Chapter 1
Review of Recent Results of Onco-Ultrasound-Tripsy on Cancer Cells

Carmine Pappalettere and Giovanni Pappalettera

Abstract In recent years, cancer research has advanced significantly, resulting in better treatments for various tumour types and extended patient life expectancy. Nevertheless, there is still considerable interest in alternative clinical approaches. A major challenge is creating treatments that precisely target tumours while preserving healthy tissue. Research has shown that low-intensity ultrasound can selectively harm tumour cells due to their unique mechanical properties compared to healthy cells. This paves the way for a technique called Onco-Ultrasound-Tripsy, OUT, which involves using ultrasound vibrations to induce selective mechanical damage to tumour cells. Many advancements have been observed in this context and this survey aims to present several results related to the employment of such approach with applications to lymphoma, breast cancer, osteosarcoma and melanoma cells.

Keywords Cancer treatment · Ultrasound · Cellular fatigue · Selective cellular damage · Onco-Ultrasound-Tripsy

Introduction

The use of ultrasound in medicine dates back to 1942 when Karl and Friederich Dussik attempted to adapt industrial techniques for detecting metal flow to visualize intracranial structures in real time [1]. Around the same time, Raimar Pohlman, influenced by Paul Langevin's discovery that fish reacted strongly to ultrasound, developed a physiotherapy protocol at Charité in Berlin [2]. This protocol set a power density limit of 5 W/cm^2 and required continuous transducer movement during treatment.

In 1942, Lynn & Putnam [3] pioneered the use of ultrasound for targeting brain tissue in animals, marking an early milestone in therapeutic ultrasound. Their equipment leveraged the piezoelectric properties of crystals first discovered by Pierre and Jacques Curie sixty years earlier [4]. They also applied Gruetzmacher's technique, which involved grounding the surface of a vibrating quartz plate to create a concave curvature, similar to a mirror, allowing ultrasound waves to be focused on a precise location.

Since then, ultrasound has been utilized in treating various conditions, including bone fractures, osteoporosis, thrombosis, glaucoma, nerve injuries, skin wounds, and cancer either as a standalone therapy or in combination with drugs [5]. Over time, ultrasound technology has seen major advancements, including miniaturization, improved functionality, more sophisticated control systems, and a wider array of probes and actuators to support expanding medical applications [6]. Some treatments have been approved for clinical use, while others remain in development.

Significant progress has been made in tumor treatment using High-Intensity Focused Ultrasound, HIFU [7, 8]. HIFU directs high-energy ultrasound waves to a small target area, preserving the surrounding tissue. The ablated tissue volume typically measures 1–3 mm (transverse) × 8–15 mm (along the beam axis) after a single exposure. The two primary mechanisms behind HIFU-induced tissue damage are mechanical energy conversion into heat and cavitation.

Additionally, low-intensity ultrasound, with drug assistance, has been investigated for anti-tumor applications, including sonodynamic therapy, which combines low-intensity ultrasound with a chemotherapeutic agent (sonosensitizer) [9]. This approach (called LIPUS) holds promise due to its ability to selectively target cancer cells while preserving healthy tissue.

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In general, when ultrasound interacts with tissue, it produces three main effects: cavitation, hyperthermia, and resonant excitation [10, 11]. Unlike cavitation and thermal effects, resonant excitation operates based on the different resonance frequencies of healthy and cancerous cells, allowing for targeted therapeutic action.

The so called Onco-Ultrasound-Tripsy approach (OUT) aims to exploit the mechanical properties of cancer cells for selective damage, emphasizing the potential of low-intensity ultrasound to inhibit cavitation and thermal effects while promoting resonant and fatigue ones. Changes in resonance frequency due to modifications in mechanical properties, such as a demonstrated reduction of up to 70% in the rigidity of cancer cells [12], support this approach.

