Differences in the Behavior of Attached and Floating Cells Subjected to Low Intensity Ultrasound

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***Abstract*—Through mechanical considerations, we will try to determine whether cancer cells die because of resonance or energy present in solution. Attached and floating cells were stressed at fixed frequencies in a range between 400 kHz and 620 kHz at 10 PRF (Pulse Repetition Frequency). In the floating cells the power and the mortality show similar variations with respect to frequency and this allows to assume a direct relationship between power and mortality. The same experiment was replicated on attached cells by exposing the cultures at US of fixed frequency (between 400 and 620 kHz, with 10 Hz Pulses Repetition Frequency) either keeping constant power output or voltage. Cell mortality was found to be more sensitive to the frequency.**

***Index Terms*—attached cells, floating cells, stiffness, amplitude, frequency**

I. Introduction

The purpose of this experiment is the comprehension of the mechanism of action of ultrasonic perturbation on cancer cell mortality. Which one is more effective, amplitude or frequency on cell survival? Does the cell die due to resonance or for a biomechanical response linked to the stress? There are evidences that low intensity ultrasound changes the mechanical property of some cancer cell [1], [2] and that can interfere with the expression of the major cytoskeletal elements [3-5] and specific integrin-binding proteins of these cells. It is known that the hyper proliferation of cancer cells is stopped by sonication [6]-[10]. In the same way, selectivity of ultrasounds is recognized: as they can destroy cancer cells preserving healthy cells [11], they are considered one of the most harmless diagnostic and treatment resources.

This study will evaluate cell mortality induced by different sonication conditions on floating (U937) and attached cancer cells (MCF-7).

Frequency and intensity have an important role in cell fate in relation to its size and mechanical characteristics [12]. In this regard, it should be specified that cell cultures, both floating and attached cells, show a high heterogeneity in cell sizes.

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II. Material and Methods

A. Ultrasound Apparatus and Intensity Measurement

The ultrasound generating apparatus is SonoPore KTAC-4000, (Nepa-Gene, Chiba, Japan). The KTAC equipment can generate frequencies from 200 kHz to 5000 kHz with a fine adjustment of 1 kHz. The probe used in the experiments is the diameter 20 mm KP-S20 (Nepa-Gene, Chiba, Japan). The instrument allows to select voltage. One of the problems related to the use of ultrasonic generators is the measure of the output power. At the same voltage during changing frequency, the power output varies in real time. The power reading is displayed during the treatment. But this measure is only indicative as it derives the power from the measurement of electrical intensity. In addition, each probe is characterized by a different output, even for the same nominal power value.

Probe output was calibrated in the range 400 kHz - 620 kHz, first by means of radiation force balance (Mettler Toledo, NewClassic MS, S/N B047090420). Power was measured and acoustic pressure was then sensed by a fiber optic hydrophone (Precision Acoustics, Dorset, UK).

B. Cell Culture and US Treatment

The U937 cell line is an oncogenic human monocyte cell line. They were isolated from the histiocytic lymphoma of a 37-year-old male patient. The interest in these cells is not related to behavior and differentiation of monocyte, but to U937 carcinogenicity and at their characteristic to be floating cells. Their behavior is counteracted at that of MCF-7, a breast cancer cell line isolated in 1970 from a 69-year-old Caucasian woman (Fig. 1).

It has high metastatic potential, invasiveness and carcinogenicity and is attached cell line.

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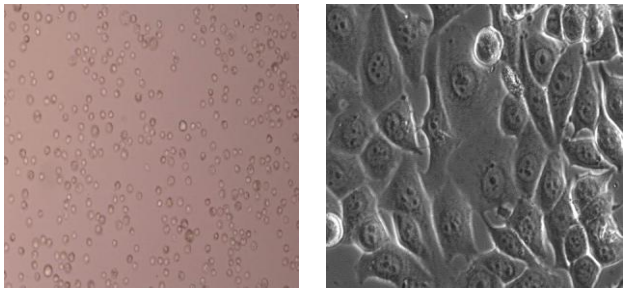

U937 MCF7

Figure 1. Cell lines used in experiment.

The human myelomonocytic lymphoma cell line U937 was cultured in RPMI 1640 (Sigma-Aldrich, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum 10% (FBS; Sigma-Aldrich, MO, USA) at 37.0 ℃ in humidified air with 5% CO_2. On the day of experiment, cells were collected and centrifuged at 1500 rpm for 5 min. The viability before treatment was always higher than 95%.

