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4aBA6. Elucidating the effects of low-intensity ultrasound on mesenchymal stem cell proliferation and viability  
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The effects of Low-Intensity Ultrasound (LIUS) on the proliferation, viability and extracellular matrix (ECM) production of Mesenchymal Stem Cells (MSCs) were investigated. Continuous-wave ultrasound was applied at 1 MHz and 350 mW/cm² to microwells, using a LIUS system assembled in the laboratory. Needle hydrophone mapping showed that pressure amplitudes ranged from 0.015 MPa at the well edge to 0.080 MPa at the centre. The LIUS group received US for 10, 20 and 30 min per day for one week. Assays were performed daily. Relative to control, 10 and 20 min LIUS very significantly stimulated MSC proliferation and ECM synthesis, while 30 min LIUS had a significant adverse effect. The phenomenon that LIUS accelerates MSC proliferation, but only for appropriate exposures, has been noted previously in the literature. However, the actual relationship between the physical forces generated by the LIUS and this phenomenon remains unknown. The fluid flow pattern created by LIUS was studied by injecting dye in the well and Eckart-streaming-like motions were observed, while thermal effects were negligible. By employing LIUS with appropriate focusing and parameters, it might be possible to exploit MSCs for tissue engineering, independently of biochemical stimuli, and in a highly spatially organised manner.  

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INTRODUCTION

Low Intensity Ultrasound has been defined as ultrasound with frequencies greater than the upper limit of human hearing (20 KHz) and intensities in the range from 30 mW/cm² to as high as 3 W/cm² 1-3, such that it does not have thermal effects 4. It has been extensively used in tissue repair processes for stimulating the proliferation of both fibroblasts (connective-tissue cells) and osteoblasts (bone cells) 5,6. LIUS has also been used for dental tissue regeneration and for tooth-root healing 7. LIUS also promotes the growth of human skin fibroblasts by activating internal biological signaling cascades 8. Furthermore, LIUS increases the proliferation 9,11 and differentiation 11 of stem cells. As detailed below, MSCs are used in tissue engineering, in which replacement human organs or tissues are grown. MSCs have the ability for self-renewal, i.e., undergoing cell division whereby their population is maintained or increased (proliferation). A particularly important feature of stem cells, including MSCs, is their ability to differentiate: to change into specialized cell types 12, which some tissue engineering processes seek to exploit. The variety of LIUS effects reported in the literature suggests the application of LIUS to stem cells stimulates proliferation and differentiation that may be useful for a broad range of tissue engineering applications.

MSCs are able to differentiate into a variety of cell types including vascular cells, neurons, osteoblasts, adipocytes (fat cells), and chondrocytes (cartilage cells). These stem cells can be isolated either from the fetus or from adult tissues such as bone-marrow. The major obstacles associated with human embryonic stem cells are the ethical issues involved and difficulties regarding immunogenicity and tumourigenicity 13,14. The stem cells derived from adult tissue such as bone-marrow requires a painful aspirating procedure, and also have a limited proliferating capacity 15. The limited proliferation capacity of the adult stem cells and the ethical issues of embryonic stem cells can be overcome by isolating the stem cells from certain tissues associated with fetal development, such as amniotic membrane, amniotic fluid, placenta, umbilical cord blood and Wharton’s jelly. The above-mentioned fetal stem cell sources have generally been discarded following birth, but several studies suggest that the stem cells have higher proliferation and differentiation capacity into multiple cell lineages either in vivo or in vitro 16,17. Owing to these advantages, along with their non-tumorigenic and low immunogenic properties, these stem cell sources could be a better source of MSCs compared with other adult tissue sources 18.

Previous studies have explained that both chemical and physical stimulations can change the behaviour of MSCs and lead to their differentiation into a specific cell lineage 19,20,21. Chemical stimuli generally include cell-specific growth factors and cytokines. They are commonly used to induce cell differentiation, ECM synthesis and the maintenance of cell morphology and functions. Their use in tissue engineering is restricted because of their side effects on the host cells. Mechanical stimulation includes different types of stress, such as tensile stress, compressive stress or shear. Mechanical stimuli also induce cell differentiation, by activating the specific cellular molecules involved in the differentiation pathway. Specific and well-defined mechanical stimuli (compressive, tensile or shear stress), can be conveniently applied to cell-culture plates and wells in vitro 22-23, but in vivo it is more problematic to arrange the same mechanical stimuli that cause differentiation. Therefore, an alternative mechanical stimulation that could operate inside the body would have great potential, provided it is understood.