After having had the idea many years ago and having studied since the 90s the possibility of selectively killing tumor cells by exploiting their different resonance frequency compared to healthy cells, Pappalettere and his collaborators from Polytechnic University of Bari began 20 years ago to deepen the research on the topic. Afterwards, experimental activities were conducted on U937 tumor cells of a myelocytic lymphoma at the University of Fukuoka in Japan (2015) [11, 13–15] and on MCF7 breast tumor cells MCF10 cells at the University of Dundee in Scotland (2016) [16–21] and the corresponding healthy MCF10A at the University of Bari (2019) [22]. Then they continued at the Polytechnic of Bari in collaboration with the University of Foggia on MG-63 osteosarcoma cells (2017) [23–25] and with the University of Bari both on the same U937 cells studied in Japan and on the corresponding healthy CD3/CD8+ Lymphocytes cells and on red blood cells (2023) [26, 27]. Finally, recently, experiments have been conducted on HBL melanoma cells.

Some more recent studies on U937 cancer cells have been developed and validated at the California Institute of Technology (CalTech) basing on the hypothesis that cellular damage to low intensity ultrasound exposure doesn't occur in a quasi-static/low cycle fatigue regime (cellular lysis) but in high cycle fatigue regime (apoptosis) (2020) [28, 29] while additional results can be found in [30].

The aim of this review is to provide an overview of major results obtained by this approach addressing new challenges and new open questions.

Materials and Methods

All the analyses have been conducted in vitro by transferring the cells grown in culture into Petri dishes and sonicating them either from the bottom or from the top of the dish, depending on whether the cells were suspended or adherent. The probe to sonicate the cells was placed in direct contact with the dish's surfaces, coupled with gel, and the dish was filled with culture medium to facilitate wave propagation and prevent air exposure.

Ultrasound waves were generated using the SonoPore KTAC-4000 device (NEPA GENE, Chiba, Japan), capable of producing frequencies between 200 kHz and 5 MHz with a fine adjustment of 1 kHz. The device allows for adjustments in pulse repetition frequency (0.5 Hz to 100 Hz), output duty cycle (0% to 100%), exposure duration, and voltage (0 V to 60 V). The voltage setting directly influences the power output, depending on the specific probe connected. A KP-S20 flat probe (NEPA GENE, Chiba, Japan) with a 20 mm emission diameter was used. For cell counting, Trypsin/EDTA was used to detach the cells from the Petri dish, and the TC20 automated cell counter (Bio-Rad, USA) was employed. Trypan blue was applied to selectively stain dead cells by permeabilizing their membranes. Following this procedure, a 100% viability rate was observed for the living cells. Additionally, the TC20 system measured the cell size, which was found to range from 10 to 15 μm in diameter. Cell mortality was calculated by Eq.1

$$cell\ mortality = \frac{live\ cells\ on\ control\text{-}live\ cells\ after\ treatment}{live\ cells\ on\ control}$$

Applications of Onco-Ultrasound-Tripsy (OUT)

Application on Lymphoma Cells

Ultrasound exposure was applied to the U937 human histiocytic lymphoma cell line. The U937 cell line, sourced from ECACC (catalog number 85011440, UK), was originally derived from the malignant cells of a 37-year-old Caucasian male diagnosed with histiocytic lymphoma. This monocytic leukemia model retains key monocytic features, including lysozyme secretion, esterase activity, and limited phagocytic capacity, consistent with its histiocytic lineage. U937 cells, widely classified as monocytic cells, were maintained at 37°C in a humidified 5% CO_2 incubator using RPMI-1640 medium (Euroclone, Italy) supplemented with 10% fetal bovine serum, L-glutamine, penicillin G, and streptomycin. The culture medium was refreshed every 24 hours to sustain a cell density between 5.0 $\times$ 105 and 1.0 $\times$ 106 cells/mL. Cells were expanded in T25

