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Ultrasound Accelerated Bone Tissue Engineering Monitored with Magnetic Resonance Microscopy

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Abstract— **Tissue engineering has the potential to treat bone loss, but current bone restoration methods, including osteogenesis from mesenchymal stem cells (MSCs), require three to four weeks for bone formation to occur. In this study, we stimulated the formation of engineered bone tissue with low-intensity ultrasound, which has been proven to accelerate bone healing *in vivo*. One group of engineered bone constructs received ultrasound stimulation 20 minutes per day over a 3-week growth period. We monitored the growth of all the engineered constructs quantitatively and noninvasively using magnetic resonance microscopy (MRM), where the T_2 relaxation times of all the constructs were measured, on a weekly basis, using an 11.74 T Bruker spectrometer. Histological and immunocytochemical sections were obtained for all constructs and correlated with the MR results. This study shows that ultrasound can accelerate osteogenesis *in vitro* for tissue engineered bone, the growth and development of which can be monitored using MRM.**

I. INTRODUCTION

MILLIONS of patients experience bone loss as a result of degenerative disease, trauma, or surgery [1]. According to Wolff's "Law of Bone Remodeling", changes occur in the bone architecture allowing restoration of its normal function to meet the mechanical demands imposed on it [2]. However, this capacity is limited when there is insufficient blood supply, mechanical instability, or competition with highly proliferating tissues [3].

The current "gold standard" for specific-site, structural and functional bone defect repair is autologous bone grafts. However, this solution presents certain complications such as donor site morbidity, infection, malformation, and subsequent losss of graft function. Another widely employed technique is transplantation of allograft bone, which presents the risks of potential disease transmission and host rejection, and suffers from limited supply [4].

Implants developed via tissue engineering may be a more viable solution to the problem of bone loss, since biocompatibility will no longer be an issue, and the implants will be more readily available to patients [5].

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One bone tissue engineering strategy currently employed is illustrated in Fig. 1. After cellular expansion of mesenchymal stem cells (MSCs) obtained from the patient, these are seeded on biodegradable and biocompatible scaffolds [6], and supplemented with growth factors that enable them to differentiate into osteoblasts (bone-forming cells) [7]. After a substantial culture period, the scaffold is implanted into the patient, leading to bone restoration [8].

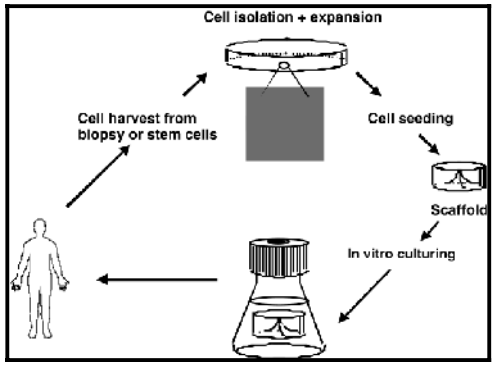


Fig. 1. A current process of bone tissue engineering [6].

Reducing the culture time of stem cells is necessary to increase the effectiveness of the implants. The use of electrical and mechanical stimulation to accelerate stem cell differentiation has been implemented**,** but the optimized usage of such techniques has yet to occur [9]. In this study, we stimulated the growth of tissue engineered bone constructs with ultrasound (US). The reasons behind exploring US stimulation were: (a) US waves are noninvasive, which is very relevant to this study to insure the integrity of the constructs; (b) previous studies showed that low-intensity pulsed US, administered with a dose as short as 20 minutes per day, activated ossification *in vitro* via a direct effect on osteoblasts and ossifying cartilage, after other animal and clinical studies showed that low-intensity US accelerated bone healing *in vivo* [10].

Magnetic resonance imaging is widely used *in vivo* to assess connective tissue degeneration [11]. It has also been effective in studying ectopic bone formation in the rat *in vivo* [12]. MRM has been used to investigate the regeneration of engineered tissue [13]. A recent study showed that MRM can be used to monitor osteogenesis in tissue-engineered constructs [8]. In this work, we study the feasibility of using US stimulation to enhance and hasten osteogenesis in tissue engineered constructs. The periodic monitoring of tissues T_2 relaxation time correlated with

histological and immunocytochemical analyses will be used to assess our results.

