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● *Original Contribution*

USING LOW-INTENSITY PULSED ULTRASOUND TO IMPROVE MUSCLE HEALING AFTER LACERATION INJURY: AN IN VITRO AND IN VIVO STUDY

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Abstract—The purpose of this study was to determine whether low-intensity pulsed ultrasound (LIPUS) could enhance the regeneration of myofibers and shorten the healing time in injured muscle. NIH C2C12 cells, a well-known myoblastic cell line, are subclones derived from the mouse myoblast cell line established from normal adult C3H mouse leg muscle. The cells differentiate rapidly and produce extensive contracting myotubes expressing characteristic muscle proteins. We exposed C2C12 cells to LIPUS therapy using the EXOGEN 2000+ system ultrasound apparatus (Exogen Inc., Piscataway, NJ, USA) with a total treatment of 20 min every 24 h. At intervals of 2, 4, 6 and 8 days, cell growth was measured by the increase in cell number and western blot analysis of myogenin and actin. Forty mice (C57BL10J+/+) were divided into five groups of eight animals each and used in the published laceration injury model. The gastrocnemius muscle of the left leg was lacerated in all the animals. The control group (sham ultrasound) did not undergo LIPUS therapy. The ultrasound 7-, 14-, 21- and 28-day groups (only changing the number of days during which the ultrasound was applied to the injured muscle) were treated with LIPUS (20 min/day) for 7, 14, 21 and 28 consecutive days, respectively. All animals were sacrificed at 4 weeks after the injury. Evaluation methods included muscle regeneration and muscle contractile properties. LIPUS therapy produced a significantly higher proliferative rate and cell number at days 6 and 8 ($p < 0.05$). Densitometric evaluation revealed an increase in myogenin and actin proteins in cells treated with LIPUS in the 4-, 6- and 8-day groups. The regeneration of myofibers, fast-twitch and tetanus of LIPUS-treated muscles (21 and 28 days) was significantly greater relative to control muscles. There was no major strength difference between the normal non-injured muscle and the group treated with LIPUS for 28 days. In conclusion, this was the first experimental study to show that LIPUS therapy is able to enhance the regeneration of myofibers with better physiologic performance in injured mice muscles after laceration, especially prior to postoperative week 4. Findings of this study demonstrate a scientific basis for future clinical trials and establish an indication for LIPUS in enhancing muscle healing after laceration injury. (E-mail: chan512@adm.cgmh.org.tw) © **2010 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Low-intensity pulsed ultrasound, Muscle laceration injury, Muscle healing, Fast-twitch, tetanus.**

INTRODUCTION

Muscle injuries are a challenging problem in traumatology and they encompass some of the most frequently occurring injuries encountered in sports medicine. The injury can occur via a variety of mechanisms, ranging from direct mechanical deformation (such as muscle laceration, strain and contusion) to indirect damage related to ischemia and neurologic dysfunction (Carlson and Faulkner 1983; Kasemkijwattana et al. 1998). Muscle injuries are frequently encountered in professional and recreational sports. The best treatment for these injuries has yet to be clearly defined and the recommended treatment regimens vary widely depending on the severity of the injury (Taylor et al. 1993). The suggested treatments currently include rest, ice, heat, water pool therapy, compression, elevation, immobilization, aggressive full range of motion using a passive motion machine, drugs and hospitalization (Aronen and Chronister 1992). Significant morbidity, such as early functional and structural deficits, re-injury, muscle atrophy, contracture and pain

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often occur after muscle injuries (Aronen and Chronister 1992). These unmet medical needs prompted us to seek novel strategies designed to improve muscle healing.

Ultrasound (US) has many medical applications, including therapeutic, operative and diagnostic procedures (Ziskin 1987). It is used both operatively and therapeutically, with intensities ranging from 0.2 to 100 W/cm^2; it achieves its biologic results by considerably increasing the temperature of the treated tissue (Wells 1985; Ziskin 1987). In contrast, safe intensities for diagnostic imaging are much lower (0.5–50 mW/cm^2) and are considered non-thermal stimuli (St. John 1984). There have been claims that therapeutic ultrasound promotes tissue repair, especially by enhancing cell proliferation and protein synthesis during the healing of skin wounds (Webster et al. 1980; Markert et al. 2005; Ikeda et al. 2006; Kobayashi et al. 2009), tendon injuries (Frieder et al. 1988; Enwemeka et al. 1990; Qin et al. 2006; Walsh et al. 2007) and fractures (Duarte 1983; Heckman et al. 1994; Lavandier et al. 2009; Wijdicks et al. 2009). Because of the lack of scientific evidence, the current use and prescription of therapeutic ultrasound as a treatment to enhance skeletal muscle regeneration is often based on the personal options and experience of clinicians (Van der Windt et al. 1999; Warden 2003; Khanna et al. 2009). There is still no consensus statement on the appropriate parameters for the treatment of muscle injuries or whether the use of therapeutic ultrasound is justified as a treatment when the goal is to influence skeletal muscle repair and regeneration.

