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Effect of Low-Intensity Pulsed Ultrasound on Nerve Repair

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1. Introduction

Low-intensity pulsed ultrasound (LIPUS) is a medical technology, generally utilizing 1-1.5 MHz frequency pulses, with a pulse width of 200 μs, repeated at 1-1.5 kHz, at an intensity of 10- 30 mW/cm^2, 20 minutes/day. There are two main types of ultrasound effects: thermal and nonthermal. Both types are thought to first "injure" the cells, resulting in their growth retardation, and then to initiate a cellular recovery response characterized by an increase in protein production(Johns 2002). Compared to high-intensity continuous ultrasound, LIPUS is much lower in intensity and has unique characteristics such as pulsed waves, which are regarded as nonthermogenic and non-destructive (Mukai, Ito et al. 2005).

Applications of LIPUS include: promoting bone fracture healing; treating orthodontically induced root resorption; regrow missing teeth; enhancing mandibular growth in children with hemifacial microsomia; promoting healing in various soft tissues such as cartilage, intervertebral disc, etc.; improving muscle healing after laceration injury. Researchers at the University of Alberta have used LIPUS to gently massage teeth roots and jawbones to cause growth or regrowth, and have grown new teeth in rabbits after lower jaw surgical lengthening (Distraction osteogenesis). As of June 2006, a device has been licensed by the Food and Drug Administration (FDA) and Health Canada for use by orthopedic surgeons. It has not yet been approved by either Canadian or American regulatory bodies and a market-ready model is currently being prepared. LIPUS is expected to be commercially available before the end of 2012. According to Dr. Chen from the University of Alberta, LIPUS may also have medical/cosmetic benefits in allowing people to grow taller by stimulating bone growth.

In recent years, data on the therapeutic effects of LIPUS have been accumulating. So far, it has been reported that LIPUS enhances cell proliferation and alters protein production in various kinds of cells such as endothelial cells, osteoblasts, chondrocytes, and fibroblasts (Ikeda, Takayama et al. 2006; Hiyama, Mochida et al. 2007; Takeuchi, Ryo et al. 2008), but there is little information on the response of Schwann cell and neurons to LIPUS irradiation. Some studies have indicated that LIPUS has positive effects on axonal regeneration during in vivo peripheral nerve injury trials (Crisci and Ferreira 2002; Chang, Hsu et al. 2005) and that its stimuli on the injured sciatic nerve can increase the number of nerve fibers compared to that of untreated injured nerves in rats (Raso, Barbieri et al. 2005). Thus, treatment with

LIPUS is likely to assist the regeneration of neuronal axons. However, the mechanism of such events is unknown.

2. Schwann cells that were subjected to LIPUS consistently demonstrated an increase in cell proliferation

Ultrasound is commonly used for diagnostic imaging and physiotherapy and can exert biological effects through either thermal or mechanical mechanisms in living tissue (Choi, Pernot et al. 2007; Nahirnyak, Mast et al. 2007). In contrast to high-intensity continuous ultrasound, LIPUS (<100 mW/cm^2) has much lower intensities, which are regarded as nonthermogenic and nondestructive (Ikeda, Takayama et al. 2006). Mechanical strains received by cells may result in biochemical events and increase membrane permeability (Danialou, Comtois et al. 2002). Despite the wide use of LIPUS for improving peripheral nerve tissue regeneration in animal models (Crisci and Ferreira 2002; Chang, Hsu et al. 2005; Raso, Barbieri et al. 2005), very little is known about its effects on the glial cells of peripheral nerves. It has been reported that Schwann cells respond somehow to LIPUS stimulation (Chang, Hsu et al. 2005; Raso, Barbieri et al. 2005). However, the results of previous investigations were somewhat inconclusive, particularly regarding the precise mechanism.

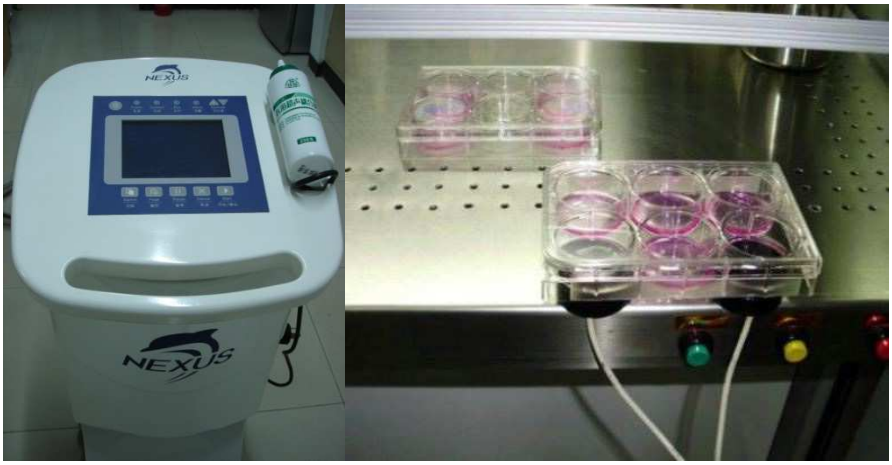

Fig. 1. Experimental apparatus for applying low- intensity pulsed ultrasound (LIPUS) which generated LIPUS with a SATA intensity of 10 mW/cm^2, pulse width of 200 microseconds, repetition rate of 1.5 KHz, and an operation frequency of 1MHz. LIPUS irradiated neurons with two probes 24 h after in culture. A six-well plate was placed on the probes. LIPUS was transmitted to culture plate via an interposed ultrasound gel. Two transducers for the control group (sham-LIPUS; LIPUS not turned on) and two probes for the LIPUS group.

Previous studies have shown that LIPUS in the cultured cells induces significant cellular responses in nucleus pulposus cells, endothelial cells, osteoblasts, chondrocytes, and fibroblasts, (Parvizi, Wu et al. 1999; Zhou, Schmelz et al. 2004; Hill, Fenwick et al. 2005; Sena, Leven et al. 2005; Hiyama, Mochida et al. 2007) but little is known about Schwann cell response to direct LIPUS stimulation. The previous work with peripheral nerve injury have

demonstrated that the magnitude and duration of LIPUS has a direct effect on whether the stimulus has a positive or negative effect on nerve regeneration (Chang, Hsu et al. 2005; Raso, Barbieri et al. 2005). Yet, there are currently no data about the actual types and levels of the ultrasound for the cells within the peripheral nerve. Although using similar values from studies on chondrocytes, endothelial cells, and osteoblasts would allow direct comparison between different cell lines, these values may not have any significance to neural tissue based upon the different physiologic demands of each tissue type. Thus, we chose the magnitude and duration of stimulation for these experiments based on previous work that demonstrated that ultrasound induced a biological response in Schwann cells (Chang, Hsu et al. 2005)

The purpose of this study was to evaluate how sustained LIPUS directly affects Schwann cell function. By evaluating for the expression of the pan-specific Schwann cell marker S-100 with immunohistochemistry, we determined whether Schwann cells de-differentiated after LIPUS stimulation. Schwann cell proliferation was explored using BrdU uptake assays to ascertain if direct LIPUS stimulation is mitogenic for Schwann cells in culture plates.

