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Applications of Low Intensity Pulsed Ultrasound for Functional Bone Tissue Engineering using Adult Stem Cells

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***Abstract*—Low intensity pulsed ultrasound (LIPUS) has been used extensively for fracture healing. Many LIPUS parameters have been studied in depth except for pulse repetition frequency (PRF). However, to examine this parameter, a custom LIPUS system needed to be designed since commercial systems have very little control over parameter modification. Specifically, the PRF of commercial systems is typically set to either 100 Hz or 1 kHz and cannot be changed. A custom system was used to explore the effects of PRF on human adipose derived adult stem cells (hASCs) and bone marrow derived mesenchymal stem cells (hMSCs). The PRF was found to significantly affect cellular response to LIPUS stimulation for both cell types. Additionally, LIPUS was shown to strongly accelerate osteogenic differentiation of hASCs based on amount of calcium accretion normalized by total DNA.**

Keywords-low intensity pulsed ultrasound; adipose derived adult stem cells; pulse repetition frequency

I. INTRODUCTION

Low intensity pulsed ultrasound (LIPUS) has been used to accelerate fracture healing for over a decade. In 1994 the FDA approved a LIPUS device designed by Exogen (Smith & Nephew, Inc) for fracture healing and in 2000 for treatment of nonunions. Despite this, there is still a lack of understanding of the effects of ultrasound (US) parameters on cell response. There are very few published studies that examine US pulse repetition frequency (PRF), a crucial parameter in US dose. Further, most studies are limited to parameter ranges available within the capabilities of commercially made systems, such as a 1 MHz sine wave, 30 mW/cm^2 intensity applied for 20 min. per day, pulsed for 200 μs with a PRF of 1 kHz, giving a 20% duty cycle (DC).

The majority of current LIPUS research is done using these commercially built US systems that have very limited parameter options. In order to study the effects of PRF and other LIPUS parameters, our group has designed a custom LIPUS system with complete parameter variability. The system consists of an arbitrary function generator (Sony AWG2021), relay circuit (using Teledyne CCR-33S60-T relays), transducer stand, RF power amplifier (ENI AP400B) and control computer. The function generator is capable of creating an US pulse with any desired waveform and parameters such as pulse repetition frequency and duty cycle. A relay circuit (Fig. 1) was designed to allow automation for exciting multiple transducers, enabling longer stimulation experiments to be run without manual interaction. The function generator and relay circuit are controlled with Labview, which is capable of loading specific pulse waveforms and stimulation parameters for each transducer. This precise control for each transducer provides the capability to highly calibrate the system so each transducer has exactly the same output intensity regardless of manufacturing tolerance differences. The transducer stand is designed to use Bioflex tissue culture plates (Flexcell, Hillsborough, NC) and work within a standard incubator. Bioflex plates have thin flexible tissue culture membranes that allow for passage of US signals through the bottom of the plate, eliminating the need for a water bath coupling system and preventing any possible contamination that could occur if the US transducers were placed in the media above the cells. The use of these unique acoustically-transparent tissue culture plates presents a significant advantage in consistency over prior studies. The system was designed so only the transducer stand is placed in the incubator to minimize the use of incubator space required and can be easily moved out by disconnecting the twist-on BNC connections for each transducer. The computer,

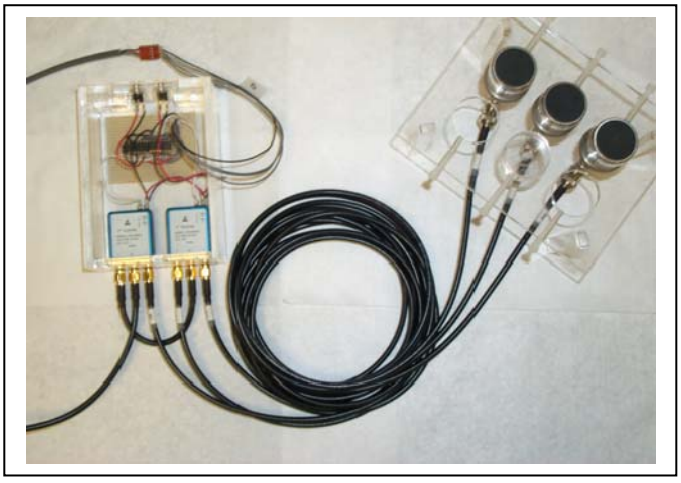

Figure 1. Relay circuit and transducer stand.

waveform generator, and amplifier can be stored in an adjacent room to further reduce the amount of lab space required to accommodate this system.