Low-intensity pulsed ultrasound (LIPUS) is used clinically for the enhancement of fracture healing. Animal studies have demonstrated the stimulation of callus tissue and the acceleration of bone healing by LIPUS (Duarte 1983; Pilla et al. 1990; Yang et al. 1996; Lavandier et al. 2009; Wijdicks et al. 2009). Scintigraphic control of the bone-healing process has revealed faster healing in ultrasonically treated animals compared with untreated control animals (Klug et al. 1986; Guerino et al. 2008). In fresh human diaphyseal tibia fractures, the application of US has been demonstrated to accelerate consolidation of the fractures by 40% (Heckman et al. 1994). Accumulating evidence suggests that therapeutic US facilitates fibroplasias and protein synthesis (Drastichova et al. 1973; Harvey et al. 1975; Morcos et al. 1978). LIPUS has been widely used in the fields of bone healing and tendon injury (Morcos and Aswad 1978; Duarte 1983; Klug et al. 1986; Pilla et al. 1990; Yang et al. 1996; Qin et al. 2006; Walsh et al. 2007). With this in mind, we hypothesized that LIPUS would be capable of enhancing the regeneration of myofibers and shorten the healing time in injured muscle. To test this hypothesis, we conducted a series of *in vitro* investigations to determine the effects of LIPUS on myoblastic proliferation and the expression of myogenic-related proteins. *In vivo*, we selected the well-established model of muscle laceration rather than other forms of muscle injury for our study because this model is most easily reproduced and repeated in the laboratory (Fukushima et al. 2001; Chan et al. 2003). We studied the positive effect of LIPUS on the development of myogenesis and evaluated its influence on muscle healing. Prior to this study, there had been no reports concerning the effects of LIPUS on muscle healing.

MATERIALS AND METHODS

The effect of LIPUS on myoblasts in vitro

Cell cultures. NIH C2C12 cells, a well-known myoblastic cell line, are subclones derived from the mouse myoblast cell line established from normal adult C3H mouse leg muscle. The cells differentiate rapidly and produce extensive contracting myotubes expressing characteristic muscle proteins. The cells were cultured in 75-mL tissue culture flasks in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 10% horse serum, 0.5% chicken embryo extract and 1% penicillin/streptomycin. All cells were grown in an incubator at 37 °C and 5% CO_2. The cells were grown for 3 days with periodic medium changes. When confluence was achieved, the cells were removed by adding trypsin/ethylenediaminetetraacetic acid (EDTA) (4 mL, 0.2% w/v in 0.02% EDTA). After washing and resuspending the cells in growth medium, aliquots (2000 cells/well) were seeded into 6-well plates.

Growth experiments. A US apparatus, the EXOGEN 2000+ US system (Exogen Inc., Piscataway, NJ, USA), was used to deliver an US signal with spatial and temporal average intensities of 30 mW/cm^2 (US group) and 0 mW/cm^2 (control). The frequency was 1.5 MHz with a 200-microsecond tone burst repeated at 1.0 kHz. The US was transmitted through the bottom of the culture dish via coupling gel (Exogen Inc.) between the ultrasonic transducer and the dish. The area of the US transducer was 5.3 cm^2. All cells were exposed to LIPUS therapy with a total treatment of 20 min every 24 h. The control cultures were subjected to the same conditions as the treated cultures, except that the US apparatus was not turned on (sham ultrasound). At intervals of 2, 4, 6 and 8 days, cell growth was measured by an increase in cell number and western blot analysis of myogenin and actin (myogenesis markers) (Lessard 1998).

