In vitro analysis of the mechanical and biological effects induced by the ultrasound-cell interaction

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Recent studies have highlighted how the exposition to megasonic fields can induce several biological effects with potential damage for human health. The more significant ones involve membrane poration, with the consequent permeabilization of cells to exogenous particles and the triggering of inflammatory and cytotoxic processes [1-3]. Nevertheless, scientific literature does not provide a univocal experimental methodology to investigate and quantitatively describe the biological risk with proper indicators.
In this context, we developed an experimental setup (Figure 1A) with the purpose to perform an *in vitro* systematic analysis of the biological effects related to the ultrasound-cell interaction. Two different cell models, human keratinocyte (HaCaT) and skin melanoma (SK-Mel28), were employed in our study. The ultrasound-induced biological effects have been investigated at two frequencies (0.5 MHz and 1 MHz) as a function of the acoustic dose, defined as the product between the spatial peak temporal average intensity (I_{SPTA}) and the treatment time.

Confocal fluorescence microscopy pointed out, for the first time even in the subcavitation regime, relevant modifications in the membrane morphology and functionality (Figure 1 B). The activation of inflammatory pathways was assayed by quantitative real-time pcr (qRT-PCR) (Figure 1C). We observed, 24 hours after treatments, a temporary overexpression of genes related to the inflammatory response, starting from a threshold acoustic dose of 54 J/cm^2 (corresponding to 30 minutes of treatment with ultrasound field of 1 MHz frequency and e I_{SPTA} = 30 mW/cm^2). The cell viability was studied by means of the MTT proliferation assay and by flow cytometry experiments to detect apoptosis, whose frequency resulted correlated to the treatment dose (Figure 1D). In the case of 0.5 MHz fields the occurrence of necrosis was observed in concomitance with the overexpression of inflammatory cytokines.
The study was performed also in the presence of cavitating microbubbles, which locally enhance the acoustic field. In this case it has been possible to recognize biological damage, with significant decrease in the cell viability, at low treatment doses, with the threshold shifted to 1.6 J/cm^2 (35 s, 1 MHz, I_{SPTA} = 45 mW/cm^2).

At doses higher than the threshold values reported, experiments pointed out a permeabilization of the cell membrane that allows the intracellular diffusion of potentially cytotoxic nanoparticles. Our study highlights how increasing the mechanical index of the ultrasound treatments (corresponding to lowering the frequency) yields an enhancement of both the membrane permeability (poration) and the biological response (cytotoxic and inflammatory). The activated inflammatory pathway could favour the tumor proliferation. Our results stress the importance to deepen the correlation between the necrotic events and the overexpression of inflammatory genes as a function of the ultrasound frequency. Whether the biological response results correlated to the mechanical index as well as to the acoustic dose, the identification as reference parameter of a novel indicator, accounting for both the contributions, would be necessary.

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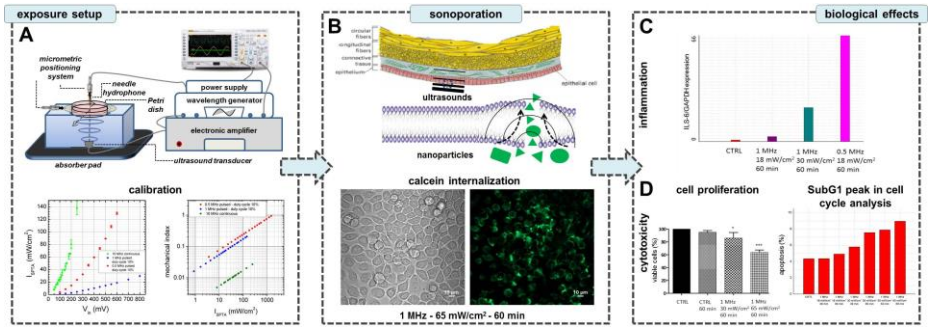

Figure 1. Analysis of the ultrasound-induced bioeffects. (A) Sketch of the exposure setup and calibration curves. (B) Sketch of the membrane sonoporation with consequent internalization of exogenous vectors and confocal fluorescence microscopy image of HaCat cells that have internalized the green dye calcein. (C) Analysis of the expression of IL-6, a proinflammatory cytokine. (D) Cytotoxicity evaluation in terms of MTT proliferation assay and distribution of apoptotic cells in SubG1 hypodiploid peak.