ORIGINAL ARTICLE

MC3T3 infiltration and proliferation in bovine trabecular scaffold regulated by dynamic flow bioreactor and augmented by low-intensity pulsed ultrasound

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Abstract   Background: Low-intensity pulsed ultrasound (LIPUS) has been used in both basic research and clinical settings for its therapeutic potential in promoting tissue healing. Clinical data has shown that LIPUS can accelerate fresh fracture healing. However, the treatment for aging osteoporosis and non-union is still unclear. In addition, the mechanism of ultrasound promoted bone healing has remained unknown.

Objective: It is proposed that noninvasive ultrasound treatment can enhance local fluid flow within the tissue to initiate remodeling and regeneration. The goal of this study was to evaluate the effects of dynamic ultrasound in promoting cellular mechanotransduction within bioengineered organic scaffolds to trigger osteogenesis and mineralization.

Methods: The experiment was designed in two-fold: to evaluate the role of LIPUS on osteoblastic-like (MC3T3) cell proliferation and mineralization in response to acoustic waves, using biomechanical rate-dependent signals in a bioreactor; and, to evaluate the new scaffold experimentation techniques, in order to generate a potential implantable biomaterial for orthopedic tissue regeneration and repair.

Results: LIPUS treatment on MC3T3 cells yielded enhanced cellular mineralization (**p < 0.001) in 3-D scaffolding, but reduced the total cell numbers (*p < 0.05), using Alizarin Red staining and cell counting analyses, respectively, in comparison to the control.

Conclusion: This study suggests that LIPUS, if applied at proper frequency and duty cycle, can promote cell mineralization within the 3-D organic scaffold under in vitro setting. The
Introduction

Osteoporosis affects millions of people worldwide by increasing fracture risk and diminishing the quality of life [1]. This systemic skeletal disease results in low bone mass and global weakening of the bone tissues through bone cell imbalances [2]. This leads to increased fracture risk in areas rich in trabecular bone, such as the wrists, hips and vertebrae [3]. Medications that regulate osteoblast and osteoclast balance have a history of success in treating patients with osteoporosis [4]. Other treatments also work by correcting hormone imbalances, improper loads of cell signalling and hyperactivation of cell activity [5]. Although these therapies have shown marked success, they require the administration of a drug or supplement; these therapies have well-established adverse side effects and are unable to specifically target bone sites that are at an increased risk for failure [6]. Furthermore, these treatments may also reflect understudied changes in the bone—hypothalamic—pituitary axis, osteocytes signalling and osteoclast secretions [7]. This experiment focuses on the osteoblast to tackle the imbalance of activity seen in patients with osteoporosis.

Relatively recently, ultrasound technology has been used for noninvasive experimentation; a modified form of it has been used to test our hypothesis [8]. Ultrasound is an acoustic wave or travelling mechanical energy that has a frequency between 20 kHz and 200 MHz [9]. Low-intensity pulsed ultrasound (LIPUS) is a specific frequency of ultrasound, most commonly studied at 1.5 MHz, that has the ability to improve fresh fracture healing, supported by both clinical evidence and basic research studies [10,11]. LIPUS manipulates mechanotransduction receptors on bone cells to activate downstream effectors that signal anabolic growth cascades [12]. LIPUS reinforces a concept embedded in the foundation of microgravity experiments. Past studies have indicated that individuals exposed to conditions of microgravity for long periods of time suffer profound bone loss [13]. Gravity is a constant source of loading linked to mechanotransduction [14]. When gravity is removed for long periods of time, e.g., astronauts during long-term space mission, the constant stimulus of mechanical loading on those receptors is lost, eventually resulting in significant decreases in bone density [15,16]. Therefore, the converse of this situation, applying extra forces (such as exercise and LIPUS), may be able to regulate the mechanotransduction pathways positively and increase bone formation parameters [17].

