Ultrasound and lipid-coated microbubbles effect on proliferation and osteogenic differentiation of mesenchymal stem cells in 3D printed tissue scaffold

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Abstract

With increasing incidence of bone disorders in a rapidly aging, sedentary, and overweight population, engineered bone tissues promise to be a better alternative for conventional bone grafts. Human mesenchymal stem cells (hMSCs) extracted from a patient's bone marrow can be cultured to create bone tissue via osteogenic differentiation of the stem cells. The engineered bone tissue may then be implanted into the injury site. However, bone tissue engineering currently suffers due to the inability to form mechanically strong, porous structures that can be grown quickly and efficiently. Low intensity pulsed ultrasound (LIPUS) stimulation has proven to enhance bone healing and recovery rates. In this study, the beneficial effects of LIPUS stimulation are harnessed to improve bone tissue engineering methods and are further improved with the addition of in-house prepared, lipid-coated microbubbles (MBs). Bone marrow hMSCs were seeded on 3D printed poly(lactic acid) (PLA) porous scaffolds and cultured to study the effects of LIPUS in the presence of MBs on proliferation and osteogenic differentiation. For proliferation studies, the samples were treated with LIPUS in the presence of 0.5% (v/v) for 3 minutes a day at 30 mW/cm2 with a frequency of 1.5 MHz and a duty cycle of 20%. A significant increase in cell number was observed after 1, 3, and 5 days of culture as compared to control. For osteogenic differentiation studies, the samples were treated with LIPUS in the presence of 0.5% (v/v) MBs for 3 days for 3 minutes a day with the same parameters as the proliferation studies. The samples were then cultured for 1, 2, and 3 weeks for osteogenic differentiation to occur. Total protein, alkaline phosphatase (ALP) activity, and total calcium content were found to increase with LIPUS with and without the presence of MBs. Integrating LIPUS and MB appears to be a promising strategy for bone tissue engineering and regeneration therapies.

Keywords: ultrasound; microbubbles; tissue engineering

Introduction

Osteoporosis and osteoarthritis are major health problems, affecting over 6 million people per year, and that number is expected to grow. With increasing incidence of these disorders, more options for therapies are desired. Tissue Engineers have been exploring the opportunity of using human mesenchymal stem cells (hMSCs) as a potential source of healthy cells that can help expedite the healing process of bone injuries and diseases. The advantage of the hMSCs is their ability to differentiate into different cells types, such as osteoblasts, chondrocytes, or adipocytes when exposed to the proper environmental factors.

3D printing technologies for tissue engineering applications have gained attention for their ability to have patient injury-specific scaffolds for replacements and grafts [1] [2]. The geometry of the patient's injury site can be obtained from a MRI or CT image and can be 3D printed from a porous, biodegradable material. These scaffolds can be used for stem cells to grow and differentiate into the desired tissue. This can then be implanted into the injury site, and as the scaffold material degrades, the tissue fills in. To induce the differentiation of the stem cells on the scaffolds, hMSCs can be exposed to osteogenic growth factors and mechanical stimuli, such as ultrasound [1].

Low intensity pulse ultrasound (LIPUS) has been shown to be effective for helping fracture healing at every phase of healing [2]. Previous studies have also shown improvements of proliferation and differentiation of hMSCs [1]. Microbubbles (MBs) have been used as ultrasound contrast agents in imaging domains. Their compressible gas core gives rise to highly echoic behavior. The compressibility also generates bubble oscillations and complex fluid patterns around the bubbles. The fluid motion can give rise to extra shear stress on the cell membranes and can lead to additional mechanical stimuli to the cells. In this study, the effects of LIPUS and LIPUS in the presence of MBs on hMSC proliferation and osteogenic differentiation were evaluated.

