Nonthermal Ultrasound and Exercise in Skeletal Muscle Regeneration

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Objective: To determine whether continuous nonthermal therapeutic ultrasound (US) and low-intensity exercise (Ex) influence skeletal muscle regeneration after a standardized contusion injury in an animal model.

Design: Randomized controlled trial with blinded comparisons in a 2×2 factorial (US by Ex) design.

Setting: Animal care facility and exercise physiology biochemistry laboratory.

Animals: Twenty male Wistar rats (age, 8mo) received a reproducible bilateral contusion injury to the gastrocnemius muscles. Ten gastrocnemius muscles from 5 noninjured, non-treated rats provided baseline control data.

Interventions: US (continuous duty cycle, 3MHz; intensity, 0.1W/cm²; transducer, 1cm²; duration, 5min/d; duty cycle, 100%) and exercise (20min/d of low-intensity treadmill walking at 14m/min). Gastrocnemius muscles from injured rats received exercise treatment alone (Ex+NoUS), exercise and US treatment (Ex+US), US treatment alone (NoEx+US), and no treatment (NoEx+NoUS).

Main Outcome Measures: Ninety-six-hour postinjury muscle mass, contractile protein concentration, fiber cross-sectional area, number of nuclei per fiber, and myonuclear density.

Results: Myonuclei per fiber were statistically greater in injured than in noninjured gastrocnemius muscle (P<.05). There were no statistical differences (P>.01) among the 4 injured treatment groups for any of the outcome measures chosen as biomarkers of skeletal muscle regeneration.

Conclusions: There is no evidence that the specific continuous US and Ex protocols investigated enhanced skeletal muscle regeneration after contusion injury.

Key Words: Muscle, skeletal; Physical conditioning, animal; Rehabilitation; Ultrasonic therapy; Wounds and injuries. © 2005 by American Congress of Rehabilitation Medicine and the American Academy of Physical Medicine and Rehabilitation

Therapeutic Ultrasound (US) is commonly used in the rehabilitative setting to elicit thermal or nonthermal physiologic effects. A recent review1 hypothesizes that it is not necessarily the heating effects of therapeutic US, but rather the nonthermal stimulus, that may produce beneficial healing effects on biomarkers of skeletal muscle regeneration in 1 specific type of musculoskeletal injury: contusion injury after blunt trauma.

Although contusion injuries are a very common form of both athletic and nonathletic injury, hospitalization is rarely required. However, these injuries affect muscle function. Structural and functional morbidity often occurs in the form of atrophy, contracture, pain, and increased likelihood of reinjury.2,3 Treatments to augment the normal repair and regenerative processes are important to a wide variety of patients, ranging from elite athletes to the elderly,4 who want to return to their previous level of function as quickly and as fully as possible. Therapeutic US is 1 such treatment. Clinicians use modalities such as US widely,5 6 and although clinical use of US is focused primarily on altering extensibility of collagenous tissues to improve range of motion (ROM), clinicians frequently use therapeutic US treatments in an attempt to enhance repair of tissue injuries in general and to reduce the associated pain.9 However, there are few data showing that US assists in skeletal muscle regeneration.

Because of the lack of scientific evidence, the use and prescription of therapeutic US as a treatment to enhance skeletal muscle regeneration is often based on the personal opinions and experience of clinicians.10 Despite the fact that this problem was noted in 1994,10 there is still no consensus statement for clinicians from any of the appropriate professional organizations on the appropriate dosage parameters for treatment of muscle injuries, or whether use of therapeutic US is even justified as a treatment when the aim is to influence skeletal muscle repair and regeneration. With more than 90% of sport-related injuries categorized as strains and contusions,11 it is clear that further study of therapeutic US as a modality to enhance skeletal muscle regeneration is necessary.

Although for ethical reasons there is understandably a paucity of research studies focusing on the effects of exercise on markers of human skeletal muscle regeneration after contusion injury, there is a growing evidence that exercise is efficacious in resolving contusion injuries to skeletal muscle.12 There is also theoretical support from the literature that exercise assists in promoting normal growth and repair of mammalian skeletal muscle.15,16 Furthermore, there is evidence that exercise promotes myonuclear accretion in injured muscle,17 although factors such as species, age, and training status also play a role.

It is clear that both US and exercise are common modalities for the management of skeletal muscle injury and are often used in an attempt to augment repair and regeneration of muscle tissue. However, it is unclear whether these modalities are specifically effective for this purpose. The fact that methods used in past studies may not have been justified18,19 (based on our understanding of the potential physiologic mechanisms by which US is supposed to influence cellular function) is but 1 reason why questions remain.

