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The Development and Validation of a LIPUS System With Preliminary Observations of Ultrasonic Effects on Human Adult Stem Cells

Skylar Marvel, Stan Okrasinski, Susan H. Bernacki, Elizabeth Loboa, and Paul A. Dayton

***Abstract*—To study the potential effects of low-intensity pulsed ultrasound (LIPUS) on cell response *in vitro*, the ability to alter LIPUS parameters is required. However, commercial LIPUS systems have very little control over parameter selection. In this study, a custom LIPUS system was designed and validated by exploring the effects of using different pulse repetition frequency (PRF) parameters on human adipose derived adult stem cells (hASCs) and bone marrow derived mesenchymal stem cells (hMSCs), two common stem cell sources for creating bone constructs *in vitro*. Changing the PRF was found to affect cellular response to LIPUS stimulation for both cell types. Proliferation of LIPUS-stimulated cells was found to decrease for hASCs by d 7 for all three groups compared with unstimulated control cells ($P = 0.008$, 0.011, 0.014 for 1 Hz, 100 Hz and 1 kHz PRF, respectively) and for hMSCs by d 14 (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, LIPUS was shown to strongly accelerate osteogenic differentiation of hASCs based on amount of calcium accretion normalized by total DNA ($P = 0.003$, 0.001, 0.003, and 0.032 between control/100 Hz, control/1 kHz, 1 Hz/1 kHz, and 100 Hz/1 kHz pulse repetition frequencies, respectively). These findings promote the study of using LIPUS to induce osteogenic differentiation and further encourage the exploration of LIPUS parameter optimization. The custom LIPUS system was successfully designed to allow extreme parameter variation, specifically PRF, and encourages further studies.**

I. Introduction

Low-intensity pulsed ultrasound (LIPUS) has been used for more than a decade to accelerate fracture healing. In 1994, the FDA approved a commercial LIPUS device designed by Exogen (Smith & Nephew, Inc., London, UK) for fracture healing, and in 2000 for the treatment of nonunions. Despite this, there is still a lack of understanding of the effects of LIPUS parameters on cell response. There are very few published studies that examine pulse repetition frequency (PRF), a crucial parameter in ultrasound dose. Further, many studies are limited to parameter choices available in commercially made systems, such as a 1 MHz sine wave, 30 mW/cm^2 spatial-averaged temporal-averaged intensity pulsed for 200 μs with a PRF of 1 kHz, giving a 20% duty cycle, applied for 20 min per day. The majority of research concerning the effects of LIPUS on specific cell lines is conducted using these commercially built ultrasound systems that have very limited parameter options. To study the effects of PRF and other LIPUS parameters, our group has designed a custom LIPUS system with complete parameter variability.

No clear set of LIPUS parameters has been found for optimizing osteogenesis; however, studies have been done on individual parameters. The most studied parameter is intensity and the widely practiced convention is to report the intensity as spatial-averaged temporal-averaged (I_{SATA}). Occasionally other representations are used or the type of intensity is not reported. Although there have been many studies focused on using different intensities [1]–[8], other LIPUS parameters used in these studies were not kept consistent and various cell types and assays were used. Ultrasound frequency has been shown to be a fairly unimportant parameter of LIPUS. Generally a frequency of 1.5 or 1 MHz is used and studies have shown little difference when using frequencies ranging from 0.5 to 4.93 MHz [9]–[11]. Duration of LIPUS stimulation, treatment times during the fracture healing process, and changes to duty cycle have also been investigated to a lesser extent [6], [12]–[15]. 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 a 100 Hz or 1 kHz PRF and although changing the PRF has been found to significantly affect amphibian embryo development [16], the effect on stem cell differentiation has only recently begun to be explored.

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 because they are readily available, relatively easily isolated from excess adipose tissue, and new lineage potentials are continually being discovered [17]. Because hASCs are capable of osteogenic differentiation [18], [19], 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. Bone is an extensively transplanted tissue with the autologous graft being the gold standard [20]. To avoid problems

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associated with harvesting autologous bone [21] or using artificial grafts [22], functional bone tissue engineering aims to create these autografts using a patient's own stem cells. 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.

