RAT CANCER CELLS NECROSIS INDUCED BY ULTRASOUND

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Sonodynamic therapy is the ultrasound dependent enhancement of the cytotoxic activities of certain drugs called sonosensitizers. The study of therapeutic efficacy of ultrasound is always preceded by in-vitro tests. In this work, two in-vitro sonication procedures were compared. One with the transducer positioned bellow the cell colony, radiating upward, with standing wave reflected from the water-air surface, the second, in the free field conditions. Efficiency of the cancer cells necrosis caused by ultrasound was compared with acoustical field intensity I_{SPTA} measured by a hydrophone. The standing wave conditions effectively increased the intensity of the ultrasonic wave at the level of cells. To achieve 50% of cell viability, the intensity I_{SATA}, decreased from 5.8 W/cm^2 to 0.3 W/cm^2. In summary, sonication in the standing wave conditions can effectively and reproducibly destroy cells by ensuring the sterility and without the risk of overheating.

INTRODUCTION

Cancer diseases are second only to cardiovascular diseases as the main cause of death both in Poland and in the world. The primary cancer therapy is surgery assisted by radiotherapy or chemotherapy. Radiotherapy and chemotherapy cause the destruction of both tumor cells and healthy ones. Currently, people are searching for new therapeutic techniques, treating tumors without damaging healthy tissue. The selective cytotoxicity of tumor tissue provides photodynamic therapy [1, 2] and sonodynamic therapy [3, 4]. Sonodynamic therapy is the ultrasound dependent enhancement of the cytotoxic activities of certain drugs called sonosensitizers. The attractive features of this modality for cancer treatment emerges from the ability to focus the ultrasound energy on malignancy sites buried deep in tissues and to locally activate a preloaded sonosensitizer. A possible mechanism of sonodynamic therapy is the

generation of sonosensitizer derived free radicals which initiate chain peroxidation of membrane lipids [5, 6, 7].

The study of therapeutic efficacy of ultrasound is always preceded by *in vitro* tests. The cells are cultured either in the Petri dish or multi-well cell culture plates or the OptiCell ® culture media. The design of the plates helps to maintain their sterility and probably because of this, cells are sonicated directly on the plates.

The natural and easiest way to reach the sonication of cells *in vitro* is to put a plate or dish with cells in water over an ultrasonic transducer [8, 9, 10, 11]. The plate can be covered or closed at the top, making it easy to maintain sterility. This enables the formation of standing waves, reflected at the water-air surface. A mismatch of the transducer acoustic impedance to the impedance of water can cause additional wave reflection at the water-piezoceramic boundary and further increase the standing wave amplitude (Fig. 1).

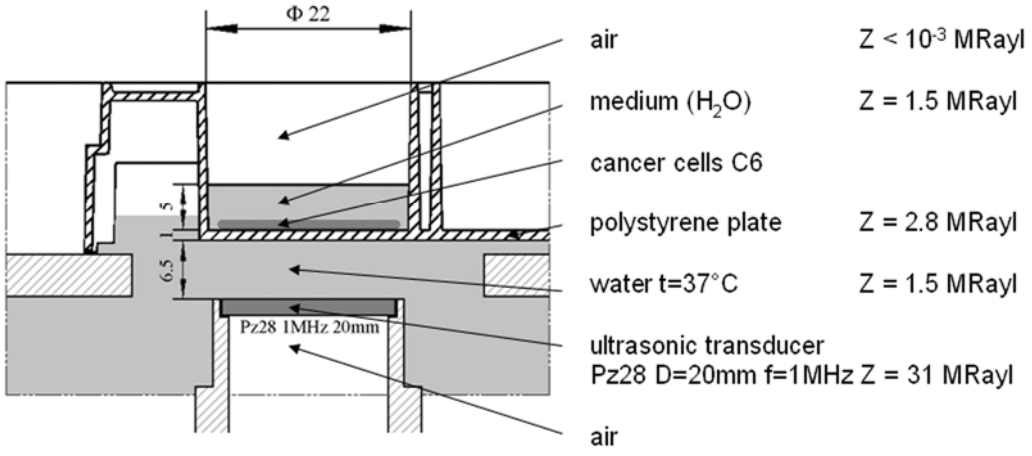

Fig. 1. *In vitro* sonication experimental setup. The setup is composed of a 1MHz ultrasonic transducer positioned from below. Cells were cultured in the 12 Well Cell Culture Plate. Distance between the cells and transducer was 7.5 mm.