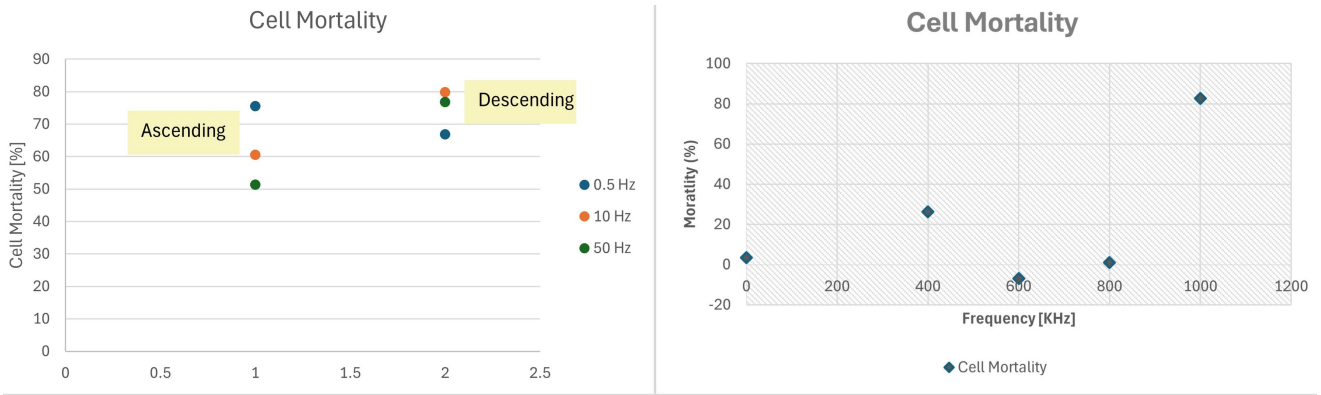

Fig. 1 Analysis of the observed mortality in human leukemic U937 cells was conducted both before (untreated) and immediately after (sonicated) ultrasound treatment. (Left) treatment at sweeping frequencies. (Right) Treatment at fixed frequencies. Results for 0 KHz are referred to untreated cells.

flasks, harvested on the day of the experiment, and resuspended in fresh medium at a concentration of 1 million viable cells/mL. This step was implemented to minimize CO2 accumulation, which may interfere with ultrasound energy propagation and subsequently impact cell mortality outcomes. U937 cells were seeded in 12-well plates at a density of 106 cells/mL in a total volume of 2 mL, with ultrasound exposure applied using a coupling gel.

First evaluations were done by exposing cell to sweeping frequency both in descending and ascending mode [11] by sweeping frequency from 400 kHz to 620 kHz and viceversa at 0.5 Hz, 10 Hz and 50 Hz burst rate.

It was observed that best results are obtained (Fig. 1 left) by working in descending mode at 10 Hz burst rate. In that situation, in fact, 79.8 % of cell mortality was observed. After these treatments the cells that remained alive were put in an incubator for 6 hours and the calculation of the living ones was re-performed. It turned out that after this time the total mortality of the U937 cancer cells rose to about 90% thus showing that the ultrasonic treatment also activated a mechanism of apoptosis of the diseased cells [11].

In a second set of experiments [26, 27] sonication of U937 cells was conducted at constant frequency values of 400, 600, 800, and 1000 kHz, employing voltage levels of 30, 40, 50, and 60 V, a 50% duty cycle, and a 10 Hz burst rate. Exposure durations of 180 and 360 seconds were assessed to identify the optimal conditions for reducing cancer cell viability.

Cell proliferation was monitored for seven days following treatment, with cell counts recorded at 0, 48, 120, and 168 hours. To ensure nutrient availability and prevent any impact on cell growth, the medium was refreshed every 48 hours.

A preliminary series of experiments was performed to assess the effect of frequency variation on U937 leukemic cells. The cells were sonicated for 180 seconds at a constant voltage of 60 V, with a 50% duty cycle and a 10 Hz burst rate, using frequencies of 400, 600, 800, and 1000 kHz (Figure 1 right). Cell mortality, measured immediately after ultrasound exposure, was significantly higher in cells treated with a 1 MHz frequency (82.73% $\pm$ 2.34) compared to untreated controls. Notably, U937 cells exposed to 400 kHz exhibited a mortality rate approximately seven times higher than untreated cells (26.24% vs. 3.50%), indicating that even lower frequencies can negatively impact cell viability.

These results suggest that among the tested sonication conditions, the 1 MHz frequency induces the highest mortality in this tumor cell type. It is important to note that mortality was calculated by Eq.1 and may be influenced by background mortality in the control group. If additional biological factors contribute to control group mortality, this could result in an apparent negative mortality rate, as observed in the case of the 600 kHz treatment.

Application on Breast Cancer Cells

Sonication experiments were conducted using the breast cancer cell line MCF7 [22]. The cells were cultured in plastic T-75 flasks (Sigma-Aldrich, USA) in RPMI 1640 medium (Gibco Thermo Fisher, USA), supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, USA) at 37 °C in a humidified incubator with 5% CO_2.