The lack of LIPUS parameter optimization encourages further research in this field and the inability of commercial LIPUS systems to provide adequate parameter variations motivates the need for a custom LIPUS system with the capability to produce a wide range of parameter settings. The development and validation of such a system was the main objective of this study; the effects of altering PRF on hASC and hMSC response were a secondary objective explored during the validation of the custom system.

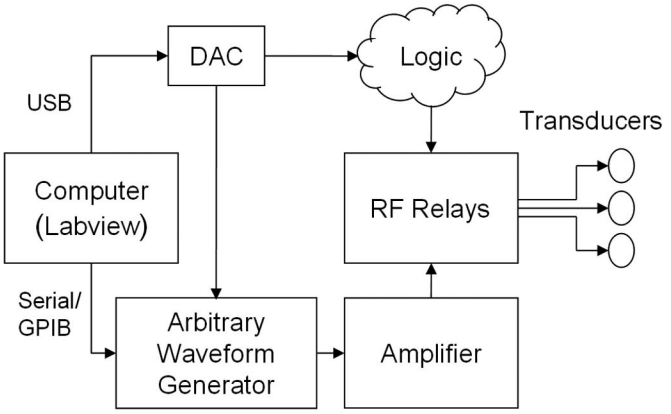

Fig. 1. Block diagram of custom LIPUS system. All components are controlled by computer running LabVIEW software. The digital-to-analog converter (DAC) selects transducers using the RF relay circuitry and sends triggering pulses to waveform generator. Waveforms are amplified by 45 dB gain and routed to appropriate transducer.

II. Materials and Methods

The LIPUS system was designed to fulfill several criteria. The first priority was to gain the ability to precisely control stimulatory waveforms, and a Sony AWG2021 (Tokyo, Japan) arbitrary waveform generator was selected to provide this aspect of the system. A second design criterion required that the system be automatable to reduce the risk of user error when loading transducer specific waveforms and lessen the need for constant user interaction. To control both waveform loading and transducer selection, a control computer running LabVIEW software (National Instruments, Austin, TX) was connected to the arbitrary waveform generator and a digital-to-analog converter (USB 6221 BNC, National Instruments). The digital-to-analog converter was used to select appropriate transducers via a custom relay circuit made with Teledyne CCR-33S60-T relays (Teledyne, Inc., Thousand Oaks, CA), and then send triggering pulses to the arbitrary waveform generator. The LIPUS signal was amplified by a 45 dB amplifier (AP400B, Electronic Navigation Industries, Rochester, NY) before reaching the relay circuit as shown in the system block diagram in Fig. 1. In addition to precise control and automation, the system was also designed to be used with standard incubators and reduce the amount of required lab and incubator space occupied by the system. To enable the use of standard incubators, Valpey Fisher 1-MHz narrow-band unfocused transducers with a 2.54 cm diameter (Hopkinton, MA) were operated in the near field directly below the target cells, which were seeded in BioFlex plates (Flexcell International, Hillsborough, NC). These plates have a flexible tissue culture membrane that is more acoustically transparent than typical hard polystyrene tissue culture plates. To reduce the amount of occupied incubator space, only the transducer stand was placed inside the incubator; the rest of the system could be stored in an adjacent room with the cables run through a port in the wall. The transducer stand and relay circuit are shown in Fig. 2.

