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Effect of therapeutic ultrasound on osteoblast gene expression

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1. Introduction

Therapeutic ultrasound (US) is a common form of treatment the repair of damaged tissues. This non-invasive application of acoustic energy at low megahertz frequencies (up to 3000 mW / cm²) has been utilized to treat over one million patients each year in the UK, constituting 20% of all treatments within hospital physiotherapy departments [1]

Beneficial responses to the application of US have been demonstrated in several soft tissue animal models, with improved healing rates [2] and tissue strength [3]. Moreover, clinical trials have also reported beneficial results at soft tissue wound sites such as venous ulcers [4] and the elbow joint [5]. In addition, US treatment of hard tissue injuries, such as bone fractures, have proved highly successful with markedly improved healing rates of human fractures (approximately 30-40%) [6,7]. Animal studies have also provided evidence of enhanced fracture healing, particularly at the lower range of clinical intensity values [8,9].

The potential for applying Therapeutic US to improve the clinical efficiency of implant materials has been demonstrated with improved rates of bone regeneration and bioabsorption of US-treated DP-Bioglass implants [10] and also improved rates and extent of bone ingrowth using US-treated porous-coated impants [11].

The studies noted above and widespread clinical experience strongly suggest that there is a clear potential of the use of US as a non-invasive method to enhance tissue repair and regeneration processes. However, the mechanisms involved in initiating favourable cellular responses from ultrasonic stimulation are poorly understood, and little is known about the optimum acoustical conditions which elicit such beneficial responses. The aim of this study was therefore to determine the relationship between intensity levels of applied US and activity of two bone-associated genes, alkaline

phosphatase (ALP) and osteopontin (OP), which play fundamental roles in the growth and function of bone.

2. Materials and methods

The osteoblast-like cell line MG63 was seeded onto 10 cm² slide chamber flasks (Nunc; Naperville; IL; USA) and grown to confluence in medium consisting of Dulbecco's Minimum Essential Medium (DMEM) (Gibco Life Technologies Ltd; Paisley, UK), 10% heat-inactivated fetal calf serum (FCS) (PAA Laboratories; Linz, Austria), 2 mM L-glutamine (Gibco), 100 U/ml of penicillin (Gibco) and 100 mg/ml of streptomycin (Gibco) at 37°C in a humidified atmosphere of 5% CO₂ in air. The flasks were filled with medium and then exposed 3.0 MHz ultrasound for 10 min, at intensity levels of 120, 390 and 1490 mW / cm². Control cultures received no exposure to US. Exposure was performed in a water bath exposure assembly, as shown in Fig. 1. Cells from within a 16 mm central circular target region of the flask were then detached by treatment with 0.25% Trypsin / 0.02% EDTA (Gibco) 6 h after exposure and used in the experiments. A 1.0 mm diameter PVdF needle hydrophone, housed within a purpose built plotting assembly, was used to calibrate the ultrasonic field from a 25 mm diameter ultrasonic probe within this target area.

In order to characterise the osteoblast gene expression, we used the reverse-transcription polymerase chain reaction (RT-PCR), a sensitive and versatile technique used to determine the presence or absence of a gene transcript and to estimate its expression levels. RT-PCR is a relatively simple technique by which a complementary DNA (cDNA) template is amplified many times quickly and reliably. The PCR process generates sufficient material for subsequent experimental analyses. The entire amplification can be performed *in vitro*. While most biochemical analyses, including nucleic acid detection with radioisotopes, require the input of significant amounts of biological material, the RT-PCR process requires very little.