Methods

A. Cell Culture

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Under an IRB-approved protocol with written, informed consent, human bone marrow mesenchymal stem cells (hMSCs) were obtained from healthy individuals at Tulane University. The cells were characterized at the Institute for Regenerative Medicine at Texas A&M Health Science Center with a fully executed Material Transfer Agreement. The cells were cultured in α-Minimum Essential Medium Eagle (α-MEM) supplemented with 16.5% (v/v) fetal bovine serum (FBS), 1% (v/v) L-glutamine, and 1% (v/v) penicillin and streptomycin. The hMSCs were incubated in a 37°C, 5% CO_2, and 95% relative humidity environment. For the studies requiring osteogenic differentiation of the hMSCs, the cells were cultured in Dulbecco's Modified Eagle Medium (D-MEM) supplemented with 10% (v/v) FBS, 50 μg/mL of L-ascorbate acid, 10 mmol/L of β-glycerophosphate, 10 nmol/L of dexamethasone, and 1% (v/v) penicillin and streptomycin. An image of the hMSCs can be seen in figure 1(a).

B. Synthesis of Microbubbles

The MBs used in this experiment were prepared locally using a lipid shell and perfluorocarbon gas. For the shell of the MBs, 0.75 mg/mL of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1.5 mg/mL of 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DPPE-PEG2000), and 3.0 mg/mL of 1,2-dipalmitoyl-3-trimethylmmonium-propane (TAP) were dissolved in heated (above 45°C) propylene glycol for 30 minutes. Then glycerol and phosphate buffer solution (PBS) were added and mixed while heated for 40 minutes. The solution was kept at 4°C overnight. The following day, 1.5 mL of the lipid solution was placed in a 2 mL vial, crimped closed, underwent a gas exchange with perflurobutane.

The bubbles were generated by mechanical agitation of the vial for 45 seconds using a Vial Mixer (Bristol Myers Squibb). The resulting bubbles are highly polydisperse in size. To narrow the range of diameters, the bubbles were diluted to 50 mL in PBS and centrifuged at 40 relative centrifugal force (RCF) for 4 minutes. The bubble size distribution was measured using qNano (Izon Science™, MA) with nanopore membrane NP2000 (1000 nm- 5000nm). The average size of the particles was roughly 1.1-1.2 μm and an average concentration of about 10^8 - 10^9 particles/mL as seen in figure 1(b).

C. Production and Characterization of Scaffolds

The 3D printing technique used to fabricate the scaffolds was fused deposition modeling (FDM) (Solidoodle, NY). The scaffolds were fabricated using poly(lactic)-acid (PLA). First, a 3D computer-aided design (CAD) model of the scaffolds was made to dictate the dimensions of the scaffolds. Then, the infill density, scaffold pore geometry, and the speed of the printer nozzle were set using Slic3r computer numerical control conversion software. We chose a square pore geometry which was previously shown to be optimal for the hMSC growth [1]. To assess the pore size, the scaffolds were imaged using scanning electron microscopy (SEM) (Zeiss Nvision) as seen in figure 1(c). The average pore dimensions of the scaffolds were 700 μm and 1000 μm.

D. Ultrasound Parameters

The ultrasound parameters for hMSC parameters were optimized previously and the setup described by Aliabouzar et al [1] was used. Briefly, a flat ultrasound transducer (Olympus NDT, Waltham, MA) was used to expose the cells from above with a frequency of 1.5 MHz, intensity of 30 mW/cm^2, and a duty cycle of 20% for 3 minutes per day. The MB concentration was also optimized (results not shown) to be 0.5% (v/v). The experimental setup can be seen in figure 1(d).

E. Proliferation of hMSCs

The effect of LIPUS with and without the presence of microbubbles on proliferation was evaluated. The hMSCs were cultures in 24 well plates with 2.5 x 10^4 cells per well overnight. The samples were divided into three different groups, control (no LIPUS or MB), LIPUS (no MBs), and LIPUS + MBs. The control cells had the LIPUS transducer dipped into the wells without any excitation. The cells were tested after 1 day, 3 days, and 5 days of treatment using Alamar Blue Assay. The cells were incubated for 2 hours at 37°C and then tested for absorbance at 570 nm and 600 nm. The absorbance measurements were converted to percent reduction of the assay using the manufacturer's instructions. The percent reduction of the assay is proportional to cell number. The cells were rinsed with PBS three times and replenished with fresh medium.