2.1 Material and methods

Schwann cells culture and low-intensity pulsed ultrasound treatment

Schwann cells were prepared using a method previously described with some modifications (Cai, Campana et al. 1999). Briefly, sciatic nerves were dissected from Wistar rats (n=30) at postnatal day 1–3. The epineural sheath was removed. Thereafter, the sciatic nerves were chopped into 200-μm pieces and enzymatically digested (collagenase/trypsin, 1 mg/ml, 1 hour, 37°C). The resulting cell suspensions were plated onto a six-well plate and cultured in Schwann cell medium (DMEM/10% heat-inactivated FCS/2 mM glutamine/pen/strep). Two different cell densities were prepared for subsequent experiments, a cell density of 5,000 cells/1.77 cm^2 for proliferation assay and immunohistochemistry assays, and a cell density of 100,000 cells/1.77 cm^2 for semiquantitative RT-PCR. The fibroblasts were eliminated by 10 μM cytosine arabinoside and complement-mediated cytolysis with the fibroblast- specific antibody Thy1.1 in conjunction with baby rabbit complement (Cedarlane, Burlington, NC). The medium was changed every other day by adding 2 μM forskolin and glial growth factor (100μg/ml) for expansion of Schwann cells for up to 14 days of cells culture. The purity of cultures was monitored by immunostaining using the Schwann cell marker S-100 and the fibroblast marker Thy-1.1.

Schwann cells were cultured and subjected to LIPUS with modifications as previously described (Takayama, Suzuki et al. 2007). This device (Nexson-The-P41, Nexus Biomedical Devices, Hangzhou, China) generated LIPUS with a pulse width of 200 microseconds, repetition rate of 1.5 KHz, operation frequency of 1 MHz, spatial average temporal average of 100 mW/cm^2, 5 minutes/day. The LIPUS treatment was started 24 hours after initiation of cells culture and repeated for 14 consecutive days. In the experimental group, LIPUS was transmitted from 35- mm diameter LIPUS transducers to the bottom of the cell culture plate via a coupling gel (Smith & Nephew, Oklahoma, CA) and was administered in an incubator (see Fig. 1). In the control group, plates were placed on the same transducers for the same duration, but the LIPUS was not administered.

Schwann cells plated at a cell density of 5,000 cells/1.77cm^2 were used for the immunocytochemistry assays. At day 14 after LIPUS treatment, a total of 18 plates (nine experimental plates and nine control plates) were analyzed for S-100, NT-3, and BDNF immunostaining, respectively. The cells were fixed in plates for 10 minutes with 4% paraformaldehyde solution and then blocked in 4% goat serum with 0.25% triton in PBS. Then, the cells were incubated with either mouse anti-S100 protein monoclonal antibody (Sigma, Saint Louis, MO), mouse anti-neurotrophin-3 monoclonal antibody (Santa Cruz, Santa Cruz, CA), or mouse antibrain-derived neurotrophic factor primary antibodies (Santa Cruz, Sant Cruz, CA), then subsequently with goat anti-mouse IgG FITC (Sigma, Saint Louis, MO) or IgG TRITC (Sigma, Saint Louis, MO) for 1 hour and counterstained with DAPI (Sigma, Saint Louis, MO). The percentage of fluorescently labeled cells/DAPI-stained nuclei was counted using a fluorescent microscope-computer interface (Zeiss, Jena, Germany).

Proliferation assay with 5-Bromo-2-deoxy-uridine

The percent of proliferation was determined by the ratio of total BrdU-positive nuclei to total number of cells (DAPI-stained nuclei) as described previously (Funk, Fricke et al. 2007). Schwann cells plated at a cell density of 5,000 cells/1.77 cm^2 were used for counting of each individual Schwann cell. A total of 12 plates per time point (six experimental plates and six control plates, respectively) were analyzed. At days 4, 7, 10, and 14 after LIPUS treatment, the cells in plates were treated by Brdu (Sigma, Saint Louis, MO) for 2 hours. The cells were then fixed in methanol for 10 minutes at 48^{o}C and treated with 1.25% proteinase K in PBS (pH 7.5) for 5 minutes. Thereafter, the cells were treated with mouse anti-BrdU monoclonal antibody (Sigma, Saint Louis, MO) for 1 hour and then with goat anti-mouse IgG FITC (Sigma, Saint Louis, MO) for 1 hour. DAPI (Sigma, Saint Louis, MO) and cover slips were added. The average proliferation percentage of the plate was counted. The average proliferation percentage was counted by examining four random images within per plate using a fluorescent microscope-computer interface (Zeiss, Jena, Germany).

2.2 LIPUS stimulus may directly trigger Schwann cell proliferation in the early phase

The Schwann cells that were subjected to LIPUS consistently demonstrated an increase in cell proliferation. Fig. 2 shows a fluorescent microscope picture demonstrating the difference in the percentage of BrdU-positive cells in both experimental and control cells at day 7. Table 1 shows the percentage of BrdU-positive cells were significantly higher in experimental groups than in control groups on day 4 ($P < 0.01$), day 7 ($P < 0.01$) and day 10 ($P < 0.01$), respectively. However, this difference between experimental and control disappeared on day 14. The percentage increase in proliferation varied depending upon the control proliferation levels of BrdU uptake. For example, when the control level of proliferation was 23.2% at day 7, there was a 100% increase in the proliferation of experimental cells. While there was a 48.6% control proliferation level at day 10, the experimental cells exhibited a 43% increase in proliferation. The variation of proliferation between both groups is not secondary to changes in media or culture media additive. Moreover, the data suggest that Schwann cells are more responsive to LIPUS at different times of their cell cycle.