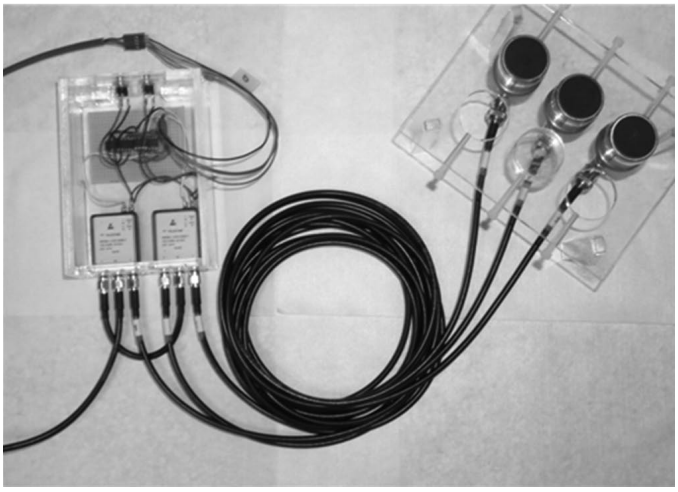

Fig. 2. Relay circuit and transducer stand with three transducers. Relay control logic is controlled by a digital-to-analog converter connected to control computer. Only the transducer stand is placed inside the incubator, with the rest of the system stored in an adjacent room.

A. Transducer Calibration

The overall calibration goal was to have each transducer output an I_{SATA} of 30 mW/cm^2, the typical value used in LIPUS fracture healing devices and research. Transducer calibration was done in several steps, repeated for each transducer.

1) BioFlex Plate Attenuation: The first calibration step was to determine the attenuation of a BioFlex plate. These plates are designed with the tissue culture membrane recessed from the bottom of the plate, which makes future calibration steps very difficult to perform. Because the system was designed to have the transducers coupled directly to the BioFlex plate, all pressure measurements would need to be done as close to the transducer as possible. However, the overhanging edges on the bottom of the BioFlex plates prevented the ability to easily scan

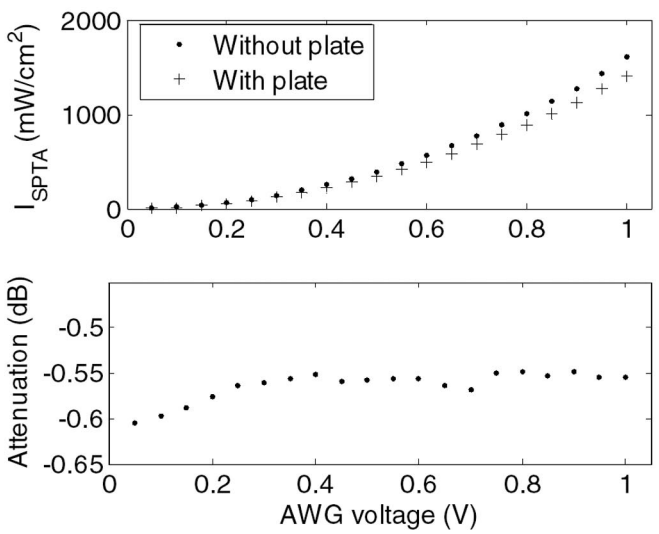

Fig. 3. Attenuation of BioFlex plate was determined by measuring spatial-peak intensity as a function of arbitrary waveform output voltage. Mean attenuation was found to be 0.56 ± 0.02 dB and was fairly consistent across all input voltages.

over the surface of the transducer. To circumvent this problem, the attenuation of the tissue culture membrane was determined and then applied to calculations in future calibration steps. A hydrophone (HNC-0400, Onda Corp., Sunnyvale, CA) controlled by a three-axis motion stage was used to measure the pressure output at the spatial peak pressure output of a transducer with and without the BioFlex plate present. Samples of the output pressure waveforms were taken at identical locations and used to calculate the spatial-peak-temporal-average intensity (I_{SPTA}) using the equation $I_{\mathrm{SPTA}} = {x_{\mathrm{rms}}}^2 \cdot d/(\rho \cdot c)$ where x_{rms} is the root-mean-square of the pressure waveform, d is the duty cycle (0.2 for these waveforms), 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 ranging from 0.05 to 1 V in increments of 0.05 V was performed using the function generator and the attenuation at each voltage was calculated with the average attenuation being found as 0.56 ± 0.02 dB, shown in Fig. 3.

