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Low Intensity Pulsed Ultrasound Stimulation: In-vitro Experiments to Study Bone Mechanotransduction

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1. Introduction

Low-intensity pulsed ultrasound (LIPUS) is a therapeutic modality arousing growing interest among clinicians to promote bone remodelling (Zong et al., 2025). Bone is a mechanosensitive tissue capable of responding to mechanical loads through a process known as mechanotransduction (Huang & Ogawa, 2010). However, the underlying mechanisms triggered by ultrasound (US) stimulation remain poorly understood and this lack of knowledge fuels controversy and prevents the development of optimized LIPUS based therapies. To improve understanding of bone cell mechanotransduction under US stimulation, in vitro studies on cell culture models are essential. For this purpose, a serial LIPUS stimulator and its digital twin have been developed to perform US stimulation of cells cultured in Petri dishes while precisely controlling both acoustic and biological conditions.

The acoustic part of the setup was validated both experimentally and numerically (Majnooni, Lasaygues et al., 2022; Majnooni et al., 2022) without cells. The device is now being tested in an incubator to confirm proper operation under controlled biological conditions.

This work presents initial experiments on bone mechanotransduction by investigating the effects of US on osteocytes. A first series of tests on MLO-Y4 cells was conducted to evaluate the influence of different US parameters. Gene expression analysis allowed to identify the effects of LIPUS stimulation on cells.

2. Materials and methods

2.1 LIPUS stimulation set-up

The set-up was developed to accommodate up to 20 Petri dishes. US stimulation was applied from below using a rack of four US transducers (Imasonic, France) immersed in a water tank filled with degassed water. Transducers were driven by a generator (Siglent, China) coupled with a power amplifier (Vectawave, UK).

The Petri dishes were maintained at the water surface using custom-designed 3D-printed holders and positioned at a distance h = 30 mm from the transducers. Stimulation took place in a cell culture incubator (Heraeus HERAcellTM 150i) in order to maintain controlled culture conditions (37°C, 5% CO2 and 95% humidity).

Each Petri dish was equipped with an anti-reflection cover to protect cells and ensure controlled acoustic stimulation (Majnooni et al., 2022).

2.2 LIPUS signal

The LIPUS signal used for stimulation is a 1 MHz burst signal with a duration τ and an amplitude V_{PP} at 50 Ω. The pulse repetition period T_{PRP} was fixed to 1 ms. The duty cycle (DC) was defined as the ratio of τ to T_{PRP}. Cells were insonified for 20 min per day over five consecutive days.

The objective of our experiments was to evaluate the effects of different spatial-average temporal-average

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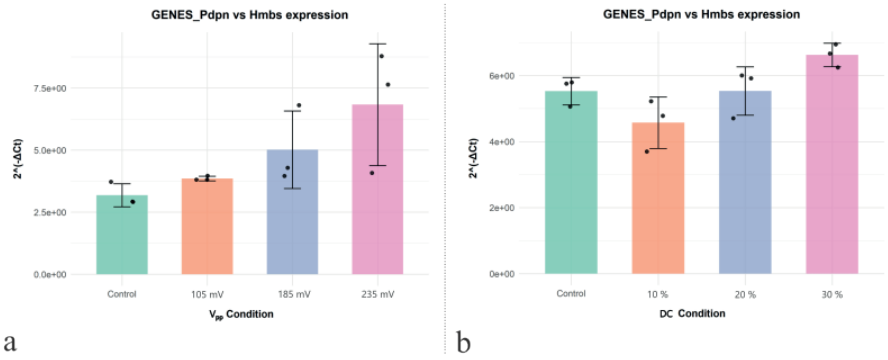

Figure 1. a. Pdpn expression for each condition of Exp 1 (DC = 20%, V_{PP} variable). b. Pdpn expression for each condition of Exp 2 (DC variable, V_{PP} = 185 mV).

intensities I_{SATA}, a common acoustic exposure metric. Since I_{SATA} depends on V_{PP} and DC, two experiments (Table 1) were conducted to reach similar I_{SATA} values using different parameter combinations. The goal was to evaluate the influence of each parameters.

Table 1. Stimulation plan for two experiments to evaluate the influence of V_{PP}, DC and I_{SATA}

	I_{SATA} [mW/cm²]	DC (%)	V_{PP} [mV]
Exp 1 (Fixed DC)	30	**20**	105
	60	**20**	185
	100	**20**	235
Exp 2 (Fixed V_{PP})	30	10	**185**
	60	20	**185**
	100	30	**185**

2.3 Control of acoustic pressure and intensity

The acoustic pressure delivered during LIPUS stimulation was experimentally measured with a 0.5 mm needle hydrophone (Precision Acoustics Ltd, UK) using the method described in (Majnooni, Lasaygues et al., 2022). Thanks to the anti-reflection cover the acoustic intensity measured corresponds to the acoustic dose received by the cells (Majnooni et al., 2022).

2.4 Cell culture

MLO-Y4 cells were cultured in 60 mm dishes (Corning 430196) with a 35 mm POM ring, seeded at about 3,118 cells/cm² in 13 mL medium. For each experiment, 16 dishes were prepared (n = 4 per condition, 4 controls).

2.5 Biological analysis

To evaluate cellular responses to LIPUS, we performed quantitative PCR (qPCR) to quantify gene expression related to bone remodeling. Gene of interest expression was normalized to a stable housekeeping gene.

3. Results and discussion

Preliminary biological results were obtained, an example of Pdpn gene expression normalized to the housekeeping gene Hmbs is shown in Figure 1. Pdpn is a marker of osteocytic activity, for this gene, no statistically significant differences were observed between conditions in the two experiments, however trends confirm that US stimulation may influence MLO-Y4 responses. Based on these results the influence of V_{pp} seems to have a greater effect on the cells than the DC. These findings are consistent with recent publications in the literature (Orthodoxou et al., 2026).

Variability is high across conditions, highlighting the need to increase the number of replicates. A second set of experiments will be planned with a limited number of conditions. The aim is to study two conditions using the same approach with n = 10, while placing controls outside the stimulator device. This will provide sufficient biological material to perform RNA-seq and explore additional genes potentially involved in bone remodelling.

Further investigation are required to determine how different US parameters correlate with biological response.

4. Conclusions

This work presents preliminary experiments conducted on a limited number of samples. These initial results provide guidance for future US stimulation protocols that will be planned on a larger number of replicates. They also confirm that the LIPUS stimulator is a suitable tool for investigating cellular mechanotransduction in controlled conditions.

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Contributor Roles

Conceptualization, PL, CB; Resources, CGC, JCS, DM, PL, CB; Methodology, MB, LO, PL, CB; Software, MB, LO, PL, CB; Validation, CGC, JCS, DM, PL, CB; Formal analysis, MB, LO, CGC, JCS, DM, PL, CB; Investigation, CGC, JCS, DM, PL, CB; Data curation, MB, PL, CB; Writing – original draft preparation, MB, PL, CB; Writing – review, MB, LO, CGC, JSC, DM, PL, CB; Supervision, PL, CB; Project administration, PL, CB; Funding acquisition, CGC, JSC, PL, CB.

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