No clear set of LIPUS parameters has been found for optimizing osteogenesis, however studies have been done on individual parameters. Ultrasound frequency has been shown to be a fairly unimportant parameter of LIPUS. Generally a frequency of 1.5 MHz or 1 MHz is used and studies have shown no significant difference between frequencies of 0.5 and 1.5 MHz [1] or between 1.65 and 4.49 MHz [2]. Li et al. tested a range of intensities (150, 300, 600, 1200 and 2400 spatial-average temporal-peak, I_{SATP}) at 1 MHz, 20% DC and concluded that 600 mW/cm^2 I_{SATP} (120 mW/cm^2 I_{SATA}) was best for osteoblast growth and PGE2 secretion using a 100 Hz PRF for 15 minutes each day [3]. A study by Hsu et al. varied the DC and intensity of a 1 MHz frequency stimulus applied for 10 minutes per day [4]. The DCs were 20%, 50% and continuous and the range of intensities was 33-100 mW/cm^2 I_{SPTA} (spatial-peak temporal-average). They found the optimum settings for cell proliferation on PLA and PLGA scaffolds to be 20% DC with an intensity of 67 mW/cm^2, however, without knowing the spatial profile of the transducer this intensity cannot be converted to I_{SATA}. Chan et al. compared the effects of applying LIPUS for 20 or 40 minutes per day for enhancing osteogenesis in bone lengthening at a fast distraction rate and found an increase in bone mineral content and bone volume in the longer duration LIPUS treatment [5]. Although many of the LIPUS parameters have been studied there is a lack of knowledge concerning the effects caused by changing the PRF. Typical commercial LIPUS systems use either the standard 1 kHz PRF or a 100 Hz PRF and no study has been done to compare the two or test other repetition rates.

Additionally, no study has been done to examine the effects of LIPUS on human adipose derived adult stem cells (hASCs). Human ASCs have gained increasing interest to researchers since they are readily available, relatively easily isolated from excess adipose tissue, and new lineage potentials are continually being discovered. One possible use of hASCs is as a source for creating autologous bone tissue, which can be used to repair critical defects or replace damaged bone. The availability and ease of access to hASCs potentially make them an ideal starting material for generating needed replacement tissue and an excellent stem cell type to explore the osteogenic differentiation capabilities of LIPUS.

II. Materials and Methods

A. Cell Culture

Human ASCs and MSCs were initially seeded at 5x10^4 cells/well in Bioflex plates with complete growth media composed of Eagle's alpha-modified Minimum Essential Medium (α-MEM) supplemented with 10% fetal bovine serum (lot selected; Atlanta Biologicals, Lawrenceville, GA), 2 mM L-glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin. After 36 hours the media was changed to osteogenic differentiation media (complete growth medium supplemented with 50 µM ascorbic acid, 0.1 µM dexamethasone and 10 mM β-glycerolphosphate) for the duration of the experiment.

B. Calibration of Transducers

All calibrations were performed with a 1 MHz sine wave stimulus. The transducers have a 1 inch diameter. Specifics for each step are covered in the respective results sections.

C. Application of Ultrasound

Cells were exposed to a 1 MHz sine wave with a spatial-averaged-temporal-averaged intensity ($I_{SATA)}$ of 30 mW/cm^2 for 20 minutes per day with a pulse repetition rate of 1, 100 or 1000 Hz, with a 20% duty cycle.

D. Alamar Blue

At days 0, 4, 7, 11 and 14 culture media was aspirated and replaced with 2.6 mL fresh media plus 10% by volume alamarBlue® and incubated for 3 hours at 37 °C. Additionally, negative control wells containing only the culture medium (i.e., no cells) were also incubated. Following incubation, 200 µL of media was removed from each well in triplicate and placed in a 96-well plate. The plate was briefly centrifuged at 1000 x g for 1 min with the cover off to remove any air bubbles and then the absorbance was measured at 570 and 600 nm using a microplate reader. Percent reduction of alamarBlue® was calculated per the manufacturer's protocol.