There are few data at the microscopic level on the independent effects of US and exercise on muscle repair after contusion injury. Furthermore, there are no data available on the...
interactive effects, if any, of US treatment and exercise on cellular markers of skeletal muscle regeneration after contusion injury. Therefore, the purpose of this investigation was to examine the effects of therapeutic US and exercise on several biomarkers of skeletal muscle regeneration after a standardized skeletal muscle contusion injury created using a reproducible drop mass technique. We hypothesized that exercise would help aid the regeneration process as would therapeutic US, and that US combined with exercise would facilitate muscle regeneration to a greater degree than would US alone.

METHODS

Design
A randomized controlled trial, with blinded comparisons in a 2×2 factorial design, was used to assess the influence of nonthermal US (levels: treatment, no treatment) and light exercise (levels: exercise, no exercise) on selected markers of skeletal muscle regeneration. The specific dependent variables measured included (1) muscle mass, (2) mean cross-sectional area (CSA) of 100 muscle cells in the injured area, (3) number of nuclei per cell in the injured area, (4) myonuclear density (CSA of each cell divided by number of myonuclei per cell [fiber CSA/myonuclei]), and (5) concentration of contractile protein. Although it is rarely disputed that satellite cells are of central importance in muscle regeneration, Rantanen et al suggested that it is possible to observe satellite cell proliferation without observing the expected differentiation into new myotubes; therefore, we assessed myonuclear density (fiber CSA/myonuclei) rather than just satellite cell proliferation.

Experimental Animals
A priori power estimations using previous data revealed that 10 muscle samples per group would likely provide statistical power of .80. Therefore, 20 experimental male Wistar rats (2 muscles per animal) were used to give 4 groups of 10 muscles. Five noninjured, nontreated rats provided 10 baseline control muscles. To control for maturation effects, all 25 rats were 8 months of age (adult). Mean body mass ± standard error of the mean (SEM) of all 25 animals was 586±17g. The university’s Institutional Laboratory Animal Care and Use Committee approved all experimental procedures. Animals were free to move about their cages, were housed 2 per cage, received food and water ad libitum, and were on a 12 hours light/12 hours dark cycle.

Interventions
Under anesthesia, all animals received standardized contusion injuries to both gastrocnemius muscles. Gastrocnemius muscles from injured rats received exercise treatment alone (Ex+NoUS), exercise and ultrasound treatment (Ex+US), ultrasound treatment alone (NoEx+US), and no treatment (NoEx+NoUS). Each animal served as its own control, in that a single exercised animal provided muscles for the Ex+NoUS and Ex+US groups, and that a single nonexercised animal provided muscles for the NoEx+US and NoEx+NoUS groups. At 96 hours postinjury, the animals were killed, and the gastrocnemius muscles were excised for analysis. Ninety-six hours after contusion injury was chosen because this is a time when proliferation of satellite cells wanes and differentiation begins in untreated injured muscle. We expected treatment(s) to have had an effect by this time. Furthermore, damaged muscle fibers need to obtain extra nuclei for repair reasonably quickly to avoid cell death, so therapeutic treatment must be given in a time frame that would enhance the ability of the injured muscle cells to repair themselves.

On the day of the blunt contusion injury, animals were anesthetized with 5% volume to volume ration of inhaled isoflurane. Because their eye-blink reflex was not present, animals were provided with an eye lubricant on the day of injury and during later US treatments. The hindlimbs of the animal were clipped to remove fur, and the midbelly of the gastrocnemius muscle was marked bilaterally 29mm from the calcaneus. Because during pilot study we found that the term “midbelly” was not specific enough, we decided to quantify where midbelly was in relation to a bony landmark. This is in line with the study by Wilkin et al, where midbelly was 22mm from the calcaneus, although the distance is different in our study because the animals were much older. The midbelly of the gastrocnemius was then positioned under the impactor of our contusion injury device. Because inducing a contusion is one of the few methods that researchers may use to cause a standardized injury, a reproducible drop mass technique (fig 1), as described by Crisco et al, and modified by Wilkin, was used to induce the injury as follows. The thigh was stabilized with a semicircular section of Tygon tube, and the gastrocnemius was clearly exposed by extending and slightly abducting the hindlimb with the aid of a rubber band and heavy metal block. With the Tygon tube in place, a 171-g mass fell through a clear Lucite guide tube from a height of 100cm onto the top of the impactor. The impactor tip (radius, 6.4mm) was in direct contact with the skin covering the midbelly of the gastrocnemius. After bilateral contusion injury, the animal was removed from anesthetic, allowed to recover, and returned to the cage.

