Low Intensity Pulsed Ultrasound for bone regeneration therapy: a controlled *in vitro* study method

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1. Low Intensity Pulsed Ultrasound

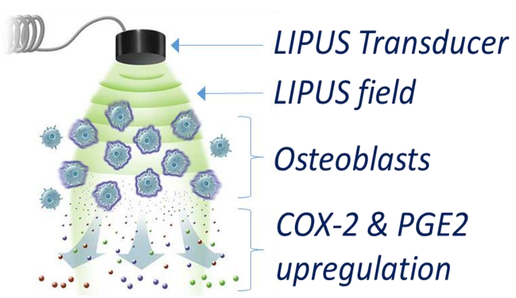
Clinical trials have shown a daily, 20-minute treatment of Low Intensity Pulsed Ultrasound (LIPUS) improves healing rates of non-healing fractures by up to 38%[1].

LIPUS field characteristics:

- Intensity 30 mWcm^{-2} (I_{SATA})
- Frequency 1.0 - 1.5 MHz
- Pulse width (PW) 200 µs
- Repetition rate (PRR) 1 kHz

In vitro trials suggest healing effects are due to mechanical stimuli[2] (Fig.1)

But in Tang's study and many others, the acoustic field applied to the cells was not fully characterised, and was affected by the ultrasound exposure set-up (Fig.2)



Tang et al[3] linked stimulation of mechano-receptors in osteoblasts to upregulation of Cyclo-Oxygenese-2 (COX-2) and Prostaglandin-E2 (PGE2), both vital for bone regrowth.

Fig.1 LIPUS->COX-2->PGE2 Pathway[4]

This study proposes a robust and repeatable *in vitro* ultrasound exposure method for investigation of the mechanisms involved in LIPUS stimulation.

2. Ultrasound exposure

Many *in vitro* LIPUS exposure set-ups (e.g. Fig.2) are affected by:

- Transmission between wells of multi-well plates
- Standing waves between transducer and bottom of plate
- Self-heating effects (when transducer not fully immersed)

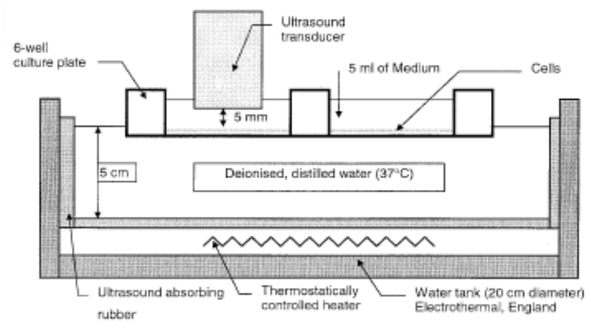


Fig.2 Typical ultrasound exposure set-up (Reher et al [5])

The proposed set-up (Fig.3) resolves the issues by:

- Cells grown in acoustically transparent 'biocell' (Fig.7) to avoid standing wave and transmission effects.
- Ultrasound passes through unchanged. Acoustic absorber (Precision Acoustics, UK) eliminates reflections.
- Transducer immersed: no self-heating.

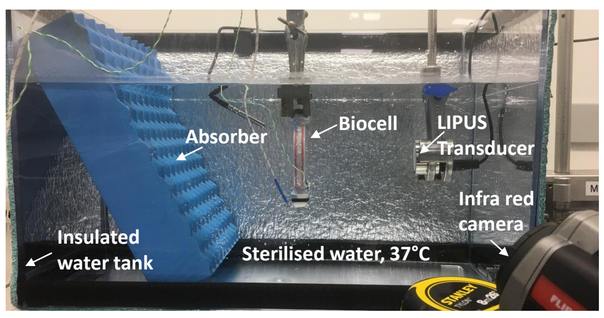


Fig.3 Proposed ultrasound exposure set-up

3. LIPUS dose

LIPUS 'dose' is most often defined by the spatial-average, temporal-average intensity (I_{SATA}), across the entire beam (defined in BS EN 61689:2013[6]).

New definitions of LIPUS dose are proposed, based on peak negative pressure (p_-), which is the accepted measure of mechanical effects [7]. :

$\hat{p}_-$ Maximum p_- across the area of exposed cells

$p_{-,SA}$ Spatial-averaged p_-, averaged across the area of exposed cells.