II. MATERIALS AND METHODS

A. Specimen Preparation

Mesenchymal stem cells (MSCs) isolated from fresh adult human bone marrow were provided by AllCells (AllCells LLC, Berkeley, CA). Nucleated cells were expanded via incubation for 3 weeks in a basic culture medium composed of Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and 1% antibiotics at 37 ^{o}C. Helistat absorbable collagen scaffolds (Integra LifeSciences Corporation, Plainsboro, NJ, USA) were trimmed into 3 mm x 3 mm x 5 mm pieces for use as the biological scaffold. Tissue engineered constructs were generated by seeding the collagen scaffolds with MSCs at a density of $2x10^6$ cells/ml with a slight vacuum created with a 20 ml syringe. The mixture of scaffolds and cell suspensions were incubated at 37 ^{o}C for 2 hours. Then, the cell-seeded constructs were divided into 3 groups: control (CON) constructs were cultured in basal media (same composition as basic culture medium described above), differentiated, non-stimulated (OB Diff No US) constructs and differentiated, ultrasound stimulated (OB Diff US) constructs were cultured in differentiating media (basal media with 100 mM dexamethasone, 100 mM β-glycerophosphate, and 50 mg/ml ascorbic acid-2-phosphate, which are factors promoting the osteogenic differentiation of human MSCs). The 3 groups were allowed to grow *in vitro* for 3 weeks.

B. Ultrasound Stimulation Treatment

The US treatment was administered in a therapy unit consisting of a sonic accelerated fracture-healing system (SAFHS) device (model 2A; EXOGEN, Memphis, TN, USA) and 6 transducers (with coupling gel), connected to a multiwell plate filled with 6 ml of tissue culture medium, and the engineered constructs, OB Diff US (Fig. 2). The transducers delivered pulsed US waves with an intensity of 30 mW/cm^2, operating frequency of 1.5MHz, pulse width of 200 μsec and pulse repetition rate of 1 kHz. The duration of each treatment was 20 minutes per day through the growth period.

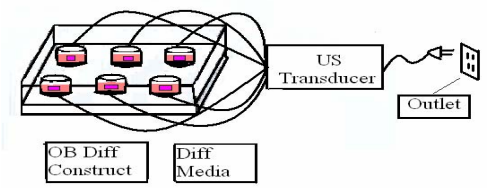


Fig. 2. Ultrasound stimulation setup.

C. Magnetic Resonance Imaging System

MRI experiments were conducted at 11.74 T (500 MHz for protons) using a 56 mm vertical bore magnet (Oxford Instruments, Oxford, UK) and a Bruker DRX Avance Spectrometer (Bruker Instruments, Billerica, MA. USA) controlled by a Silicon Graphics SGI2 workstation (Mountain View, CA, USA). MR images were acquired using a Bruker Micro 5 imaging probe with triple axis gradients (maximum strength 2 T/m), and a 10 mm diameter RF saddle coil was used to transmit/receive the nuclear magnetic resonance signals.

D. MRI and Measurements of MR parameters

OB Diff No US, OB Diff US, and CON constructs (n=2) were studied at 4 growth stages, referred to as weeks 0, 1, 2, and 3. Axial slices were taken along the axis of the test tube and positioned at the center of each specimen. For each sample, the spin-spin relaxation time (T_2) was measured and averaged for specific regions of interest (ROIs) localized at the periphery of each construct. T_2 was measured using a spin echo imaging pulse sequence with 32 echoes (repetition time TR = 4 s, echo time TE = 7 ms, NEX = 1, matrix dimensions = 128 x 128).

The T_2 values were calculated using laboratory built software in MATLAB 7.0 (MathWorks INC., Natick, MA), which performs least square fitting of the experimental MR data for each ROI to calculate the mono-exponential T_2 relaxation time (1).

$$SNR(TE) = SNR_0 e^{-TE/T_2} \quad (1)$$

where $SNR(TE)$ is the SNR value at a specific TE value, and SNR_0 is the initial SNR value.

In addition, pixel by pixel T_2 mapping was produced for all the imaged constructs using a laboratory built MATLAB software, in order to maximize the contrast information throughout the construct. It should be noted that all the data in this study is presented without statistical significance.

E. Histological and Immunocytochemical Analyses

Following MR testing, one set of tissue engineered constructs containing the 3 different experimental groups was washed with phosphate buffered saline (pH 7.4), then fixed in 10% formalin, every week throughout the growth period. All fixed samples were sent to Histoserv, INC. (Germantown, MD. USA), sectioned at 5 μm thickness, and stained for histological and immunocytochemical analyses. Hematoxylin and Eosin (H&E) staining was performed to detect cell nuclei in purple, over the collagen matrix, in pink. This would prove the increase of cell proliferation with time. Also, von Kossa staining was performed to examine the mineralization during osteogenic differentiation due to calcium deposition. The tissue sections were treated with a sliver nitrate solution and the silver was deposited by replacing the calcium [8]. Furthermore, staining for osteocalcin (OCN), a bone matrix protein, was performed to ascertain the presence of bone tissue in the differentiated constructs. In this stain, the copper regions are a mark of OCN presence.