Western blot analysis. C2C12 cells were treated 4, 6 and 8 times with LIPUS (once per day). The cells were then washed with 1× phosphate-buffered saline (PBS) and lysed in RIPA buffer. Ten micrograms of total protein extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using

10% gel and the separated proteins were transferred to polyvinylidine difluoride (PVDF) membranes for immunoblot analysis. Western blot analysis of myogenin was performed by incubation with anti-myogenin mAb and detected by enhanced chemiluminescence reaction (ECL). To evaluate whether LIPUS influences the intracellular myogenic protein content, we set up similar amounts (10^5 cells) of C2C12 cell cultures seeded in different T-25 flasks and treated them 3, 6 and 9 times with LIPUS (once per day) in parallel with the growth experiments. The expressions of myogenin and actin (myogenesis markers) were examined in cells treated with different dosages of LIPUS using anti-myogenin (MAB3876; Chemicon, Billerica, MA, USA) and anti-actin (MAB1501; Chemicon) antibodies, respectively. After a 72-h incubation, the cells were washed twice with PBS and lysed using a lysate buffer [β-mercaptoethanol: sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol) = 1:20]. After boiling, the whole cell lysates were centrifuged at 3000 $\times$ *g* for 5 min and the supernatants were collected and stored at 4 °C. Lysate supernatants were analyzed by running on 12% SDS-PAGE gels. Protein concentrations were determined using the Bradford method (Bradford 1976). Four micrograms of protein from each sample was run at a voltage of 55 V for 1 h and then 75 V for 3 h. We then transferred the total protein to a nitrocellulose membrane at 60 V overnight. Membranes were washed in PBS for 5 min and then blocked with 1% non-fat dry milk and 2% horse serum in T-PBS (0.01% Triton in PBS) for 1 h at room temperature (shaken at 60 cycles/min). A α-smooth muscle actin primary antibody (1:1000; Chemicon) was applied at room temperature for 1.5 h, followed by four 15-min washes in T-PBS. A horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (AP124P; Chemicon) was applied for 1 h, followed by four 15-min T-PBS washes. After washing, the blots were developed using enhanced chemiluminescence (Supersignal West Pico Chemiluminescent Substrate; Pierce, Rockford, IL, USA), and positive bands were detected using X-ray film. The filter was scanned and evaluated using North Eclipse software (version 6.0; Empix Imaging, North Tonawanda, NY, USA), which calculated the density in each protein band.

Determination of the biologic and physiologic effects of LIPUS on muscle healing after laceration

Animal model. A muscle laceration model was developed in mice (C57BL10J+/+) based on previously described studies (Fukushima K et al. 2001; Chan et al. 2003). Forty mice, with an average age of 8 weeks and an approximate weight of 18 to 22 g, were used in this experiment. The animals were housed in cages and fed with commercial pellets and water *ad libitum*. The policies and procedures of the animal laboratory were in accordance with those detailed by the Taiwan Department of Health and Human Services. The Animal Research and Care Committee of the authors' institutions approved the research protocols used for these experiments (protocol no. AN-93069). The mice were anesthetized using a low dose (80 mg/kg) of pentobarbital sodium (Anpro Pharmaceuticals, Arcadia, CA, USA) administered by intraperitoneal injection. The gastrocnemius muscles of the 40 mice were lacerated with a surgical blade (No. 11) at their largest diameter through the lateral 50% of their width and 100% of their thickness (Fukushima K et al. 2001; Chan et al. 2003). Polydioxanone suture material (PDSII 5-0; Ethicon, Somerville, NJ, USA), 4 mm in length, was placed at the medial edge of the lacerated side of each leg as a marker of the laceration site. After the laceration was made, the skin was closed with 4-0 black silk. Only the left gastrocnemius muscle was lacerated in all the animals. Ultrasound treatment using the EXOGEN 2000+ US system (Exogen Inc.) was initiated 24 h post-laceration injury. Ultrasonic coupling gel was placed on the skin overlying the laceration site. The US therapy was performed once daily on anesthetized animals for 20 min per session. The control group was subjected to the same conditions as the treated groups, except that the US apparatus was not turned on (sham ultrasound for 28 days). The 7-day US group was treated with LIPUS for 7 continuous days and with sham ultrasound for the rest of 21 days after laceration. The 14-day US group was treated with US for 14 continuous days and with sham ultrasound for the rest of 14 days. The 21-day US group was treated for 21 continuous days and with sham ultrasound for the rest of the 7 days. The 28-day US group was treated with 28 continuous days of LIPUS therapy. The exposures and analyses were double blinded. Two animals per group were assessed histologically. All the animals were sacrificed for the evaluation of healing and regeneration at 4 weeks after the injury. The gastrocnemius muscles were isolated and frozen in 2-methylbutane pre-cooled in liquid nitrogen.

