day treatment period

LIV treatments at 45 Hz/0.4 g also improved healing of lacerated gastrocnemius muscle **(Figure 3 A, B)**. Compared to non-LIV but injured control mice, the cross-sectional area but not the minimum fiber diameter of individual myofibers was larger in mice treated with LIV at 14 days post-injury **(Figure 3 C, D)**. When centrally-nucleated and peripherally-nucleated myofibers were assessed separately, cross-sectional area of both but not minimum fiber diameter was significantly larger in LIV treated mice (**Figure 3 E, F)**. There was no significant difference in the percent area occupied by centrally-nucleated myofibers or damaged area between LIV-treated and non-LIV control mice, however there may be a trend towards an increase in percent area of peripherally-nucleated fibers with LIV ($p = 0.2$) **(Figure 3 G)**. Considering the increases in fiber diameter and/or area between each of the LIV protocols, these morphological data suggest that LIV may not influence the formation of regenerating fibers, but instead enhances myofiber growth after formation.

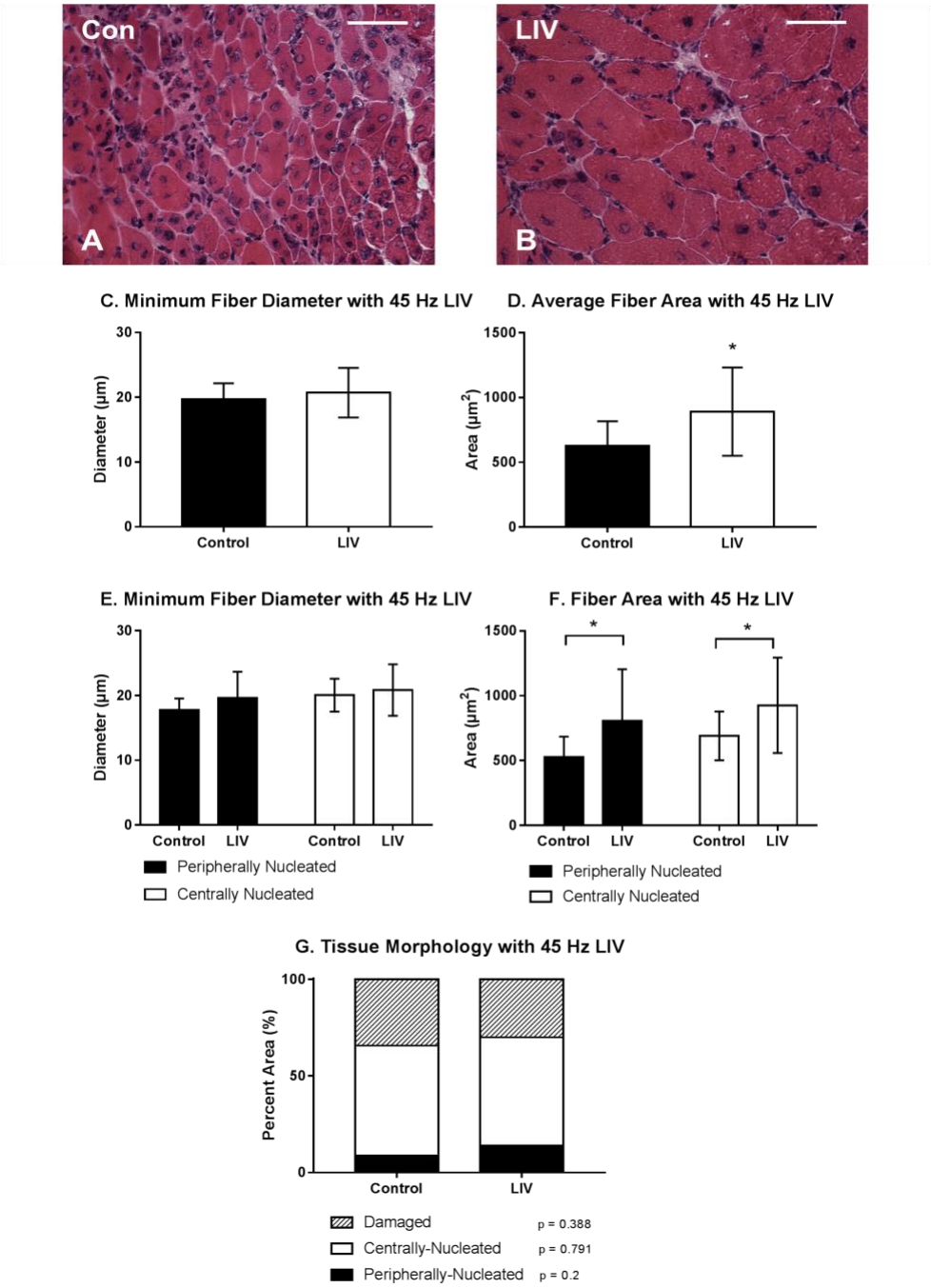

Figure 3.3. ***Low-intensity vibration (LIV) at 45 Hz and 0.4 g enhances myofiber growth following laceration muscle injury in mice at 14 days post-injury***. Gastrocnemius muscles were lacerated and collected for histological analysis at day 14 post-injury. (A, B) Representative images of hematoxylin and eosin-stained sections (scale bar = 50 μm, 40x magnification). (C, E) Average minimum myofiber diameter, (D, F) average cross-sectional area of individual myofibers, and (G) percent area of injury that consists of peripherally-nucleated fibers, centrally-nucleated fibers, or damaged tissue was quantified in five 40x fields per muscle in hematoxylin and eosin-stained sections. (C and D) All fiber types averaged together. (E and F) Myofibers grouped by type. Data are presented as mean ± SE. *$P \leq 0.05$.

Effects of LIV on Fibrosis for 14 day treatment

Since lacerated gastrocnemius muscle heals by a combination of regeneration and fibrosis, we also assessed the effects of LIV on muscle fibrosis. Trichrome staining in muscle cryosections revealed a trend of reduced collagen deposition in mice treated with 45 Hz/0.4 g LIV vs. non-LIV controls on day 14 following injury. This same effect was not replicated with the 90 Hz/0.2 g LIV protocol (**Figure 4 A-C**). When considered alongside the LIV-induced increase in myofiber cross-sectional area, these findings suggest that LIV, at least the 45 Hz protocol, may improve muscle healing by enhancing myofiber growth and reducing fibrosis.

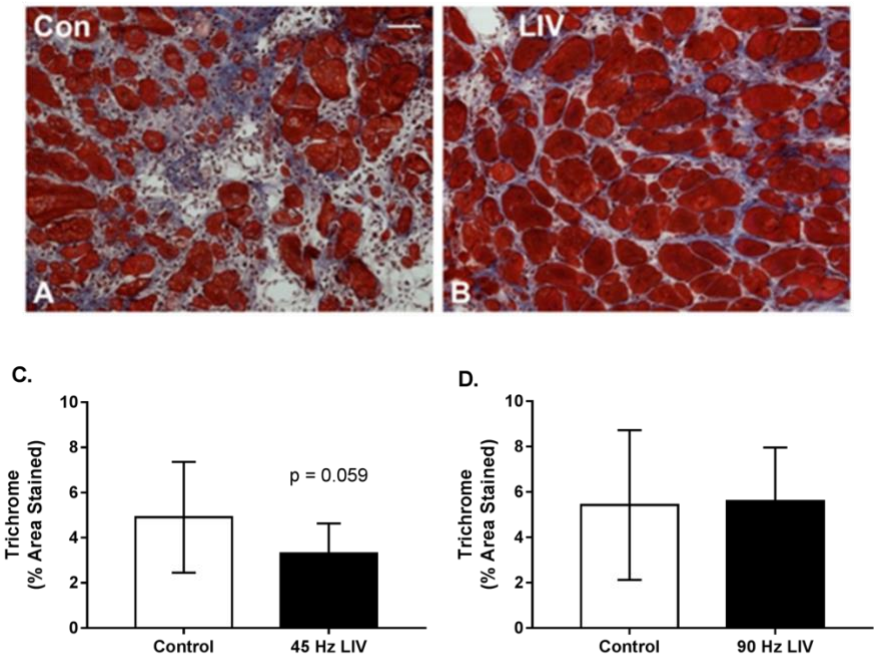

Figure 34. ***Fibrosis may be reduced in lacerated muscle following low-intensity vibration***. (A and B) Representative images of trichrome-stained sections at day 14 following laceration of the gastrocnemius muscles (scale bar = 50 μm, 20x magnification). (C, D) Collagen accumulation was quantified as percent blue pixels in three to six 20x fields per muscle in Masson's trichrome-stained sections. Data are presented as mean ± SE. $*P \leq 0.05$.

LIV Protocol using 90 Hz and 0.2 g for 7 day treatment period

Since LIV increased muscle fiber size and tended to reduce fibrosis after 14 days of treatment (**Figures 2-4**), the experiment was repeated and muscles were harvested at day 7 to determine whether LIV induces early improvements in muscle regeneration. After 7 days of treatment, LIV at 90 Hz/0.2 g did not noticeably improve healing of lacerated gastrocnemius muscle **(Figure 5 A, B)**. Compared to non-LIV but injured control mice, both the minimum fiber diameter and the cross-sectional area of individual myofibers were not different in mice treated with LIV at 90 Hz/0.2 g on day 7 post-injury (**Figure 5 C, D**). When centrally-nucleated and peripherally-nucleated myofibers were assessed separately, neither minimum fiber diameter, fiber area, nor morphological characteristics were different between treatment groups (**Figure 5 E-G**). Additionally, no differences were found in markers for angiogenesis or macrophage accumulation as assessed histologically by staining with CD31 and F4/80, respectively (**Figure 6**). Taken together, these data indicate that LIV does not influence the early regenerative phase of healing and instead improves healing through an influence on the remodeling phase of healing. Alternatively, 7 days of LIV treatment may not be sufficient to induce observable improvements in the healing process.