This experiment will study those pathways by concentrating on the three components of the tissue engineering triad, which includes the cell, the growth-stimulating signals (provided physically by the bioreactor) and the scaffold [18]. Most commonly, a scaffold is a microscopic structure that abets the development of tissues in a given 3-D cellular space [19]. A scaffold that develops seeded cell does this by fulfilling the in vivo environment, which, in nonpathological conditions, supplies vascularisation, cell attachment points and optimised migration [20]. This experiment decellularised the extracellular matrix of bovine trabecular bone to produce a macroscopic scaffold.

LIPUS is known to enhance mesenchymal stem cell recruitment and differentiation through a variety of mechanotransductions [21,22]. There is evidence that LIPUS can induce cavitation and acoustic streaming in interstitial fluid and mechanical vibrations in the extracellular matrix [23]. This yields shear stresses and strains to osteoblasts and deformation to cell membranes, which can then activate mitogen-activated protein kinase (MAPK), angiotensin I (ATI ) mechanoreceptors, integrin, mechanosensitive calcium channels, G-proteins, insulin-like growth factors (IGF) and a variety of other downstream effectors [24,25].

Another component of our experiment is the bioreactor, which simulates the biological environment of the sample, a bovine trabecular bone scaffold, for our experiment. The bioreactor we used supported cell life by nourishing it with media at a constant rate [26]. This is analogous to how blood from capillary beds supply human cells with oxygen and nutrients [27]. We use the three components of the tissue engineering triad here by treating the cells seed in the scaffold, inside the bioreactor, with LIPUS probes.

The effect of LIPUS as a noninvasive modality for tissue regeneration and cellular proliferation in a tissue engineering setting has not been investigated. Therefore, the primary objective of this study was to test the hypothesis that LIPUS treatment on scaffolds embedded with MC3T3 cells in a bioreactor setting enhances both cell proliferation and bone formation parameters, namely matrix mineralisation. We found our hypothesis to be true regarding LIPUS treatment on MC3T3-yielded enhanced cellular mineralisation (“*p < 0.001) but false regarding its effect on the total cell numbers, which was reduced (“p < 0.05). The results section further elaborates on the LIPUS- versus control-treated groups’ cellular proliferation rate in the
scaffold and cell mineralisation as a function of calcium deposition. Results of this experiment also include the preliminary data on cell counts over 2 weeks as an assessment of the scaffolds and cells for experimentation.

**Materials and methods**

**Scaffolds and cells**

Six trabecular scaffolds were isolated from the proximal head of bovine femur (purchased from United States Department of Agriculture (USDA)-approved local market). Bovine trabecular bone was used because of its high porosity, a critical factor in promoting cell seeding, vascularisation and waste removal [28]. The culture media used for this experiment was a mixture of 89% alpha-MEM (minimum essential medium, Gibco, Life Technologies, Grand Island, NY, USA), 10% foetal bovine serum (Gibco, Life Technologies) and 1% Penicillin-Streptomycin (Gibco, Life Technologies). MC3T3, mouse osteoblastic precursor cell line (Sigma Inc., USA), was cultured in 11 mL of this media at a passage of 23 and 13 (in separate experiments) to 85% confluency. The plates were then incubated under standard conditions (95% humidity, 5% CO₂ and 37°C) for 24 h to secure firm attachment.

**Bioreactor and LIPUS**

TGT’s OsteoGen bioreactor (Instron TGT, MA), a device that uses a system of cartridges, tubing and chambers to simulate fluid perforation by controlling the flow rate of media, was used to perfuse the seeded scaffolds with cell culturing media. We used a rate of 0.1 mL/min as that rate permits a balance between sufficient vascularisation and seeding efficacy [29]. The Sonicator 740 (Mettler Electronics Corporation, CA, USA) with gel-coupled plane wave US applicator (ME7410) was used to apply ultrasound stimulation to the bioreactor chambers (Fig. 1).