In this study, we observed that LIPUS increased Schwann cell proliferation indicating that LIPUS is mitogenic for Schwann cells in vitro (See Fig.3). The LIPUS treatment could effectively improve Schwann cell proliferation at an early stage (day 4, day 7 and day 10), while at later stages (day 14) self-renewal ability of these cells reached to a much higher level but there was no obvious difference between experimental and control groups. This increase in proliferation confirmed results with previous in vitro data, which proposes that cultured cells may be mitogenic in response to LIPUS stimulation (Mukai, Ito et al. 2005; Iwashina, Mochida et al. 2006) Furthermore, these data lend credence to the possibility that the LIPUS stimulus may directly trigger Schwann cell proliferation in the early phase.

		Day 4	Day 7	Day 10	Day 14	F	P
group	Control group	13.3± 1.67	30.53±4.98	51.93±11.56	76.70±9.67	147	0.00
	experiment group	16.33±2.68	40.73±7.45	71.07±9.03	80.20±11.68		
Comparison at same time point	LSD-t	2.35	2.788	3.20	0.06		
	P	0.04	0.02	0.01	0.58		

The mitotic cells were identified by metabolic BrdU labeling. The results showed that experimental groups display a higher proliferation rate. LSD-t, Least Significant Difference t test; F, F value of One-Way ANOVA; p, the p-value is the probability that the null hypothesis is true.

Table 1. the effect of LIPUS on the cell proliferation rate of cultured Schwann cells.

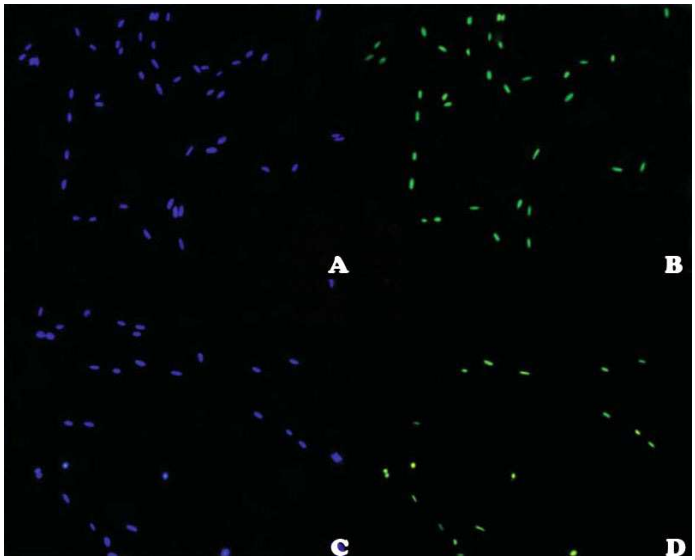

Fig. 2. LIPUS induces increased mitogenesis of in vitro cultured Schwann cells. Fluorescent images depicting the increase in the ratio of the number of BrdU-stained nuclei (green) to DAPI-stained nuclei (blue) in experimental or control cells. Note the increased number of BrdU-positive cells in experimental cells versus the control cells.
(experimental A, B; control C, D) at day 4.

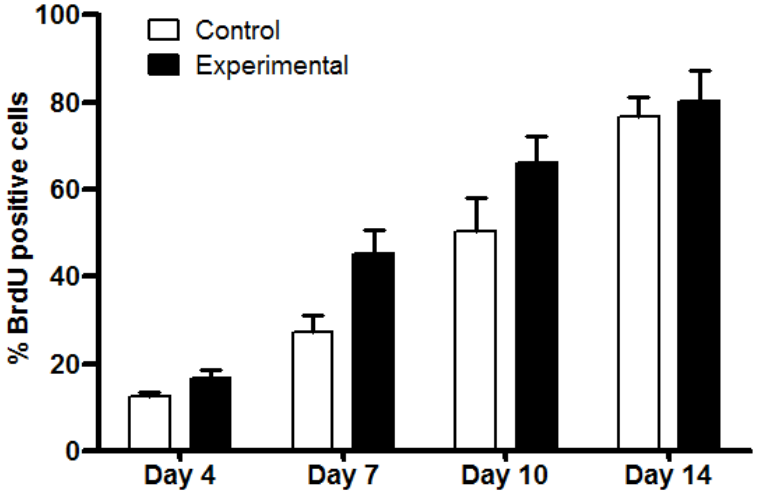

Fig. 3. Increase in proliferation of cultured Schwann cells in response to LIPUS at day 4, day 7 and day 10. No significant difference at day 14.

2.3 LIPUS treatment does not change the phenotype of the Schwann cell

In addition to the increased cell proliferation, LIPUS stimulation of cell cultures has previously been demonstrated to induce an alteration of cellular phenotype,(Ikeda, Takayama et al. 2006; Schumann, Kujat et al. 2006), but little is known about the effects of LIPUS stimulation on a Schwann cell phenotype. Thus, the initial experiments were used to determine whether LIPUS would induce a phenotype alteration to the Schwann cell or not. S-100 immunostaining results showed that Schwann cells do not de-differentiate into another cell type following LIPUS stimulation.

The immunohistochemistry study showed that more than 98% of Schwann cells were positive for the pan-specific Schwann cell marker S-100 at day 14, with or without LIPUS treatment. Moreover, immunostaining for NT-3 and BDNF shows that Schwann cells were positive in more than 98% of the evaluated cells in both the experimental and control cells at day 14. Additionally, the distribution of the positively stained cells was uniform for both the inner and outer areas of the circular plated region. These results further demonstrated that LIPUS treatment does not change the phenotype of the Schwann cell.

3. Effect of LIPUS on the expression of neurotrophin-3 and brain derived neurotrophic factor in cultured Schwann cells

Both neurotrophin-3 (NT-3) and brain-derived neurotrophic factor (BDNF) are two of key neurotrophins constituents in peripheral nervous system, NT-3 is an important regulator of neural survival, development, function, and neuronal differentiation (McAllister, Lo et al. 1995; McAllister, Katz et al. 1999). Hess (Hess, Scott et al. 2007) et al observed that NT-3 expression may modulate the number of Schwann cells at neuromuscular synapses. Otherwise, neurotrophin-3 is an important autocrine factor supporting Schwann cell

survival and differentiation in the absence of axons (McAllister, Katz et al. 1999), Schwann cells also contribute to the sources of BDNF during nerve regeneration and the deprivation of endogenous BDNF results in impairment in regeneration and myelination of regenerating axons (Zhang, Luo et al. 2000), BDNF also plays a role in activity-dependent neuronal plasticity (Schmidhammer, Hausner et al. 2007). The exogenous administration of these factors has protective properties for injured neurons and stimulates axonal regeneration (Lykissas, Batistatou et al. 2007). Based on these properties, these molecules may be used as therapeutic agents for treating degenerative diseases and traumatic injuries of both the central and peripheral nervous system.