Evaluation of muscle regeneration after LIPUS therapy. Hematoxylin and eosin staining was used to monitor the number of regenerating myofibers within the injured site treated with the different dosages of LIPUS, and the results among the different groups were compared. Centronucleated cells were considered to be regenerating myofibers (Hurme and Kalimo 1992; Bischoff 1994). Nuclei with no discernible surrounding cytoplasm were discarded. The observer performing the counting was blinded to the source of the samples. Five random fields were selected for each sample and the total number of regenerating myofibers within the injured site

was measured using a previously described protocol (Fukushima K et al. 2001; Chan et al. 2003).

Physiologic evaluation of muscle contractile properties after LIPUS therapy. Thirty mice were enrolled in the physiologic test. The gastrocnemius muscle of the left leg in each mouse was lacerated, as described earlier, and the treated with different dosages of LIPUS after injury. The gastrocnemius muscle of the right leg in each mouse was simply exposed and kept intact. Six mice in the groups treated with 7, 14, 21 or 28 doses of LIPUS (one treatment per day) were examined by physiologic testing for functional recovery at 28 days after injury. The mice were anesthetized using a low dose (80 mg/kg) of pentobarbital sodium administered by intraperitoneal injection. Both gastrocnemius muscles were removed and mounted in a 5-mL double-jacketed organ bath at 36 °C in Krebs solution (mmol/l: NaCl, 113; KCl, 4.7; $CaCl_2$, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 25; KH_2PO_4, 1.2; glucose, 11.5) and constantly bubbled with a mixture of 95% oxygen and 5% carbon dioxide. The initial tension was set at 20 mN; isometric contractions were measured with strain-gauge transducers coupled with a TBM4 strain gauge amplifier (World Precision Instruments Inc., Sarasota, FL, USA) and recorded on a computer using a data acquisition program (Windaq; DATAQ Instruments Inc., Akron, OH, USA). The sampling rate per channel was set at 500 Hz. The amplitude of the stimulation-evoked contractions was computed using a calculation program (WindaqEx, DATAQ). After 20 min of equilibration, electrical field stimuli were applied through two platinum wire electrodes positioned on the top and bottom of the organ bath and separated by 4 cm. The muscles were stimulated with square-wave pulses of 0.25-millisecond duration with maximal voltage (50 V). Initially, 1 Hz stimulation was applied, and the muscle twitches were recorded. Then, six tetanic stimulations were applied with a 0.5-s strain duration at 100 Hz every 10 s. Finally, the muscle was weighed using a microbalance (Mettler Toledo Inc., Greifensee, Switzerland). The strength was measured per unit weight and expressed in milli-Newtons per gram.

Statistical analyses. The average and standard deviation of the cell proliferation, densitometric evaluation, number of regenerating myofibers, fast-twitch strength and tetanus strength were compared among the different groups using a Manny-Whitney test for statistical analysis. Statistical significance was defined as $p < 0.05$.

RESULTS

The Effect of LIPUS on myoblasts in vitro

Effects of LIPUS on the myoblast growth rate. The effect of LIPUS on the proliferation of NIH C2C12 myoblasts is shown in Figure 1. The cell growth curve showed that the experimental group treated with LIPUS had a significantly higher proliferative rate and cell number at days 6 and 8 ($p < 0.05$).

Effect of LIPUS on intracellular myogenin and actin: Western blot analysis. To elucidate whether LIPUS acts on the production of myogenin and actin proteins, we investigated the effect of LIPUS on the intracellular myogenin and actin protein contents in the samples. To ensure that cells for intracellular myogenin and actin protein analysis responded to LIPUS, their growth rate was determined in parallel. Densitometric evaluation revealed increases in myogenin and actin proteins in the cells treated with LIPUS in the 4-, 6- and 8-day groups (Fig. 2). The densitometry also revealed a 25% ($p = 0.07$) and 24% ($p = 0.08$) increase in myogenin and actin proteins, respectively, in the cells treated with 4 doses of LIPUS, 34% ($p = 0.036$) and 37.5% ($p = 0.02$) increases in the cells treated with 6 doses of LIPUS, and 40% ($p = 0.005$) and 47% ($p = 0.003$) increases in the cells treated with eight doses of LIPUS compared with the control. Western blot analysis showed that the experimental group treated with LIPUS had a significant increase in myogenin and actin proteins at days 6 and 8 ($p < 0.05$).