To investigate the effects of intensity and frequency, cell viability after sonication was evaluated using trypan blue dye exclusion tests on macroscopic samples. For this, the cells were plated in 35 mm Petri dishes and incubated for 24 hours before sonication experiments. Prior to the experiments, MCF7 cells had a confluence of over 90%, while MCF10 cells reached around 60% confluence. After sonication at various voltage/frequency settings, the cells were immediately washed with phosphate-buffered saline (PBS) and detached from the dish bottom by treating with 0.250 mL of 0.05% trypsin for 3

minutes. Then, 1.75 mL of medium was added, mixed, followed by the addition of the previous supernatant, PBS, and trypan blue and finally centrifugate at 1200 rpm for 3 mins.

The following sonication conditions were chosen: a sinusoidal wave in pulsed wave mode, with a pulse repetition frequency of 10 Hz, a 50% duty cycle, and 7 fixed frequencies of 400 kHz, 436 kHz, 472 kHz, 510 kHz, 546 kHz, 582 kHz, and 620 kHz. The tests were conducted either at a constant voltage of 60 V or at a constant power of 0.1 W. This power value represents the average output power across the frequency range of 400–620 kHz and also the median power among the 7 sonication frequencies. Results in terms of observed mortality are shown in Fig. 2.

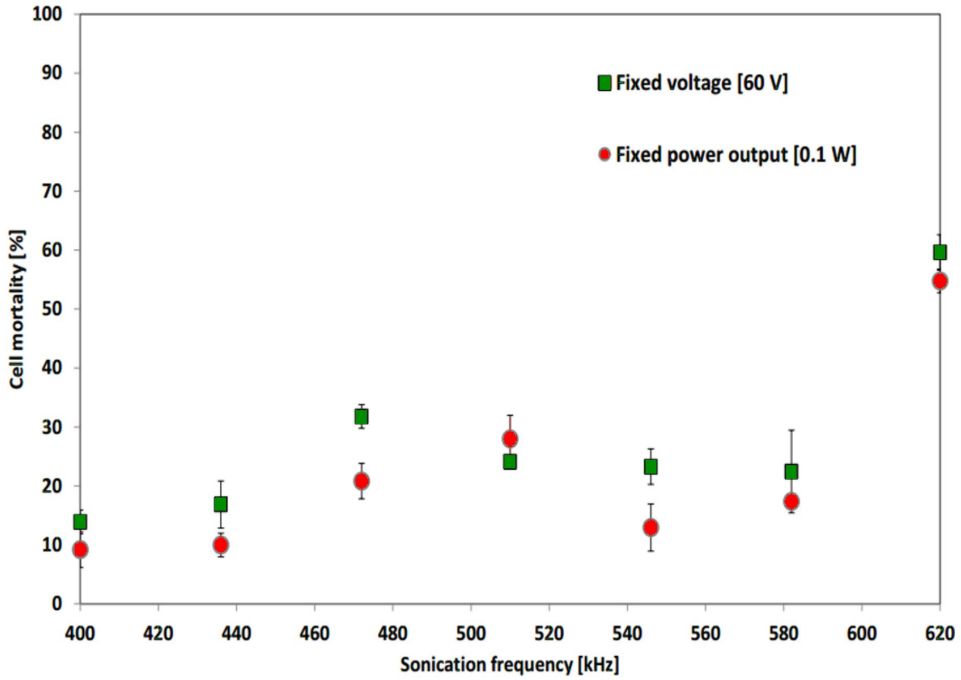

Fig. 2 Response in terms of cell mortality for the fixed voltage condition (green square) and the fixed power condition (red circles)

Figure 2 shows the trend of cell mortality while varying the frequency. The two tested conditions show quite similar trends indicating that cell mortality is mostly correlated with frequency. More specifically cell mortality rises 10% to 15% at 400 kHz to 55% to 60% at 620 kHz. However, this increase is concentrated in the 540–620 kHz frequency range.

Application on Osteosarcoma Cells

The MG-63 osteosarcoma (OS) cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) [25]. Cells were seeded at a density of 8 × 104 cells per T25 flask and incubated at 37 °C in a standard environment with 95% air and 5% CO2. To detach the cells, Trypsin/EDTA was used, after which the cells were re-plated in Petri dishes and returned to the incubator. Cell confluence was monitored using a Nikon Eclipse Ti-U inverted microscope with a C1 Digital Eclipse modular confocal system.