2) Spatial-Average Temporal-Average Intensity: As a starting point for determining a transducer's I_{SATA}, the I_{SPTA} was found for each transducer over the voltage range from 0.05 to 1 V in steps of 0.05 V. I_{SPTA} was calculated using pressure waveforms measured at the peak output pressure location during the transducer steady-state output from 100 to 150 μs when stimulated by a 200-μs pulse of a 1-MHz sine wave. Based on the I_{SPTA} values, voltage ranges were selected for performing beam field scans to determine the spatial average output for each transducer. Each beam field scan was performed by using the three-axis stage to scan across a 35 × 35 mm area, 1 mm from the transducer surface. Pressure measurements were taken at every location of a 20 × 20 grid over the scanning area where the peak pressure for each point was captured during the steady state operation of the transducer between 100 and 150 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 I_{SPTA} versus maximum output pressure plots generated from the I_{SPTA} data measured previously. The resultant interpolated intensity was used as the I_{SATA} value for the initial system validation. For the transducers used during the following system validation experiments, the typical input voltage to the amplifier was 0.33 ± 0.01 V after including BioFlex plate attenuation and was amplified 45 dB to reach the desired 30 mW/cm^2 I_{SATA}.

B. System Validation

Two experiments were performed to validate the custom LIPUS system. The first experiment investigated the previously unstudied effects of LIPUS on human adipose derived stem cells (hASCs) using three different PRF settings: 1, 100, and 1000 Hz. The 100 Hz and 1 kHz PRFs were chosen to study any differences that might be caused when using different commercial LIPUS systems and the 1 Hz PRF was chosen to mimic the loading and unloading seen by bone during gait (assuming $f = 1$ Hz for normal walking gait). The second experiment studied the effects of LIPUS on human bone marrow derived mesenchymal stem cells (hMSCs) from two donors when exposed to the same three PRF settings.

1) Stem Cell Isolation: Human ASCs were derived from excess human adipose tissue from abdominoplasty procedures and isolated from the tissue using a method founded on density and differential adhesion as first described by Zuk *et al.* [23]. Human MSCs were derived from extraneous trabecular bone and isolated from the tissue using a method similar to that developed by Sakaguchi *et al.* [24]. Both stem cell types were characterized via immunohistochemical analysis of surface markers present (CD105, CD166) and absent (CD34, CD45) and their ability to differentiate down oteogenic and adipogenic pathways. The cultures were prepared following a strict protocol outlined in previous studies by the Loboa laboratory [25]–[33].

2) Cell Culture: Human ASCs and MSCs were initially seeded at 5 × 10^4 cells/well, 3 wells per experimental condition and control, in BioFlex plates with complete growth media comprised of Eagle's α-modified minimum essential medium 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 h, 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. Once the media was changed, cells were exposed to a 1-MHz sine wave with an I_{SATA}

of 30 mW/cm^2 for 20 min per day with a pulse repetition rate of 1, 100, or 1000 Hz and a 20% duty cycle.

3) Cell Proliferation: Cell proliferation was monitored throughout the duration of the experiments using a non-destructive alamarBlue assay (AbD Serotec, Raleigh, NC). As in previous studies by the Loboa laboratory [26], [34], the percent reduction of alamarBlue, which measures metabolic activity, was used to indirectly estimate cell proliferation. At d 0, 4, 7, 11 and 14, culture media was aspirated and replaced with 2.34 mL of fresh media plus 10% by volume alamarBlue, then incubated for 3 h 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 mixture was removed from each well in triplicate and placed in a 96-well plate. The plate was briefly centrifuged at 1000 × *g* for 1 min with the cover off to remove any air bubbles and then the absorbance measured at 570 and 600 nm using a microplate reader. Percent reduction of alamarBlue was calculated per the manufacturer's protocol.

4) Calcium Accretion: Calcium content was quantified using a Stanbio Total Calcium LiquiColor kit (Stanbio Laboratory, Boerne, TX). Cells were washed twice with 1 mL phosphate buffered saline (PBS) per well. The Bio-Flex plate tissue culture membranes were visually inspected for homogeneous cell growth, then carefully cut out and divided into two halves using a razor blade. One half was used for calcium measurements and the other was used for DNA measurements to ensure representation of true cell response in the exact same environment when calculating calcium accretion per quantity of DNA. The first half was transferred into a microcentrifuge tube, where 0.5 mL of 0.5N HCl was added. Samples were incubated overnight on an orbital shaker at 4°C, and then centrifuged at 500 × *g* for 2 min and supernatant transferred to a new tube. Calcium concentration was calculated per the manufacturer's protocol.