Determination of the biologic and physiologic effects of LIPUS on muscle healing after laceration

Evaluation of muscle regeneration after LIPUS therapy. The muscles that had been treated with LIPUS exhibited numerous regenerating myofibers at the site of laceration (Fig. 3). These were uniformly present throughout the injured region in the superficial and in the deep part of the muscle. However, the control muscles also contained regenerating myofibers, although these

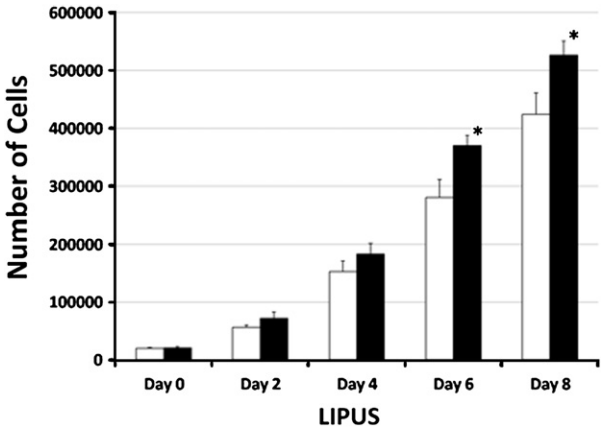

Fig. 1. A cell growth curve showing that the experimental group treated with low-intensity pulsed ultrasound (LIPUS) had a significantly higher proliferative rate at days 6 and 8 ($p < 0.05$). White bars: control group; black bars: experimental group.

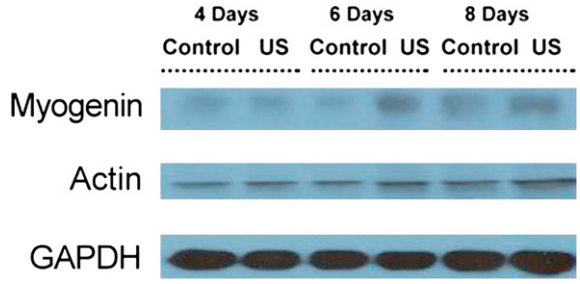

Fig. 2. Effect of low-intensity pulsed ultrasound (LIPUS) on myogenin and actin protein level after 4, 6 and 8 (once per day) treatments. Densitometric evaluation revealed increases in myogenin and actin proteins in the cells treated with LIPUS in the 4-, 6- and 8-day groups. Western blot analysis showed that the experimental group treated with LIPUS had a significant increase in myogenin and actin proteins at days 6 and 8 ($p < 0.05$). US: ultrasound. GAPDH = glyceraldehyde-3-phosphate dehydrogenase (loading controls for quantitative Western blotting).

were predominantly located in the deep parts of the laceration. In the superficial region of the control muscles, there were infiltrations of mononucleated cells with only a few regenerating myofibers and most of this area was occupied by fibrosis tissue. Figure 4 shows the mean number of regenerating myofibers. All the centronucleated myofibers present in the injured muscle were counted. We observed an increased number of regenerating myofibers in all the LIPUS-treated groups when compared with the controls. However, only high dosages of LIPUS (14, 21 and 28 days) led to a significantly higher number of regenerating myofibers when compared with controls ($p < 0.05$).

Physiologic evaluation of muscle contractile properties after LIPUS therapy. Four groups of mice treated with LIPUS (7, 14, 21 and 28 days) after the laceration injury were examined by physiologic testing for functional recovery at 28 days after injury. The physiologic evaluation of contractile properties was performed on six animals from each group. The effect on the lacerated muscle was observed by physiologic testing (tetanus strength and fast twitch) and compared with that in the control group (no LIPUS treatment). The fast-twitch muscle strength was 106.5 ± 12.5 mN/g in the normal noninjured muscle group, 20.3 ± 4.9 mN/g in the control group, 24 ± 4.8 mN/g in the group treated with US for 7 days, 31.8 ± 5.5 mN/g in the group treated with US for 14 days, 50.3 ± 5.8 mN/g in the group treated with US for 21 days and 95 ± 5.9 mN/g in the group treated with US for 28 days. There was a significant difference between the control group and the 14-, 21- and 28-day US groups. There was no major strength difference between the normal noninjured muscle and the group treated with US for 28 days. We also observed a significant difference in strength after comparing the injured control muscles and the muscles treated with US for 7, 14 and 21 days with normal noninjured muscle (Fig. 5A).

The tetanus muscle strength was 602.7 ± 34.4 mN/g in the normal muscle, 250 ± 14.8 mN/g in the control group, 300.5 ± 19.9 mN/g in the 7-day US group, 411.2 ± 30.5 mN/g in the 14-day US group, 502.5 ± 35.8 mN/g in the 21-day US group and 570 ± 21.9 mN/g in the 28-day US group. There was a significant difference between the control group and the groups treated with US for 21 and 28 days. There was no major strength difference between the normal noninjured muscle and the group treated with US for 28 days. We also observed a significant difference in muscle force (tetanus strength) after comparing the injured control muscles and the muscles treated with US for 7, 14 and 21 days with the normal noninjured muscle (Fig. 5B).