The solution would be to uncover the plate or dish with the cells and merge the ultrasonic absorber into the medium. The absorber should be acoustically matched to the water [12]. An alternative solution is to place the ultrasonic transducer directly in the medium and an ultrasound absorbing material below the cell colony [13]. Such arrangements make it rather difficult to maintain the sterility and there is a risk of overheating of cells both by the heat emitted by the transducer and the heat generated by the ultrasonic absorber, if it is placed too close to the cells. In addition, the diameter of the transducer must be less than the inside diameter of the opening in the plate or dish, which results in reduced intensity of the ultrasonic wave at the edges and reduced efficacy of cell destruction.

A good solution would be placing cells in a special container with walls made out of thin foil with negligible attenuation and placing it in a large water tank with walls covered with material absorbing ultrasound [14]. Similarly, cells might be placed in a small, sealed polyethylene bag [15]. Another solution is to use the OptiCell® box, whose walls are made of a thin film [16, 17]. The disadvantage of this solution is the large size of the box, 7x7 cm, requiring a very large ultrasonic transducer.

An acoustic wave, after encountering an obstacle, is reflected from it and returns towards the source. The interference between the incident and the reflected waves generates a temporary increase in the intensity, due to the phenomenon known as a standing wave. The reflection coefficients R and transmission coefficient T (both for intensity) at the interface of the media with different acoustic impedances is given by these formulas [18]:

$$R = \left(\frac{Z_2 - Z_1}{Z_1 + Z_2} \right)^2 \tag{1}$$

$$T = \frac{4 Z_1 Z_2}{\left(Z_1 + Z_2 \right)^2} \tag{2}$$

where Z_1 – acoustic impedance of the medium where ultrasonic source is placed and Z_2 is acoustic impedance of the second medium. For water-air interface $Z_1 = 1.5 \cdot 10^6$ Rayl and $Z_2 = 4.3 \cdot 10^2$ Rayl. For our case $Z_1 >> Z_2$ and R = 1, T = 0 and $I_M/I_0 = 4$, where I_M – is maximum standing wave intensity and I_0 is the intensity of incident wave.

There is also the additional reflection at the water-piezoceramic interface, further increasing the pressure and standing wave intensity. For piezoceramic Pz28 (Meggitt, Denmark), $Z_{Pz} = 31 \cdot 10^6$ Rayl and the intensity reflection and transmission coefficients are equal R = 0.82 and T = 0.18, respectively. Then, according to the energy conservation law $I_M/I_0 = 5.7$ for lossless medium and plane wave.

In summary, we expect a 4 - 5.7 fold increase in the intensity of the acoustic wave in the case of a standing wave.

1. MATERIALS AND METHODS

The rat C6 glioma cells obtained from American Type Culture Collection (Manassas, VA, USA) were used in the study. The cells were grown on a Petri dish after the addition of Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were grown at 37°C in a humidified atmosphere containing 5% carbon dioxide. DMEM medium was changed every 3-4 days. Then the cells were sown in every second well of 12 Well Cell Culture Plate 665-180 (Greiner, USA).

$2 \cdot 10^5$ cells and DMEM medium supplemented with 10% FBS serum and penicillin and streptomycin were put into one well. After a 24 hr incubation at 37°C, the medium was replaced with a new DMEM without FBS serum. The cells were then sonified for 3 min. After the experiment, the cells were returned to the incubator for another 24 hrs and then 100 ml solution of Cell Counting Kit 8 (CCK - 8, Sigma, USA) was added to each well containing cells. After a 3 hr incubation, the plates with cells were placed on a shaker for 15 min. The percentage of viable cells was determined by measuring the absorption of 570 nm light in a spectrophotometer Epoch Microplate Reader (BioTek, USA).