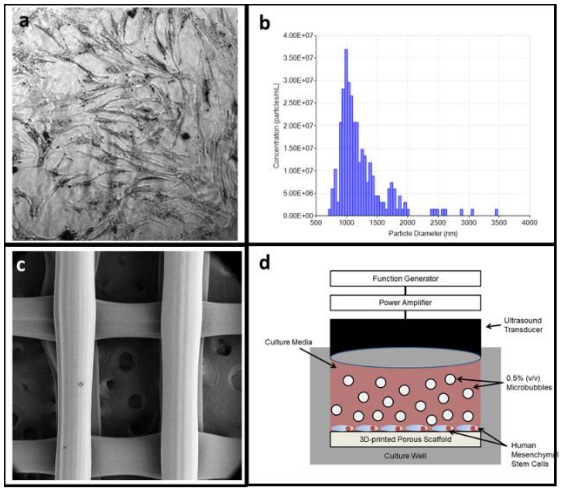

Figure 1. (a) The hMSCs in culture, (b) The size distribution as measured by qNano of the centrifuged microbubbles (c) An SEM image of the 3D printed PLA scaffolds, (d) the experimental setup of the ultrasound exposure of the hMSCs

F. Osteogenic Differentiation of hMSCs

For osteogenic differentiation studies, the hMSCs were cultured in 24 well plates with 1 x 10^5 cells per well overnight. The cells were divided into the same three groups and were then immersed in osteogenic media and exposed to ultrasound with the parameters mentioned above for 3 days. After that, the cells were incubated in osteogenic media for 1, 2, or 3 weeks. Alkaline phosphatase (ALP) activity and extracellular calcium deposition are two biomarkers that are indicative of osteogenic differentiation of hMSCs. ALP activity was tested using a standard assay kit (Bioquest, Sunnydale, CA) according to the manufacturer instructions. Calcium deposition was also tested using a standard assay kit (Pointe Scientific, Canton, MI) according to the manufacturer instructions.

Results and Discussion

For the effects of LIPUS and LIPUS in the presence of 0.5% (v/v) MBs on hMSC proliferation, the percent reduction of Alamar Blue assay was measured after 1, 3, and 5 days of treatment. The results can be seen in figure 2 for all three groups (control, LIPUS, and LIPUS+MBs). After one day, there was 8% increase in percent reduction when the cells were exposed to LIPUS in the presence of microbubbles as compared to control ($p < 0.001$). With only LIPUS stimulation, there was a 4% percent increase as compared to control ($p < 0.05$). After three days the cells with LIPUS stimulation in the presence of microbubbles had a 11% increase in percent reduction as compared to control, while LIPUS alone had a 5% increase ($p < 0.001$). After five days, a 12% increase in percent reduction was observed after 5 days in the LIPUS in the presence of MBs when compared to control and only 7% with LIPUS alone ($p < 0.05$). The addition of the LIPUS in the presence of microbubbles improved cells number significantly.

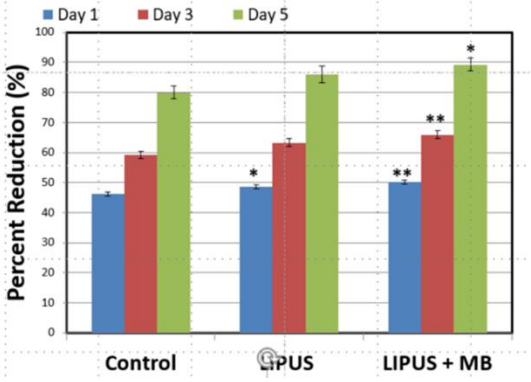

Figure 2. The percent reduction of the Alamar Blue assay after 1, 3, and 5 days of treatment. (*p<0.05, **p<0.001)