DISCUSSION

One common treatment for laceration injury is ultrasound; however, a complete understanding of the underlying science is not well established. Several studies have aimed to discover new data about the biologic effects of therapeutic pulsed ultrasound regarding its use in the treatment of muscle injury. The exact mechanisms underlying ultrasound effects on tissue repair are probably related to its mechanical effects as opposed to the thermal effect (Dinno et al. 1989; Karnes and Burton 2002). In this

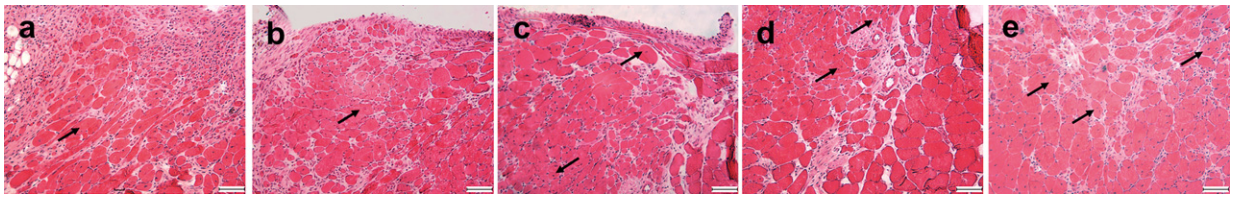

Fig. 3. Histologic evaluation of the muscle healing process in the lacerated muscle 4 weeks postinjury at 5 time points after four different doses of low-intensity pulsed ultrasound (LIPUS) therapy. Panels (a) (control), (b), (c), (d) and E show lacerated muscle treated immediately after injury with 7, 14, 21 and 28 days of LIPUS therapy, respectively. Centronucleated cells were considered to be regenerating myofibers (black arrows in (a), (b), (c), (d) and (e)). An increased number of regenerating myofibers in all the LIPUS-treated groups when compared with the controls. (original magnification, × 200), Bars = 250 μm.

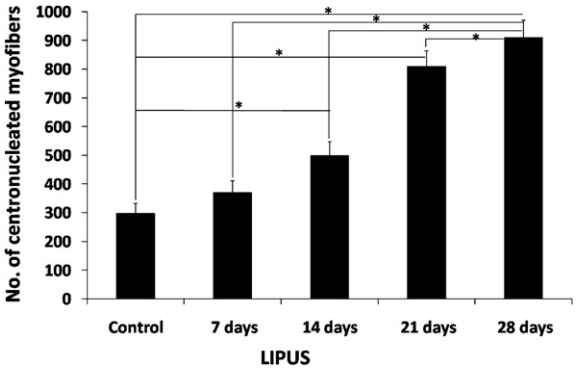

Fig. 4. Panel demonstrates a comparison of the numbers of regenerating myofibers observed in the control and treatment groups with various dosages of low-intensity pulsed ultrasound (LIPUS) at 4 weeks after laceration. *$p < 0.05$.

context, it is believed that the mechanical stimulus provided by ultrasound waves may activate signal-transduction pathways involved in healing (Markert et al. 2005; Ikeda et al. 2006; Kobayashi et al. 2009). It has already been shown that ultrasound alters the activity of platelets and neutrophils and macrophages