5) Quantification of DNA: The second half of the membrane obtained during the calcium quantification method was transferred into a separate microcentrifuge tube where 0.5 mL of papain digest (5 mL PBS, 25 μL papain (Sigma-Aldrich, St. Louis, MO), 7.3 mg EDTA, and 4.4 mg cysteine HCl) was added. Samples were incubated overnight at 60°C, then vortexed and the 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, Carlsbad, CA) and fluorescence was read at 460 nm in a microplate reader when excited at 350 nm. The fluorescence value was compared with a standard curve to calculate the amount of DNA.

6) Statistical Analysis: All data were compared using ANOVA with a Tukey-Kramer *post hoc* analysis. Significance was accepted at $P < 0.05$.

III. Results

A. hASC and hMSC Proliferation

Percent reduction of alamarBlue is a measurement of metabolic activity associated with cell proliferation, with more reduction indicative of greater proliferation. Percent reduction of alamarBlue for the hASC cell line [Fig. 4(a)] shows that the LIPUS-treated cells had a lower percent reduction by d 7 of treatment. Although there were no significant differences on d 0 and 4, by d 7 all three stimulated groups were significantly lower than the unstimulated control ($P = 0.008$, 0.011, and 0.014 for 1 Hz, 100 Hz, and 1 kHz, respectively) with no significant differences among the different PRF settings. The percent reduction of alamarBlue for hMSC donors 1 and 2 are shown in Fig. 4(b) and 4(c), respectively. In both cell lines, all three PRF settings produced significant differences compared with the control cells by d 14 (donor 1: $P = 0.0005$, 0.0002, 0.0003; donor 2: $P = 0.0003$, 0.0002, and 0.0001; for PRFs of 1 Hz, 100 Hz, and 1 kHz, respectively). All three stimulated groups had significantly lower percent reduction compared with control cells by d 4 for donor 1 and d 11 for donor 2. Additionally, by d 14 donor 2 had a significant difference between the two common PRF settings used in commercial systems (100 Hz and 1 kHz) with the 1 kHz stimulated cells having significantly less percent reduction ($P = 0.005$).

B. Calcium Accretion Normalized by Total DNA

LIPUS was shown to have a significant effect on cell-mediated calcium accretion per amount of DNA for hASCs by d 7 as shown in Fig. 5. There was no significant difference among control and stimulated cells after 4 d of LIPUS treatment; however, by d 7 all calcium per DNA measurements were significantly different among each experimental group except between the control/1 Hz and 1 Hz/100 Hz PRF. All others were significantly different with a trend of increased calcium per DNA with increased PRF setting ($P = 0.003$, 0.001, 0.003, and 0.032 between control/100 Hz, control/1 kHz, 1 Hz/1 kHz, and 100 Hz/1 kHz, respectively). Calcium accretion per amount of DNA for both hMSC cell lines on d 14 is shown in Fig. 6. Human MSCs from donor 1 produced much less calcium than those from donor 2 but the trends of calcium production in response to LIPUS PRF settings were similar. Both demonstrated a trend of improved calcium accretion for the 1 kHz PRF, however there was no significant difference among the groups.

IV. Discussion

Although many commercial LIPUS systems have either a PRF of 100 Hz or 1 kHz, little research has been done to study the effects of PRF on cell differentiation. Because of the fairly rigid parameter settings on commercial LIPUS