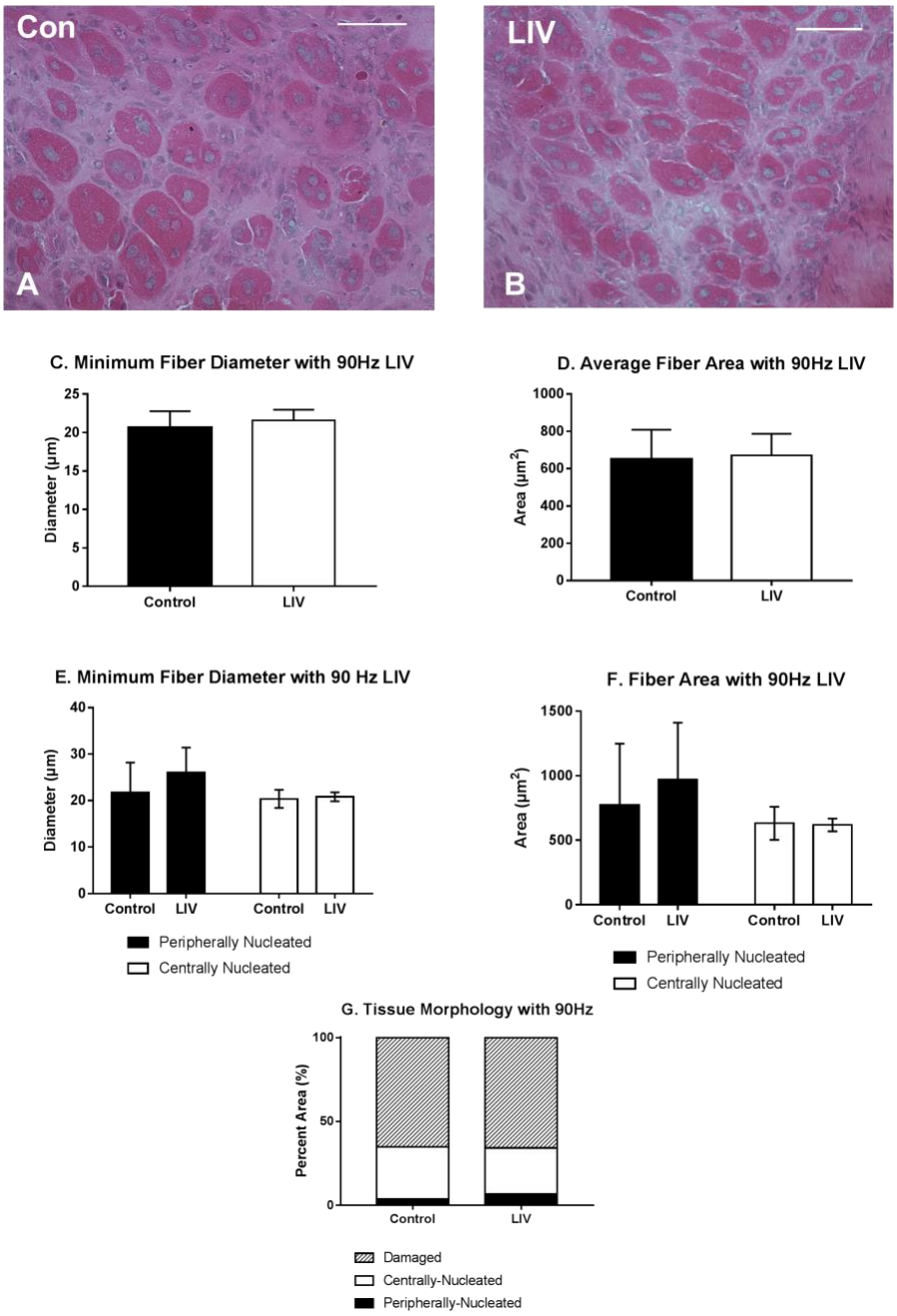

Figure 3.5. ***Low-intensity vibration (LIV) at 90 Hz and 0.2 g does not influence muscle regeneration on day 7 post-injury following laceration muscle injury in mice***. Gastrocnemius muscles were lacerated and collected for histological analysis at day 7 post-injury. (A, B) Representative images of hematoxylin and eosin-stained sections (scale bar = 50 µm, 40x magnification). (C, E) Average minimum myofiber diameter, (D, F) average cross-sectional area of individual myofibers, and (G) percent area of injury that consists of peripherally-nucleated fibers, centrally-nucleated fibers, or damaged tissue was quantified in five 40x fields per muscle in hematoxylin and eosin-stained sections. (C and D) All fiber types averaged together. (E and F) Myofibers grouped by type. Data are presented as mean ± SE. $*P \leq 0.05$.

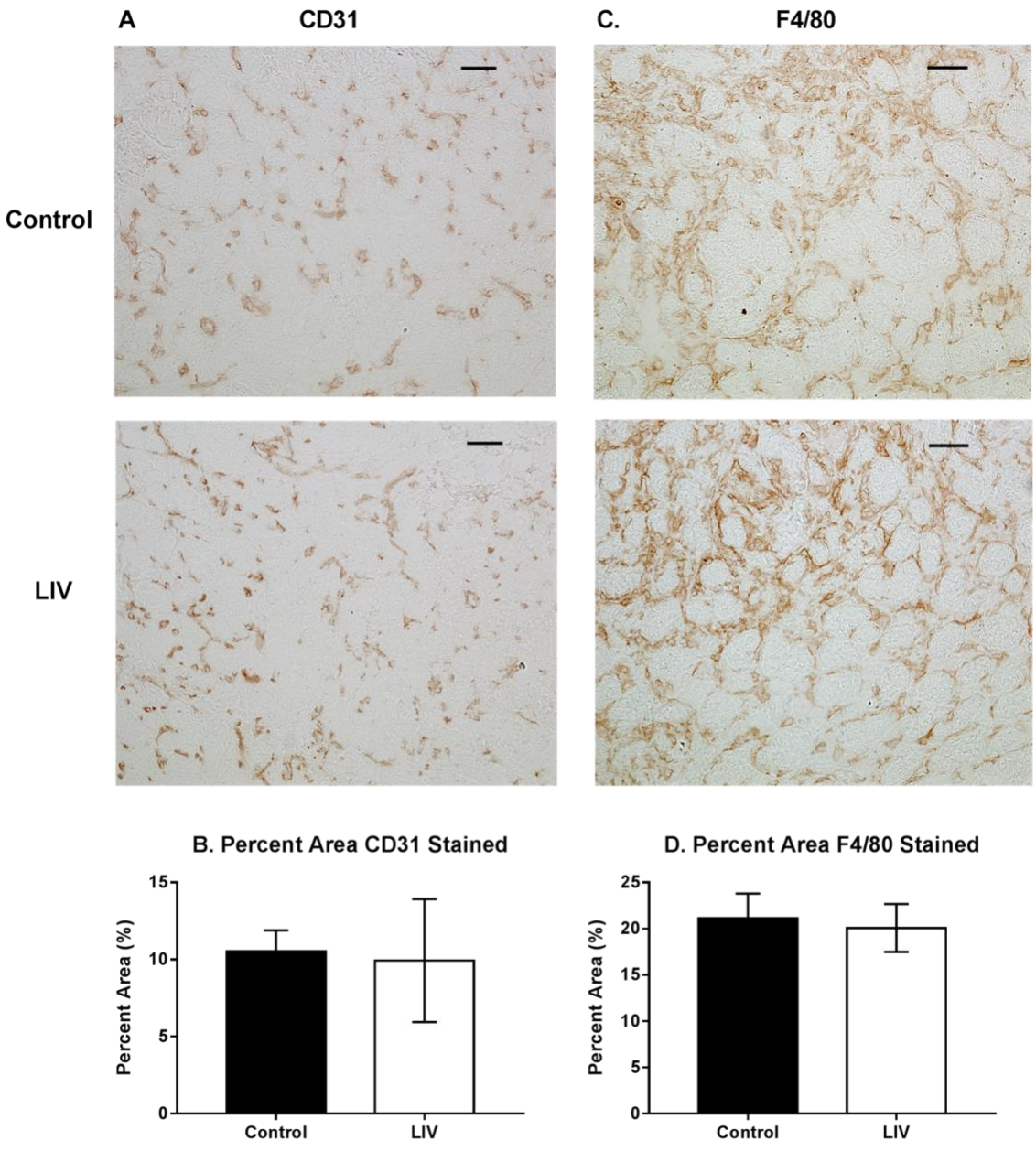

Figure 3.6. ***Effects of Low-intensity vibration (LIV) at 90 Hz and 0.2 g on angiogenesis (CD31) and macrophage accumulation (F4/80) at day 7 post-injury following laceration muscle injury in mice***. Gastrocnemius muscles were lacerated and collected for histological analysis at day 7 post-injury. (A) Representative images of CD31-stained sections (scale bar = 50 μm, 20x magnification). (B) Percent area that stained positive for CD31. (C) Representative images of F4/80-stained sections (scale bar = 50 μm, 20x magnification). (D) Percent area that stained positive for F4/80. Data are presented as mean ± SE. N=6 per group.

Effects of LIV on uninjured muscle

Interestingly, in uninjured mice, LIV did not increase average myofiber cross-sectional area after 20 days of LIV treatments, suggesting that the beneficial effects of this LIV protocol do not accrue to non-injured skeletal muscle but likely require prior muscle damage and subsequent regeneration (**Figure 5**).

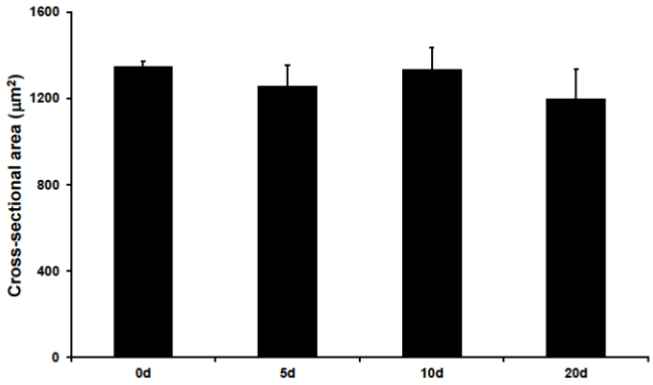

Figure 3.7. ***Low-intensity vibration does not enhance myofiber size in uninjured mice***. Uninjured control mice were subjected to the LIV protocol and gastrocnemius muscles were collected at the indicated time points. Average cross-sectional area of individual myofibers was quantified in five 40x fields per muscle in hematoxylin and eosin-stained sections. Data are presented as mean ± SE.

5. Discussion

Unlike toxin- or exercise-induced muscle injuries, recovery from traumatic muscle injuries is typically prolonged and incomplete[2,149,150], resulting in permanent impairments of muscle and joint function[3–6]. This impaired healing results in significant costs for rehabilitation, loss of time for work and reduced combat readiness in military personnel[149]. Thus, effective therapies for promoting healing of traumatic muscle injuries are needed. Interestingly, mechanical stimulation via LIV has been shown to ameliorate bone loss and to enhance bone regeneration[32–34,55]. Furthermore, we have recently shown that LIV improves the delayed healing of skin wounds in diabetic mice[35]. However, little is known about the effects of LIV on the healing of damaged muscle. We therefore determined whether mechanical stimulation via LIV could

1) improve muscle regeneration and 2) reduce muscle fibrosis following traumatic injury in mice. Our findings provide evidence that LIV indeed improves muscle repair by influencing the remodeling phase of healing.

To our knowledge, this is the first study to assess the effects of mechanical stimulation via LIV on muscle healing. We observed larger myofiber size in mice that received LIV treatment protocols at 90 Hz/0.2 g or 45 Hz/0.4 g for 14 days post-injury, but not for 7 days post-injury, compared to non-LIV controls. LIV did not promote myofiber hypertrophy after 20 days of LIV treatments in uninjured mice. These findings indicate that prior muscle damage and subsequent regeneration is likely required for the beneficial effects of LIV. While the pathways that modulate the cellular response to LIV remain to be elucidated, we can speculate that LIV may exert local and/or systemic effects and that these effects are likely at later stages of healing since improvements were not seen until 14 days post-injury. LIV may increase fiber size via direct mechanical effects on muscle cells, since muscle is particularly sensitive to mechanical stimuli, or indirectly via the production of cytokines and growth factors that promote muscle growth. Alternatively, it is well documented that LIV can be anabolic to bone, and thus, LIV may promote the mobilization and/or homing of bone marrow-derived cells to the injured tissue. These cells include progenitor cells and monocytes/macrophages, which are important during tissue repair as they release growth factors and cytokines that promote tissue healing[137,138,142,151]. Our findings suggest that LIV may have anabolic effects on regenerating muscle and elucidating mechanisms underlying the local and/or systemic effects of LIV warrants further investigation.