**Time course**

The first set of samples was tested five months before experimentation with the bioreactor and LIPUS. This set was used to determine the efficacy of bovine trabecular bone as a scaffold and MC3T3 as seeding material. After obtaining results for this part of the experiment, we cultured the cells again and made new scaffolds for both the first experiment and the second experimentation. One month of preparation was required to allow appropriate incubation time of the cells in the scaffold before experimentation with the bioreactor and LIPUS.

**Treatment regimen**

In experiments with MC3T3, the control group (scaffolds 1–3) received no ultrasound treatment. Reher et al’s study on mouse calvaria bone in tissue culture found that transducer output energy, using a spatial-averaged and temporal-averaged intensity, of 100 mW/cm² can provide approximately 30 mW/cm² acoustic energy in tissue with acoustic coupling distance, which can provide significant bone formation stimulation [30]. Therefore, scaffolds 4–6 received a sinusoidal ultrasonic pulse at 1 MHz with a repetition frequency of 100 Hz and at an intensity of 30 mW/cm², with

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Figure 1  *Bioreactor setup in incubator and probes*. The control panel was installed into the hood for technical reasons. The Masterflex rotor could then be operated using the control panel to circulate media at the specified flow rate of 0.1 mL/min to the OsteoGen chambers. This setup has the LIPUS console outside of the incubator. This allowed control LIPUS stimulation without opening the incubator, thus minimizing environmental perturbations. Probes were coupled to the chambers through a polyurethane mold and gel to remove airspace and seal coupling. To ensure the consistency of the treatment, we have set the ultrasound exposure in the far field, which operated by an acoustic lens (~7 mm), acoustic coupling gel (~1–2 mm) and the thickness of the bioreactor chamber (~3 mm). Within the ultrasound far field zone, the energy is consistent and is controlled at approximately 30 mW/cm². LIPUS = low-intensity pulsed ultrasound.
MC3T3 cell proliferation in scaffold regulated by bioreactor fluid flow using LIPUS

20% duty cycle for 20 min each day for 5 days total. After the first experiment was completed, the scaffolds were stored to be reused for future experiments.

Count and quantification

Cell counting and Alizarin Red staining were used to determine if LIPUS treatment promoted effective tissue engineering. The cells were counted with the Scepter 2.0 Handheld Automated Cell Counter (Millipore Corporation, MA, USA). After cells (from first experiment with MC3T3) were counted, they were stained with 40 mM Alizarin Red staining solution (pH 4.2) and imaged using an Axioskop 2000M Inverted Microscope (AxioCam MRc; Carl Zeiss Inc., Thornwood, NY). After imaging, the stain was removed from the cells using 10% cetylpyridinium chloride (CPC) and 10 mM sodium phosphate solution for 20 min. This eluted stain was then measured at 592 nm in the Bio-Tek EL800 spectrophotometer (Winooski, VT, USA). Quantification was achieved through an Alizarin Red standard curve in 10% CPC and normalised to the total number of cells [21].

Statistical analysis

GraphPad QuickCalcs (GraphPad Software, La Jolla, CA, USA) was used to perform all unpaired t-tests. The student t-test was then used to define all values of significance. A p-value < 0.05 was considered significant. Averaged data were presented with error bars equal to the averaged data’s standard deviation [21].

Results

Assessment of scaffold and MC3T3 cells

Preliminary data demonstrated good MC3T3 seeding in bovine trabecular bone and longitudinal growth over a 2-week period. Previous studies have indicated that decellularised bovine trabecular bone can support bone cells; therefore, this part of the experiment was more important for determining if cell seeding and culturing were possible in an incubator before using a bioreactor [31]. Furthermore, we estimated that successful testing of both LIPUS and the bioreactor with MC3T3 cells would require a minimum of 10,000 cells to seed in the bovine trabecular bone scaffold. Our preliminary tests yielded an average of 10% seeding success rate as a percentage of total cells in the dish. This count proved to be more than sufficient in every single scaffold, which seeded from 13,000 to 86,400 cells (Table 1). This part of the experiment was also a longitudinal study to test approximately when the scaffolds would have the most optimal confluency. Plates 1 and 2 were counted after 3 days. Plates 3 and 4 were counted after 7 days. Plates 5 and 6 were counted after 14 days (Table 1). Results from 3 to 7 days demonstrated the best confluency. The cell count seemed to decrease around 14 days but not to numbers below a tolerable confluency for an experiment with the bioreactor.