Despite a substantial body of studies in which LIUS was demonstrated to affect stem cells, the actual mechanism by which LIUS affects stem cell proliferation and differentiation is not fully understood. Furthermore, the effects of LIUS stimulation on Amniotic Membrane (AM) derived MSCs, one of the most appropriate stem cell types, has not been explored yet.

Most research involving LIUS has been done using commercially built LIUS systems that have very limited parameter options, mostly 1 MHz frequency, 20% duty cycle, 30 mW/cm² intensity, and a duration of 20 min 26-28. Furthermore, the actual parameters used are not consistently reported in the literature. Few studies have been done to test the effects of US on stem cell response by independently varying the frequency, exposure time, intensity or duty cycle 29,30. This means that there is no agreed optimum set of LIUS parameters for stem cell proliferation and differentiation. Neither has a study been done to examine the effects of LIUS system on the proliferation and ECM production of AM-MSCs.

In the present study, a custom LIUS system was assembled that gives control over the LIUS parameters. We report a preliminary experiment in which the US exposure time was varied, to check the effects of LIUS on AM-MSC proliferation and viability, and also the synthesis of ECM material.

MATERIALS AND METHODS
Isolation of AM-MSCs

AM-MSCs were isolated from amniotic membranes obtained following written informed consent obtained from women delivered at term by caesarean section at Royal Women’s Hospital. The cells were isolated using a standard protocol. Cells from four individual donors were pooled. Cells were cultured in a tissue culture flask in an incubator at 37°C in a humidified atmosphere under 5% CO₂ in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS). A population density of 1 x 10⁵ cells/cm² was reached. Once the culture was approximately 50% confluent, the medium was changed every other day and then, once the culture reached 80% confluence, the medium was changed every day. When the cells reached 90% confluence, they were trypsinized and subcultured to reach a passage number 2.

Preparation of AM-MSCs Culture for Ultrasound Procedure

Cells seeded at a density of 50,000 cells/well were cultured in Dulbecco’s Modified Eagle medium (DMEM). The culture medium was supplemented with 10% FBS, 500 µg/ml fungizone, 500 µg/ml antibiotic/antimitotic mixture and 50 µg/ml vitamin C. A total of 40 wells were prepared using this cell density to carry out the experiment. The wells were non-coated tissue culture six-well plates (Becton, Dickinson and Company, Cat No. 353046) with each well 35 mm in diameter and filled to a depth of 10 mm, giving a volume of 5 ml. The cells were maintained in the incubator in a humidified atmosphere under 5% CO₂ at 37°C. To replenish the nutrients needed by the cells, the culture medium was changed every two to three days.

Measurements of the Pressure Amplitude

The LIUS system (figure 1) consists of a function generator (Tektronix AFG 3021B), a transducer (Olympus C-302), a RF power amplifier (ENI model 550L), and an oscilloscope. The transducer was 25.4 mm in diameter and thus had a slightly smaller diameter than the well. The function generator used is capable of creating an US pulse with any desired waveform (sine, square, pulse, arbitary) and is capable of changing the pulse repetition frequency (PRF) and duty cycle. Applied power can also be varied using a RF amplifier.

The pressure amplitude was measured throughout the microwell volume of 5 ml at 1 MHz frequency using a needle hydrophone (Model HPM1/1, Precision Acoustics, Dorchester, UK). The needle hydrophone has sensitivity of 1681.1 mV/MPa and the signal-to-noise ratio was limited by the accuracy of the digital oscilloscope (±0.5mV or ±0.3kPa), which was sufficient for this application. The function generator was capable of generating 5 V pk-pk pulse signals at 1 MHz with a duty cycle of 97%. The rise and fall time of the pulse was 18 ns. To boost the excitation signal to the ultrasonic transducer, the RF amplifier which has a gain of 50 dB, was inserted between the function generator and the ultrasonic transducer, as shown in figure 1. The amplifier required an input signal with peak amplitude of 1V and a frequency greater than 500 kHz. For equipment protection, a 6 dB attenuator was placed in series on the output of the amplifier. This system provided a total amplitude gain of 44 dB or 158.5 times. The resulting pressure signal as measured was effectively Continuous Wave (CW).

**FIGURE 1.** Ultrasound system, comprising function generator, RF amplifier, transducer and the needle hydrophone and oscilloscope.
The pressure measurements were carried out in 5 ml of water placed in one well using the above-noted set up. It was measured throughout the well from the centre to the edge, defining the pressure field experienced by cells at different points in the well.

