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# EXPERIMENTAL EVALUATION OF MG-63 CELLS BIOLOGICAL RESPONSE TO LOW INTENSITY ULTRASOUNDS EXPOSURE

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***Abstract.*** *The purpose of this study is to evaluate effect of low intensity ultrasounds (US) on MG-63 osteosarcoma cell lines. It is known that low intensity ultrasound can induce mild thermal, apoptosis and sono-mechanical effects. However, response to ultrasonic treatment depends at a great extent on the type of tested biological tissues. In order to find the frequency or the range of frequency yielding most significant biological effects on MG-63 cells, experimental tests are performed by sonicating MG-63 cells at fixed frequency. Cell cultures are grown in 60 mm Petri dishes and then exposed to US for 3 minutes. US waves are generated by a SonoPore KTAC-4000 sonoporator and then transferred to specimens by KP-S20 probes. The selected frequencies are 400, 510, 582, 800 and 1.000 kHz. Cell viability is measured after sonication using a TC20 cell counter. The number of counted cells is found to decrease with sonication frequency. In particular, it drops to about 30% at 1.000 KHz. Interpretations of experimental evidence are attempted in the article.*
***Keywords****: low-frequency ultrasounds, osteosarcoma, ultrasound-biophysics mechanisms.*

## 1. INTRODUCTION

A big amount of genetic lesions, caused from both exogenous and endogenous sources, occur during cell life. Normally DNA repair mechanisms can manage to these occurrences. Different studies found that cancer is a genetic disease, where chromosomal lesions and changes lead to cell abnormal proliferation and alteration of apoptosis processes. As human cancers may present different morphologies and diversity of mutations, it is necessary to find a personalized therapy for each of them [1].
Apoptosis restoration is one of the most important results that treatments such as chemotherapy are trying to achieve. However, many of these therapies are really dangerous for the whole organism. The new challenge in cancer treatment is therefore to find therapies that can selectively act on cancer cells.
An interesting subject in this field is the interaction of ultrasounds (US) with biological tissues. Ultrasounds can produce thermal and non-thermal effects (e.g. cavitational and non-cavitational effects [2]). Different studies demonstrate that heat, cavitation and hyperthermia [3-4] induced by ultrasounds can produce antitumor effects. Low-intensity long-time and high-intensity short-time protocols yield good results in cancer treatment [5]. For example, Hrazdira et al. [6] treated HeLa cells with US at a frequency of 0.8 MHz, SA-intensity of 50, 100 and 500 mW/cm$^2$, for 5 or 10 min. This treatment affected cell proliferation and on cytoskeleton. The latter is probably due to the immediate action of mechanical forces of the ultrasound field on cellular structures [6]. Feril et al. [7] investigated how low intensity ultrasounds can induce cell mortality and synergistic effects between ultrasounds and other agents [7]. Ivone et al. [8-10] analysed mechanical and biological effects of US on floating (U937) and attached (MCF-7) cancer cells. They used fixed or variable frequencies finding frequency values that yield the highest cell mortality [8-10]. Another interesting study is the one carried out by Lagneaux et al. [11] that treated human leukaemia cell lines with ultrasound at a 1.8 MHz frequency different exposure times. They observed cell damage associated with the apoptotic process.
In this paper, we investigate the interaction between low intensity ultrasounds and a particular type of osteosarcoma cell line called MG-63. Previous studies investigated chromosomal alterations of osteosarcoma cell lines, and pointed out differences in gene expression profiles between these cells and normal osteoblasts [12-13]. Pautke et al. [14] compared osteosarcoma cell lines with human osteoblasts. They discovered that attached osteosarcoma cells present a different morphology compared to osteoblasts: MG-63 cells are oval to spindle-shaped, without branching cell processes. Furthermore, growth characteristics changes from osteosarcoma cells

to osteoblasts: all osteosarcoma cells showed a 2 to 3-fold greater mean doubling-time and a 15 to 20-fold higher saturation density than osteoblasts. Moreover all osteosarcoma cell lines lose physiological features and typical cell-to-cell communication. Contact inhibition is lost and cell growth is not restricted to monolayers.