US Treatment
Therapeutic US was initiated 24 hours after contusion injury unilaterally on the right gastrocnemius muscle. Each left gastrocnemius muscle received only sham US treatment, in which every detail of US treatment was mirrored, except that electric power was not supplied to the applicator. The area of the US transducer was 1cm². US treatment (continuous duty cycle, 3MHz; intensity, 0.1W/cm²; duration, 5min) occurred on 4 separate occasions, once every 24 hours postinjury, until the animal was killed at 96 hours postinjury. The US device was calibrated during pilot study. This specific nonthermal US...
protocol was based loosely on recently reported work, given our constraint of maintaining a 100% duty cycle, we compromised intensity to deliver an amount of total energy, which was slightly lower than used previously.

Assuming that the effective radiating area of the US crystal roughly equals the area of the US transducer, the US duration of 5 minutes per 24 hours follows a common practice of treating an area 2 times the area of the sound head (applicator) for 10 minutes. Because the US transducer and the rat gastrocnemius are approximately equal in surface area, it was not possible to treat an area twice the size of the transducer. Therefore, the duration was reduced by half to 5 minutes per treatment, to prevent overdosing of the muscle tissue compared with common treatments.

A continuous rather than pulsed US protocol was chosen to maximize any potential effects of treatment as follows. A typical 20% duty cycle pulsed US protocol would consist of the US being on for 20 ms, off for 80 ms; this clearly limits exposure time of the muscle to any potential effects from energy delivery. Our protocol used a 0.1 W/cm² × 100% duty cycle, which elicits a spatial averaged-temporal averaged intensity comparable to that of a 0.5 W/cm² × 20% duty cycle. Thus, although the protocol uses continuous US, the low-intensity setting ensures that effects will be primarily nonthermal (ie, mechanical).

Treatment was initiated at 24 hours postcontusion injury, to maximize the potential regenerative effect of treatment. We assumed that commencement of treatment before 24 hours might enhance (rather than inhibit) the degenerative action of neutrophils, but this occurs at the expense of activating damaging reactive species. One study documented significant neutrophils, but this occurs at the expense of activating damage to the muscle. Therefore, the duration was reduced by half to 5 minutes per treatment, to prevent overdosing of the muscle tissue compared with common treatments.

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Exercise Treatment

Rats in the Ex group exercised individually at low intensity (walking) on a calibrated motor-driven rodent treadmill on the same days as US treatments. Exercise duration was 20 minutes. When not on the treadmill, all rats were free to move about their cages. For consistency, exercise always followed US treatment. The treadmill velocity was held constant at 14 m/min, and the animals averaged about 110 steps/min at this velocity. When necessary, rats were gently prodded in the hindquarters, to assure exercise compliance.

Surgery and Euthanasia

On the day the rats were killed, the animals were weighed to determine an appropriate volume of sodium pentobarbital for intraperitoneal injection. Most rats required 50 to 70 mg/kg sodium pentobarbital to induce loss of righting, toe-pinching, and eye-blinking reflexes. Once in the surgical plane of anesthesia, the gastrocnemius muscles of each animal were excised, trimmed of excess fat and connective tissue, and weighed on an analytic balance. Distal and proximal orientation of the muscle was carefully monitored during weighing. A portion of the damaged midbelly area of the muscle was cut and placed in a precooled labeled microcentrifuge tube for later analysis of contractile protein concentration. This tube was then flash frozen in liquid nitrogen. The gastrocnemius was quickly frozen in isopentane cooled in liquid nitrogen for later analysis. Gastrocnemius muscles were embedded in tissue freezing medium, mounted on a labeled board of cork to ensure optimal preservation and maintenance of orientation of the gastrocnemius, frozen, and wrapped in aluminum foil.

Although complete fractures of the tibia were not observed during any of the surgeries, no attempt was made to assess whether hairline fractures occurred as a consequence of the impact that produced the contusion injury. The impact force applied in creating the injury was unlikely to cause fracture, and no fractures have been observed in any other studies from our lab that have used this protocol. Animals were killed by exsanguination after bilateral excision of the gastrocnemius muscles.