Cells were exposed for 180 s at a fixed frequency in pulse mode, with a burst rate of 10 Hz, 50% duty cycle and 60 V voltage. In an initial phase, different frequencies were tested to identify the ones able to induce the lowest % of live cancer cells after sonication. Five emission frequencies were analyzed: 400, 510, 582, 800 and 1000 kHz. The power density was, in any case, lower than 5.4 W/cm^2. After these preliminary tests, two frequencies were found associated with the lower percentage of living cells after sonication: 800 and 1000 kHz. The sonication tests for these two frequencies were then repeated on two different days with three replications of the same experimental conditions for each day. Moreover, we the growth index of the cells after ultrasound treatment in real time was analyzed. This was done by using a device able to measure variation in cellular impedance. In fact, an increment of the number of adherent cells results in an increment of the measured impedance and this can be used as an indicator of the cellular growth. These growth indexes were then compared with the growth index of the untreated cells. Experiments were carried out using the xCELLigence RTCA instrument (ACEA Biosciences, Inc., CA, USA), placed in a humidified incubator maintained at 37 °C with 95% air and 5% CO_2.

Cells were exposed for 180 seconds at a fixed frequency in pulse mode, with a burst rate of 10 Hz, a 50% duty cycle, and 60 V voltage. Initially, different frequencies were tested to identify those that caused the lowest percentage of live cancer cells post-sonication. Five emission frequencies were evaluated: 400, 510, 582, 800, and 1000 kHz, with a power density always below 5.4 W/cm^2. After these preliminary tests, two frequencies (800 and 1000 kHz) were found to result in the lowest percentage of surviving cells post-sonication. These two frequencies were then tested again on two separate days, with three replicates of the same experimental conditions for each day. Additionally, the cell growth index was monitored in real time following ultrasound treatment. This was achieved by using a device that measures changes in cellular impedance, where an increase in the number of adherent cells corresponds to a rise in impedance, serving as an indicator of cell growth. These growth indices were then compared to that of untreated cells. The experiments were conducted using the xCELLigence RTCA instrument (ACEA Biosciences, Inc., CA, USA), in a humidified incubator maintained at 37°C with 95% air and 5% CO_2.

Figure 3 (Left) presents the percentage of dead cells following sonication for five different tested frequencies: 400, 510, 582, 800, and 1000 kHz. The evaluation of dead cells was carried out using Trypan blue assays, as dead cells are permeable to the dye and can thus be counted. This procedure was performed immediately after the sonication process, and the number of dead cells was compared with the initial number of treated cells to calculate the percentage shown in Figure 3. The data reveals a general increase in the percentage of dead cells as the frequency rises, particularly for frequencies above 600 kHz. The results at 400 kHz should be considered an outlier and warrant further investigation. The highest cancer cell mortality was observed at the two highest frequencies: 45% of cells were dead after sonication at 800 kHz, and 60% of cells were dead at 1000 kHz.

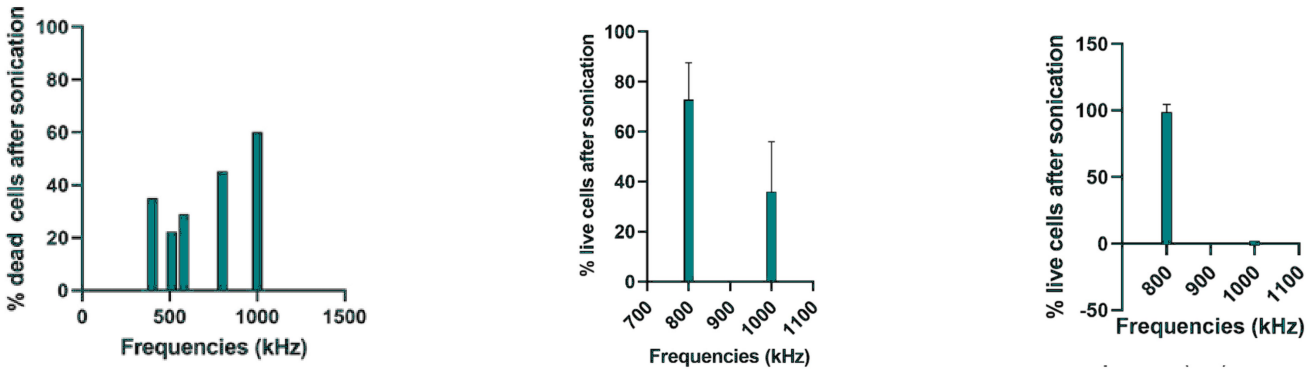

Fig. 3 (Left) Percentage of dead cells after sonication at the five selected frequencies; (Middle) follow-up of the live cells percentage two days after the sonication; (Right) Recalculation of the live cells percentage including considerations about cell/fragment diameter.