## 2. MATERIALS AND METHODS

### 2.1 Cell culture

The osteosarcoma cell line MG-63 were cultivated in DMEM containing 10% FBS. Cells were seeded at 8 x $10^4$ cells/T25 flasks and they were cultured in a humified incubator at 37°C using a standard mixture of 95% air and 5% CO2. When confluence was reached, cells were detached with trypsin/EDTA and subsequently re-plated in 60mm Petri dishes.
A 60mm Petri dish was analyzed after confluence was reached. We first checked out visually the confluence using a Nikon Eclipse Ti-U inverted microscope equipped with a C1 Digital Eclipse Modular Confocal System. We also measured the number of living cells by using a TC20 Cell Counter. Cells were detached with trypsin/EDTA and 5 µl of cultured cells treated with Trypan Blue were put on a cell counting slide. Trypan Blue selectively colors dyed cells by permeating their membrane. With this method we found 100% living cell and a total count of 1,52 x $10^6$ cells/ml. TC20 also allows to measure cell diameter which was found to vary between 10 and 15 µm.

### 2.2 Ultrasound generator equipment

Ultrasound waves were generated by a SonoPore KTAC-4000 device (Figure 1). This equipment can generate US at frequencies between 200 kHz and 5 MHz. It is possible to operate at fixed frequency, with continuous wave or pulsed wave, changing the frequency with which pulses are repeated (burst rate) between 0.5 and 100 Hz. It is also possible to operate at increasing frequency (sweep 1) and decreasing frequency (sweep 2). It is possible to set the duration of the experiment and the voltage (between 0 and 60 V). The output power may range between 0 and 5.00 W, depending on the probe used. Here, we used a flat probe KP-S20 (i.e. a 20 mm diameter transducer) to transfer ultrasounds to the specimens.

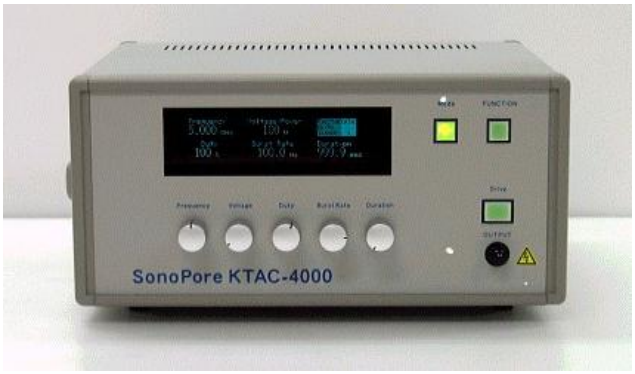


**Figure 1**: The SonoPore KTAC - 4000

### 2.3 Experimental tests

The purpose of this study was to understand the behaviour of MG-63 cell cultures treated with ultrasounds after sonication. We sonicated cells at fixed frequencies for 3 minutes, using 10 Hz pulse repetition frequency, 50% duty cycle and a constant voltage of 60 V. The output power was between 0,1 – 0,3 W (low intensity range). The selected fixed frequencies were 400 kHz, 510 kHz, 582 kHz, 800 kHz and 1.000 kHz.
Cells were sonicated by introducing the US probe in the Petri dish. The probe was in contact with the liquid solution but not totally immersed in it, maintained on the surface through a supporting structure (Fig. 2). All experiments were executed directly on 60mm Petri dishes come to confluence, leaving cells in the attached configuration.

**Figure 2**: The experimental setup

After each sonication, cells were detached with trypsin/EDTA and a 5 µl sample was put on a cell counting slide and treated with Trypan blue. This agent immediately colors death cells, thus giving the number of live and death cells in a very short time. Each slide was put inside the TC20 Cell Counter. We gathered information about: *total count* (cell/ml) which is the total number of counted cells (living and dead) by the device; *live count* (cell/ml), which is the number of living cells counted by the device; *live cells* (%), which is the percentage of living cells out of the total number of cells.