The development of fibrosis likely contributes to the impaired healing of traumatic muscle injuries. As such, experimental therapeutic approaches have attempted to improve healing by blocking actions of transforming growth factor (TGF)-β1 and the associated fibrosis; these anti-fibrotic agents have included suramin, interferon (IFN)-**γ**, decorin and losartan[152–155]. While these agents have shown promise in animal

studies in reducing fibrosis and improving regeneration following traumatic muscle injury, many of these agents have serious side effects and would likely not be an option for treating muscle injuries. In the current study, the trend of reduced collagen deposition following injury in the LIV-treated mice at 14 days post-injury with the 45 Hz protocol suggests that LIV may serve as a safe, non-pharmacological therapy for reducing fibrosis. Because LIV was initiated within hours of the injury, our findings suggest that LIV may be effective in attenuating or preventing fibrosis. Whether or not LIV can reverse fibrosis after it has been established warrants further investigation.

One reason that healing is impaired in models of traumatic injury (such as laceration) compared to other injury models (such as toxin-induced injury) may be the disruption of blood supply to the muscle. Thus, improving perfusion of damaged muscle may be an additional mechanism by which LIV can improve healing. Although LIV did not improve CD31 staining, a marker for angiogenesis, at day 7 in the current study, we have recently shown that LIV improves the delayed healing of skin wounds in diabetic mice, which was associated with an increase in CD31 staining[35]. LIV can also acutely increase blood flow in the skin of the ear of hairless mice, the skin of the dorsal side of the lower leg of healthy human subjects, and the skin of the underside of the forearm of both healthy and Type 2 diabetic human subjects[107,124,156]. Furthermore, nitric oxide (NO) is well-known for its vasodilatory effects. Serum nitrite levels, a marker for NO signaling, increases with the application of LIV in juvenile pigs[120,157]. L-NAME, a NO synthase inhibitor, blocked the LIV induced increase in skin blood flow in the ear of hairless mice[120,156,157]. LIV has been shown to improve healing of pressure ulcers in humans by upregulating NO and improving blood supply[158]. Relatedly, LIV also slowed the progression of pressure ulcers into deep tissue injury in a rat model[136]. Thus, future studies should further investigate the influence of LIV on blood vessel formation and perfusion of damaged skeletal muscle.

Our study is limited in that the effects of LIV on muscle healing were only assessed at two time points. Skeletal muscle repair following injury occurs in four overlapping phases: hemostasis, inflammation, new muscle fiber formation and subsequent remodeling. Our findings are likely relevant to the remodeling phase as we have previously shown that new myofiber formation predominates during the first two weeks following muscle laceration, while myofiber maturation and collagen deposition typically occur thereafter[147]. We are currently performing a time course study that investigates the effect of LIV on muscle healing during each of the different phases of healing. This study is also limited in that mechanisms underlying LIV-induced improvements in healing were not thoroughly investigated. The purposes of this initial study were to evaluate whether or not whole-body LIV could be a feasible and effective strategy for improving healing of a traumatic muscle injury and whether varying LIV parameters (frequency and amplitude) had an impact on improving healing of a traumatic muscle injury. Further optimization of the LIV protocol may yield even better results. Now that we have established LIV as a potential therapeutic strategy for muscle healing, mechanistic studies are ongoing. Finally, this study is limited by the lack of assessment of muscle functional recovery. We plan to determine the effect of LIV on the time course of functional recovery in a future study.

In summary, our findings are consistent with our hypothesis that LIV improves muscle regeneration and reduces fibrosis following traumatic injury. Thus, LIV may provide a novel, non-pharmacological therapeutic approach for improving the prolonged and incomplete healing typically seen with these injuries. The LIV protocol used in this study is simple, inexpensive and safe; at the amplitude employed (<100 μm), the vibration is barely perceptible to human touch. Furthermore, the LIV protocol could be easily translated to use for human studies, since the equipment utilized has already been used to ameliorate bone loss in human subjects[33,159–162].

6. Acknowledgments

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7. Contribution of Authors

TK and SJ conceived and designed the experiments; TC and EW performed the experiments; TC, EW, TK and SJ analyzed the data; TC, EW, TK and SJ wrote the paper.

8. Conflicts of Interest

TJK and SJ have a patent pending regarding the application of vibrations for therapeutic treatment. TC and EW certify that he or she, or a member of his or her immediate family, has no funding or commercial associations (e.g., consultancies, stock ownership, equity interest, patent/licensing arrangements, etc.) that might pose a conflict of interest in connection with the submitted article.

IV. MANUSCRIPT 2

Manuscript 2 Title: Improved healing of laceration injury in mice by Low-Intensity Vibration is transduced locally by muscle cells

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1. Abstract

Recovery from traumatic muscle injuries is typically prolonged and incomplete leading to impaired muscle and joint function and whole-body low-intensity vibration (LIV) enhances healing in a mouse laceration model. We sought to determine whether mechanical stimulation via locally-applied LIV could 1) improve muscle regeneration following injury in mice, 2) increase differentiation in cultured myotubes, 2) and if these effects were associated with IGF-1. C57BL/6J mice were subjected to laceration of the gastrocnemius muscle and were treated with LIV applied to the lower leg for 30 min/day or non-LIV sham treatment (controls) for 7 or 14 days. LIV was also applied to differentiating myotubes in culture for 30 min/day or not vibrated for 3 or 6 days. Muscle morphology, myotube morphology, protein concentration, and mRNA expression were assessed. Compared to non-LIV control mice, myofiber cross-sectional area, diameter, and % area of peripherally-nucleated fibers increased while % damaged area decreased in mice treated with local LIV after 14 days of treatment. Cultured myotubes exposed to LIV had increased fusion and diameter after 6 days. Although these changes were not associated with any changes in IGF-1, total Akt increased at day 7 in injured muscle and day 3 in cells, while phosphorylated to total Akt ratio increased at day 14 in injured muscle and day 6 in cells when treated with LIV. These changes were also associated with LIV-induced suppression of FOXO1 and Atrogin-1 gene expression at day 7 in injured muscle. These findings demonstrate that muscle cells directly transduce local LIV signals into increased growth and differentiation, and this effect is associated with increased Akt signaling.

Key words: skeletal muscle injury; laceration; low-intensity vibration; muscle regeneration; signal transduction

2. Introduction

Traumatic muscle injuries are a devastating consequence of motor vehicle accidents, limb salvage surgeries for the removal of tumors[1] and military combat[2]. They are characterized by a significant loss of tissue and inadequate healing leading to impaired muscle function, joint stiffness, and loss of mobility[3–6]. The regenerative capacity of muscle is highly dependent on the severity of the injury with less severe injuries able to regenerate fully and more severe injuries resulting in functional deficits due to denervation and scar formation. The only current clinical option for traumatic muscle injury is autologous muscle transfer using muscle flaps or vascularized tissue[163]. These surgical procedures, however, are technically difficult, have limited success and result in donor site morbidity[1]. Due to these limitations, experimental approaches in the form of tissue engineered xenotransplants made from decellarized tissue[164] and cell therapy[165] are currently being explored. Thus, there is an urgent need for new therapies that can restore functional muscle tissue following traumatic injury.

Physical therapy can be an adjunct treatment for patients with traumatic muscle injury. Skeletal muscle is known to respond to changes in mechanical loading with changes in muscle mass; resistance exercise can cause muscle hypertrophy, while unloading leads to atrophy[143–145]. Standard of care for patients with less severe muscle injuries, such as sports-related strains, involves early mobilization of the muscle[21,22]. Unfortunately, patients suffering from traumatic muscle injury often have limited use of the affected limb and limited mobility due to the severity of the injury and accompanying pain. Alternate forms of physical therapy that do not require active muscle force production by the patient and that can be applied passively could overcome this obstacle to physical therapy treatment. Low-intensity vibration (LIV) defined as a vibration signal with a magnitude less than 1g acceleration represents such a passive mechanical therapy[81]. We have shown that LIV applied to the whole-body via a vibrating plate daily to mice recovering from laceration injury increases myofiber size by day 14 compared to

sham LIV, injured controls [81]. However, little is known about which cells that respond to this stimulus or the mechanisms involved.

Insulin-like growth factor 1 (IGF-1) is a well-known regulator of skeletal muscle mass via activation of Akt/mTOR signaling and the downregulation of myostatin signaling[39]. Muscle hypertrophy is associated with increased IGF-1 expression in models of stretch-induced hypertrophy[44], overload-induced hypertrophy[45], and compensatory hypertrophy[46] in rodents and transgenic mice overexpressing IGF-1 specifically in muscle showed an increase in muscle size[47]. In addition, two isoforms, IGF-1Ea, a muscle-specific isoform, and mechanogrowth factor (MGF) are upregulated in muscle after mechanical stimulation; both isoforms increased within 4 days of stretch in rabbit EDL muscles[48] and in older humans on a prolonged resistance exercise program[49]. Thus, the expression of local IGF-1 appears to depend on mechanical signals received by the muscle and may play a role in mechanical load induced changes in muscle mass.

The purpose of this study was to explore the mechanism(s) by which LIV improves muscle healing following laceration injury and if muscle cells themselves directly transducing these mechanical signals into increased growth. We hypothesized that locally-applied LIV would (1) improve muscle regeneration similar to whole-body application, (2) enhance growth and fusion of cultured myotubes, and (3) increase levels of IGF-1 and associated downstream markers of hypertrophy in muscle and cells. In this manuscript, we present our results bearing on these hypotheses.

3. Methods and Materials

Animals

C57BL/6J mice were obtained from Jackson Laboratories and housed individually in a pathogen-free, barrier facility with a 12 hour light/dark cycle at a constant temperature and humidity. Experiments were performed on male mice 11-13 weeks old. Following traumatic muscle injury, mice were randomly assigned to the LIV treatment group or the non-LIV sham control group, starting on the day of wounding. All procedures involving animals were approved by the Animal Care Committee (17-067) at the University of Illinois at Chicago.

Muscle Injury

Bilateral laceration of the gastrocnemius muscle was used as a model of traumatic injury and was performed as previously described[147]. Briefly, mice were anesthetized with isoflurane, and a longitudinal incision was made through the skin on the posterior of the leg to expose the gastrocnemius muscle. A scalpel was used to lacerate the lateral gastrocnemius transversely at its widest point from the central neurovascular complex (taking care to preserve the integrity of this complex) to the lateral edge of the muscle. The laceration goes through the entire thickness of the mid-belly of the muscle which is approximately 2-3 mm thick. The skin was closed, and the procedure was repeated on the contralateral leg. For histological assays, gastrocnemius muscles were harvested, embedded in freezing medium and flash frozen in 2-methylbutane cooled on dry ice. Serial transverse 10μm-thick cryosections were taken throughout the entire injured portion of the muscle. Sections with the greatest percentage of damaged, non-regenerated area were then selected for further analysis by staining with hematoxylin and eosin. For experiments involving protein and RNA analysis, muscles were stored in liquid nitrogen until processing.