We therefore measured how LIPUS affects Schwann cells neurotrophic function by evaluated the mRNA expression of NT-3 and BDNF, two members of the neurotrophic factor family of the Schwann cells.

3.1 Semiquantitative RT-PCR for detecting the BDNF and NT-3 mRNA expression

Schwann cells plated at a cell density of 5,000 cells/ 1.77 cm^2 were used for the RT-PCR assays. At day 14 after LIPUS treatment, a total of 12 plates (six experimental plates and six control plates) were analyzed for NT-3 and BDNF mRNA expression, respectively. The cells were then incubated for 12 hours at 37°C to allow for gene transcription. Cells were then trypsinized, collected as pooled samples, and RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA) following the protocol. The cDNA was prepared for experimental and control samples using 3μg of RNA with SuperScript II RNase reverse transcriptase (Invitrogen, Oklahoma, CA) and specific primers for NT-3 (forward: 5'- CTTATCTCCG TGGCATCCAAGG-3', reverse: 5'- TCTGAAGTCAGTGCTCGGACGT-3'), BDNF (forward: 5'-ATGGGACTCTGGAGAGCGTGAA-3', reverse: 5'-CGCCAGCCAATTCTCTTTTTGC-3'), and b-actin (forward: 5'-CCCAGAGCAAGAGAGGCATC-3', reverse: 5'-CTCAGGAGGAG CAATGATCT-3') (Hatami, Oryan et al. 2007).

The PCR reaction conditions were consisted of one cycle of 94°C for 5 minutes, followed by 30 cycles of thermal cycling 30 seconds at 94°C, 30 seconds at T_0°C, and 1 minute at 72°C. The T_0 was 60°C for BDNF, 64°C for NT-3, and 58°C for b-actin. The final cycle was followed by a 5-minute extension at 72°C. Ten microliters of PCR product was then differentiated on a 1.5% agarose gel and the gel image was taken with a digital camera. ImagQuant analysis software (Stratagene Company, La Jolla, CA) was used to determine the densities of the NT-3 and BDNF bands when compared with the b-actin control for both experimental and control samples.

3.2 Effect of LIPUS on the expression of NT-3 and BDNF mRNA in Schwann cell

Schwann cells that were subjected to sustained LIPUS exhibited an increase in NT-3 mRNA expression, and a decrease in BDNF mRNA expression (Fig. 4). The NT-3/β-actin ratio of RT-PCR products in the experimental group was 0.56±0.13 and 0.41±0.09 in the control group. However, the BDNF/β-actin ratio of RT-PCR products in the experimental group was 0.51±0.05 and 0.60±0.08 in the control group. The differences in NT-3 and BDNF products for experimental and control groups were found to be statistically significant ($p<0.01$ and $p<0.05$, respectively). Reverse transcriptase controls with no reverse transcriptase enzyme confirmed that there was no genomic DNA contamination.

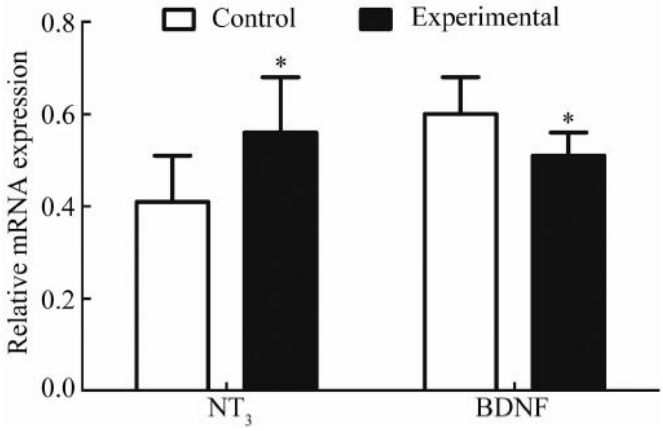

Fig. 4. Results from RT-PCR analysis of NT-3 and BDNF are expressed relative to β-actin mRNA expression 14 days after the LIPUS stimulation. There was significantly upregulated in experimental groups compared with the control in NT-3 mRNA expression ($t=2.324$, $P<0.05$), and significantly downregulated in BDNF mRNA expression ($t=2.337$, $p<0.05$).

4. LIPUS enhances elongation of neurites in rat cortical neurons through inhibition of GSK-3β

Intracellular mechanisms that enhance neurite outgrowth evidently require the reorganization of the neurite cytoskeletons including the microtubules and actin filaments (Dent and Gertler 2003). Recently, a cytoskeletal-related signaling pathway: PI 3-kinase/Atk/glycogen synthase kinase (GSK-3)/collapsin response mediator protein (CRMP-2) was reported to be important for the outgrowth of neurite, with GSK-3 being a central regulator (Jiang, Guo et al. 2005; Yoshimura, Kawano et al. 2005). GSK-3 is a multifunctional serine/threonine kinase found ubiquitously in eukaryotes (Jiang, Guo et al. 2005) and it plays key roles for various biological processes, such as the canonical Wnt signaling pathway, microtubule dynamics, and astrocyte migration (Doble and Woodgett 2003; Etienne-Manneville and Hall 2003). GSK-3 phosphorylates at least have four types of microtubule-associated proteins (MAPs), CRMP-2(Yoshimura, Kawano et al. 2005), tau (Jiang, Guo et al. 2005), adenomatous polyposis coil gene product (APC)(Frame and Cohen 2001; Grimes and Jope 2001) and MAP1B (Lucas, Goold et al. 1998; Goold, Owen et al. 1999). It modulates axial orientation during the development, differentiation, and neurite outgrowth in neurons through phosphorylation of these MAPs (Jiang, Guo et al. 2005; Yoshimura, Kawano et al. 2005; Chen, Yu et al. 2007; Conde and Caceres 2009). Some research have proved that the local inhibition of GSK-3 effectively enhances neurite/axon elongation (Kim, Zhou et al. 2006), whereas overexpression of GSK-3 could impair neurite/axon elongation (Munoz-Montano, Lim et al. 1999). During peripheral nerve regeneration, some factors such as BDNF, NT3, and laminin, locally activate the PI3-kinase/Akt/GSK-3 pathway and inhibit GSK-3, which favors neurite elongation (Kim, Zhou et al. 2006).