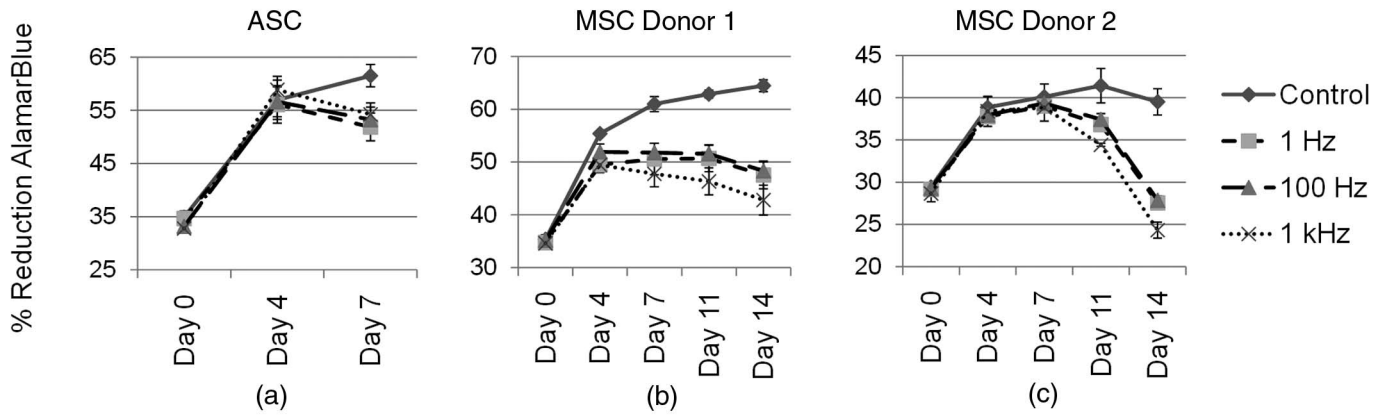

Fig. 4. Cell proliferation as monitored by percent reduction of alamarBlue for both hASC (a) and hMSC (b) and (c) cell lines. A higher percent reduction indicates more metabolic activity and more proliferation. All LIPUS treated cells have a lower percent reduction compared with unstimulated control cells after 4 or 7 d. By d 14, the 1 kHz PRF treated cells have significantly lower percent reduction than 1 and 100 Hz PRF treated cells.

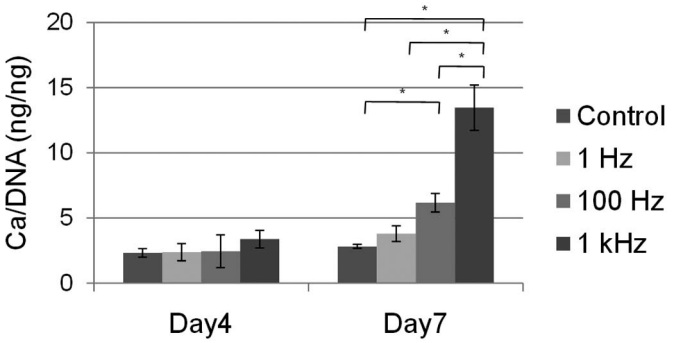

Fig. 5. Calcium accreted per amount of DNA for hASCs on d 4 and 7. No significant difference at d 4, but by d 7 all are significantly different except between control/1 Hz and 1 Hz/100 Hz PRF. Asterisk indicates $P < 0.05$.

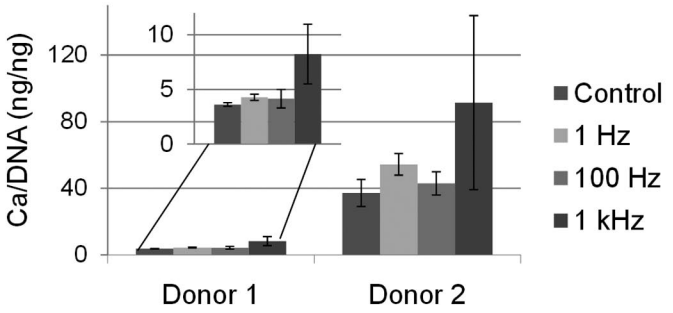

Fig. 6. Calcium accreted per amount of DNA for both hMSC cell lines on d 14. Although little significant difference is seen, there is a similar trend in calcium per DNA response for both donors with the largest mean resulting from stimulation with 1 kHz PRF.

systems, we designed an extremely flexible system that uses a unique acoustically-transparent tissue culture plate. The system was then validated when 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 d 4 and 11 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 the hASC cell line on d 4 and 7 and both hMSC cell lines on d 14. Although the hMSC calcium per DNA values were not significantly different, there was a trend of similar response for both cell lines when stimulated with different PRF settings. Both had the highest mean value of calcium per DNA with the highest PRF setting, indicating that of the three tested, a 1 kHz PRF may be best for production of calcium. By d 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 with unstimulated control cells. Both the 100 Hz and 1 kHz PRF stimulated cells produced significantly more calcium per DNA, with the 1 kHz PRF resulting in the highest amount of calcium per DNA between the two. 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 these stem cells.