Cell count and analysis

To evaluate the anabolic nature of LIPUS treatment on MC3T3 cells in bioreactor conditions, we measured cell proliferation in each scaffold after experimentation. The results initially showed a surprising decrease in cell count when the chambers were treated with LIPUS. This was seen consistently throughout the two sets of triplicates, with scaffold 2 yielding numbers around the average LIPUS experimental group count. The control MC3T3 groups on average had a higher count of cells than the average of the LIPUS experimental group (Fig. 2).

LIPUS exposure significantly increases calcification

To establish the anabolic effects of LIPUS on matrix mineralisation, matrix calcification was determined using Alizarin Red staining. At 4× magnification, it was difficult to discern noticeable differences in levels of Alizarin Red when comparing the control and LIPUS groups (Fig. 3). At 10× magnification, there is a similar difficulty as both seem to have strong reddened areas. Qualitative analysis thus showed no significant difference in matrix mineralisation between the control- and LIPUS-treated samples.

Quantitative data, however, give strong statistical evidence supporting the hypothesised anabolic effect of LIPUS on MC3T3 cells in the bioreactor. High absorbance from this stain reflects an increase in Alizarin Red stain, which measures calcium deposition. This quantification was achieved through comparison of our results with an Alizarin Red standard curve in 10% CPC and normalised to the total number of cells. 0.69 is the average absorbance of the two control groups; 2.18 is the absorbance average of the two LIPUS groups (Fig. 4). LIPUS treatment showed a significantly higher absorption than the control group (Fig. 4). These data support that LIPUS treatment increased the matrix mineralisation in MC3T3 cell cultures seeded in scaffolds under conditions of the bioreactor.

Discussion

Past studies on LIPUS have demonstrated its anabolic effect both in vivo and in vitro [32]. Stimulating the

Table 1 Test for longitudinal growth. Six scaffolds were tested over a 2-week period to see when MC3T3 cells reached an optimal confluency in the bovine trabecular bone scaffold. Results from 3 days to 7 days were optimal and seemed to decrease around 14 days but not to values that would be considered below a tolerable confluency for an experiment with the bioreactor.

| Plate number | 1 (3 day) | 2 (3 day) | 3 (7 day) | 4 (7 day) | 5 (14 day) | 6 (14 day) |
|--------------|-----------|-----------|-----------|-----------|------------|------------|
| Plate count ($\times 10^5$) | 4.267 | 4.940 | 5.627 | 4.615 | 4.247 | 4.443 |
| Scaffold count ($\times 10^3$) | 0.4184 | 0.8640 | 0.6000 | 0.6460 | 0.2266 | 0.1382 |
mechanoreceptors of bone cells, both osteoclasts and osteoblasts, has proven to be just as important as maintaining biochemical/hormonal signal transduction homoeostasis [33]. This concept led us to the expectation that ultrasound should enhance cell proliferation and mineralisation. The statistically significant results of the Alizarin Red stain agreed with our hypothesis regarding the anabolic actions of LIPUS treatment. Although qualitative analysis from the picture could not help us truly differentiate which plate had calcium deposits, it was suggested from quantitative analysis that the LIPUS treatment increased matrix mineralisation in the MC3T3 cells under conditions of the bioreactor.

The discrepancy between cell proliferation and matrix mineralisation can be due to a range of factors. The additional shear stress and deformation of the cell membranes may not have been well favoured in a bioreactor environment, where the cells are already experiencing such deformation from a fluid flow shear stress. This could potentially explain why fewer cells were able to survive or proliferate.