We measured the length of neurites to examine whether LIPUS is effective for the elongation of the neuronal processes. Then we examined the change in the activity and the

mRNA expression of GSK-3β to determine the intracellular mechanism of neurite outgrowth following irradiation by LIPUS. It is concluded that LIPUS can enhance elongation of neurites and it is possible through the decreased expression of GSK-3β (Ren, Li et al. 2010).

4.1 Effect of LIPUS treatment on neurite outgrowth

4.1.1 Materials and methods: Cell culture and ultrasound treatment

Cortical neurons isolated from the brain of Wistar rats were bought from ScienCell Research Laboratories (San Diego, USA). These cortical neurons were subcultured with a density of 20 000 cells/1.6 cm^2 in poly-L-lysine coated 6-well plates (Costa, USA) for immunoblot and semi-quantitative RT-PCR analysis, and a density of 100 cells/0.32 cm^2 in poly-L-lysine coated 96-well plates (Costa, USA) for the measurement of neurite length. The cells were cultured in neuronal medium (3 mL medium per well in the 6-well plates and 0.1mL per well in the 96-well plate; ScienCell Research Laboratories, San Diego, CA, USA) in a humidified atmosphere of 5% CO_2 in air at 37 ℃. The medium was refreshed every 3 d.

A LIPUS-therapeutic apparatus, Nexson-The- P41 was constructed according to instructions from Nexus Biomedical Devices (Hangzhou, China). There were two LIPUS probes in the apparatus, both of which generated LIPUS with a SATA intensity of 10 mW/cm^2, pulse width of 200 microseconds, repetition rate of 1.5 kHz, and an operation frequency of 1 MHz. The LIPUS was applied to the cultured cortical neurons after 24 h in culture through the bottom of the 6-well plates via a coupling gel (Smith & Nephew, Oklahoma, CA, USA) and was administered for 5 min every day during the span of this experiment (Fig. 1). Ultrasound signals from this generator were detected by a hydrophone system (Model OS-111; Hewlett-Packard, Japan), and the wave amplitudes of the signals passing through the tube wall were more than 90%, which resulted in more than 85% energy irradiated. Control samples were prepared in the same manner with the exception of no LIPUS treatment.

Neurite length measurement protocol

Cultured cortical neurons in 96-well plates were randomly divided into two groups: the LIPUS-treated group and the control group. After being subcultured for 24 h, the LIPUS treatment began and was administered for 5 min every day. On the third day, both the LIPUS-treated and control groups were photographed 2 h after the treatment. A Nikon Diaphot inverted microscope with a Nikon Plan 20× objective (Nikon, Tokyo, Japan) coupled to a video camera was used to obtain cell images (Carl Zeiss, Germany). Images of at least 200 neurons for each group were obtained. For each neuron, we measured its longest neurite with the software Image-Pro Plus 6.0 (Media Cybernetics, USA).

4.1.2 Neurites in LIPUS-treated group were significant longer

There are no significant difference in morphology between LIPUS-treated group and control group except the length of neurites. In both LIPUS-treated group (Fig. 5a) and control group (Fig.5b), there were many neurons with 2-7 processes; some were thick fibers, or some were thin fibers with varicosities. We measured the length of 200 neurites in each group and most neurite measured have a length between 50 μm to 80 μm. Data showed that compared with control group, neurites in LIPUS-treated group were significant longer [(73.14 ±8.32) μm vs.

(68.18 ±8.96) μm, P<0.01](Fig. 5c). We attempted to investigate how processes of the cultured neurons were extended under the influence of LIPUS. Morphological changes revealed that LIPUS could effectively enhance elongation of neurites after three days of treatment compared to the control group. However, we failed to measure the length of neurites on the seventh or tenth day because after the fifth day, most neurites reached another neurite and, consequently, the growth of those neurites stopped. Although the mechanism by which LIPUS affects the neuronal processes is likely to be complex, the regulation of the cytoskeleton is crucial for the proper growth cone motility (Dent and Kalil 2001). To clarify the intracellular mechanism of this effect, we examined the proteins related to the cytoskeletal-related signaling pathway to determine whether the proteins in the cultured neurons were changed following the LIPUS treatment.

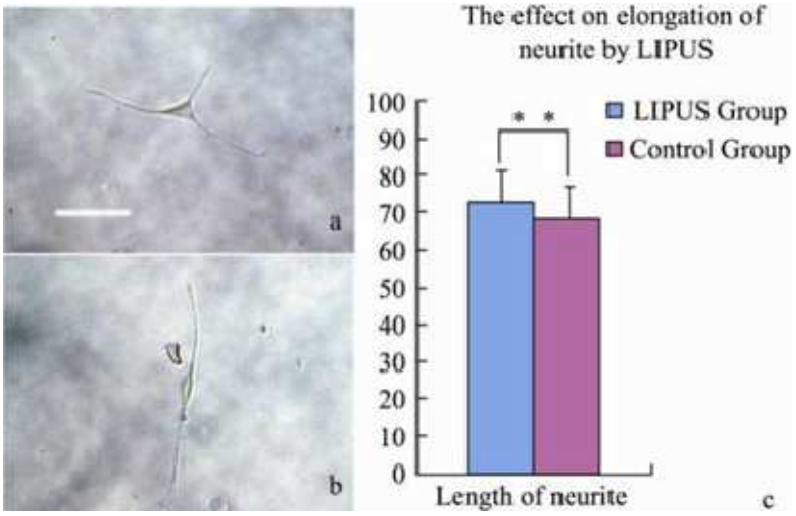

Fig. 5. On the third day, both the LIPUS-treated and control groups were photographed with a Nikon Plan 20× objective coupled to a video camera. In both LIPUS-treated group (Fig. 5a) and control group (Fig. 5b), there were many neurons with 2-7 processes; some were thick fibers, and some were thin fibers with varicosities. Bar=50 μm. Images of at least 200 neurons for each group were obtained. For each neuron, we measured its longest neurite with the software Image-Pro plus 6.0. Most neurite measured have a length between 50 μm to 80 μm. Data showed that neurites in LIPUS-treated group were significant longer than that in control group [(73.14 ±8.32) μm vs. (68.18 ±8.96) μm, P<0.01] (Fig. 5c).

4.2 Changes in protein activity related to the Cytoskeletal-signaling pathway caused by LIPUS treatment

To investigate changes in protein activity related to cytoskeletal-signaling pathway caused by LIPUS, total proteins were extracted on the third, seventh, and tenth days following daily LIPUS treatment and their acticity were examined using Western blot analysis (Fig. 6).