The observed decrease in proliferation is the opposite of the effect commonly seen by other researchers [3], [35]–[38], with many studies reporting an increase in proliferation specifically for chondrocytes [13], [39]–[41]. Sena *et al.* investigated the temporal expression of both early response genes indicative of proliferation and bone differentiation marker genes in rat bone marrow derived stromal cells when exposed to LIPUS [42]. The authors found that after a single treatment of LIPUS, several genes from both

groups were significantly upregulated 3 h post stimulation, showing both an increase in proliferation and differentiation. However, this increase in proliferation is not always seen, as other studies have reported no change in proliferation when using LIPUS stimulation [8], [43]–[47]. The effects of LIPUS on proliferation and osteogenic differentiation of a rat osteosarcoma cell line (ROS 17/2.8) was studied by Takayama *et al.* [47]. Cell proliferation was monitored by a cell-counting reagent where the intensity of the reaction products was measured spectrophotometrically, similarly to how cell proliferation was monitored in this study. Over the 14 d treatment, no significant change was seen in cell proliferation. Several osteogenic differentiation marker genes were monitored and found to be significantly increased by LIPUS ranging from 6 to 24 h post treatment, depending on the gene. Calcium content was measured after 14 d and found to be significantly increased by LIPUS treatment. In a study by Zhang *et al.* on White Leghorn chick chondrocytes, a decrease in proliferation was seen when applying a 30 mW/cm^2 LIPUS stimulus [48].

Although calcium content was significantly increased after 7 d for the hASCs, the hMSCs only showed a trend of increased calcium after 14 d. One possible explanation for non-significant increases in calcium may be substrate stiffness. Substrate stiffness has been shown to have a significant effect on cell differentiation where cells grown on stiffer substrates tend to osteogenically differentiate [49], [50]. In current literature, LIPUS treated cells are typically seeded on polystyrene tissue culture plates which have a stiffness of ∼2.6 GPa, whereas the stiffness of the BioFlex plates is ∼1.6 MPa, several orders of magnitude lower [51]. The decrease in substrate stiffness may be counteracting the differentiation cues caused by LIPUS stimulation. Substrate stiffness may also be affecting how LIPUS signals are transmitted into the cell. In a recent review paper, Pounder *et al.* present several integrin-initiated pathways for LIPUS transduction [52]. Although the BioFlex plates are acoustically favorable, the flexible membrane may be diminishing the effect of LIPUS treatment if the membrane and stem cells are able to move more in unison to the ultrasound wave. However, if the membrane stiffness is detrimental to stem cell response, the custom system can easily be adapted to use standard hard plastic tissue culture plates by simply recalibrating the transducers through the new interface surface. The data presented by this paper was primarily used to validate the custom system design and is still in the pilot stage of research. The reason for reporting the initial results is to show that there is a significant difference in stem cell response to different PRF settings, which cannot be studied by using the current commercial LIPUS systems.

V. Conclusion

Significant differences for cell proliferation (for donor 2 hMSCs) and calcium per total DNA (for hASCs) were observed when using the two commonly used PRF settings in commercial LIPUS systems, 100 Hz and 1 kHz. 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 and the effects of altering LIPUS parameters. To enable the precise control of LIPUS parameters, a custom system was designed to be used in standard incubators, automatically load and produce tailored waveforms, and transmit through acoustically transparent BioFlex tissue culture plates. This system was validated on hMSCs and hASCs. 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 because 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 determined, LIPUS may be incorporated into modern bioreactors as an additional stimulus to increase osteogenic differentiation.

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