The acoustic field of a custom-built LIPUS transducer (Fig.4) was measured at the focal point (100mm) at $\hat{p}_-$ up to 500kPa (Fig.5). The $p_{-,SA}$ and an estimate of I_{SATA} were derived (Fig.6).

Fig.4 Custom-built LIPUS transducer

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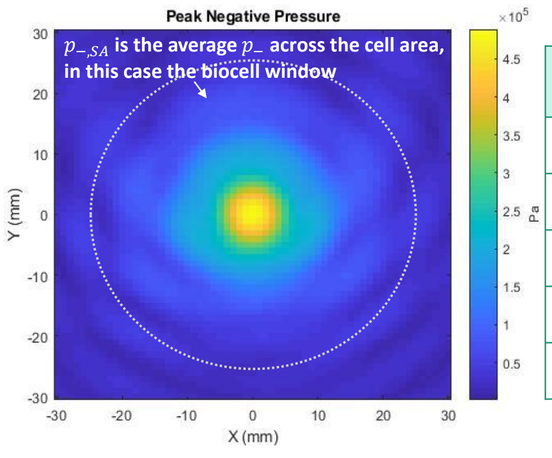

Fig.5 p_- and $p_{-,SA}$ of the LIPUS transducer

$\hat{p}_-$ (kPa)	$p_{-,SA}$ (kPa)	I_{SATA} (mWcm^{-2})
50	10	4.5
100	21	18
200	42	75
350	71	216
500	101	475

Fig.6 LIPUS Transducer $\hat{p}_-$, $p_{-,SA}$ and I_{SATA}

4. The biocell

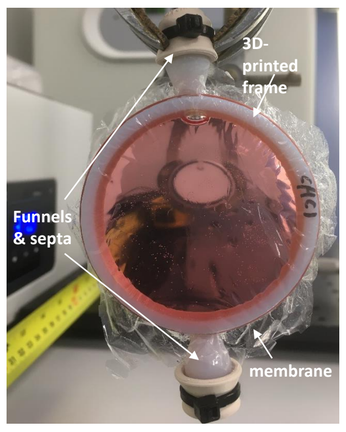

Fig.7 The biocell

- 6µm mylar membranes (Goodfellow, UK) stretched across a 3D-printed frame (VeroGray™) –Fig.7.
- Funnels on either side allow injection of cells and growth media via self-sealing septa (Suba-Seal®, Merck, UK).
- Easy and economical to produce: use once or sterilise and re-use.
- Easily adapted for different beamwidths – simply change the radius of the circular window (Fig.8).
- Testing indicates no significant difference in acoustic field with and without the biocell (Fig.9).