**Low Intensity Ultrasound Stimulation of AM-MSCs**

The wells containing the cultured AM-MSCs were placed on the ultrasonic transducer that had been coated with an ultrasonic coupling gel (PUSG-Sportek Othersonic); thus there was no coupling water-bath or sac. One of the 40 wells was then stimulated at 1 MHz and 350 mW/cm² CW intensity for identical time periods (10 min/day, 20 min/day and 30 min/day) for six consecutive days. Untreated cells in the control sets were also placed onto the ultrasonic transducers each day, without applying the ultrasound. The applied conditions, including the frequency, pressure amplitude, overall power and the exposure time, were designed to fall within the ranges that had been reported, albeit inconsistently, in the previous literature \(^{26,27,22}\). The literature had suggested no adverse effects on the stem cell proliferation and viability\(^5\).

Checks on the same well-plates, liquid and ultrasound parameters confirmed that there was no measurable heating within +/-0.5 degrees. Moreover, the experimental procedure involved removing all well-plates, including the controls, from the incubator, thus cooling the cells from 37 °C to room temperature, before the ultrasound was applied. All well-plates were then returned to the 5% CO2 incubator, which entailed reheating them to 37 °C, a temperature excursion much greater than any that might have been produced by the ultrasound.

The experimental sets, with 10 wells in each set were,

i) Set I: control with no LIUS treatment  
ii) Set II: US-1 with 10 min LIUS treatment;  
iii) Set III: US-2 with 20 min LIUS treatment;  
iv) Set IV: US-3 with 30 min LIUS treatment;

The treated and control AM-MSCs from each set were removed every day for cell counting and cell proliferation assays. The spent DMEM medium was collected in a 15 ml Falcon tube for the measurement of ECM components.

**Analysis of Cell Viability and Cell Proliferation**

After each ultrasonic treatment, the cultured AM-MSCs were removed from the incubator and then detached with trypsin and stained with trypan blue stain to count the number of viable and non-viable cells. The cellular proliferation was measured on days 1-6 and assays using the trypan blue were performed in triplicates.

**Quantitative Analysis of Extracellular Matrix Components (ECM)**

The ECM components collagen, elastin, and glycosaminoglycans (GAGs), were then analysed quantitatively using the protocols detailed in the assay kits (Biocolor, S1000, F1000 and B1000). Total collagen in each sample was measured by using a Sircol assay and the absorbance of dye-sample complex was read at 555 nm. The elastin in the sample was measured using a Fatin elastin assay, which is a quantitative dye binding method. The absorbance was read at 513 nm. The Blyscan assay was used for the analysis of sulphated proteoglycans and glycosaminoglycans using 1,9-dimethyl-methylene blue dye in an inorganic buffer. The absorbance of the dye-sample complex was read at 656 nm. These assays were performed every other day for 6 days.

**Fluid Flow Pattern Studies**

The fluid flow pattern created by LIUS was studied by injecting red coloured food dye (Pillar-box red food colour, QRE 50, Queen Fine Food Pty Ltd. Australia) in the well filled to the same volume as in the experiment, and applying the same ultrasound parameters used in the experiments for 10, 20 and 30 min. The fluid flow pattern was also studied in the control set without ultrasound, to assess the extent of natural diffusion.

**RESULTS**
Pressure Amplitude Measurement

Pressure amplitude was found to vary across the well with maximum pressure $0.075 \pm 0.005$ MPa in the centre of the well. This gradually decreased towards the edge with minimum $0.015 \pm 0.005$ MPa, as shown in figures 2(a) and 2(b) respectively. The variation in the pressure amplitude implies that the cells in different regions of the well experienced different pressure amplitudes. If, in the future, it appeared that pressure amplitude affected the final cell type after stem cell differentiation, it might be possible to differentiate different cell lines in a single well or in vivo by applying an appropriate ultrasonic field shape. In the literature, the pressure amplitude applied to stem cells was in the range of 0.03 MPa to 0.065 MPa.

![Pressure amplitude measurement](image)

**FIGURE 2.** Pressure amplitude measured at the centre of the well in 5 ml of water. (a) Pressure amplitude in the centre; (b) pressure amplitude at the edge.

Cell Viability and Cell Proliferation

Most significantly, the proliferation of AM-MSCs exposed for 10 min and 20 min LIUS was found to be higher than that of the control cells (figure 3). Meanwhile, the proliferation of cells exposed for 30 min LIUS was lower than the control as shown in figure 3. Cell viability was also measured from the 2nd day of culture and was highest in the 20-min LIUS-stimulated cells than any of the other sets.