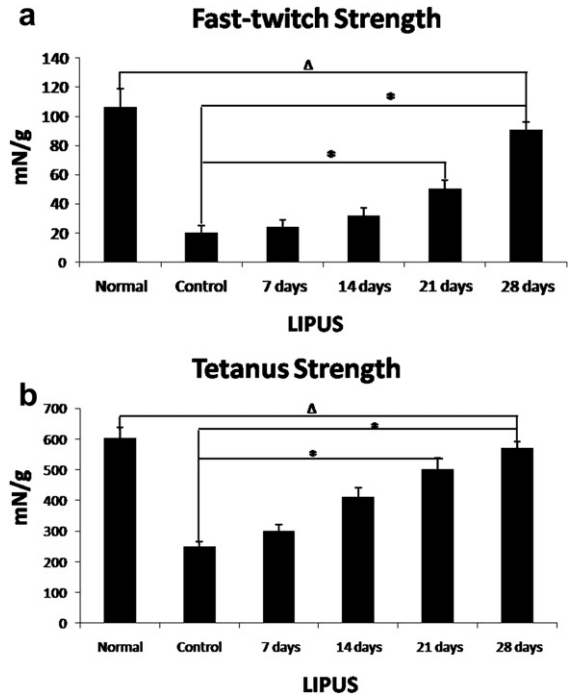

Fig. 5. Physiological analysis of low-intensity pulsed ultrasound (LIPUS) therapy on muscle strength. The results of fast twitch (A) and tetanus strength (B) showed that there was no major difference between the normal age-matched control group and the groups treated with LIPUS for 7 and 14 days. However, there was a significant difference between the control group and the groups treated with US for 21 and 28 days. Moreover, there was no major fast-twitch and tetanus strength difference between the normal noninjured muscle and the group treated with LIPUS for 28 days. *$p < 0.05$; Δ $p > 0.05$.

involved in the inflammatory phase of the healing muscle process, speeding up this process. Another effect is the increase in the speed of angiogenesis (Rantanen et al. 1999). Results from previous studies of the effectiveness of ultrasound in helping tissue repair are still contradictory because some investigations found no effect on regenerating skeletal myofibers, fibroblasts or ulcers and wounds. The different findings may be explained by the different types of tissue examined, the models of injury and the mode, intensity and frequency of ultrasound treatment. There seems to be little doubt, however, that ultrasound treatment accelerates cell proliferation, at least for fibroblasts (Webster et al. 1980; Young and Dyson 1990) and satellite cells (Rantanen et al. 1999).

Skeletal muscle is capable of extensive regeneration after injuries, such as contusions, lacerations and strains (Carlson and Faulkner 1983; Taylor et al. 1993; Kasemkijwattana et al. 1998). The myogenic cells responsible for this regeneration are the mononucleated satellite cells, which are located between the basal lamina and plasma membrane of the muscle fiber (Hurme and Kalimo 1992; Bischoff 1994). After muscle injury, it is hypothesized that the disruption of the basal lamina and the plasma membrane releases and activates the satellite cells. The satellite cells begin to proliferate and differentiate into multinucleated myotubes and eventually into myofibers (Quinn and Haugk 1996). The growth of these regenerating myofibers into the injured site promotes the healing of the muscles. The parallel occurrence of muscle regeneration and scar tissue formation in the injured muscle may lead to proliferative competition between both systems during the healing process. Many growth factors can stimulate the growth and protein secretion of many musculoskeletal cells. It is presumed that during muscle regeneration, growth factors and cytokines released by the injured muscle activate the satellite cells. Preliminary data have further suggested that growth factors play various roles during muscle regeneration. Of particular interest are the insulin-like growth factors (IGF-1 and 2), which have been found to be mitogenic for myoblasts (Florini and Magri 1989; Florini et al. 1996; Quinn and Haugk 1996).

The mechanism through which ultrasound facilitates tissue repair is likely related to its mechanical effects (ultrasonication) (Dinno et al. 1989; Karnes and Burton 2002). Ultrasonication, or ''micromassage'' of the tissue, produces a change in membrane permeability and stimulates the transport of second messenger substances, such as calcium, across the cell membrane (Mortimer and Dyson 1988; Dinno et al. 1989). These second messengers may stimulate the proliferation of myogenic cells, in the case of skeletal muscle, the satellite cells (Mauro 1961). The nest stage of the repair process, cell fusion and differentiation, is regulated by IGF-I

(Allen et al. 1990). Rantanen et al. (1999) observed an increase in satellite cell number in regenerating skeletal muscle after pulsed ultrasound treatment, compared with injured untreated muscle. Others also noted an increase in the cell proliferative stage of tissue repair because of ultrasound treatment (Webster et al. 1980; Young et al. 1990). Freitas et al. (2007) demonstrated the effect of therapeutic pulsed ultrasound on parameters of oxidative stress in skeletal muscle after laceration injury. They concluded that LIPUS seems to protect the muscle from oxidative injury and diminished catalase and superoxide dismutase activity, especially on the first day following muscle laceration.