E. Calcium Accretion of Osteogenic Differentiated Cells

Calcium content was quantified using Stanbio Total Calcium LiquiColor kit (Stanbio Laboratory). Cells were washed twice with 1 mL PBS per well. The tissue culture membranes were carefully cut out and divided into two halves using a razor blade. One half was then transferred into a microcentrifuge tube where 1 mL of 0.5N HCl was added. Samples were incubated overnight on an orbital shaker at 4 °C, and then centrifuged at 500 x g for 2 minutes and supernatant transferred to a new tube. Calcium concentration was calculated per the manufacturer's protocol.

F. Total DNA

The second half of the membrane obtained during the calcium quantification method above was transferred into a separate microcentrifuge tube where 0.5 mL of papain digest (5 mL PBS, 25 µL papain (Sigma-Aldrich), 7.3 mg EDTA, 4.4 mg cysteine HCl) was added. Samples were incubated overnight at 60 °C, and then vortexed and supernatant transferred to a new tube. Triplicates of 100 µL per sample were added to 100 µL of 0.2 µg/ml Hoechst 33258 dye (Invitrogen) and fluorescence read at 460 nm in microplate reader when excited at 350 nm. Fluorescence value was compared to a standard curve to calculate amount of DNA.

G. Statistical Analysis

Student's T-tests were used to compare results with significance accepted at $p<0.05$.

III. Results

A. *Bioflex plate attenuation*

As expected, calibration steps were affected by the presence of the Bioflex plate. To make measurements easier, the attenuation of the tissue culture membrane was determined and then applied to calculations in future calibration steps. A hydrophone was used to measure the pressure output at a specific location on a transducer with and without the plate. Samples of the output pressure waveform were taken at identical locations and used to calculate the spatial-peak-temporal-average intensity (I_{SPTA}) using the equation:

$$I_{SPTA} = x_{rms}^2 \cdot d/(\rho \cdot c), \quad (1)$$

where x_{rms} is the root-mean-square of the pressure waveform, d is the duty cycle (0.2), ρ is the density of water (1000 kg/m^3) and c is the speed of sound in water (1500 m/s). A voltage sweep was done over a range of function generator output voltages. Mean attenuation was found to be 12.1±0.3 % or 0.56±0.02 dB and was constant regardless of input voltage.

B. *Calculation of spatial-peak-temporal-average intensity*

The overall calibration goal was to have each transducer output a spatial-average-temporal-average intensity (I_{SATA}) of 30 mW/cm^2, the standard used for LIPUS research. First, the I_{SPTA} was found for each transducer over the voltage range from 0.05 to 1 V in steps of 0.05 V. To calculate I_{SPTA}, (1) was used where the pressure waveform was specified as the transducer's steady state output from 1 to 1.5 µs when using a 2 µs stimulus pulse. The location of the hydrophone was manually placed at the peak output location for each transducer. A steady waveform section was used to prevent the requirement of generating a new calibration waveform for changes in duty cycle or PRF.

C. *Calculation of spatial-average temporal-average intensity*

Based on the I_{SPTA} results, voltages were selected for performing beam field scans to determine the spatial average output for each transducer. Each beam field scan was performed using a computer-controlled scanning system across a 35x35 mm scanning area with 20x20 sample points where the peak pressure for each point was captured during the steady state phase of the transducer (1 to 1.5 µs). Pressure outside a 15 mm radius was used to determine the DC offset of the pressure profile and the average pressure was calculated for values inside a 12.5 mm radius. The average pressure for each beam scan was then used to interpolate the corresponding intensity from the I_{SPTA} vs maximum output pressure plot generated during the I_{SPTA} calculation. The resultant I_{SPTA} using the spatially averaged pressure corresponds to the spatial-average-temporal-average intensity (I_{SATA}) for the specific input voltage.