Locally-applied Low-Intensity Vibration

For in vivo LIV treatment, mice were anesthetized with isoflurane and placed in a supine position with their feet attached directly to the actuator of the vibration device (Figure 4.1). LIV was applied horizontally at a frequency of 90 Hz and peak acceleration of 0.2 *g* (amplitude < 0.1 mm) for 30 min per day for either 7 or 14 days (Figure 4.1). Non-vibrated sham controls were treated identically but were not subjected to LIV. For in vitro LIV treatment, cell culture plates were vibrated with the same parameters using specially designed plate holders (Figure 4.2). For cell stimulation, LIV was applied daily for either 3 or 6 days. LIV signals were calibrated using an accelerometer attached directly to actuator of the vibration device. The combination of 90 Hz frequency and 0.2 g acceleration was chosen because it has been used to improve healing following muscle injury[81] and ameliorate bone loss in rodents[54].

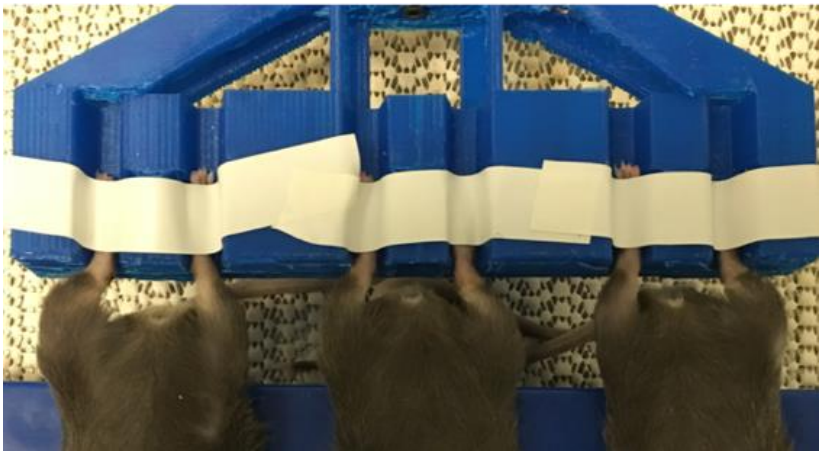

Figure 4.1. *Equipment used to deliver local low-intensity vibration (LIV) to mice.* Mice were anesthetized and placed in a supine position with their feet attached directly to the actuator of the vibration device. LIV was applied horizontally at 90 Hz with peak acceleration of 0.2 *g* (amplitude < 0.1 mm) for 30 min/day. The non-vibrated controls were treated identically but were not subjected to LIV.

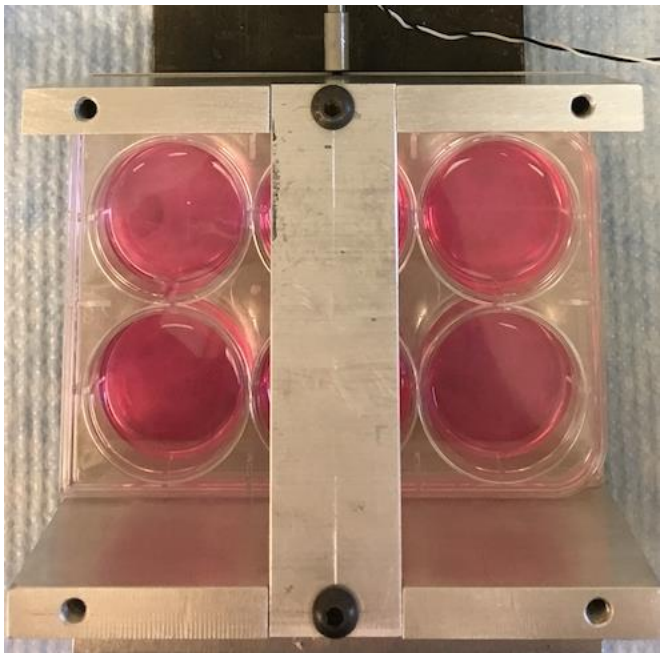

Figure 4.2. *Equipment used to deliver low-intensity vibration (LIV) to cell culture plates.* The same device was used to deliver LIV to cells. Cell culture plates were place on the portion of the device specifically designed to fit culture plates. LIV was applied horizontally at 90 Hz with peak acceleration of 0.2 *g* for 30 min/day. The non-vibrated controls were treated identically but were not subjected to LIV.

Histology

Muscle regeneration was assessed by histological analysis as previously described[147]. Regeneration was quantified in hematoxylin and eosin-stained sections by morphological analysis on five representative images of each muscle section obtained using a Nikon Instruments Eclipse 80i microscope with a 40x objective, a DS-Fi1 digital camera, and NIS Elements software (Nikon, Melville, NY). Images were taken within the muscle belly and care was taken to avoid extramuscular connective tissue. Fibers were identified as either centrally-nucleated or peripherally-nucleated with no evidence of damage. Centrally-nucleated fibers likely represent both fibers that have undergone denervation and those in the process of regeneration[148]. Percent of total fibers that were classified as centrally- or peripherally-nucleated were then quantified using ImageJ (NIH). Damaged area was quantified by subtracting the area of all fibers from the total area within the field of view.

ELISA

Upon collection, injured muscle tissue was snap frozen in liquid nitrogen. Samples were homogenized in ice-cold buffer (40mM Tris (pH 7.5), 1 mM EDTA, 5 mM EGTA, 0.5% Triton X-100, 25 mM β-glycerophosphate, 25 mM NaF, 1 mM Na3VO4, protease inhibitor cocktail) using a dounce homogenizer. After centrifugation, supernatants were diluted 1:4 and protein concentrations for IGF-1 were determined by enzyme-linked immunoassay (R&D Systems, Minneapolis, MN, USA).

Cell culture

C2C12 mouse myoblasts (CRL-1772; American Type Culture Collection, Manassas, VA, USA) were grown on tissue culture treated plates and maintained in DMEM (#10-014-CV, Corning, Manassas, VA, USA) containing 10% FBS in a humidified incubator at 5% CO2 and 37 degrees C. All experiments were performed with cells between passage 5 and 7. For all experiments, cells were seeded at a density of 10^4 cells/cm^2. Cells were maintained in DMEM with 10% FBS (#26140; Gibco, Grand Island, NY, USA) until they were 90 to 100% confluent (approximately 48 hours). Culture medium was then changed to DMEM with 2% horse serum (#26050; Gibco, Grand Island, NY, USA) to induce differentiation of myoblasts into myotubes. Immediately following the change in media, cells were treated daily with LIV for 30 minutes at room temperature or treated identically without LIV for the indicated time points.

Immunocytochemistry

Cells were stained directly on culture plates. Cells were washed with cold PBS, fixed with 4% paraformaldehyde for 20 minutes, permeabilized and blocked in 0.1% Triton X-100 X% bovine serum albumin (BSA), incubated at room temperature for 1 hour with primary antibody for myosin heavy chain (MHC) (MF-20; Developmental Studies Hybridoma Bank, Iowa City, IA, USA), washed with PBS, incubated at room temperature with FITC-conjugated goat anti-mouse secondary antibody (Jackson Immunoresearch), washed with PBS, and mounted in mounting medium with DAPI (Vectashield, H-1200; Vector, location).

Myotube size and fusion were quantified in wells double stained for MHC (MF20) and nuclei (DAPI) by morphological analysis on five representative images of each sample obtained using a Keyence BZ-X710 All-in-One Fluorescence Microscope with a 20x objective. Images were analyzed using ImageJ (NIH, Bethesda, MD, USA). Measurements were taken for total nuclei count, myotube count (# of MHC+ cells with 2+ nuclei), myotube nuclei count (# of nuclei in MHC+ cells that contain 2+ nuclei), and diameter. Diameter measurements were taken by averaging 3 separate measurements taken along the entire length of the myotube. Fusion index is defined as the ratio of myotube nuclei to total nuclei per field.

SDS-PAGE and Western Blotting

Injured muscle samples were homogenized in ice-cold buffer (40mM Tris (pH 7.5), 1 mM EDTA, 5 mM EGTA, 0.5% Triton X-100, 25 mM β-glycerophosphate, 25 mM NaF, 1 mM Na3VO4, protease inhibitor cocktail) using a dounce homogenizer. Cells were collected in the same buffer using a cell scraper. All tissue homogenates and cell lysates were centrifuged, and protein concentration was

determined using Pierce 660nm Protein Assay Kit (Thermofisher Scientific, Waltham, MA, USA). Homogenates were prepared with equal protein concentrations and then dissolved in 4x Laemmli buffer (#1610747; BioRad, Hercules, CA, USA) and heated at 95 degrees C for 5 minutes. Samples were immediately subjected to separation via SDS-PAGE, transferred to a nitrocellulose membrane, blocked with Odyssey TBS blocking buffer (LI-COR Biosciences, Lincoln, NE, USA) for 1 hour at room temperature, incubated overnight with primary antibody (Akt, #9272; Phospho-Akt (S473), #9271; p70S6K, #9202; Phospho-p70S6K (T389), #9205; Cell Signaling Technology, Inc.; β-Tubulin I, T7816, Sigma Aldrich) in blocking buffer at 4 degrees C, washed in TBS containing 0.1% Tween 20 (TBST), incubated for 30 minutes in secondary antibody (anti-mouse, #925-68070; anti-rabbit, #925-32211; LI-COR) in blocking buffer at room temperature, washed in TBST, and finally imaged on the LI-COR Odyssey CLx. Densitometry of blots was measured using ImageJ (NIH, Bethesda, MD, USA).

RNA Isolation, Reverse Transcription, and Polymerase Chain Reaction

Injured muscle samples were homogenized in Trizol (#15596018, Thermofisher, Waltham, MA, USA) using stainless steel beads in a bead homogenizer. Cells were washed in PBS and collected in Trizol. All tissue homogenates and cell lysates were centrifuged, and the supernatants were used for RNA isolation. Briefly, chloroform was added for phase separation, the aqueous phase was removed, isopropanol was added for precipitation of RNA, washed in 75% ethanol, and reconstituted in DEPC-treated water. Purified RNA concentration was measured using a Nanodrop 2000 (Thermofisher Scientific, Waltham, MA, USA). Equivalent concentrations of RNA were prepared, and cDNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (#4368814; Applied Biosystems, Waltham, MA, USA). mRNA expression was measured with Power SBYR Green PCR Master Mix (#; Applied Biosystems, Waltham, MA, USA) using the ViiA7 Real-Time PCR System

(Applied Biosystems, Waltham, MA, USA). Relative ΔΔCT values were calculated by determining the difference in CT value compared to the housekeeping gene (ΔCT) and then calculating $2^{(\Delta CT \text{ sample-average } \Delta CT \text{ control group})}$.