To measure the length of neurites to examine whether LIPUS is effective for the elongation of the neuronal processes, we examined the change in the activity and the mRNA expression

of GSK-3beta to determine the intracellular mechanism of neurite outgrowth following irradiation by LIPUS (Ren, Li et al. 2010).

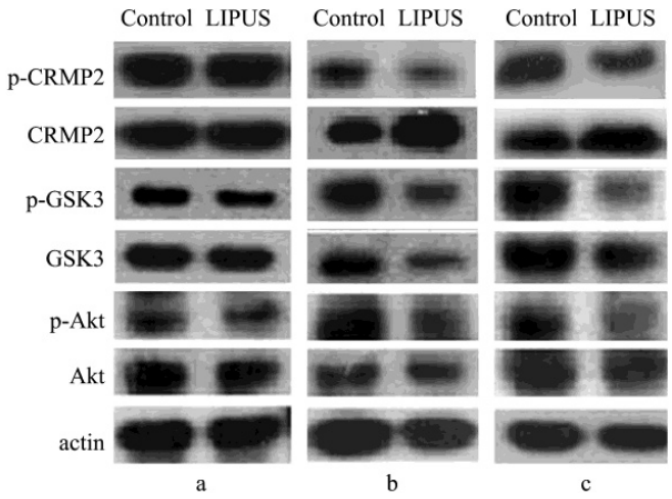

Fig. 6. Total proteins were extracted on the third, seventh, and tenth days following daily LIPUS treatment and their activity were examined using Western blot analysis. (a) On the third day, there was no significant difference in protein levels between the control and LIPUS groups. (b) On the seventh day, the levels of p-Akt, GSK-3beta, p-GSK-3beta, and p-CRMP-2 were decreased in the LIPUS group compared to the controls. (c) On the tenth day, a remarkable decrease of p-Akt, p-GSK-3beta, and p-CRMP-2 were observed while it appeared that GSK-3beta was slightly decreased. The beta-actin in each lane served as an internal control.

4.2.1 Materials and methods: Western blot analysis

For Western blot analysis, the treated and untreated cultured cells were harvested at third, seventh, and tenth days. LIPUS group cells were harvested 2 h after the last LIPUS treatment. Whole cell extracts were prepared by boiling the cells in lysis buffer (2% SDS; 10% glycerol; 10 mmol/L Tris, pH 6.8; 100 mmol/L DTT) for 10 min. Proteins were separated by electrophoresis on 4%-12% Bis-Tris gels (Novex; Invitrogen, Carlsbad, CA, USA). Separated proteins were then transferred onto PVDF membranes. The membranes were blocked with 5% nonfat dry milk in PBS, pH 7.4, and 0.1% Tween 20 (PBS-Tween) for 1 h at room temperature. The membranes were incubated with primary antibodies diluted in 5% BSA overnight at 4 ℃. The blots were washed in PBS-Tween and then incubated with diluted secondary antibodies (HRP, 1:10 000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. Reactive proteins were visualized with SuperSignal West Pico chemiluminescence's reagent (Pierce Biotechnology, Rockford, IL, USA) followed by exposure to x-ray film.

The primary antibodies used for the Western blot analysis were as follows: rabbit anti-GSK-3beta antibody (21001-1; Signalway Antibody, Pearland, TX, USA), rabbit anti-

phospho GSK-3beta (Ser 9) antibody (11002-1; Signalway Antibody), rabbit anti-CRMP-2 antibody (SC-30228, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-phospho CRMP-2 (Thr 514) antibody (9397, Cell Signaling Technology, Beverly, MA, USA), goat anti-Akt antibody (SC-1618; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and rabbit anti-phospho-Akt (Ser 473) antibody (SC-101629; Santa Cruz Biotechnology, Santa Cruz, CA, USA). All of these primary antibodies were polyclonal and used at a dilution of 1:500. Mouse anti-beta actin polyclonal antibody (SC-81178, 1:1000, Santa Cruz, CA, USA) was used at a dilution of 1:1 000. As secondary antibodies, HRP-conjugated goat anti-rabbit (SC-2004; Santa Cruz Biotechnology, Santa Cruz, CA, USA), donkey anti-goat (SC-2020; Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat anti-mouse (SC-2005; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used at a dilution of 1:10 000.

Semi-quantitative RT-PCR analysis

For RT-PCR analysis of GSK-3β gene expression, neurons were cultured in two 6-well plates. One of the plates was irradiated by LIPUS for 7 d (5 min/day; 10 mW/cm^2); the other was the control group without LIPUS treatment. Cultured cells were harvested 2 h after the last irradiation. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the instruction manual, then resuspended in diethylpyrocarbonate (DEPC)-treated water. The extracted RNA was used to synthesize first strand cDNA with the PrimeScript™ RT-PCR Kit (Takara Biotechnology, Dalian, China) according to the kit's manual. Aliquots of synthesized cDNA were added to PCR mixtures containing sense and antisense primers (0.1 μmol/L each) for GSK-3beta, dNTP mixture (0.2 mmol/L of each dNTP), 1.5 mmol/L MgCl2, and rTaq DNA polymerase (1 unit) (Takara Biotechn ology, Dalian, China). The primers for GSK-3β were 5' -AGCCAGTGCAGCAGCCTTCAG C-3' for the sense strand and 5' -TCTCCTCGGACCA GCTGCT TTG-3' for the antisense strand. The primers for β-actin were 5' -GAGCTACGAGCTGCC TGACG-3' for the sense strand and 5' -CCTAGAA GCATTTGC GGTGG-3' for the antisense strand. The PCR products were electrophoretically separated in a 2% agarose gel and then visualized and photographed with an imager (Alhpa-imagerTM 2200; Alpha Innotech Corporation, San Leandro, CA, USA).

4.2.2 LIPUS enhances neurite outgrowth through the down-regulation of GSK-3β activity

On the third day, there was no significant difference in protein levels between the control and LIPUS groups. However, on the seventh and tenth days after irradiation by LIPUS, the levels of p-Akt, GSK-3beta, p-GSK-3beta, and p-CRMP-2 were decreased in the LIPUS group compared to the controls. On the tenth day, a remarkable decrease of p-Akt, p-GSK-3β, and p-CRMP-2 were observed while it appeared that GSK-3beta were slightly decreased.