![Cell proliferation analysis](image)

**FIGURE 3.** Analysis of stem cell proliferation. The yield of MSCs and their viability increased from day 2 in the 10 and 20 min LIUS stimulated cells, significantly more than the control sets, while adverse effects were observed in the 30 min LIUS stimulated cells. Therefore, 20 min of LIUS stimulation was the optimum dose at these LIUS parameters. Bars shown are 95% confidence limits based on the Student’s t-test.

The data represents the means ± standard error. The paired Student’s t-test was used to compare the data from four different groups of LIUS treatment sets (control, US-10, US-20 and US-30 min). A p value <0.05 (95%
confidence) was considered statistically significant and the only error bars and bounds shown in the present paper were for 95% confidence.

Quantitative Analysis of ECM Components

The extracellular matrix results (Table 1) were consistent with the proliferation findings. The most ECM components were produced by the 20 min exposure group. Again, a significant reduction relative to control was observed in cells exposed for 30 min.

<table>
<thead>
<tr>
<th></th>
<th>Quantity (µg/ml)</th>
<th>Day 2</th>
<th>Day 4</th>
<th>Day 6</th>
</tr>
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<tr>
<td></td>
<td>Collagen</td>
<td>Control</td>
<td>72.2 ± 2.1</td>
<td>123.1 ± 1.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>US-1</td>
<td>102 ± 1.9</td>
<td>142.2 ± 2.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>US-2</td>
<td>133.4 ± 2.6</td>
<td>163 ± 1.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>US-3</td>
<td>44.2 ± 1.6</td>
<td>52.5 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>GAGs</td>
<td>Control</td>
<td>27.3 ± 0.8</td>
<td>48.1 ± 2.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>US-1</td>
<td>31.1 ± 1.2</td>
<td>53.4 ± 1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>US-2</td>
<td>42.4 ± 1.8</td>
<td>76.5 ± 2.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>US-3</td>
<td>20 ± 1.5</td>
<td>33.1 ± 1.8</td>
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<td>Elastin</td>
<td>Control</td>
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<td>6.2 ± 0.4</td>
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<td>3.6 ± 0.3</td>
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<td></td>
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<td>US-2</td>
<td>11.3 ± 0.2</td>
<td>19.1 ± 0.3</td>
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<td></td>
<td></td>
<td>US-3</td>
<td>1.2 ± 0.3</td>
<td>5.3 ± 0.3</td>
</tr>
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</table>

TABLE 1. Analysis of ECM production. Bounds shown are 95% confidence limits based on Student’s t-test.

Fluid Flow Pattern Studies

A preliminary study of fluid flow pattern was made by injecting the dye in both control and LIUS-stimulated wells. Eckart-streaming-like motions were observed, as shown in figure 5. Water was used in both control and LIUS tests and the diffusion in the control showed that thermal or other diffusive effects were negligible throughout the stimulation. Dye initially at the edge of the well at the bottom was drawn towards the centre, from where it was lifted to the surface and distributed over the whole well. There was also some swirl motion in the plane of the well bottom. Thus, the acoustic streaming fluid flow created by LIUS in these wells appears to take the form of a weak broad jet directed upwards at the centre of the well, creating a toroidal vortex within the well, modified by swirl. More research has to be done to determine the significance of the stress being applied to stem cells by such streaming motions.

FIGURE 5. Eckart-streaming-like motions owing to LIUS applied to the well.

CONCLUSION
A customized LIUS system with appropriate pressure and power measurements to study stem cell proliferation and viability was assembled. It was found that LIUS stimulation increased the yield and viability of amniotic membrane derived MSCs. This is consistent with findings in the literature on the effects of LIUS on MSCs of other sources. Intra-well pressure measurements indicate that it the cells would experience significantly different pressures at different points in the well. LIUS stimulation for 10 min and 20 min increased the proliferation of MSCs to a significantly greater extent than the control, while 30 min LIUS stimulation has adverse effects on the stem cell fate. Furthermore, the concentration of ECM components produced by MSCs was much higher in 10 min and 20 min LIUS stimulated cells than the control. In future studies, it would be worth studying the differentiation of AM-MSCs using the same LIUS parameters. LIUS as a mechanical stimulation promises many useful clinical applications, especially the formation of tissue-engineered vascular grafts, cartilage and bone repair, using the enhanced MSCs yield both in vivo and in vitro. Amongst future work should be a determination if the mechanism of the results reported is the acoustic streaming observed, or a direct effect of the ultrasound.

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