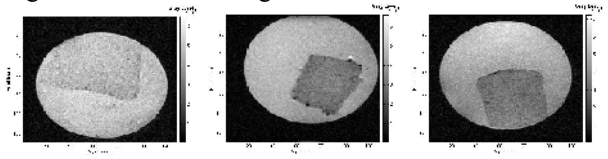
III. RESULTS

A. *Dependence of MR Parameters on Engineered Bone Tissue Formation and Comparison of US Stimulated Constructs with Non-Stimulated Ones*

T_2-weighted MR magnitude axial images of US stimulated osteogenic constructs at weeks 0, 1, and 2 are shown in Fig. 3. Spin-echo imaging pulse sequence was used with TR = 4000 ms, TE = 140 ms, slice thickness = 0.5 mm, and matrix dimensions = 128 x 128. The intensity of the MR images for the osteogenic constructs decreased with tissue development.

Fig. 4 compares the variation of T_2 relaxation time calculated weekly, using mono-exponential fitting, for peripheral ROIs in the constructs, for each of the 3 different experimental groups, over the 3-week developmental period. The T_2 relaxation time decreases with time for the osteogenic constructs (OB Diff No US and OB Diff US), whereas that for the control constructs (CON) does not show any similar decrease. Starting with initial values of 96.6 ms and 97.8 ms at week 0, the T_2 relaxation time reaches a value as low as 56.0 ms for the US stimulated osteogenic constructs, whereas it only reaches values of 72.1 ms and 74.1 ms for the non-stimulated osteogenic constructs; the lowest T_2 values calculated for the control group are 80.2 ms and 83.1 ms.

Fig. 5 displays pixel by pixel T_2 relaxation time maps of the same samples shown in Fig. 3. The color bar to the right of each map shows the T_2 values in ms that each color represents. The variation of the dominant color from red, yellow, to green-blue, in the region of the map occupied by the construct, demonstrates a decrease in the computed T_2 values when going from week 0 to week 2. This effect is actually present for both osteogenic construct groups, as shown in Fig. 4. It can be noticed by examining those maps that they contain better contrast information than what is displayed in the T_2-weighted MR magnitude images shown in Fig. 3, and therefore, provide better tissue characterization throughout the construct region.



(a) (b) (c)

Fig. 3. T_2-weighted MR images of OB Diff US constructs acquired for weeks 0 (a), week 1 (b), and week 2 (c).

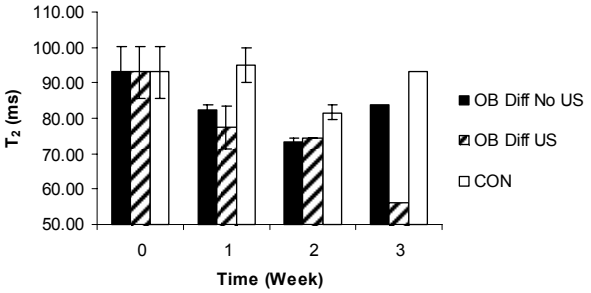
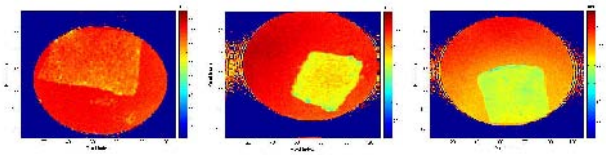


Fig. 4. Graph of the variation of the average T_2 values for peripheral ROIs selected in all 3 of the construct groups over the 3-week incubation period.



(a) (b) (c)

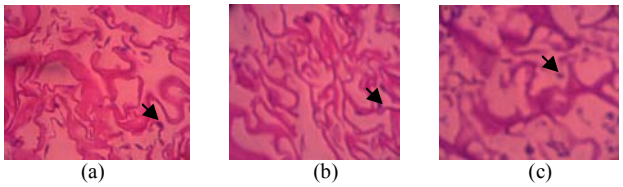
Fig. 5. Pixel by pixel mono-exponential T_2 maps for the same constructs shown in Fig. 3: (a) Week 0, (b) Week 1, and (c) Week 2.

B. *Correlation of Histological and Immunocytochemical Results with T_2 Relaxation Time Measurements*

Fig. 6 shows the H&E staining results at week 2 for the 3 construct groups. In all constructs, collagen is stained in pink, and cell nuclei in purple. A black arrow points to a nuclei. The US stimulated osteogenic construct has more cells than the non-stimulated osteogenic construct at week 2, indicating greater cell proliferation in the construct receiving ultrasound treatment.