The experimental results from the following two days of testing for the selected frequencies of 800 and 1000 kHz are presented in Figure 3 (Middle). The sonication experiments confirm that 1000 kHz is the frequency that results in the lowest percentage of live cancer cells post-sonication, with an average value of under 36%.

Further evaluation was conducted to investigate the possibility that live cells might be present in the culture medium after treatment, potentially detached from the bottom of the dish by the ongoing pressure wave but not damaged by the ultrasound. To rule out this hypothesis, which could result in an overestimation of the number of killed cells, the culture medium was centrifuged to collect the pellet. The pellet was then cultured, and the corresponding growth index was assessed. For all the analyzed dishes, the growth index was found to be zero, indicating the absence of live cells in the medium. Results of this new calculation are presented in Fig. 3 (Right) where analysis is restricted only to cells in the range between 12 and 25 μm.

Application on Melanoma Cells

In order to assess the effects of low-intensity ultrasound on melanoma cells, the HBL metastatic cell line was utilized, known for its high proliferative capacity. These cells were derived from human biopsies at the Department of Translational Biomedicine and Neuroscience (DiBraiN) at the University of Bari "Aldo Moro."

Cells were cultured in Dulbecco's Modified Eagle's Medium High Glucose (DMEM H.G.), supplemented with 10% fetal bovine serum (FBS), 1% L-Glutamine (Euroclone), and 1% Penicillin/Streptomycin (Full DMEM/HG). All cultures were maintained in an incubator at 37 °C with 95% humidity and 5% CO_2.

The culture medium, containing a pH indicator, was refreshed every two to three days based on the requirements of the cell culture. Cells were seeded in four 12-well plates at a density of 100,000 cells per well. These were incubated until reaching a confluence of 70–80%, a prerequisite for initiating ultrasound treatment. Confluence was assessed via optical microscopy. To analyze cell viability and mortality pre- and post-treatment, two control wells were included for each condition.

All plates were exposed to ultrasound using the following sonication parameters: Duty Cycle 50%, Burst Rate 10 Hz, Voltage 40 V, Frequency 1 MHz, and treatment duration of 180 s. These parameters were selected based on preliminary investigations identifying the optimal frequency for inducing cell mortality.

Following treatment, melanoma cells were detached from the wells, washed with 1X PBS, treated with 1X Trypsin-EDTA solution (0.05%/0.02%), and centrifuged at 1100 rpm for 5 minutes.

A cell count was subsequently performed for both control and treated wells using the TC-20 automated cell counter (Bio-Rad) in the presence of Trypan Blue vital dye. Cells were resuspended in 1 mL of complete DMEM/HG medium, and 10 μL of the resulting cell suspension was mixed with 10 μL of Trypan Blue (1:1 dilution). The mixture was then loaded into the counting slide chamber and analyzed using the TC-20 instrument. Cell mortality was evaluated following Eq.1

The findings for HBL melanoma cells are summarized in Figure 4. As depicted, plate #1 exhibited a mortality rate of 100%, while plate #3 and plate #4 showed mortality rates of 83.5% and 85.5%, respectively. However, plate #2 displayed a markedly different outcome, with a significantly lower mortality rate of only 3.33%.