Statistical analyses

Values are reported as means ± standard error. Data were tested for homoscedasticity and those passed were compared using two-sided t tests and those that did not pass were compared using the nonparametric Mann-Whitney test. Differences between groups were considered significant if $P \leq 0.05$. Graphpad Prism Version 7.00 was used to generate all figures.

4. Results

Locally-applied LIV enhances muscle fiber regrowth following traumatic injury

Consistent with our hypothesis, locally applied LIV treatment improved healing of lacerated gastrocnemius muscle at day 14 post-injury **(Figure 4.3)**. Daily local LIV treatment increased both the diameter (by 12%) and cross-sectional area (by 18%) of individual myofibers compared to sham treated, but injured, control mice (**Figure 4.3**). Furthermore, the area occupied by peripherally-nucleated myofibers in muscle cross-sections was significantly increased from 8% to 14% of total muscle area and damaged area was significantly decreased from 53% to 45% in mice treated with LIV compared to controls. Percent area of centrally-nucleated myofibers was not different between LIV-treated and control mice (**Figure 4.3**). In our previous study, whole body LIV treatment increased diameter by 12% and area by 34%, however there were no significant changes in damaged area, or the area occupied by peripherally-nucleated myofibers. These data indicate that locally applied LIV improves muscle healing perhaps to a greater degree than WBV.

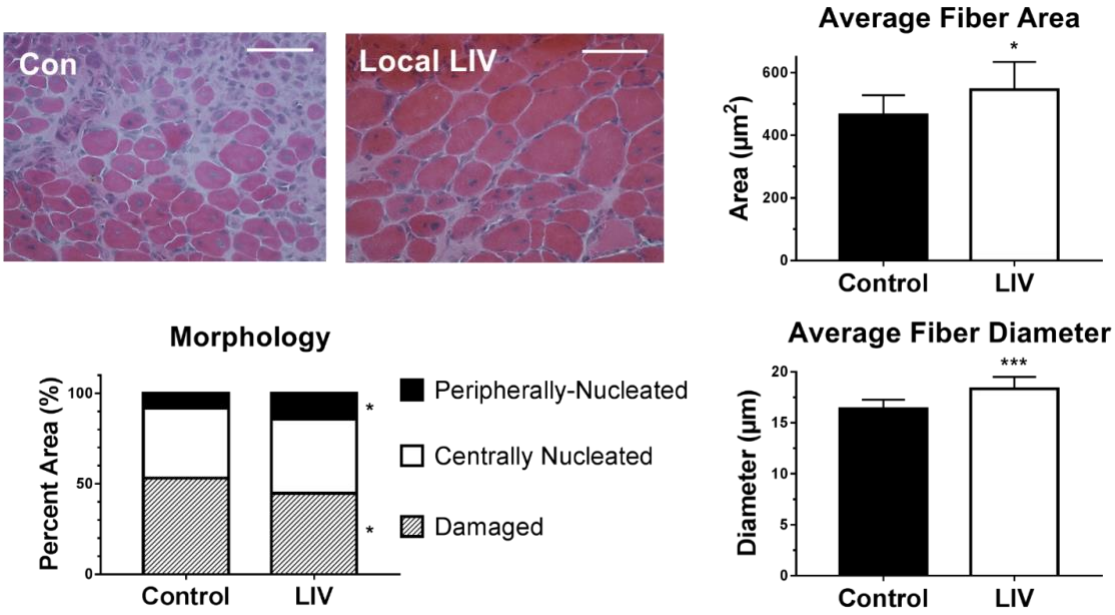

Figure 4.3. ***Locally-applied low-intensity vibration (LIV) at 90 Hz and 0.2 g enhances myofiber growth at day 14 post-injury following laceration muscle injury in mice***. Gastrocnemius muscles were lacerated and collected for histological analysis at day 14 post-injury. (A, B) Representative images of hematoxylin and eosin-stained sections (scale bar = 50 μm, 40x magnification). (C) Average cross-sectional area of myofibers, (D) percent area of injury that consists of peripherally-nucleated fibers, centrally-nucleated fibers, or damaged tissue, and (E) average minimum diameter of myofibers was quantified in five 40x fields per muscle in hematoxylin and eosin-stained sections. Data are presented as mean ± SE. *$P \leq 0.05$.

Locally-applied LIV does not alter IGF-1 protein or mRNA levels in damaged muscle

Since we previously reported that LIV increased IGF-1 levels in skin wounds associated with improved healing[35] and IGF-1 is a well-known promoter of muscle growth and regeneration[166], we sought to determine if the local LIV-induced improvements in muscle healing were mediated by IGF-1. Total IGF-1 protein concentration was measured by ELISA and IGF-1, IGF-1R1, IGF-1Ea, and MGF mRNA expression were measured by qPCR. IGF-1R1 is the tyrosine kinase receptor that is activated by IGF-1 and is upregulated in L6 myoblasts exposed to mechanical stimulation via cyclic stretch[1] and IGF-1Ea and MGF are individual isoforms of IGF-1 that are known to be expressed locally in skeletal

muscle in response to mechanical stimulation[48]. Local LIV treatment had no effect on total IGF-1 protein levels or mRNA expression of total IGF-1, IGF-1Ea, MGF, or IGF-1R1 after 7 or 14 days of treatment (Figure 4.4). These data indicate that, unlike skin wound healing, increases in IGF-1 do not appear to mediate local LIV-induced improvements in muscle healing.

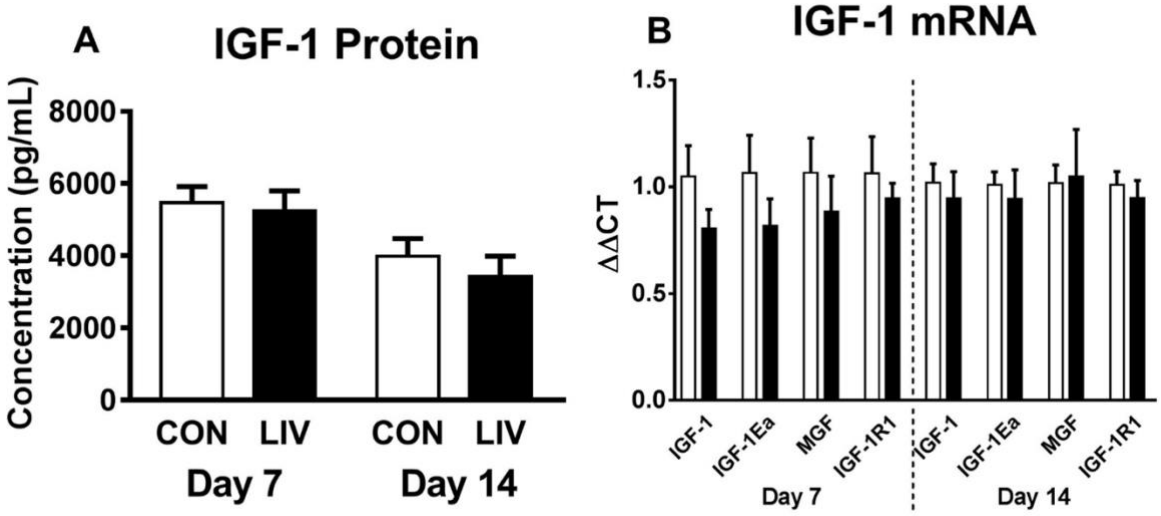

Figure 4.4. ***The effects of locally-applied low-intensity vibration (LIV) on IGF-1 at day 7 and 14 post-injury following laceration muscle injury.*** Gastrocnemius muscles were lacerated, and the injured portions of the muscles were collected for protein and mRNA analysis at day 7 and 14 post-injury. (A) Total IGF-1 protein concentration in injured muscle at day 7 and day 14 post-injury. (B) mRNA expression levels of total IGF-1, local IGF-1 isoforms IGF-1Ea and MGF, and IGF-1R1 at day 7 and 14 post-injury. Data are presented as mean ± SE. No significant differences were observed between sham controls and LIV treated mice.

Locally-applied LIV enhances Akt signaling in damaged muscle

The Akt/mTOR/p70S6K pathway is well-known for playing a central role in the complex network of hypertrophic and atrophic signaling in skeletal muscle, both in response to changes in mechanical loading and following injury[39]. The Akt/mTOR/p70S6K pathway can be activated by IGF-1 and though we did not find any effect of LIV on IGF-1 levels, this pathway can also influence muscle

growth via IGF-1-independent mechanisms[167,168].Thus, we sought to determine if components of the Akt/mTOR/p70S6K pathway are upregulated in response to local LIV using Western blots. At day 7 post-laceration injury, there was a significant increase in protein levels of total Akt (by 22%) and total p70S6K (by 27%) but no increase in the ratio of phosphorylated to total protein ratio for either molecule (Figure 4.5 A-E). In contrast, at day 14 post-laceration injury, there was no change in levels of total Akt or p70S6K; however, there was a trend towards an increase in phosphorylated to total Akt ratio (by 29%, p = 0.057) (Figure 4.5 F-J). These data suggest that local LIV may first increase the signaling capacity of the Akt/p70S6K pathway at day 7 and then may increase signaling through this pathway at day 14. These changes may occur independently of mTOR as no changes in the phosphorylation of p70S6K were observed.

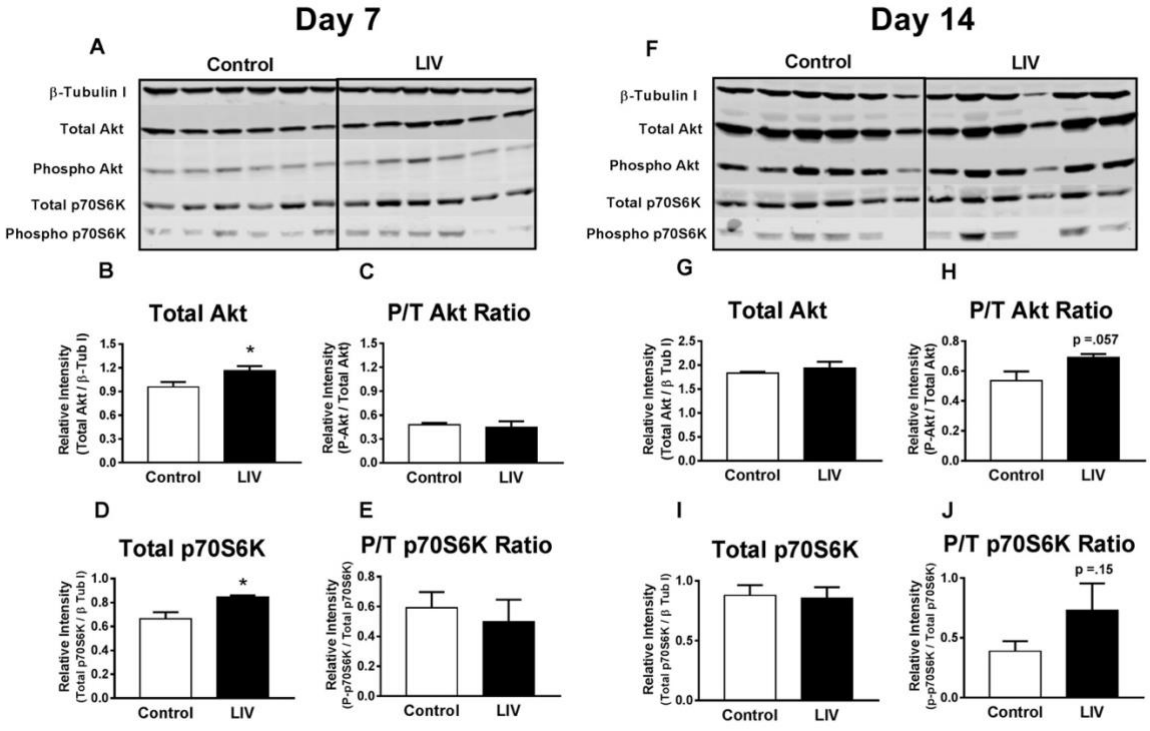

Figure 4.5. ***The effects of locally-applied low-intensity vibration (LIV) on Akt and p70S6K at day 7 and 14 post-injury following laceration muscle injury.*** Gastrocnemius muscles were lacerated, and the injured portions of the muscles were collected for SDS-PAGE/western blot analysis at day 7 and 14 post-injury. (A,F) Western blot images