Cells were harvested by trypsinisation and subsequent centrifugation. RNA was isolated from cells within the slide chamber target region by the single step method of Chomczynski and Sacchi [12]. Briefly, it was important to achieve this rapidly and efficiently to inhibit the endogenous ribonucleases, which are present in virtually all living cells. High-quality RNA greatly increases the efficiency of RT-PCR. The isolated RNA was then reverse transcribed into first strand complementary DNA (cDNA), and then amplified using oligonucleotide primers specific for the bone-associated proteins ALP and OP, using a previously published protocol [13]. Glyceraldehyde-3-phosphate hydrogenase (GAPDH), a 'housekeeping' gene whose activity remains constant in all mammalian cells, was also amplified as an internal control. The PCR process amplifies short (approximately 100-1000 base pairs) segments of the longer cDNA molecule. The components of the reaction are mixed and the reaction is placed in a programmable thermal cycler, which is an automated instrument that takes the reaction through a series of different temperatures for varying amounts of time. This series of temperature and time adjustments is referred to as one cycle of amplification. Each PCR cycle theoretically doubles the amount of targeted template sequence in the reaction. Ten cycles theoretically multiply by a factor of about one thousand; 20 cycles, by a factor of more than a million in a matter of hours. Each cycle of PCR amplification consists of a number of steps. Each step denatures the template producing two oligonucleotide-primed single-stranded DNA templates, sets up the polymerisation reaction, and synthesises a copy of each strand of the template being targeted. These steps were optimised for each template and primer pair combination. Thus, for each primer 40, 36 and 28 cycles were used for ALP, OP and GAPDH primers respectively. The amplified products are loaded on the gel along with a reference DNA 'ladder' containing known DNA fragments of particular sizes (in base pairs). The gel also contains ethidium bromide so that it can be viewed under UV illumination. Thus, if the sequences under investigation are present, the illuminated bands will be revealed and their sizes checked against the DNA ladder.

3. Results

Fig. 2 shows representative agarose gels of the RT-PCR products of ALP and OP with their corresponding GAPDH gels obtained from the mRNA transcripts of cells incubated for 6 h after insonation with 0 (control), 120, 390 and 1490 mW / cm² at 3.0 MHz. The resultant gels were subject to image analysis using Scion Image software to record band intensities relative to background intensity levels, which are presented in Fig. 3. for ALP and Fig. 4. for OP, respectively. From the area of the histogram, the ratio of ALP or OP gene expression to GAPDH gene expression at each dose of ultrasound was calculated, and presented as an Expression Index (E.I.). From this E.I., expression at each US intensity, relative to non-exposed control, was calculated.

Thus, although the GAPDH remained at the same band intensity for all doses of ultrasound (and the same as the control cultures which recorded no ultrasound exposure), the expression of the both the ALP and OP genes were found to vary at different US intensities. As seen in Fig. 3., ALP expression relative to GAPDH expression was found to be 10% above control levels for 120 mW / cm², in excess of double at 390 mW / cm² and more than four times greater at 1490 mW / cm². However, OP expression (Fig. 4.) was down-regulated by 40% at the lowest dose of 120 mW / cm², while up-regulation by 30% and 60% was measured at 390 mW / cm² and 1490 mW / cm² respectively. .

4. Discussion and conclusion

Exposure to therapeutic doses of US was found to alter the expression of the bone-associated genes ALP and OP at 6 h post-insonation in osteoblast-like cells. At the highest two doses, ALP and OP expression were both clearly up-regulated, particularly for ALP. However at the lowest dose, a differential response was found with down-regulation of OP to little variation from control for ALP.

These results suggest that clinical therapy using ultrasound may be mediated, at least partly, by altered expression of these genes due to ultrasound stimulation, such as up-regulation of ALP and OP at higher doses. However, the down-regulation of OP at the lowest intensity exposure may infer that the response of cells to ultrasound may be highly dose-specific. Such an observation is consistent with findings of optimal fracture healing *in vivo* at the lower end of the therapeutic US intensity range.

These results suggest the possibility that ultrasound exposure parameters could be modulated to optimise the clinical efficacy of ultrasound for engineering new bone and improving implant biocompatibility.

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Figure Legends

Fig. 1. A schematic diagram describing the US exposure apparatus.

Fig. 2. Representative electrophoretic agarose gels of the PCR products of ALP and OP genes in MG63 cells exposed to different intensities of US. For each bone marker, the results are expressed relative to the expression of the housekeeping gene, GAPDH, used as an internal standard, and amplified by PCR from the same cDNA samples. The size, in base pairs (bp) of each product is indicated.

Fig. 3. Relative expression of GAPDH and ALP mRNA transcripts for different US intensity exposures. The band intensity histograms show the Expression Index (E.I.) of ALP relative to GAPDH for each US dose.

Fig. 4. Relative expression of GAPDH and OP mRNA transcripts for different US intensity exposures. The band intensity histograms show the Expression Index (E.I.) of OP relative to GAPDH for each US dose.