Fig. 7 shows the von Kossa staining results at week 2 for the 3 construct groups. Black nodules indicate calcium deposition, which increases with osteoblast formation; the black arrow points to one nodule. Black nodules are absent from the control constructs, but apparent in the osteogenic ones (US stimulated and non-stimulated) at week 2, indicating calcium deposition in these only. The US stimulated construct, as shown, has more black nodules than the non-stimulated one, indicating greater calcium deposition in the construct receiving US treatment.

Fig. 8 shows the results of staining for OCN at week 2 for the 3 construct groups. Copper colored regions indicate the presence of OCN. One of the copper colored regions is indicated by the black arrow. Copper colored regions are absent from the control constructs, but apparent in both osteogenic constructs, indicating the presence of OCN in these only. The US stimulated construct has more copper colored regions than the non-stimulated osteogenic one, indicating greater presence of osteocalcin in the construct receiving US treatment.

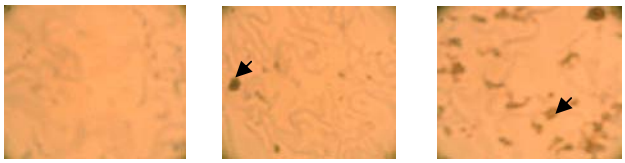


(a) (b) (c)

Fig. 6. H&E staining results at week 2 for CON (a), OB Diff No US (b), and OB Diff US (c) constructs. Image magnification is 20x.

(a) (b) (c)

Fig. 7. von Kossa staining results at week 2 for CON (a), OB Diff No US (b), and OB Diff US (c) constructs. Image magnification is 20x.



(a) (b) (c)

Fig. 8. OCN staining results at week 2 for CON (a), OB Diff No US (b), and OB Diff US (c) constructs. Image magnification is 20x.

Both von Kossa and OCN stains show further mineralization and bone formation in the US stimulated osteogenic constructs than in the non-stimulated ones, while having a faster decrease in T_2 values measured for the US stimulated constructs. Therefore, there is good correlation between the histological/ immunocytochemical and the MR results, implying that the faster decrease in T_2 values over the growth period can be directly correlated to the faster increase in stiffness, due to improved mineral deposition.

IV. DISCUSSION

Both the quantitative MR and the qualitative histological/ immunocytochemical results show that low-intensity pulsed US has the potential to accelerate mineralization of tissue engineered osteogenic constructs *in vitro*. In addition, because of the good correlation between the results, this study confirms the effectiveness of the T_2 measurement protocol for characterizing the growth and development of engineered osteogenic tissue, as previously shown in [8], where the T_2 relaxation time decreased from 67.7 ± 6.1 ms at week 0 to 24.2 ± 8.9 ms at week 4 upon mineralization. Note that the absolute, quantitative values of T_2 depend on the initial cell seeding density of the engineered constructs.

This work is unique in two aspects: First, it shows that MRM is sensitive enough to characterize the acceleration of osteogenic constructs growth, by sensing the difference in T_2 values between the US stimulated and non-stimulated constructs. Second, it shows that US is effective in accelerating osteogenesis *in vitro*. The decrease in the T_2 values over time for the osteogenic constructs can be explained by the introduced magnetic susceptibility upon mineral deposition [8], and translated into an increase in stiffness of the tissue.

Furthermore, a decrease in the size of the osteogenic constructs over the incubation period was observed, and was more evident for the US stimulated constructs than the non-stimulated ones. This may be due to normal growth consolidation or non-uniform cell seeding in the collagen scaffold [8]. However, the degradation of collagen scaffolds by osteoclasts along with the formation of new bone matrix by osteoblasts has been proven in a recent study [14]. Therefore, the MSCs possibly differentiating into both of these bone cell types allow degradation of the collagen scaffold matrix upon mineralization. This fact is most likely the major reason for the decrease in size observed in the osteogenic constructs, and offers an advantage for bone remodeling using tissue engineered constructs, where biodegradability of the scaffold material is very important [14]. In addition, we noticed a better decrease in T_2 values over time in the peripheral than in the central parts of the constructs, which means that more mineralization must have occurred at the periphery of the constructs where the highest cell density probably resided. This problem may be overcome by improving the cell seeding procedure, which we suggest be done in future studies. Also, statistical validity was not achieved in this study, and all experiments need to be repeated with a higher number of samples in order to statistically confirm the effect of US stimulation on the ossification of engineered constructs *in vitro*.

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