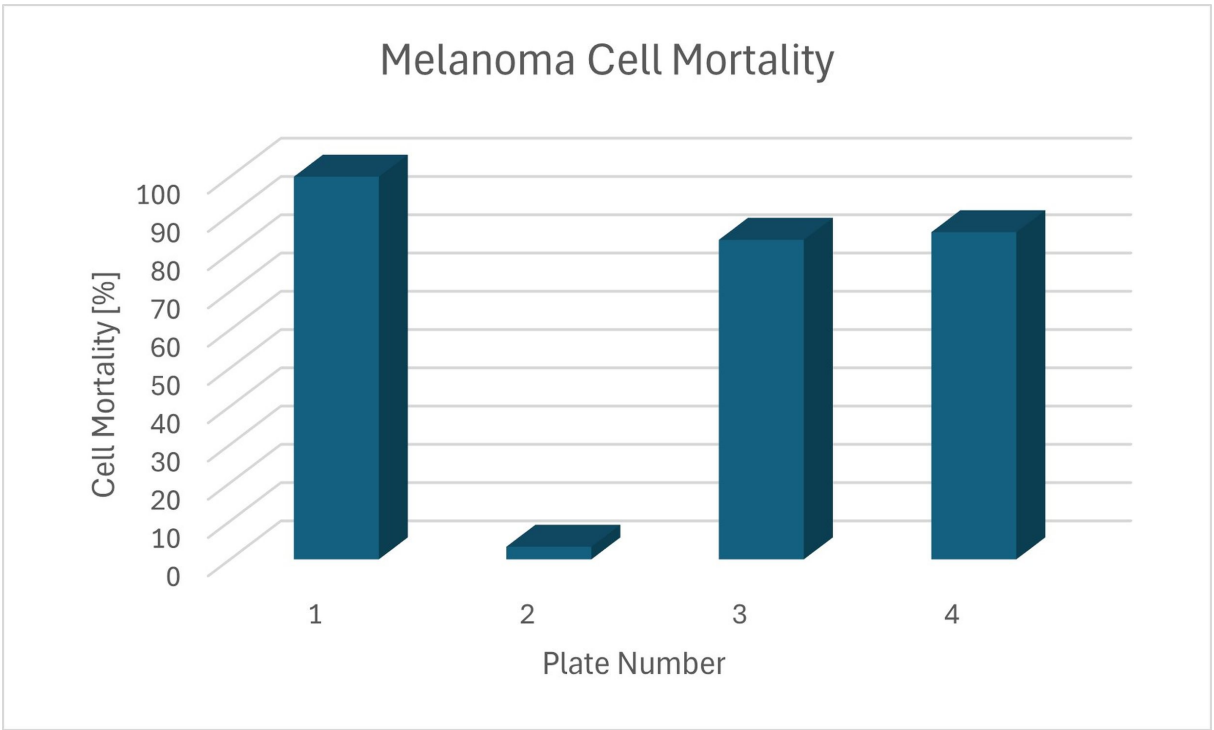

Fig. 4 Evaluation of the cell mortality in the four tested plates

The results obtained for HBL melanoma cells appear somewhat inconsistent and warrant further investigation. While three of the analyzed plates exhibited significant mortality rates, plate #2 showed no such effect. This discrepancy highlights the need for additional studies to identify potential factors underlying these findings.

A closer examination of plate #2's specific conditions is necessary. As described before in this case, cells remained in culture for four days, reaching 100% confluence and displaying a slightly acidified culture medium compared to the previous day. A possible explanation for the lack of effectiveness could be alterations in the culture medium, which also serves as the propagation medium for sound waves. Such changes may have interfered with the resonance regime through absorption and dispersion mechanisms. Furthermore, additional research is required to investigate other potential contributing factors, whether biological or ultrasound-related, that may have influenced the high mortality observed in plates #1, #3, and #4. Ultimately, these findings should be validated through studies with a larger statistical sample.

Conclusion

The overview presented in this paper is testifying the capability of the onco-ultrasound-tripsy approach to face with different kinds of cancer cells at least for what is related to the in-vitro operations. Even if effectiveness of the approach is strictly related to the specific targeted cancer cells in all the presented situation it was found a response in terms of mortality of the cancer cells after exposure. Even more interestingly this response exhibits a frequency dependent behaviour which is fundamental to guarantee selectivity of the approach. Starting from this a lot of efforts are still required to completely understand the whole mechanism of ultrasound-cell interaction, to model them, to understand the role of the cellular micro-environment paving the way of possible preliminary in-vivo investigations. It worth underlining, however, that first evidence of applicability in vivo were shown in [31] where it was observed the capability of the low intensity ultrasound approach in inhibiting cancer growth in mice.

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