During nerve regeneration, GSK-3β is locally inhibited by some factors at the growth cone through the PI3-kinase/Akt/GSK-3β signaling pathway which favors neurite outgrowth (Chen, Yu et al. 2007). The overexpression of active GSK-3beta blocks neurite growth in cultured neurons (Munoz-Montano, Lim et al. 1999). In the PI3-kinase/Akt/GSK-3beta/CRMP-2 pathway, active Akt inhibits GSK-3beta through phosphorylation at Ser 9 and GSK-3beta inhibits CRMP-2 though phosphorylation at Thr 5. If LIPUS enhances

neurite elongation though this pathway, the phosphorylation of GSK-3beta should be up-regulated and the activity of GSK-3beta should be inhibited. However, in this research, the activity of GSK-3beta was inhibited by LIPUS and the phosphorylation of GSK-3beta by Akt was inhibited, too. This conflict of results revealed that LIPUS enhances neurite outgrowth through the down-regulation of GSK-3β activity but not through the PI3-kinase/Akt/GSK-3beta pathway. Therefore, we employed semi-quantitative RT-PCR to examine the mRNA of GSK-3beta. The results of the semi-quantitative RT-PCR revealed that the expression of GSK-3beta mRNA decreased after LIPUS irradiation on the seventh day. From these findings, we postulate that when neurons are irradiated by LIPUS, an unknown intracellular mechanism may be activated as a response to this "injury" and, consequently, neurons reduce the mRNA expression of GSK-3β. The decrease of GSK-3beta activity comes from reduced expression, but not through the PI3-kinase/Akt/GSK-3β signaling pathway (Ren, Li et al. 2010).

4.2.3 The expression of GSK-3β mRNA decreased after LIPUS irradiation

The mRNA expression of GSK-3beta in the cultured neurons following LIPUS treatment was examined using a semi-quantitative RT-PCR. For this analysis, the LIPUS-treated cultured neurons on the seventh day were selected as they showed a significant decrease in their mRNA levels compared to the control. Data from analysis of the imager indicated mRNA of GSK-3β decreased about 4 folds [(1.001 ±0.017) vs. (0.627 ±0.037), P<0.001] (Fig. 7). As a result, mRNA expression of GSK-3beta was also decreased on the seventh days compared to the control.

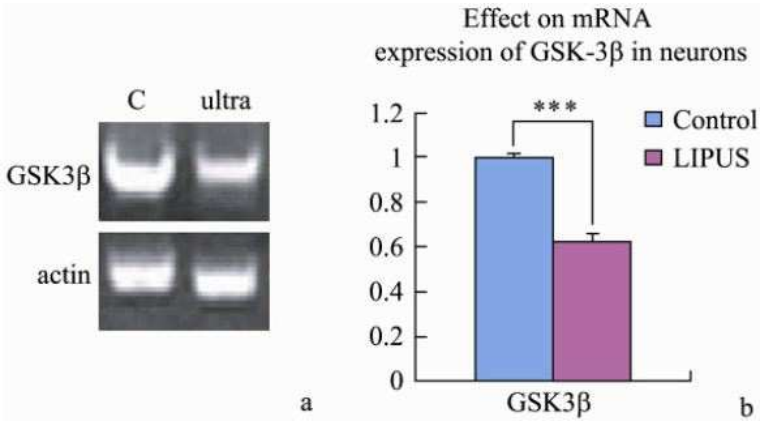

Fig. 7. Expression of GSK-3β in neurons was evaluated by semi quantitative RT-PCR. Neurons were irradiated for 7 d and harvested 2 h after the last LIPUS irradiation. The right lane represents the experimental mRNA expression, and the left lane corresponds to the control mRNA expression (Fig. 7a). Data showed that expression of GSK-3β decreased about 4 folds [(1.001 ±0.017) vs. (0.627 ±0.037), P<0.001] (Fig.7b). The β-actin in each lane served as an internal control.

The reduced expression is a kind of global inhibition of GSK-3beta that has a complex effect on neurite elongation. It favors neurite elongation at a low level of inhibition whereas it

impairs neurite elongation at a high level of inhibition (Munoz-Montano, Lim et al. 1999). Strong global GSK-3beta inhibition results in excessive microtubule stability all along the neurite shaft due to the inhibition of MAP1B, which eliminates dynamic microtubules, and the abnormal distribution of APC that stabilizes microtubules all along the neurite shaft. In this case, there was no pool of dynamic microtubules at the growth cone, which are necessary for growth cone advancement, and no localization of APC to microtubule plus ends (Kim, Zhou et al. 2006).

In our research, significant morphological changes were found on the third day whereas significant changes in the activity of GSK-3beta were found on the seventh and tenth days. We postulate that daily treatment of LIPUS would result in neurons' response accumulation. Morphological changes were observed on the third day when the inhibition of GSK-3beta is not significant enough to be found. Since overly strong global inhibition of GSK-3beta impairs neurite elongation, whether LIPUS could impair neurite elongation needs further study.

5. Conclusion

5.1 LIPUS stimulation induced an alteration in Schwann cell function as demonstrated by promoted cell proliferation and NT-3 gene expression

LIPUS is one of the physical agents that is known to accelerate bone and tissue regeneration following injury (Heckman, Ryaby et al. 1994; Lu, Qin et al. 2006). Consequently, it has been accepted as an effective therapy for nonunion fractures and fresh fracture healing through an easy and non-invasive application (Azuma, Ito et al. 2001; Schortinghuis, Bronckers et al. 2005). Previous studies indicate that LIPUS has positive effects on axonal regeneration by in vivo peripheral nerve injury trials (Crisci and Ferreira 2002; Chang, Hsu et al. 2005). Raso (Raso, Barbieri et al. 2005) et al have demonstrated that the locally applied ultrasound stimuli on the injured sciatic nerve rather than the untreated nerves of rats can effectively enhance the number of Schwann cell nuclei. LIPUS has been used in conjunction with tissue engineered nerves in repairing peripheral nerve defect, Chang (Chang, Hsu et al. 2005) et al demonstrated that applying low-intensity ultrasound on seeded Schwann cells within poly (D, L-lactic acid-co-glycolic acid) conduits have a significantly greater number and area of regenerated axons compared to the sham groups. Although this secondary response by Schwann cells has been well characterized, there is still limited information as to how Schwann cells would directly respond to LIPUS stimulation. Therefore, we cultured Schwann cells in plate as an in vitro model, and applied LIPUS in the model to demonstrate the direct effects of physical stimulation on Schwann cells (Zhang, Lin et al. 2009).