Figure 2  LIPUS decreases the cell count after 5 days of stimulation. The average number of cells counted after control ($5.70 \times 10^4$) and LIPUS treatment ($3.52 \times 10^4$) on the scaffolds in conditions of bioreactor ($p < 0.05$) demonstrates results in favour of the null hypothesis regarding the effect of LIPUS treatment on cell proliferation.

Figure 3  Alizarin Red Stain on fixed MC3T3 cells. Images A and C depict the control under 4× and 10× magnification, respectively. Images B and D depict the LIPUS-treated cells under 4× and 10× magnification, respectively. It is difficult to qualify the difference in calcific deposition by the images alone. Scale bar 4× = 5000 μm and 10× = 2000 μm. (For interpretation of the references to color/colour in this figure legend, the reader is referred to the Web version of this article.)

LIPUS = low-intensity pulsed ultrasound.
There must be some limit to cell membrane deformation that the MC3T3 can survive before it starts to become harmful. However, for those that were able to survive, the additional acoustic streaming in the interstitial fluid of the MC3T3 cells should explain the increase in matrix mineralisation as LIPUS increases activation of downstream activators [21]. Furthermore, from previous 2-D osteoblast-like cell studies, ultrasound exposure showed maintenance or increased cell differentiation and enhanced mineralisation [34,35]. Although this 3-D cell culture has shown significance with LIPUS in promoting cell mineralisation, the cell counts was reduced. Perhaps, MC3T3 is most likely comparable with the 2-D cell dish environment but operates at different optimised differentiation duration in the 3-D scaffold environment. Future studies would be needed to explore the duration or time-dependent differentiation in such 3-D scaffold conditions. Further research should also concentrate on defining a set upper limit to the cell membrane deformation that a preosteoblastic cell can survive. Furthermore, the sources of deformation should vary in stimulus form as LIPUS- and bioreactor-induced fluid flow shear stress differs. A limitation of this study was the inability to perform multiple tests on the scaffolds with seeded MC3T3 cells to see if unoptimised LIPUS parameters could be responsible for the decreased cell count. We understand that repeated experimentation could further elucidate how matrix mineralisation could increase in the presence of decreased cell count; however, technical difficulties prevent such repetition.

The primary osteogenic assessment was using cell counts and Alizarin Red. Previous studies from our laboratory on ultrasound-regulated stem cell differentiation and mineralisation have shown that ultrasound has the potential to promote both osteogenesis and mineralisation on human mesenchymal stem cells (hMSC), in which analyses of collagen, alkaline phosphatase (ALP), osterix (OSX), receptor activator of nuclear factor kappa-B ligand (RANKL), runt-related transcription factor 2 (RUNX2), and osteoprotegerin (OPG) have shown significance in cell viability and mineralisation (assessed by Alizarin Red) [18]. While this work was the first attempt to extend the study from 2-D cell culture into 3-D cell motility in the scaffold, the analyses were mainly focused on mineralisation and motility. In the future, we will extend the analyses to include extensive broader assessment.

Conclusion

The objective of this experiment was to study the effects of LIPUS on MC3T3 cells in bovine trabecular bone scaffolds under conditions of dynamic flow bioreactor. It was discovered that LIPUS decreased cell proliferation but significantly increased extracellular matrix mineralisation. Future studies should investigate the role of LIPUS in bioreactor environments to better understand how dynamic fluid flow interacts with LIPUS treatment. From the gathered information, we conclude that LIPUS stimulation was critical in contributing to the mechanical signalling transductions that activated bone enhancement parameters and thus has essential potential to create a change in how we pretreat scaffolds for orthopaedic surgery in the future.

Conflict of Interest

The authors certify that they have no affiliations with or involvement in any organisation/entity with any financial interest or nonfinancial interest in the subject matter or materials discussed in this manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jot.2018.02.002.

Author contributions

SSM and YXQ conceived and designed the experiments. SSM performed the experiments, analysed the data, contributed reagents/materials/analysis tools, and wrote the article. YXQ edited and reviewed the article.

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