Cells were then resuspended in fresh medium at a final concentration of 10^6 cells/ml and 2 ml were aliquoted in each well of Lumox Multiwell 24 TC Quality (Greiner-Bio). The dishes were sonicated at PRF (Pulse repetition frequency) 10 Hz, DC (duty cycle) 50%, at fixed frequencies 400 kHz, 436 kHz, 472 kHz, 510 KHz, 546 kHz, 582 kHz, 620 kHz for 180 s. The low values of power recorded during the experiment and the value of intensity recorded in the probe calibration phase let us presume to be below inertial cavitation threshold. The cells were subjected to sonication in pulsed wave, with duration 180 seconds, and at 60 V. Cell viability was immediately assessed after sonication by means of Trypan blue dye exclusion test. Cell suspension containing 10 µl was mixed with an equal volume of 0.4% Trypan Blue solution. After mixing, 10 µl of solution were pipetted in a counting slide and introduced in TC20™ Automated Cell Counter (Bio-rad, USA). The number of total and alive cells were displayed immediately on the monitor.

The attached cells culture is human breast adenocarcinoma cell line MCF-7. MCF-7 cells were grown in plastic T-75 flasks (Sigma-Aldrich). They were cultured in RPMI 1640 (Gibco Termofisher, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich, MO, USA) at 37℃ in humidified air with 5% CO_2.

Cell viability was assessed after sonication by means of Trypan blue dye exclusion test on a macroscopic sample, to know at macroscopic level the effect of intensity and frequency.

For this test, the cells were cultured in 35 mm diameter Petri dish the day before the experiment. The cells confluence before the experiment was almost 90%. After different sonication protocols, cells in the dish were immediately washed with PBS (Phosphate-buffered saline), detached from the dish bottom by 0.250 ml 0.05% Trypsine for 3 min, added 1,75 ml medium, mixed, added again old supernatant, PBS and Trypan Blue centrifuged for 3 min at 1200 rpm and counted by Luna count cell (Logos Biosystem).

The same procedure was also performed on the non-sonicated cells of controls dish.

In the second experiment (probe characterization by viability), cells were sonicated introducing the probe in the dish in touch with solution by a support that kept the probe always in the same liquid relative position.

The sonication condition for this treatment were the following: sinusoidal wave in PW (pulsed wave), 10 Hz PRF 50%, DC at the fixed frequencies of 400 kHz, 436 kHz, 472 kHz, 510 KHz, 546 kHz, 582 kHz, 620 kHz. The tests were conducted once at the constant voltage of 60 V and once maintaining constant power of 0.1 W. In the latter case, voltage values (set to have 0.1 W output on the KTAC) were:

Frequency	Voltage
400	75
436	66
472	81
510	61
546	56
582	51
620	42

III. Results and Discussions

In the U937 experiment, cell killing rate was determined as the ratio of the difference between average number of alive cells of the day control (non-ultrasound treated cells assumed to have 100% viability) and average number of alive cells after sonication, to the number of control cells.

Killing rate was related to the probe output, calibrated by means of an oscilloscope in series with a KP-S20 probe used as a microphone, connected to probe to characterize.

For AC measures, the root mean squared signal (V_{RMS}) is proportional to the power of the signal. The conversion factor was experimentally determined between the obtained data and the data in W by a radiation force balance with suspended target (Mettler Toledo, NewClassic MS).

The results showed a direct relation between killing rate and power (Fig. 2).

This lets us assume that, in this case, the intensity and hence the amplitude had a greater role than frequency in the cell mortality.

The investigation was continued on attached cells. The viability test was performed at constant voltage (60 V) and at constant power (0.1 kW).

Due to the different type of cells, in this experiment mortality (the complement to one of the viability) was compared with the power. The attached cells, although resulting from the same crop, bred overnight with speeds slightly different and could not be compared with the non-sonicated control.

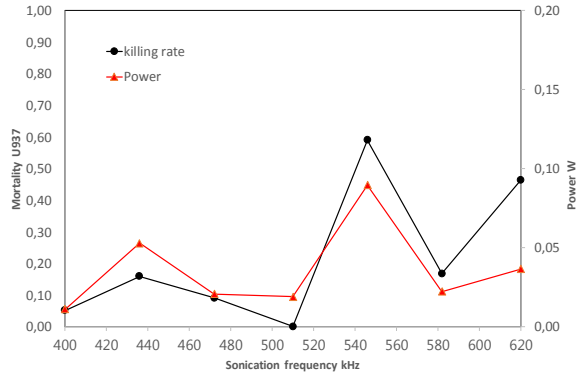

Figure 2. U937 killing rate and probe output at fixed frequencies.

The probe calibration gave results a little bit different due to the different probe used and the dependence by power was not so evident as shown in Fig. 3. The frequency seems to affect mortality much more significantly (see Fig. 4).