D. *Alamar Blue*

The percent reduction of alamarBlue® for hMSC donors 1 and 2 are shown in Fig. 2A and 2B, respectively. In both cell lines, all three PRF settings produced significant differences

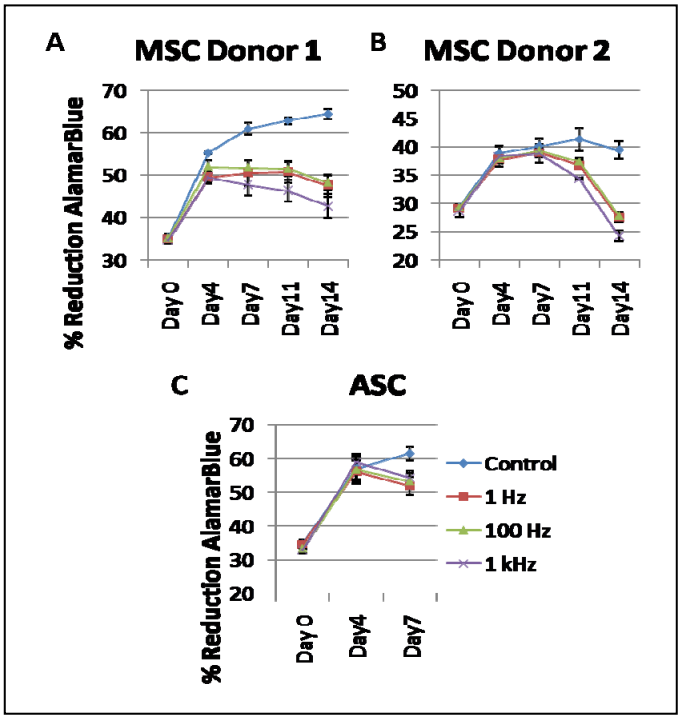

Figure 2. Percent reduction of alamarBlue® for both hMSC and hASC cell lines. A higher percent reduction indicates a higher proliferation rate.

compared to the control cells (donor 1: p=0.0005, 0.0002, 0.0003; donor 2: p=0.0003, 0.0002, 0.0001; for PRFs of 1 Hz, 100 Hz and 1 kHz, respectively). Additionally, both cell lines had significant difference between the 100 Hz and 1 kHz PRFs (p=0.045 and 0.005 for donor 1 and 2, respectively). For the hASCs (Fig. 2C) there was no significant difference at day 4, however by day 7 all three PRF settings were significantly different from the control wells (p=0.008, 0.011, 0.014 for 1 Hz, 100 Hz and 1 kHz, respectively) with no significant difference among the PRF settings.

E. *Calcium accretion normalized by total DNA*

Calcium accretion per total amount of DNA for both hMSC cell lines on day 14 is shown in Fig. 3. Human MSCs from Donor 1 produced much less calcium than those from Donor 2

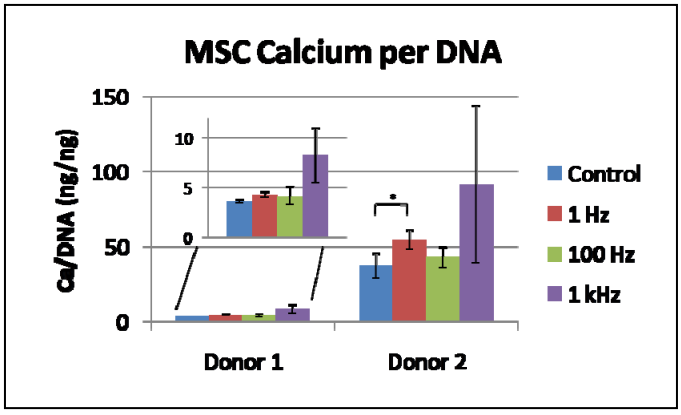

Figure 3. Calcium accreted per total DNA for hMSC cell lines by day 14.

but the trends of calcium production in response to LIPUS were similar. Both demonstrated a trend of improved calcium accretion for the 1 kHz PRF, however the only significant difference occurred between the control cells and 1 Hz PRF for donor 2 hMSCs (p=0.045).