In this respect, there exists a theoretical construct for the use of low-intensity pulsed US. This theory is based largely on the extrapolation of *in vitro* studies and animal studies that have examined the effects of US on surgically induced skin wounds and tendon transactions. *In vitro* studies have demonstrated that exposure to ultrasonic energy stimulates the proliferation of fibroblasts (Young and Dyson 1990; Ramirez et al. 1997) and increases their metabolic activity, primarily the synthesis of collagen (Harvey et al. 1975; Young and Dyson 1990; Ramirez et al. 1997; Kobayashi et al. 2009). In animals with surgically induced skin wounds, low-intensity US applied in the early postinjury period has been demonstrated to stimulate angiogenesis (Young and Dyson 1990), decrease wound size, increase collagen deposition and increase the ability to withstand tensile loading (Byl et al. 1992, 1993). Previous animal and clinical studies (Duarte 1983; Pilla et al. 1990; Lavandier et al. 2009; Wijdicks et al. 2009) have clearly revealed a positive effect of US on the rate of osseous repair. Kristiansen et al. (1997) demonstrated that US stimulation in the range of diagnostic imaging (30 mW/cm^2) could promote fracture healing. In earlier studies, Sun et al. (1999) demonstrated that US treatment in the lower range of therapeutic intensity (330–770 mW) accelerated the repair of femoral defects in an *in vitro* tissue culture model. The US device used in this study applied 0.068 W/cm^2, which is in the upper range of diagnostic intensity, to the bone cell culture. Wijdicks et al. (2009) also demonstrate that LIPUS enhances bone formation induced by recombinant human bone morphogenetic protein-2 (rhBMP-2). In the present study, for the administration of LIPUS treatment, we selected the EXOGEN 2000+ ultrasound system (Exogen Inc.), which is an FDA-approved clinical treatment used for the promotion of fracture healing in fresh fractures and nonunion (Rubin et al. 2001). The device delivers US of high quality and stability, is easy to operate and incorporates its own battery system. We used US signals with a spatial and temporal average intensity of 30 mW/cm^2 with a frequency of 1.5 MHz, and with a 200-mirosecond tone burst repeated at 1.0 kHz. The EXOGEN 2000+ system (Exogen Inc.) can be used a maximum of 300 times for therapy.

To the best of our knowledge, this is the first study to describe the positive effect of LIPUS on injured skeletal muscle *in vitro* and *in vivo*. By measuring cell numbers, we demonstrated that LIPUS is a highly proliferative agent in NIH C2C12 myoblast cultures when LIPUS therapy was administered for 6 and 8 days. Myogenin and actin have been implicated in the pathology of myogenesis and are marker proteins for the myoblastic phenotype (Lessard1998). Western blot analysis showed that the experimental group treated with LIPUS had a significant increase in myogenin and actin proteins at days 6 and 8 ($p < 0.05$). Wilkin et al. (2004) observed no evidence to suggest that pulsed therapeutic ultrasound treatment (5 min duration, frequency of 3.3 MHz, intensity of 1W $\times$ cm^{-2}, ERA of 1 cm^2 and duty cycle of 20%, once daily after injury for 7 days) as administered enhances the examined biologic markers (muscle mass, total protein concentration or myonuclear number) of skeletal muscle of regeneration following a contusion injury. However, the authors did not perform functional studies on the regenerating muscle. In our animal study, we found an increase in significant functional recovery by 21 days postinjury and there was no major strength difference between the normal noninjured muscle and the group treated with US for 28 days. Moreover, the positive effect of LIPUS on the development of muscle proliferation appeared to be dose-dependent. This suggests that direct application of LIPUS is capable of increasing myogenic protein formation and, consequently, is capable of improving muscle regeneration. The results we obtained for physiologic testing are also comparable with the previous data.

In conclusion, the results of this study suggest that LIPUS therapy enhances regenerative myofiber formation in injured muscles. In the LIPUS-treated groups, the repaired muscles had a better physiologic performance and displayed earlier histologic evidence of healing. However, the effect was dependent on the dosage of LIPUS. LIPUS therapy may have considerable clinical potential for use in shortening the healing time of injured muscle. In the future, we will attempt to study the physical mechanisms of LIPUS on muscle healing. The authors will consider exploring different ultrasound parameters in addition to the exposure time and attempt to determine ultrasound mechanisms leading to the observed effects, such as the ultrasound intensity, frequency, measurement of the temperature increase due to ultrasound application, the effect of cavitation and other mechanical ultrasound mechanisms.

We will also attempt to study the effect of LIPUS on other muscle injuries, particularly muscle strain. Muscle strain is the most common type of muscle injury

encountered in sports medicine. The healing process in muscle after a strain injury is similar to that following laceration, *i.e.*, the development of significant fibrosis and subsequent muscle regeneration (Kasemkijwattana et al. 1998; Taylor et al. 1993; Chan et al. 2005). Therefore, we will apply similar techniques of LIPUS therapy in future studies of muscle strain injuries. The advantage of LIPUS is that it is a useful tool for the clinics and a more applicable clinical therapy.

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