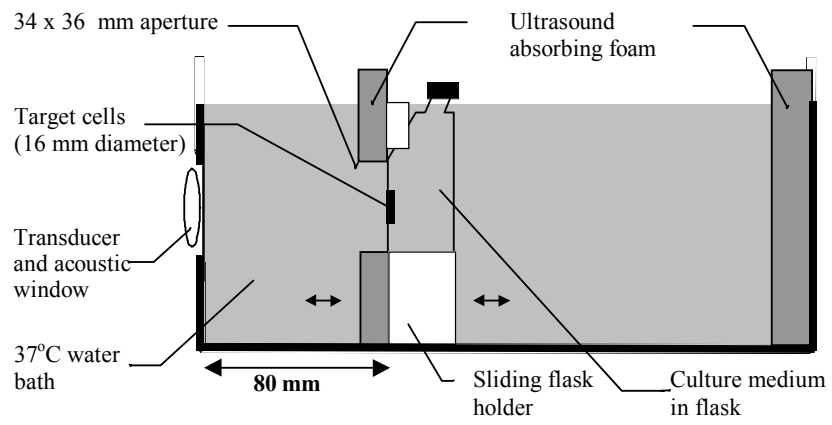


Fig. 1. Harle et al.

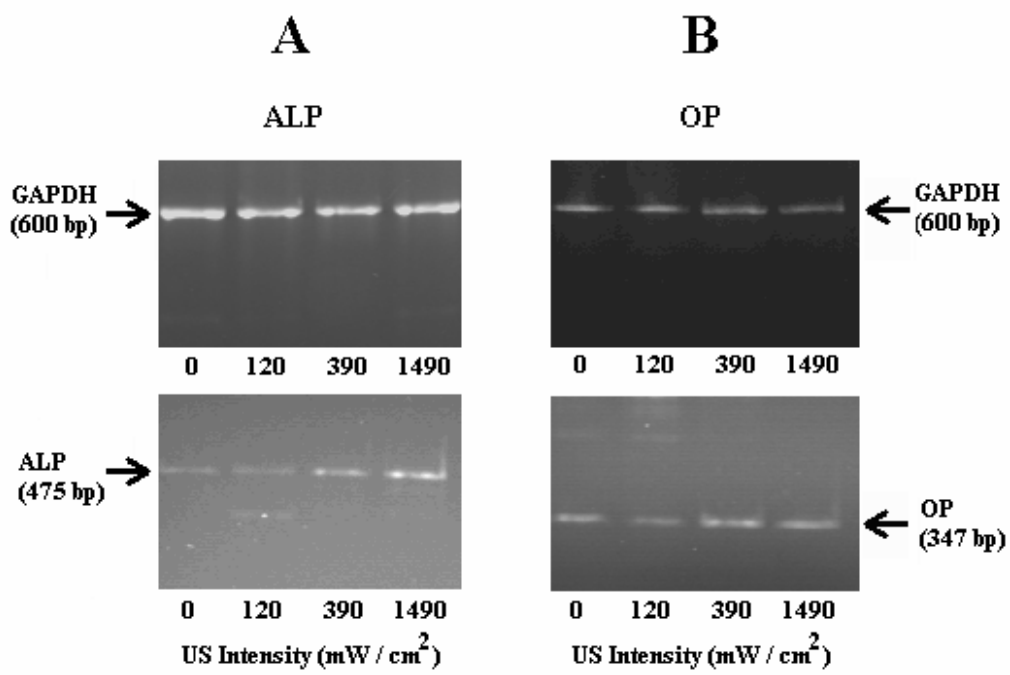


Fig. 2. Harle et al.

Intensity (mW/cm ²)	GAPDH mRNA expression	ALP mRNA expression	E.I.	% E.I. Change
0			13.2	100
120			14.7	110
390			32.6	240
1490			55.8	420

Intensity (mW/cm ²)	GAPDH mRNA expression	OP mRNA expression	E.I.	% E.I. Change
0			18.9	100
120			11.7	60
390			24.4	130
1490			30.7	160

Fig. 3. Harle et al.

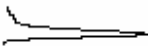

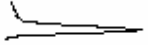

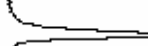

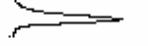

Intensity (mW/cm ²)	GAPDH expression	O P expression	E.I.	% cont
0			0.189	100
120			0.117	60
390			0.244	130
1490			0.307	160

Fig. 4. Harle et al.