The Plexiglas container (230x160x70 mm), designed for the experiment of cells sonication is shown in Fig. 2. The vessel was filled with distilled, degassed water. The device had a built-in electronic thermostat stabilizing water temperature at 37°C and a magnetic stirrer. An ultrasonic transducer or a cone-shaped (diameter 50 mm and a height 30 mm) silicone rubber ultrasonic absorber, were immersed in the water container. The top cover of the vessel had a 40 mm diameter hole and a recess milled to 5 mm depth, designed in such a

way that shifting 12 Well Cell Culture Plate to one of the corners of the recess, the next well could be placed symmetrically over the ultrasonic transducer or absorber.

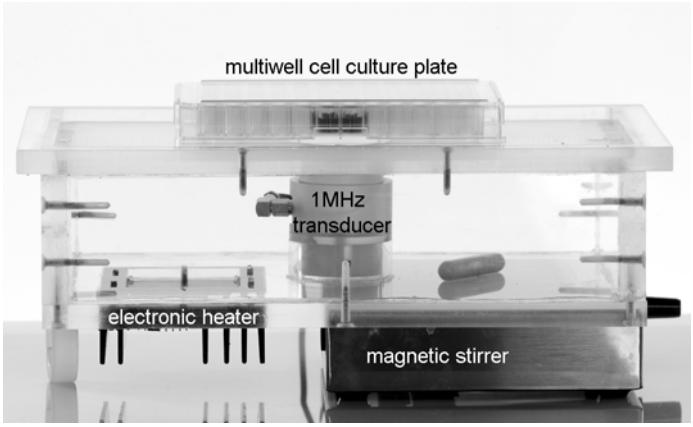

Fig. 2. Experimental setup. Plexiglas container filled with distilled, degassed water with electronic thermostat and magnetic stirrer.

For measurements, a 1 MHz ultrasonic transducer and 20 mm diameter (Pz28, Meggitt, Denmark) was used. The transducer was placed either under the bottom of the Plexiglas box in order to make use of the standing wave (Fig. 1), or was inserted into the hole in the plate, and the absorber was placed in the box to prevent the formation of standing waves.

12Well Cell Culture Plate was sonicated from the bottom with acoustic wave intensities I_{SATA} = 0.16 to 0.64 W/cm^2 in the presence of the standing wave conditions and from the top with intensities 4 to 7 W/cm^2 - under no standing wave conditions. Cells were sown in every second well, altogether six wells were used. Four wells were insonified with the same intensities, leaving two wells as a reference.

An ultrasonic transducer was excited by the RF power amplifier (3100LA RF Power Amplifier, ENI, USA). The amplifier was controlled by a generator 33250, Agilent, USA. The transducer was excited with bursts of 1 MHz sine wave pulses, 1000 periods long, repeated every 2.5 ms (duty factor = 40%). The acoustic power was adjusted by changing the voltage amplitude at the output of the signal generator. The total acoustic power generated by the transducer was measured using Ultrasound Power Meter UPM model DT1A, (Ohmic Instruments, USA). The acoustic wave intensity I_{SATA} - space averaged, time averaged, was calculated according to the formula [19]:

$$I_{SATA} = \frac{P_A}{S} \tag{3}$$

where P_A is acoustic power and S is the transducer active aperture surface.