for total and phosphorylated Akt and p70S6K at day 7 and day 14 post-injury. (B-E) Quantification for day 7 western blots, (G-J) Quantification of day 14 western blots. Total protein amounts were normalized to β-Tubulin I and phosphorylated protein is represented as the ratio of phosphorylated protein/total protein. Data are presented as mean ± SE. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

Locally-applied LIV downregulates atrophy gene expression in damaged muscle

Since Akt plays a central role in regulating changes in muscle mass, we also measured the mRNA expression of several markers associated with myogenesis, muscle growth and atrophy including those for satellite cell activation (Pax7), myoblast proliferation (M-cadherin, MyoD), and differentiation (MyoD, myogenin) as well as genes related to atrophy (Myostatin, FOXO1, FOXO3a, Atrogin-1, Muscle Ring Finger-1). Interestingly, despite the increase in muscle fiber size, none of the myogenesis related genes (Pax7, M-cadherin, MyoD, myogenin) were upregulated in response to local LIV either at day 7 or 14 post-injury. Among the atrophy related genes, FOXO1 ($p=0.05$) and Atrogin-1 ($p<0.01$) mRNA expression were decreased by 45% and 59%, respectively, with the application of local LIV on day 7 post-injury (Figure 4.6A); the others were not significantly altered. Interestingly, FOXO1 and Atrogin-1 are known to be downregulated when Akt activity is upregulated[41] suggesting a possible role of these atrophy genes early in LIV-induced muscle fiber growth following laceration injury.

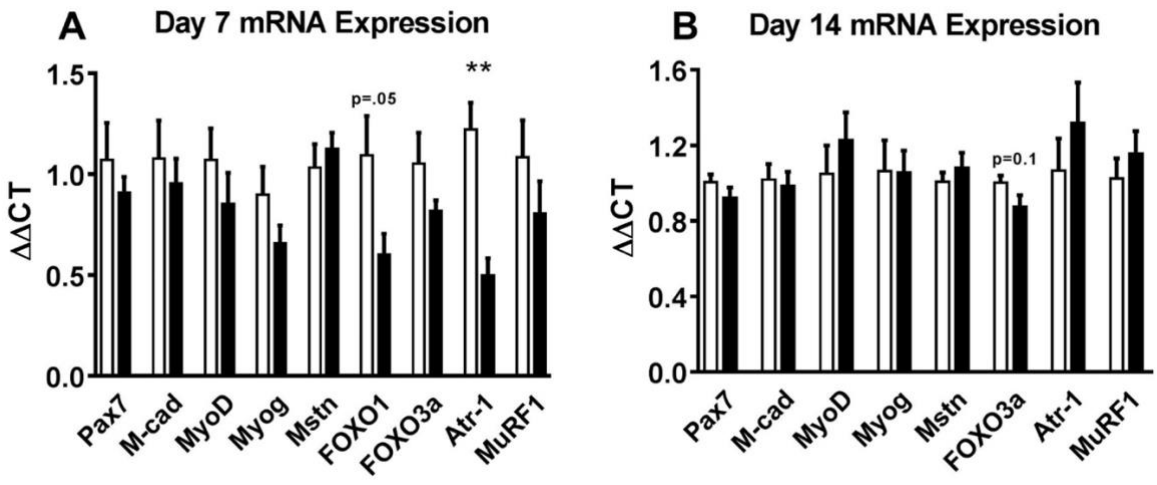

Figure 4.6. ***The effects of locally-applied low-intensity vibration (LIV) on hypertrophic and atrophic gene expression at day 7 and 14 post-injury following laceration muscle injury.*** Gastrocnemius muscles were lacerated and the injured portions of the muscles were collected for qPCR analysis at day 7 and 14 post-injury. Genes measured: Pax 7, m-cadherin (M-cad), MyoD, Myogenin (Myog), Myostatin (Mstn), FOXO1, FOXO3a, Atrogin-1 (Atr-1), Muscle Ring Finger 1 (MuRF1). (A) Day 7 and (B) Day 14 mRNA Expression. GAPDH was used as the house-keeping gene and all samples are represented as ΔΔCT which represents the fold-expression compared to the average of the control group. Data are presented as mean ± SE. * p≤0.05, **p≤0.01, ***p≤0.001

LIV enhances growth of differentiating C2C12 cells

Since locally applied LIV increases growth of muscle fibers after injury, we sought to determine if LIV signals are transduced directly by muscle cells. To accomplish this goal, we applied LIV daily to cultured C2C12 mouse muscle cells following the initiation of differentiation and measured markers of myoblast fusion into myotubes as well as myotube size at days 3 and 6. On day 3, LIV increased myotube diameter by 17% compared to control cells with no changes in total nuclei count, myotube nuclei count, or fusion index (ratio of myotube nuclei count / total nuclei count) (Figure 4.7 A-E). By day 6, LIV-treated cells had a significantly greater total nuclei count (by 11%), fusion index (by 39%), and myotube diameter (by 13%). These data indicate that muscle cells are capable of directly transducing LIV signals into enhanced differentiation and growth.

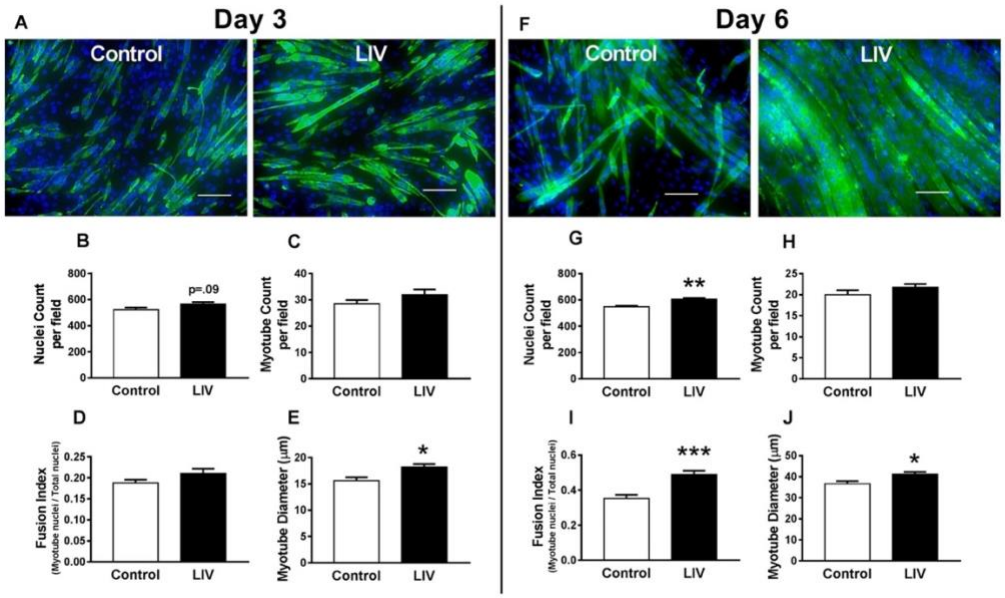

Figure 4.7. ***The effects of low-intensity vibration (LIV) on C2C12 cell morphology at day 3 and 6 of differentiation.*** Differentiation was initiated in C2C12 cells and LIV was applied 30 min/day for either 3 or 6 days. (A,F) Representative images of C2C12 cells stained with MF-20 antibody for myosin heavy chain (green) and nuclei stained with DAPI (blue) at day 3 and day 6 of diffentiation. (B, G) Average total nuclei count per field. (C, H) Average myotube count per field. Myotubes determined as MF-20+ cells with 2+ nuclei. (D, I) Average Fusion Index calculated by nuclei in myotubes / total nuclei. (E, J) Myotube Diameter calculated by averaging the diameter at 3 locations across the length of the myotube. All values were quantified in five 20x fields per sample, n = 6 per group. Data are presented as mean ± SE. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

LIV enhances Akt signaling in differentiating myocytes

Since LIV enhanced C2C12 myotube differentiation and growth, we aimed to determine if Akt signaling might mediate this response. Cells treated with LIV had signficantly greater levels of total Akt (by 10%) than control cells at day 3, while the ratio of phosphorylated to total Akt showed a trend towards an increase ($p=0.1$). At day 6, there was no difference in total Akt levels but there was a 40% increase in phosphor/total Akt ratio in cells treated with LIV compared to controls. No differences were observed between groups at either day 3 or 6 for total p70S6K or the phospho/total ratio. Similar to what we observed in injured muscle; these data suggest that LIV-induced increases in myotube size might be mediated by Akt signaling in a manner that is independent of changes in p70S6K signaling.