Contractile Protein Concentration

Contractile protein concentration was determined, after fractionation via serial centrifugations, with a bicinchoninic acid protein assay (BCA), following the method of Linderman et al. Briefly, 80 to 120 mg of tissue were minced in 0.5 mL of homogenization buffer (sucrose, 250 mM; potassium chloride, 100 mM; ethylenediaminetetraacetic acid, 5 mM; Tris, 20 mM), ground on ice with an overhead stirrer, and centrifuged 10 minutes at 5000 rpm in a refrigerated centrifuge. The pellet from the final centrifugation was diluted to fit within the BCA standard curve, and the BCA was performed using a microplate reader. Analysis of each sample was performed in triplicate. Contractile protein percentage was determined as the quotient of contractile protein and muscle wet weight.

Fiber

To determine fiber CSA, 10-μm-thick cross-sections of tissue were cut on a cryostat and stained with hematoxylin and eosin. The cross-sections were analyzed using available free-ware. One hundred cells were counted per sample, to minimize error.

Myonuclei

Number of myonuclei was determined using the same cross-sections and methodology as described above for determination of fiber CSA. Nuclei per fiber were counted and recorded. One hundred cells were counted per sample to minimize error. These data were used to calculate mean myonuclear density (indicated by fiber CSA/myonuclei). A single blinded investigator assessed number of myonuclei, fiber CSA, and fiber CSA/myonuclei.

Statistical Analysis

Five separate 2 × 2 analyses of variance (ANOVAs) were used to identify differences between the 4 injured treatment groups in terms of the 5 dependent variables. Analyses were performed using JMP software. To protect against inflation of the experimentwise α level, a Bonferroni adjustment was applied, and individual comparisonwise α level for each ANOVA was established a priori at P < .05. To assess whether injury status had an effect, muscle from injured rats from the

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The nontreated experimental group (NoEx + NoUS) was compared with muscle from the noninjured rats for the number of myonuclei with an independent t test. We chose number of myonuclei as a marker of injury based on the premise that nuclei, as carriers of genetic information, ultimately drive the other dependent variables we assessed. Here, the level was established a priori at P less than .05.

RESULTS

All data are presented as mean ± SEM. The mean body weight of all rats was 586 ± 17 g. Although all rats were 8 months of age, they exhibited a range (410–750 g) of body weights; however, there were no body-weight differences between the randomly assigned groups (table 1). Furthermore, the gastrocnemius muscle mass (3.3 ± 0.01 g; fig 2) did not differ (F = 1.33, P = .257) among groups.

Myonuclear Number per Fiber

To assess whether injury occurred and whether injury status had an effect, muscle from injured rats from the nontreated experimental group (NoEx + NoUS) was compared with muscle from the noninjured baseline control rats for the number of myonuclei with an independent t test. Number of myonuclei as a criterion for assessment of injury status was chosen based on observations cited in a recent review of literature focused on the topic of myonuclear domain.29 Furthermore, researchers have suggested that in normal adult rat skeletal muscle, no more than 1% to 2% of myonuclei are replaced per week,30 so we did not expect any confounding effects due to cell turnover in the data from the noninjured control gastrocnemius muscles. There were statistically fewer (P < .05) myonuclei per fiber section in the nontreated injured group than in the noninjured control group (fig 3).

There were no statistical differences in myonuclear number per fiber among the 4 experimental groups. The statistical analysis hinted toward a main effect of US on myonuclear number (F = 6.21, P = .017). Because the P value was low, we had some concern that we committed a type II error (ie, concluding there is no difference when there might be one); however, post hoc power analysis31 indicated that this was probably not the case. Likewise, it is noted that the mean number of myonuclei observed in sections of US-treated injured muscle was 2.06 ± 0.07, whereas in non-US-treated injured muscle the value was 1.85 ± 0.06.

Contractile Protein Concentration

There were no statistical differences (F = .015, P = .904) among any of the 4 treated groups in percentage of contractile protein (fig 4).

Fiber CSA

There were no statistical differences (F = .780, P = .383) among any of the 4 treated groups in fiber CSA (fig 5).

Fiber CSA/Myonuclear Number

There were no statistical differences (F = .510, P = .480) among any of the 4 treated groups in the ratio of fiber CSA/myonuclear number (fig 6). Note that the fiber CSA/myonuclear number ratio and myonuclear density have an inverse relationship; that is, as the ratio decreases, density increases.