## 3. RESULTS

For each sonication, we first registered the variation of the following values: frequency (kHz), total count (cell/ml), live count (cell/ml). It appears that the percentage of live cells does not decrease significantly after sonication. In order to investigate this evidence, we compared live count registered for an unsonicated sample (i.e. 1.520.000 cell/ml, indicated as "Control") with live counts registered for sonicated samples (indicated as "SLC"). By subtracting SLC live count from Control live count, we found that the total number of cells in significantly decrease. The maximum reduction was observed for 1000 kHz. The (Control–SLC) difference is denoted as "Dead or Disappeared cells", to underline that these cells could be not detected from TC20. Since the Trypane Blue colors only dead cells, it is not possible to identify cell's scraps suspended in the culture liquid. We calculated the percentage of "Dead Cells" by dividing the number of "Dead or Disappeared cells" by the Control live count. All these results are reported in Table 1.

**Table 1:** Experimental results

| Frequency (kHz) | Total count (cell/ml) | Live count [SLC](cell/ml) | (Control – SLC) (cell/ml) | Dead Cells (%) |
|---|---|---|---|---|
| 400 | 989,000 | 962,000 | 558,000 | 37% |
| 510 | 1,080,000 | 1,050,000 | 360,000 | 24% |
| 582 | 1,180,000 | 1,160,000 | 470,000 | 31% |
| 800 | 831,000 | 831,000 | 689,000 | 45% |
| 1000 | 603,000 | 581,000 | 939,000 | 62% |

The trend shown by these experimental data indicates that the percentage of dead cells increase with the frequency. The 400 kHz is an outlier as there are much more dead cells that at higher frequencies. Figure 3 shows the variation of cell mortality with respect to sonication frequency. It can be seen that data are fitted by a linear regression with $R^2$=0.8937 including the outlier and, more interestingly $R^2$=0.9968 considering only higher frequencies than 500 kHz.

## 4. CONCLUSION

In this paper we investigated the effects of low-intensity ultrasound on osteosarcoma MG-63 cell cultures. For that purpose, cells were sonicated at five fixed frequencies. Experimental data indicate that cell mortality seems to increase with frequency. However, there are at least four issues to be addressed in future studies.
1) The behavior of MG-63 cells to low intensity US will be analyzed at other frequencies to fully understand how these cancer cells respond to sonication. This will help us to assess behavior at the outlier point of 400 kHz.

2) To investigate on the presence of cell's scraps suspended in the culture liquid. Different biological techniques may turn useful for this purpose. It would be also important to verify confluence after sonication to understand interaction between low-intensity ultrasounds and intercellular adhesion molecules.
3) To investigate how ultrasounds damage MG-63 cells with special focus on resonance, impact and apoptosis effects.
4) To verify selectivity of US treatment by performing sonication on osteoblast healthy cells.

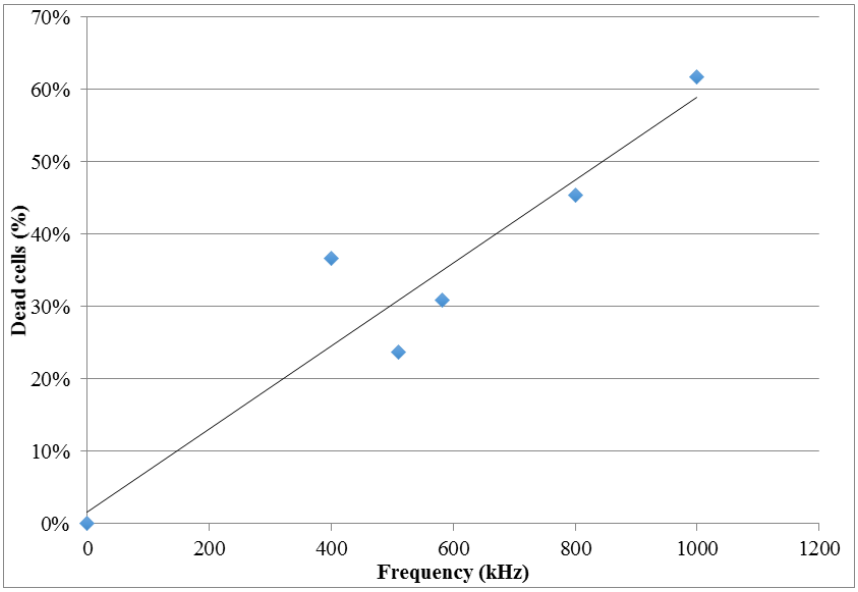


**Figure 3**: Variation of cell mortality with respect to sonication frequency

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