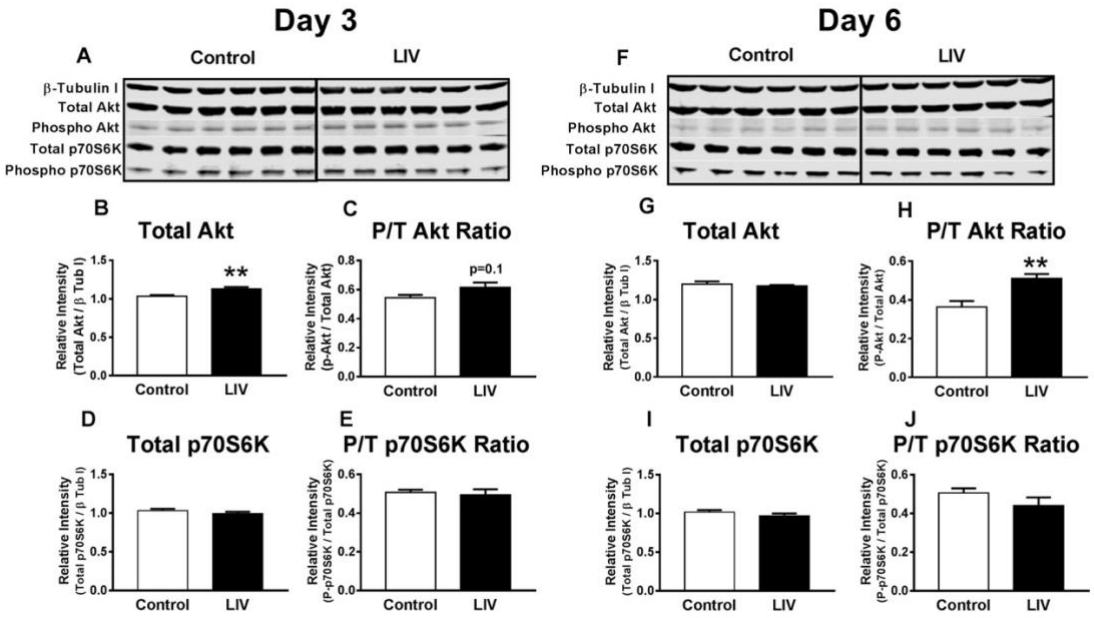

Figure 4.8. ***The effects of low-intensity vibration (LIV) on C2C12 cell Akt and p70S6K at day 3 and 6 of differentiation.*** Differentiation was initiated in C2C12 cells and LIV was applied 30 min/day for either 3 or 6 days. (A,F) Western blot images for total and phosphorylated Akt and p70S6K at day 3 and day 6 post-differentiation. (B, G) Total Akt normalized to β-Tubulin I. (C, H) Phosphorylated Akt/Total Akt Ratio. (D, I) Total p70S6K normalized to β-Tubulin I. (E, J) Phosphorylated p70S6K/Total p70S6K Ratio. n = 6 per group. Data are presented as mean ± SE. * p≤0.05, **p≤0.01, ***p≤0.001

LIV does not change myogenic gene expression in differentiating myocytes

We again measured the expression of genes related to satellite cell activation, myoblast proliferation, differentiation, growth and atrophy. At day 3, myostatin was significantly increased with the application of LIV compared to controls which is unexpected considering myostatin is a marker associated with muscle atrophy and is known to be downregulated by Akt[71]. However, none of the other atrophy-related genes (FOXO1, FOXO3a, Atrogin-1, Muscle Ring Finger-1) that are downstream from myostatin were changed with LIV. At day 6, MyoD expression was significantly upregulated while there was a trend towards a decrease in FOXO3a expression (p=0.07). These data suggest that LIV-induced increases in myotube size were not strongly associated with changes in expression of genes related to myogenesis or atrophy.

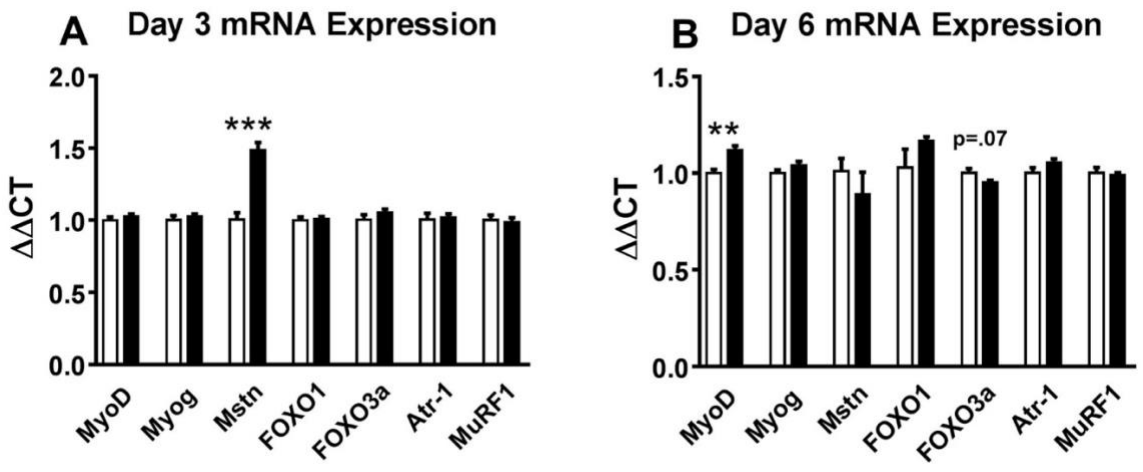

Figure 4.9. ***The effects of low-intensity vibration (LIV) on C2C12 cell mRNA expression at day 3 and 6 of differentiation.*** Differentiation was initiated in C2C12 cells and LIV was applied 30 min/day for either 3 or 6 days. mRNA expression measured by qPCR. Genes measured: MyoD, Myogenin (Myog), Myostatin (Mstn), FOXO1, FOXO3a, Atrogin-1 (Atr-1), Muscle Ring Finger 1 (MuRF1). (A) Day 3 mRNA expression. (B) Day 6 mRNA expression. n = 6 per group. Data are presented as mean ± SE. * p≤0.05, **p≤0.01, ***p≤0.001

5. Discussion

Traumatic muscle injuries can result in persistent disability as recovery from these injuries is prolonged and incomplete[2,149,150], resulting in long-lasting impairments of muscle and joint function[3–6]. This impaired healing negatively impacts quality of life. Thus, there is a need for regenerative therapies capable of restoring functional muscle tissue. Consequently, early mobilization of muscle following injury leads to improved regeneration[21,22], however limited use of the affected limb, limited mobility, and pain often prevent this treatment option. LIV represents a non-invasive physical therapy strategy that circumvents these obstacles. Previously, we showed that whole-body LIV enhances myofiber growth in a mouse laceration injury model by day 14 post-injury[81], however little is known about the mechanisms involved. In the present study, we demonstrate that locally-applied LIV improves muscle regeneration similar to whole-body application, and that muscle cells can directly tranduce LIV signals

into enhanced myofiber growth, associated with increased Akt signaling, but independent of changes in IGF-1 levels.

A review of the literature indicates that this is the first study to assess the mechanisms by which LIV enhances muscle healing after injury. Locally applied LIV induced larger myofiber size and improved muscle morphology, compared to injured, but sham treated controls (Figure 4.3). These improvements in muscle healing were not associated with any changes in IGF-1 protein or mRNA expression (Figure 4.4). However, they were associated with changes in proteins associated muscle hypertrophy; total levels of Akt and p70S6K were increased at day 7 post-injury (Figure 4.5) and the ratio of phosphorylated Akt to total Akt trended towards an increase at day 14 (Figure 4.5). These data suggest that LIV may increase the signaling capacity of Akt by day 7, and increase signaling through this pathway by day 14. To further evaluate the pathways involved, we measured the expression of genes associated with muscle regeneration and atrophy. Notably, there were no changes in any genes associated with muscle regeneration; however, there were significant decreases in FOXO1 and Atrogin-1 expression (Figure 4.6), which are associated with muscle atrophy. When active, FOXO1 induces transcription of Atrogin-1 which is an E3 ubiquitin ligase that causes atrophy via increased protein degradation[41,69]. When activated, Akt phosphorylates FOXO1 and sequesters it in the cytoplasm, inhibiting its transcriptional activity[39]. Thus, the improvements in muscle healing caused by LIV may be mediated by Akt through the inhibition of FOXO1 and Atrogin-1 activity.

To determine if LIV signals are directly transduced by muscle cells, we applied LIV to differentiating C2C12 mouse myoblasts in culture. Our data show that LIV significantly increased myotube differentiation and diameter after six days of treatment. Similar to its effects in muscle injury, LIV increased total levels of Akt on day 3, and the ratio of phosphorylated to total Akt on day 6. However, in cultured myotubes, increased Akt activity was not associated with reduced FOXO1 or

Atrogin-1 expression. In fact, expression of genes related to muscle regeneration and atrophy were largely unaffected by LIV; only MyoD was increased at day 6. Taken together, muscle cells are capable of directly transducing LIV signals into enhanced differentiation and growth, and Akt may be involved in this process.

Previous studies have tested the effects of vibration on cultured myoblasts, and muscle of humans and mice, albeit with much different vibration protocols. Wang et al applied vibration at 5, 8, and 10 Hz with an amplitude of 0.4 mm (compared to <0.1 mm in our study) for 10 minutes per day for up to 6 days to confluent C2C12 myoblasts in growth medium and observed an increase in myotube number, size, and fusion [43]. These measurements corresponded with an increase in gene expression of MyoD and myogenin; both markers of myogenic differentiation. This study differed from ours in that LIV was applied while cells were in growth medium supplemented with 10% FBS rather than differentiation medium supplemented with 2% HS. Furthermore, although the vibration parameters used would be classified as "low-intensity", the frequencies used were lower and the amplitude was higher than those used in our study. In another study, Ceccarelli et al applied vibration in vivo to developing mice and in vitro to primary myoblasts[42]. Vibration was applied at 30 Hz and 11 mm amplitude for 1 hour per day over a period of 7 days. This vibration signal would not be classified as LIV due to the increased intensity with such an amplitude. This higher intensity vibration showed an increase in cell fusion when applied directly to cells in culture[42]. This study also found a significant increased expression of M-cadherin, an indicator of satellite cell activation, in mouse TA muscles, primary myoblasts, C2C12 myoblasts, and L6C11 myoblasts when treated with vibration. Further studies have investigated the effects of LIV on muscle in aging individuals in humans and mice. Three months of whole-body vibration in older adults with sarcopenia increased skeletal muscle mass index[37], however six months of local vibration in postmenopausal women had no effect on muscle mass in the

quadriceps[38]. Lastly, vibration decreased damage from pressure ulcers and associated oxidative damage in senescence-accelerated mice, but not controls[36]. The differences in the outcomes and mechanisms implicated in these studies may be due to the differences in study design and vibration parameters. Despite these differences, it appears that that there is a range of vibration signals that can induce myogenesis or growth of muscle fibers in vitro and in vivo.

Although our data show that LIV acts directly on muscle cells, LIV may also have beneficial effects on other cells involved in muscle repair. One reason that healing from traumatic muscle injury is impaired compared to less severe injury is the disruption of blood supply. LIV can improve skin wound healing in diabetic mice, which was associated with new blood vessel formation[35]. LIV can also positively effect circulation in the skin of hairless mice, the lower leg of healthy human subjects, and the forearm of both healthy subjects and those with Type 2 diabetes[107,124,156]. Furthermore, LIV can improve healing of pressure ulcers in humans by nitric oxide induced improvements to blood flow[158]. Thus, the effect of LIV on neoangiogenesis and blood flow within injured muscle warrants further study.

Our study has several limitations. Although we assessed potential mechanisms in a number of ways, the data are essentially correlative. Further experiments using pharmacological or genetic approaches to target factors or pathways are necessary to demonstrate their role in LIV-induced healing. Additionally, the response of cells grown in culture likely depends on growing conditions, notably substrate stiffness. We grew cells directly on cell culture plastic, which is much stiffer than normal biological substrates and responses to LIV may differ on softer substrates. Regardless, we saw remarkably similar effects of LIV in culture as we did in mice. A final limitation is that we did not assess functional characteristics related to recovery. We plan to investigate how LIV influences neuromuscular functioning in a future study.