Table 1: Descriptive Statistics

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Mass (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonexercised rats (n=10)</td>
<td>563±20</td>
</tr>
<tr>
<td>Exercised rats (n=10)</td>
<td>604±32</td>
</tr>
<tr>
<td>Control rats (n=5)</td>
<td>595±29</td>
</tr>
<tr>
<td>Pooled data (n=25)</td>
<td>586±17</td>
</tr>
</tbody>
</table>

NOTE. Values are mean ± SEM. All rats were 8 months of age.

Fig 2. Gastrocnemius (GTN) muscle masses. No significant (P < .01) differences were noted among the 4 treatment groups.

Fig 3. Myonuclear number per fiber section. No significant (P < .01) differences were noted among the 4 treatment groups. *A significant (P < .05) difference was noted between the injured NoEx + NoUS group and the noninjured baseline control group.

Fig 4. Percentage of contractile protein in the gastrocnemius muscle samples. No significant (P < .01) differences were noted among the 4 treatment groups.
controls. There was a statistically significant difference from the NoEx compared the number of myonuclei per fiber in injured rats—readily be distinguished as either injured or noninjured—we hypothesized that the number of myonuclei per fiber would be increased in the Ex+US group compared with the 3 other treatment groups. However, this hypothesis was not supported. Likewise, our hypothesis that the density of myonuclei (decreased fiber CSA/myonuclei ratio) in Ex+US treated muscle would be increased was not supported. Rantanen et al suggest that their specific US protocol has the ability to increase satellite cell proliferation after contusion injury, but that production of myotubes does not improve. Furthermore, consistent with the findings of Rantanen, we observed no effect on cell fiber CSA. One might expect that both treatments could affect the normal regeneration process and could either (1) decrease fiber CSA if there was internal damage to the muscle cell without sarcolemmal damage, because the treatments could hasten resolution of cellular swelling; or (2) increase fiber CSA if there was both internal and sarcolemmal damage, because the treatments could lead to hastened regeneration. The assertion that sarcolemmal damage may be the deciding factor whether the injured cell gains or loses fiber CSA may explain why the injury appeared to have little effect on mean fiber CSA. In other words, the magnitude of membrane damage may determine the magnitude of a swelling, shrinking, or regenerative response. Myonuclei, protein concentration, and fiber CSA would undergo obligatory changes.

There was no difference in terms of gastrocnemius mass among any of the 4 groups. Our hypothesis that the Ex+US muscles would exhibit increased mass compared with the 3 other contusion-injured groups was not supported. We had expected that muscle mass in this group would be increased because of the theorized effects of nonthermal US, combined with the effects of exercise. The US effects include possible heightened permeability of muscle cells and fibroblast cells, which could permit increased activity of macro-

**DISCUSSION**

It is theorized that US may influence cellular function because (1) it may cause resonance of the cells, and that the resonance may open protein channels that need to be opened to ultimately promote the movement of healing substances; or (2) the mechanical stimulus provided by US waves may cause inhibitor molecules of a multimolecular complex to dislodge, making the complex functional and leading to activation of signal-transduction pathways involved in healing. There are few data at the microscopic level to support the frequency resonance theory. Furthermore, there are no data available on interactive effects, if any, of US treatment and exercise on cellular markers of regeneration after contusion injury. Although use of nonthermal therapeutic US in the clinical treatment of muscular injuries, such as contusions, remains widespread, there is a growing body of literature that questions the effectiveness of the modality. Therapeutic exercise is a commonly prescribed treatment for contusions. Furthermore, a review of 13 studies in which both US and exercise were used as interventions concluded that US is not useful as a clinical adjuvant to exercise therapy. Thus, our purpose in performing this research was to use a reproducible drop-mass technique to create a standardized skeletal muscle contusion injury, in order to examine the effects of a common nonthermal therapeutic US protocol and a common Ex protocol on several markers of regeneration.

Our results suggest that the intervention did not alter any markers of regeneration in the 96 hours after contusion injury. In the context of previous time course data, the nonthermal US results are not surprising. However, previous time course data, which take both therapeutic exercise and markers of regeneration into account, are not available. Therefore, we speculate that the efficacy of exercise is a question of time, requiring more than 96 hours to observe an effect. Gregory et al waited 32 days postcontusion injury to assess erythrocyte count as a measure of resolution and concluded that exercise is preferable to no exercise.