By contrast, the PRF setting had a major effect on the Ca/DNA production for the hASC cell line by day 7 (Fig. 4). On Day 4 there was no significant difference among control and stimulated cells. However, by day 7 all measurements were significantly different except between the control and 1 Hz PRF. For the others, p=0.003, 0.001, 0.027, 0.003 and 0.032 between control/100 Hz, control/1 kHz, 1 Hz/100 Hz, 1 Hz/1 kHz and 100 Hz/1 kHz.

IV. Discussion

Although many commercial LIPUS systems have either a PRF of 100 Hz or 1 kHz, no study has been done to compare the two. Because of the fairly rigid parameter settings on commercial LIPUS systems, we designed an extremely flexible system that uses a unique acoustically-transparent tissue culture plate. The system was then used to study the effects of PRF on hMSCs and hASCs.

The percent reduction of alamarBlue® is a measurement of metabolic activity associated with cell proliferation. For both the hMSC and hASC cell lines LIPUS clearly reduced the percent reduction of alamarBlue®, indicating that those stimulated cells have less proliferation than the unstimulated control cells. The deviation generally occurred between days 4 and 7 after the media was changed from growth media to osteogenic differentiation media. This data could potentially indicate that LIPUS-stimulated cells may be induced down an osteogenic differentiation pathway earlier than unstimulated cells, resulting in a decrease in proliferation rate.

Calcium accretion levels were normalized by the amount of total DNA to accommodate for the differences in proliferation among the stimulated and control cells. The resultant calcium per total DNA values were reported for both hMSC cell lines on day 14 and for the hASC cell line on days 4 and 7. Although the hMSC calcium per DNA values were significantly different only between the control and 1 Hz PRF stimulated cells for donor 2, there was a trend for the mean calcium/DNA for both donors with 1 kHz PRF resulting in the highest mean, indicating that 1 kHz PRF may be best for production of calcium. By day 7, the calcium per total DNA values for the hASC cell line showed a clear stimulation of calcium production by all three PRF settings compared to unstimulated control cells. While the calcium per DNA for the 1 Hz PRF wasn't significantly higher than the control, both the 100 Hz and 1 kHz were, with the 1 kHz PRF resulting in the highest amount of calcium per DNA. The combination of data from both hMSCs and hASCs indicates that, of the pulse repetition frequencies tested here, the 1 kHz PRF may be best for stimulating osteogenic differentiation of the stem cells.

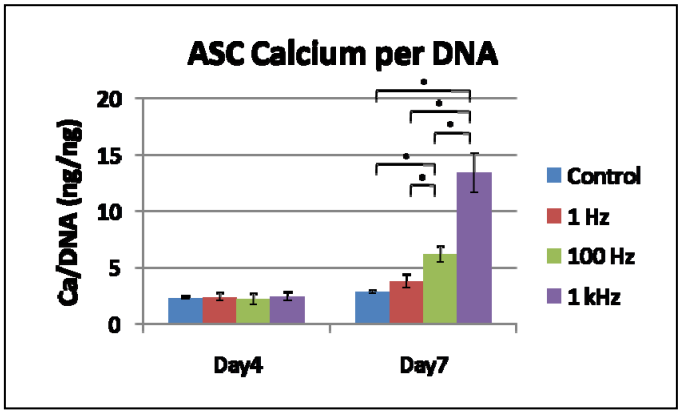

Figure 4. Calcium accreted per total DNA for hASCs on days 4 and 7.

Both the hMSC alamarBlue® measurements and hASC calcium per total DNA ratio showed significant differences between the 100 and 1 kHz PRFs. The data showed an increased response with higher PRF and future studies may involve extending the PRF to higher values.

Clearly more research needs to be done exploring the effects of LIPUS on stem cells. Both hMSCs and hASCs can be used for creating autologous bone tissue to fill critical bone defects and would benefit greatly from increased osteogenic differentiation from LIPUS stimulation. Our results are particularly exciting for hASCs since they are more easily obtained than hMSCs and had a strong response to LIPUS stimulation. One of the significant factors for LIPUS has been determined to be the PRF. Once proper parameters have been found, LIPUS may be incorporated into modern bioreactors as an additional stimulus to increase osteogenic differentiation.

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