In summary, our findings are partially consistent with our hypothesis that local LIV improves muscle regeneration following injury and myotube growth and differentiation in culture; however, this does not appear to be mediated by IGF-1. Muscle cells were able to directly transduce LIV signals into growth and differentiation, which was associated with increased Akt signaling. Since Akt plays a central role in maintaining muscle mass and works through multiple pathways, future studies could be focused on further elucidating the role of Akt in LIV-induced muscle repair.

6. Acknowledgements

The institution of one or more of the authors has received funding from the Department of Defense (W81XWH-14-1-0281 to TJK).

7. Contributions of Authors

TC performed the experiments. TK and TC conceived and designed the experiments, analyzed the data, and wrote the paper;

8. Conflict of Interest

TJK has a patent pending regarding the application of vibrations for therapeutic treatment. TC certifies that he, or a member of his or her immediate family, has no funding or commercial associations (e.g., consultancies, stock ownership, equity interest, patent/licensing arrangements, etc.) that might pose a conflict of interest in connection with the submitted article.

V. DISCUSSION

1. Significance

Traumatic muscle injuries are a devastating consequence of motor vehicle accidents, limb salvage surgeries for the removal of tumors[1] and military combat[2]. These injuries are characterized by a significant loss of tissue and inadequate healing leading to impaired muscle function, joint stiffness, and loss of mobility[3–6]. The regenerative capacity of muscle is inversely related to the severity of the injury with less severe injuries able to regenerate fully and more severe injuries resulting in permanent functional deficits due in part to inadequate blood supply, denervation, and scar formation. Patients who suffer from traumatic muscle injury typically experience impaired mobility and are in need of treatment that does not require active muscle contraction. LIV is a promising treatment option due to its non-invasive application of mechanical stimulation that does not require active muscle contraction. Muscle is known to respond to mechanical stimulation by increasing muscle size; however, the effects of vibration on injured muscle have yet to be elucidated. The goal of this study was to determine if LIV improves the healing of injured muscle and if these effects are mediated by IGF-1 and associated downstream pathways.

2. Specific Aims

Specific Aim 1: Determine the effect of LIV on muscle repair in a mouse model of traumatic muscle injury.

Aim 1.1. Determine whether altering amplitude, frequency and/or mode of application alters efficacy in the treatment of traumatic muscle injury.

A review of the literature revealed that no studies have tested the effect of LIV on the healing of muscle injury. Therefore, our initial studies were focused on identifying the optimal vibration

parameters based. WBV at 45 Hz/0.4 g increased myofiber area, while WBV at 90 Hz/0.2 g increased both myofiber area and diameter at day 14 post-muscle injury. Neither protocol, however, changed the percent of damaged area in the injury. There were no differences in fiber size after 7 days. These data suggest that LIV enhances myofiber growth by influencing the later stages of the healing process since differences in myofiber size were only apparent by day 14. Although we do not have sufficient evidence to conclude which vibration protocol is optimal for muscle healing, we proceeded with the 90 Hz and 0.2g protocol for future experiments since there was an increase in both area and diameter whereas the 45 Hz and 0.4g protocol only showed an increase in area, not diameter.

Next, we wanted to determine if LIV-induced healing occurred when applied locally to the injury site similar to when it is applied to the whole-body. We found that local LIV at 90 Hz and 0.2g increased both myofiber area and diameter in injured muscle at day 14 post-injury. There was also a significant increase in percent area of peripherally-nucleated myofibers (associated with mature muscle) and a decrease in percent damaged area suggesting that local LIV improves muscle healing to a similar, if not greater, extent as WBV.

Aim 1.2. Determine the effect of LIV on expression of total IGF-1, local IGF-1 isoforms, and related hypertrophic/atrophic downstream targets in lacerated gastrocnemius muscle.

Contrary to our original hypothesis, local LIV did not change the protein concentration of total IGF-1 or the mRNA expression of total IGF-1, IGF-1R, IGF-1Ea, or MGF in lacerated gastrocnemius muscles on days 7 or 14 post-injury. However, there was an increase in total Akt in at day 7 post-injury and phosphorylated to total Akt ratio at day 14 post-injury in muscle. Additionally, total p70S6K also showed an increase at day 7 similar to Akt, however the phosphorylated to total ratio remained unchanged at day 14. Furthermore, these changes in Akt were associated with a downregulation of

mRNA expression of FOXO1 and Atrogin-1 at day 7 with no change to any other genes related to muscle growth and differentiation. When active, FOXO1 induces transcription of Atrogin-1 which is an E3 ubiquitin ligase that causes atrophy via increased protein degradation[41,69]. When activated, Akt phosphorylates FOXO1 and sequesters it in the cytoplasm, inhibiting its transcriptional activity[39]. Thus, the improvements in muscle healing caused by LIV may be mediated by Akt through the inhibition of FOXO1 and Atrogin-1 activity, which would subsequently suppress atrophy.

<u>Specific Aim 2</u>. Determine whether muscle cells directly sense LIV signals and transduce them into increased growth.

Aim 2.1. Determine the effect of LIV at 90 Hz and 0.2g on of myotube differentiation and size in C2C12 cells.

Now that we have data to suggest that LIV induces growth in damaged myofibers, we sought to establish whether LIV was acting directly on muscle cells or through a secondary cell population (i.e. macrophage, neuron, etc.). We applied LIV to differentiating C2C12 myoblasts in culture for 30 min/day for either 3 or 6 days. LIV increased growth of fibers at day 3 and LIV increased both differentiation and growth of cultured myotubes at day 6 post-differentiation. Thus, muscle cells are capable of directly receiving LIV signals and transducing them into increased growth.

Aim 2.2. Determine the effect of LIV on expression and/or accumulation of total IGF-1, local IGF-1 isoforms, and related hypertrophic/atrophic downstream targets in C2C12 cells.

Since LIV had no effect on IGF-1 in vivo, we did not measure it in vitro, however, we did measure the associated downstream markers that are relevant to differentiation, hypertrophy, and atrophy. Similar to the in vivo experiments, there was an increase in total Akt at day 3 and

phosphorylated to total Akt ratio at day 6 post-differentiation in cultured myotubes. These changes in Akt were not associated with changes in p70S6K signaling or atrophy-related gene expression, however. Nonetheless, these data suggest that LIV increases growth and differentiation in cultured myotubes, and Akt may be involved in this process.

3. **Limitations**

3.1 Model Selection

Our results suggest that mechanical stimulation such as LIV is promising as an adjunct therapy in combination with other regenerative therapies in traumatic muscle injuries. Such injuries often involve damage to surrounding bony and connective tissues in addition to those contained within the musculature. These complex injuries present complications that would require treatments to address these extra-muscular issues. While our model of laceration does not incorporate damage to the surrounding tissues, it does recapitulate certain aspects of traumatic injuries such as increased fibrosis and impaired muscle fiber regeneration which allowed us to explore the effects of LIV specifically on the muscle tissue without extraneous complications. This is the first step in establishing LIV as a treatment modality for these types of injuries, and future studies can apply LIV to more complex injury models with or without other treatment options.

3.2 Experimental limitations

Our experiments were limited in a number of ways. First, our claims regarding the LIV-induced mechanisms are primarily based on correlative data. To confirm these findings, experiments that inhibit and/or activate these pathways using pharmacological or genetic techniques are required. Specifically, experiments are needed to assess if the targeted inhibition of Akt using LY29004[169] or the secondary

inhibition of Akt by inhibiting the IGF-1/PI3K/Akt/mTOR pathway using wortmannin or rapamycin, prevents the LIV-induced enhancements in muscle growth. However, such experiments would not be easy to interpret because inhibiting Akt pharmacologically would likely influence growth of non-LIV treated muscle cells as well.

In addition, functional measurements would improve the validity of our claims as functional deficits are inherent to traumatic muscle injuries. Future studies should address effects of laceration on force production and if LIV improves these outcomes. Next, muscle repair is a complex process governed by a variety of spatiotemporal factors. Furthermore, our study only collected data at day 7 and day 14 post-injury in mice. Collecting data at earlier, intermediate, and later time points on outcomes related to inflammation, angiogenesis, innervation, and fibrosis would give a more comprehensive view of the effects of LIV. Finally, our cultured cells were all grown and treated directly on plastic dishes. This substrate is much stiffer than biological substrates in vivo and may alter the way signals are received and transmitted. Future experiments could test the effects of LIV on cells when grown on a variety of extra-cellular matrix substrates that more closely model biological substrates in vitro.

4. Future Directions

Our data from cultured cells demonstrates that muscles cells can directly receive and transduce LIV signals. This does not, however, rule out other beneficial effects that LIV may have on muscle repair in addition to enhanced growth. The first way to address this would be to explore the changes in cell populations in damaged muscle with the application of LIV using flow cytometry. This could identify key players in regeneration based on common surface markers seen in inflammatory, endothelial, or neuronal cell types. Additionally, extensive gene expression analysis could help narrow down the possible mechanisms involved in the many phases of muscle repair. Those selected for the

current study were based on their prominent roles in healing or muscle growth from the literature, but only represent a small percentage of possible candidates. Future studies could assess this in an unbiased fashion using single-cell analysis techniques which could elucidate cell populations whose gene expression changes in response to LIV.

Based on our measurements of muscle cells in vivo and in vitro, it is apparent that LIV enhances growth of muscle fibers and this effect is observable at later time points of repair or differentiation. This observation, along with the increased fusion of myotubes at day 6 in vitro suggest that LIV may increase fusion of myotubes during the intermediate to later stages of repair. Several mechanisms could be explored to determine their role in this process. NFATc2 is a transcription factor that is activated by calcineurin. NFATc2 has been shown to increase secondary fusion (the fusion of nascent myotubes with myoblasts) through the production of prostaglandin F2α and IL-4[170,171]. Additionally, serum response factor (SRF) is another transcription factor involved in myoblast fusion as well as satellite cell recruitment. Independent of IGF-1, SRF controls muscle growth in a model of overload-induced hypertrophy through the expression of IL-6 and Cox2/IL-4[172]. Lastly, another candidate involved in myoblast fusion is focal adhesion kinase (FAK). FAK is necessary for myofiber fusion following injury as well as myotube differentiation in cultured cells through the expression of caveolin-3 and β1 integrin[173]. Interestingly, FAK has also been reported as a sensor for mechanical stimuli in cells, receiving signals at the cell surface and transducing them directly to the nucleus via the cytoskeleton and the linker of nucleoplasm and cytoplasm complex (LINC)[174]. Future studies should explore the effects of LIV on the potential mechanisms for increased fusion.

5. Conclusion

In summary, these findings suggest that LIV improves muscle repair following traumatic injury by enhancing myofiber growth by acting directly on muscle cells. These beneficial effects are associated with an increase in Akt signaling albeit independent of IGF-1, but more studies are needed to confirm this as well as unveil any other possible mechanisms involved. Traumatic muscle injury is a complex problem, and LIV may serve as a low-cost, non-invasive physical therapy that can increase muscle regeneration and improve functional recovery.

VI. Appendix

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> [Quoted text hidden]

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