LIPUS stimulation of cultured Schwann cells induced an alteration in cell function as demonstrated by promoted cell proliferation and NT-3 gene expression, which is consistent with that LIPUS enhances peripheral nerve regeneration that was observed from in vivo models (Lowdon, Seaber et al. 1988; Raso, Barbieri et al. 2005). It has been documented that NT-3 has a strong effect on neurite outgrowth (Markus, Patel et al. 2002; Sahenk, Nagaraja et al. 2005). Additionally, some studies using genetically modified Schwann cells to overexpress the NT-3 gene have examined the role of NT-3 in the neuron survival and axonal regeneration/remyelination (Zhang, Zeng et al. 2007; Pettingill, Minter et al. 2008). It has been reported that Schwann cells transduced ex vivo with adenoviral (AdV) or lentiviral

(LV) vectors encoding a functional NT-3 molecule led to the presence of a significantly increased number of axons in the contusion site (Golden, Pearse et al. 2007). The results of Yamauchi(Yamauchi, Miyamoto et al. 2005) et al showed that NT-3 activation of TrkC stimulates Schwann cell migration through two parallel signaling units, Ras/Tiam1/Rac1 and Dbs/Cdc. Poduslo(Poduslo and Curran 1996) et al observed that NT-3 has a higher permeability coefficient across the blood–nerve barrier, and would contact sensory axons soon after reaching the circulation of adult rats. The increase in NT-3 expression might lead to an increase in the number of nerve regeneration in the axons. LIPUS-induced increase in NT-3 expression, as demonstrated in this model, may produce a microenvironment that is permissive for axonal sprouting and Schwann cells migration after peripheral nerve injury.

Neurotrophin–neurotrophin interactions are regulated by neurotrophin levels, NT-3 and BDNF in particular can be co-expressed and each can regulate the levels of the other. The relative expression level of the neurotrophins is thought to be mediated through receptor tyrosine kinase (Trk) activity (Mallei, Rabin et al. 2004). NT-3 infusion caused a significant decrease in the level of BDNF proteins in both kindled and non-kindled hippocampus, likely via down-regulation of TrkA (Yamamoto and Hanamura 2005) Furthermore, a study of Karchewski (Karchewski, Gratto et al. 2002) et al showed that NT-3 can act in an antagonistic fashion to NGF in the regulation of BDNF expression in intact neurons, and mitigate BDNF's expression in injured neurons. It is also consistent with a study in which, in contrast, deletion of the NT-3 gene in transgenic mice increased BDNF and TrkB mRNA synthesis, suggesting that decreased NT-3 may disinhibit BDNF expression (Elmer, Kokaia et al. 1997). Similarly, our model has demonstrated an up-regulation of NT-3 mRNA and down-regulation of BDNF mRNA expression after the LIPUS stimulation. Hence, it is possible that NT-3 acts in an opposite fashion result in a down-regulation in BDNF expression in intact Schwann cells. Further investigation is necessary to determine the molecular mechanisms of NT-3 and BDNF signaling pathway by the data presented in our study (Zhang, Lin et al. 2009).

5.2 LIPUS enhances neurite elongation in rat cortical neurons

LIPUS enhances neurite elongation in rat cortical neurons, indicating that LIPUS could be a potential application for clinical treatment of nerve regeneration in both the central and peripheral nervous systems. The intracellular mechanism also indicates that LIPUS has the same action as the neurotrophic factors, laminin and LiCl. Compared to other pharmacological inhibitors of GSK-3beta, LIPUS has some advantages: (1) LIPUS is considered to be nontoxic, thus it has a wide margin of biologic safety; (2) It directly irradiates target neurons and does not affect other tissues; and (3) The decreased expression comes from a response of neurons and is not affected by the metabolism or blood brain barrier. However, further investigation is required to identify an accurate and continuous application of LIPUS treatment to achieve constant and reproducible results prior to clinical use.

The results suggest that LIPUS may have several different clinical applications in the improvement of peripheral nerve regeneration. First, given its nontoxicity and a wide margin of biologic safety, it may be used as an effective physical stimulant when engineering peripheral nerve tissue. Schwann cell-based therapies that use transplantation techniques for the treatment of nerve tissue repairing are being widely investigated for their

potential as clinical applications (Rochkind, Astachov et al. 2004; Li, Ping et al. 2006; Gravvanis, Lavdas et al. 2007). LIPUS applied in conjunction with other forms of biologic stimulation, is worth considering when optimizing an innovative "multi-level" form of treatment. Second, application of LIPUS in vivo is likely to be considered to stimulate repair of damaged peripheral nerve tissues. Some experimental studies supported the result that both end-to-side and tubulization repair of peripheral nerves led to successful axonal regeneration along the severed nerve trunk as well as to a partial recovery of the lost function (Geuna, Nicolino et al. 2007; Lloyd, Luginbuhl et al. 2007). With the availability of the LIPUS as an activator of Schwann cells, it can be an effective alternative in nerve reconstruction and be of great value in various kinds of peripheral nerve microsurgery. Further investigation is required to identify an accurate and continuous application of LIPUS treatment, in order to achieve constant and reproducible results prior to clinical use.

As demonstrated in the current study, NT-3 and BDNF mRNA expression in Schwann cell response to LIPUS may be independent of the reciprocal regulation between the glial cells and neurons. Normally, during development and axonal injury, this reciprocal relationship between the glial cells and neurons causes a response in the glial cells, which occurs secondary to the neuron. However, data from the in vitro model indicate otherwise. The Schwann cells responded robustly in the absence of neurons, suggesting that Schwann cell responses may be directly elicited through LIPUS stimuli in the model.

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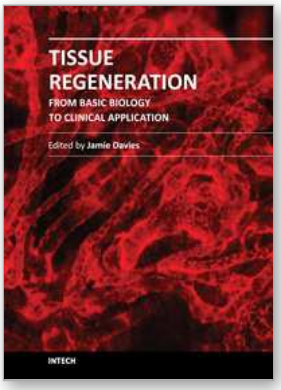

Tissue Regeneration - From Basic Biology to Clinical Application
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When most types of human tissue are damaged, they repair themselves by forming a scar - a mechanically strong 'patch' that restores structural integrity to the tissue without restoring physiological function. Much better, for a patient, would be like-for-like replacement of damaged tissue with something functionally equivalent: there is currently an intense international research effort focused on this goal. This timely book addresses key topics in tissue regeneration in a sequence of linked chapters, each written by world experts; understanding normal healing; sources of, and methods of using, stem cells; construction and use of scaffolds; and modelling and assessment of regeneration. The book is intended for an audience consisting of advanced students, and research and medical professionals.

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