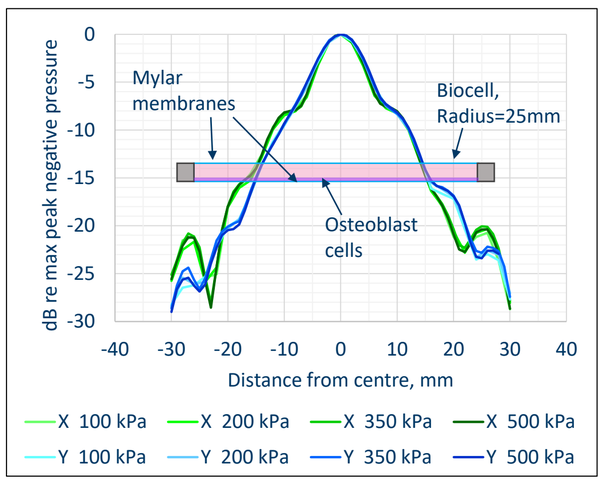

Fig.8 Beam plots in horizontal (X) and vertical (Y) with biocell

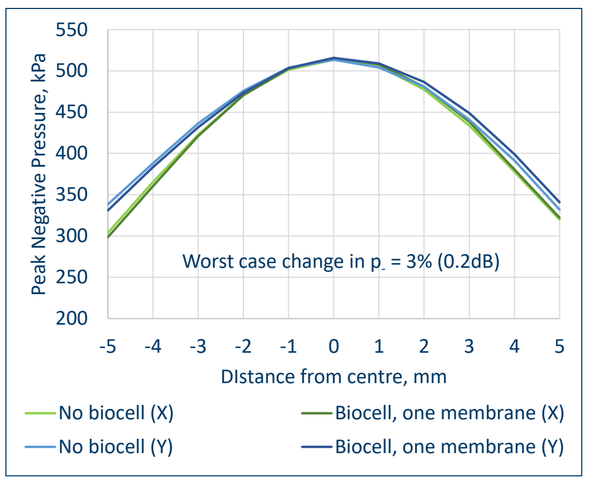

Fig.9 p_- with and without biocell (one membrane)

5. Pilot study

A pilot study was conducted to test the ultrasound exposure method. The protocol was as follows:

- Six biocells seeded with MC3T3-E1 osteoblasts and incubated overnight.
- Each biocell filled with new growth media, mounted in the heated test tank (Fig.3) and exposed to 20 minutes of LIPUS (200µs burst at 1MHz, PRR = 1kHz and range of $\hat{p}_-$ as in Fig.6. Controls treated the same but with no output (0kPa).
- After 20 hours incubation, PGE2 concentration in the growth media was measured with an Enzyme-Linked Immunosorbent Assay (Abcam ab133021).

Results (Fig.10) show $\hat{p}_-$ = 100kPa produces significant upregulation of PGE2. This $\hat{p}_-$ corresponds to 18 mWcm^{-2} I_{SATA} - close to the LIPUS intensity (30 mWcm^{-2}). Future trials will focus on this range.

Cell count estimates before exposure (Fig.11) exhibit high standard deviation (21%). This is thought to be due to inconsistent cell adhesion.

Comparing before and after cell counts (Fig.11), higher PGE2 levels seem to correlate to increased cell proliferation.

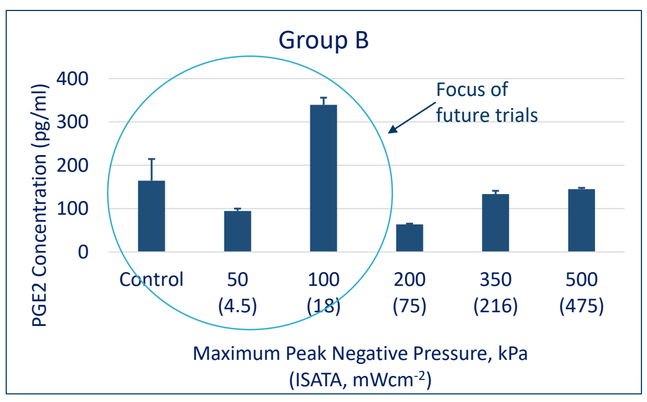

Fig.10 PGE2 Concentrations for all $\hat{p}_-$(and I_{SATA}). Error bars show sample standard deviation in mean ELISA result

$\hat{p}_-$ (kPa)	#Cells before	#Cells after
0	454,000	420,000
50	401,000	240,000
100	469,000	1,700,000
200	630,000	340,000
350	475,000	540,000
500	336,000	60,000

Fig.11 Cell counts before and after Exposure. 'Before' estimated from microscope pictures. 'After' counted on haemocytometer

Further work will be conducted to improve consistency of cell adhesion through membrane pre-treatments such as collagen. Future trials may focus on measuring cell proliferation rather than PGE2. Once the method is fully developed it will be used to test optimum LIPUS fields in terms of p_- , PW, PRR and frequency.

6. References

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3. Tang, C.-H., et al., *Ultrasound Stimulates Cyclooxygenase-2 Expression and Increases Bone Formation through Integrin, Focal Adhesion Kinase, Phosphatidylinositol 3-Kinase, and Akt Pathway in Osteoblasts.* Molecular Pharmacology, 2006. **69**(6): p. 2047-2057.
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6. British Standards Institute, *BS EN 61689:2013 Ultrasonics - Physiotherapy systems - Field specifications and methods of measurement in the frequency range 0,5 MHz to 5 MHz*. 2013, BSI Standards: UK.
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