For osteogenic differentiation, the two biomarkers that were tested were ALP Activity (figure 3(a)) and calcium deposition (figure 3(b)). ALP is an enzyme that is important in the formation and mineralization of the bone extracellular matrix (ECM) [1] . The ALP activity is an early stage indicator of osteogenic differentiation [1]. The

addition of LIPUS in the presence of MBs improved the ALP activity within the samples. After 2 weeks, there was 74% increase as compared to control, while LIPUS alone there was only a 7% increase. After 3 weeks, there was a 20% increase as compared to control and LIPUS alone had no statistical significant increase in ALP activity.

Extracellular calcium deposition is another biomarker for osteogenic differentiation of hMSCs. However, calcium deposition is a late-stage differentiation marker. Calcium has shown to be important for bone strength and stability [5]. After 3 weeks, there was a 7% increase in calcium concentration in samples that were exposed to LIPUS in the presence of MBs as compared to control. While LIPUS alone only had a 4% increase. Overall, LIPUS in the presence of MBs has shown to enhance proliferation and differentiation potential of the hMSCs. The mechanism behind LIPUS stimulated cell behavior is not fully understood. There are several pathways that LIPUS may stimulate, such as RhoA-GTP, p-ERk1/2, p-FAK, p-MEK1/2, p-p38, p-Akt, p-IKKα/β, and NK-kB [1]. The mentioned pathways are involved with bone regeneration and repair.

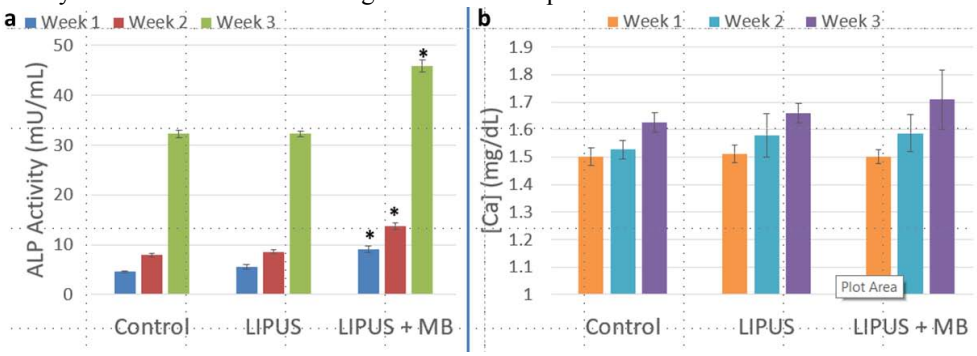

Figure 3. (a) The ALP Activity of the three groups after 1, 2, and 3 weeks (*p<0.05), (b) the calcium deposition of the three groups after 1, 2, and 3 weeks.

Conclusion

Overall, LIPUS in the presence of MBs has shown to enhance proliferation and differentiation potential of the hMSCs as shown by ALP Activity and Calcium Deposition. After 5 days of culture, there was a 12% increase in percent reduction of Alamar Blue, which is proportional to cell number, as compared to control. While LIPUS alone, only had a 7% increase after 5 days. For osteogenic differentiation of the hMSCs, two biomarkers were quantified, ALP Activity and extracellular calcium deposition. The ALP Activity increased by 25% after 3 weeks of culture in the samples exposed to LIPUS in the presence of MBs, while LIPUS did not have a statistically significant effect. For calcium deposition, there was a 7% increase after 3 weeks in samples exposed to LIPUS in the presence of microbubbles and only a 4% increase in LIPUS alone. The addition of LIPUS in the presence of MBs shows to be a promising enhancement of the osteogenic differentiation of hMSCs and could be a promising strategy for future bone tissue engineering applications.

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