To assess whether the injury methodology was effective—that is, capable of producing muscle samples that could readily be distinguished as either injured or noninjured—we compared the number of myonuclei per fiber in injured rats from the NoEx+NoUS group with the noninjured baseline controls. There was a statistically significant difference \(P < .05\) between injured and noninjured rats: roughly 5 myonuclei were counted in noninjured sections, whereas roughly 2 were counted in injured sections. This is of importance if one accepts the previously mentioned argument that a primary driving force behind variables, such as contractile protein concentration, fiber CSA, and muscle mass itself, is the genetic machinery contained in the nuclei. There were 2 other indications that our injury methodology was effective. First, there was the “ghost-like, moth-eaten appearance” in our stained sections of injured muscle, which is consistent with the description of the damaged area called the retraction zone. Second, a “wavy” appearance of the sarcolemma has been noted after loss of myofibrillar protein, which may lead to an increased extracellular space; a majority of the injured muscle sections exhibited the same characteristics.

It is reasonable to suggest that a loss of fiber CSA and contractile protein will follow a loss of myonuclei. We hypothesized that the number of myonuclei per fiber would be increased in the Ex+US group compared with the 3 other treatment groups. However, this hypothesis was not supported. Likewise, our hypothesis that the density of myonuclei (decreased fiber CSA/myonuclei ratio) in Ex+US treated muscle would be increased was not supported. Rantanen et al suggest that their specific US protocol has the ability to increase satellite cell proliferation after contusion injury, but that production of myotubes does not improve. Furthermore, consistent with the findings of Rantanen, we observed no effect on cell fiber CSA. One might expect that both treatments could affect the normal regeneration process and could either (1) decrease fiber CSA if there was internal damage to the muscle cell without sarcolemmal damage, because the treatments could hasten resolution of cellular swelling; or (2) increase fiber CSA if there was both internal and sarcolemmal damage, because the treatments could lead to hastened regeneration. The assertion that sarcolemmal damage may be the deciding factor whether the injured cell gains or loses fiber CSA may explain why the injury appeared to have little effect on mean fiber CSA. In other words, the magnitude of membrane damage may determine the magnitude of a swelling, shrinking, or regenerative response. Myonuclei, protein concentration, and fiber CSA would undergo obligatory changes.

![Fig 5. Mean fiber CSA of the muscle samples. No significant \(P < .01\) differences were noted among the 4 treatment groups.](image)

![Fig 6. Fiber CSA/myonuclear number. No significant \(P < .01\) differences were noted among the 4 treatment groups.](image)
phages and various other white blood cells. The effects of exercise include increased satellite cell activation\textsuperscript{13,17} as well as neural recruitment\textsuperscript{16} and mechanical loading,\textsuperscript{14} which all play key roles in growth and regeneration. The cascade of events leading to muscle regeneration\textsuperscript{35,41-43} was expected to be hastened compared with the 3 other experimental groups. Although it may be possible that differences might be observable at another time point, we specifically chose the 96 hours postinjury time point based on other work\textsuperscript{23} that suggests that satellite cells begin differentiating into myotubes at this time.

The hypothesis that treatment with Ex+US would increase gastrocnemius contractile protein concentration was not supported. Data on the critical influence of mechanical force in myofiber morphogenesis\textsuperscript{15} suggest that exercise would be beneficial not only to the organization of muscle proteins, but also to their synthesis. However, there were no differences in contractile protein concentration among the groups. Although functional measurements of muscle mechanical properties were not assessed in our study, we did assess the percentage of contractile protein.

An article reviewing effectiveness studies\textsuperscript{18} identified several important methodologic points that we incorporated into our experimental design. These include: (1) adequate controls, including placebo treatment and randomized group allocation; (2) adequate blinding of the experimenter; (3) adequate description of treatment variables, including calibration of the ultrasound machine; (4) meaningful outcome measures (ie, valid for addressing the research question); (5) adequate sample size; and (6) acceptable statistical analysis of the results.

However, caution is warranted in extrapolating these results to human clinical treatments, because of the limitations of our study and clinical use of US. It is possible that our exercise intensity (walking) was not adequate to stimulate a meaningful effect, or that protein synthesis measurable with our methodology was not adequate at 96 hours and would be evident at some later time. Other than measuring gross mass of the gastrocnemius muscles, there was no attempt made in our study to measure edema,\textsuperscript{44} which is a limitation. We also made no attempt to measure angiogenesis, which may be viewed as a limitation.\textsuperscript{45-48} Clearly, our findings are limited by the variables studied, the treatment protocols, and the specific injury. The Ex treatment may not be comparable between this (quadruped) rodent model and a (biped) human model. Furthermore, US treatment may not be comparable between our specific US and Ex protocols investigated interact to enhance skeletal muscle regeneration after contusion injury.

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