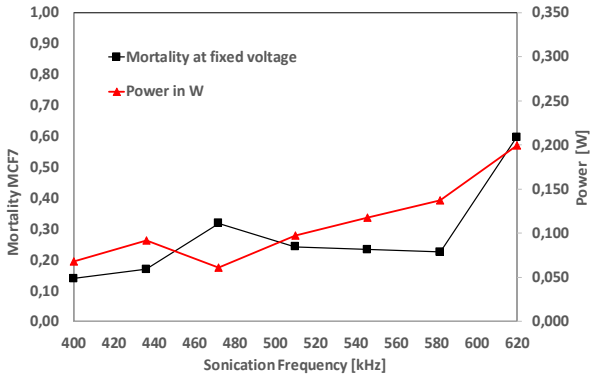

Figure 3. MCF7 mortality at fixed voltage and probe output at fixed frequencies.

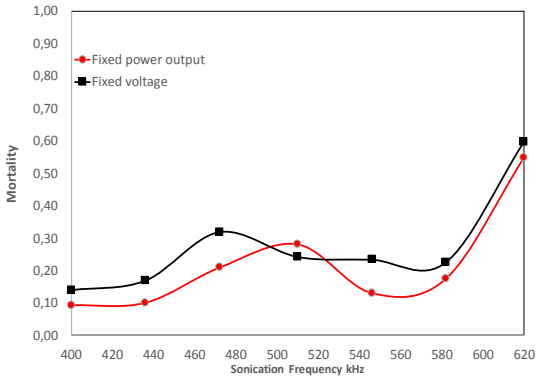

Figure 4. Mortality at fixed power output and at fixed voltage.

The frequency had a strong influence on the diameter. Regardless of the sonication protocol adopted in the experiments, cell diameter always was found to decrease with frequency and this trend became more marked at the very high frequencies, as shown in Fig. 5. Probably, since adhered cells are somehow similar to semi-ellipsoids, it will be easier to put into vibration bigger cells along some preferential direction and cause early mortality for resonance.

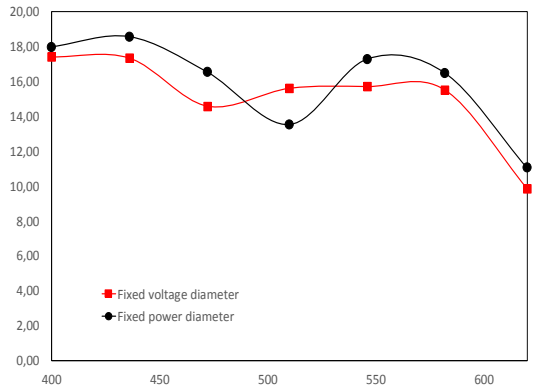

Figure 5. Diameter at 60 V fixed voltage (square) and diameter at 0.1W fixed power (circle).

It is really difficult to hypothesize a different mechanism of death for these cells. What we can see is that in the first experiment, the medium in which are suspended the U937 cells has a mass, a rigidity, an elasticity and a thermal inertia. So the solution absorbs power from probes and gives back it to cells. Therefore, in this case, the cells are more sensitive to power.

In the second experiment, cells were constrained to the substrate and to the neighboring cells. These kinematic constrains do not allow each cell to displace independently of the other cells and the whole culture starts to vibrate simultaneously.

IV. Conclusions and Future Directions

The present results suggest that the synergistic action of intensity and frequency induces mortality.

Each biological process is influenced by the mechanical properties of the surrounding environment: mechanical cues play an integral role in maintaining and influencing the cell fate and tissue maintenance throughout life.

The investigation is still in its infancy. In the next future it would be interesting to deepen the correlation between dimensional and mechanical property like size and cell stiffness. For which it would be necessary to repeat the experiments on different types of cells with different geometries and mechanic features. It would allow us to determine an empirical relationship between the type of cells and the best conditions of sonication to destroy them.

In particular healthy cells (platelet-rich plasma, erythrocytes, monocytes) will be tested: our sonication parameters are commonly used in different medical applications, like in physiotherapy, in lithotripsy and in aesthetic cavitation, therefore a destructive effect on healthy blood cells can be ruled out, but it need other confirms.

It seems that the floating cells behavior is different from attached cells behavior: in the first ones amplitude effect is dominant, in second ones the frequency is prevailing on mortality and cell diameter. In future constant power tests will also be performed on floating

cells, they will provide better information about the correlation between size and frequency

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She began her professional activity like installations and structures free lancer planner at family enterprising activity, after she worked like mechanical engineer in some factories and at this time she became Mechanical disciplines tenured teacher in public high school.

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