The next step was the determination of the ultrasonic intensity in the condition of free space and also with the standing wave. Measurements were performed at the distance of 0.75 mm above the bottom of 12 Well Cell Culture Plate. The distance of 0.75 mm corresponds to half a wavelength ($\lambda/2$) for a frequency of 1 MHz in water. Since the amplitude of the standing wave is repeated cyclically every $\lambda/2$, so the intensity of the wave at a distance of 0.75 mm from the bottom is the same as at the bottom of the plate, at the level of cells. Measurements were performed using the needle hydrophone, 0.2 mm diameter S/N 1661 (Precision Acoustics, UK) preamplifier and DC coupler with power supply DCPS142 (Precision

Acoustics, UK). Signal from the hydrophone was recorded using 62Xi Waverunner digital oscilloscope, (LeCroy, USA). The transducer was excited with bursts of 1 MHz sine wave pulses, 1000 periods long, repeated every 2.5 ms as for cell sonication. When measuring in free space, the pulses were repeated every 100 ms in order to eliminate multiple reflections in the container. The standing wave intensity was measured with 2.5ms pulse repetition rate as it was during the experiments with cells.

The intensity I_{SPTA} - space peak, time averaged was calculated according to the formula [19]:

$$I_{SPTA} = \frac{t_d}{T}\frac{1}{Z_0\, t_d}\int_{t_d} p^2(t)\,dt \quad = \frac{t_d}{T}\frac{1}{Z_0}\frac{v_{RMS}^2}{s^2} \tag{4}$$

where td – pulse duration, T – pulse repetition time, Z_0 – acoustic impedance of the medium, p(t) – acoustic pressure, V_{RMS} – voltage RMS value at the hydrophone output at time t_d, and s is hydrophone sensitivity (V/Pa).

2. RESULTS

The efficacy of cancer cell destruction (necrosis) as a result of applied ultrasonic intensity I_{SATA} is shown in Fig.3. Intensity thresholds were calculated at the level for which the cell viability was 50%. For 12 Well Cell Culture Plate this threshold was $I_1 = 0.3$ W/cm^2 for the standing wave and $I_2 = 0.3$ W/cm^2 without standing waves.

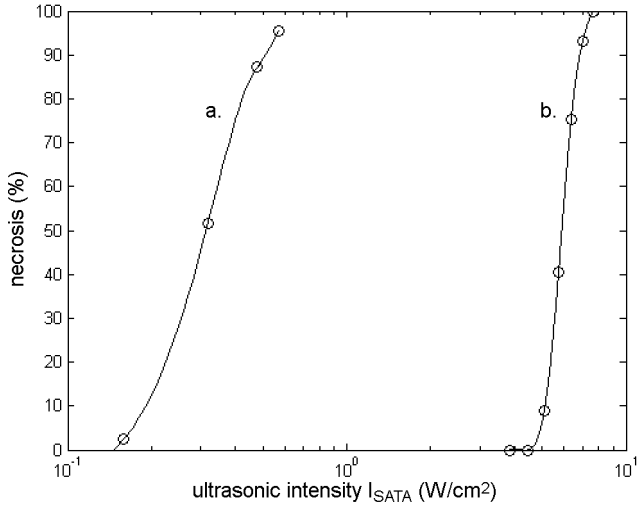

Fig. 3. Necrosis rate of C6 glioma cells after ultrasound treatment at 1 MHz for various intensities I_{SATA} from 0.16 to 7 W/cm^2. a. Standing wave condition, transducer positioned below the plate, reflection from the water-air surface. b. Free field condition, transducer inserted into the plate and absorber placed below the plate.

The I_{SPTA} intensities (Eq. 4) distribution across the ultrasonic beam were measured in both cases, in free field, 0.75 mm ($\lambda/2$) above the bottom plate without standing wave and with standing wave reflected from both surfaces, water and water–transducer interface. The 12 Well Cell Culture Plate is corresponded to a 8.25 mm distance from the surface of the transducer. The results of ultrasonic intensity I_{SPTA} measurements are shown in Fig. 4. For I_{SPTA} measurements, the results could be compared with the results of measurements of the degree of necrosis, so measurements were performed for the intensities $I_{SATA} = 0.3$ W/cm^2 and

5.8 W/cm^2. Those results are presented on Fig. 4, and images of the single well of 12 – well plate during sonication at different acoustical intensities are presented on Fig. 5.

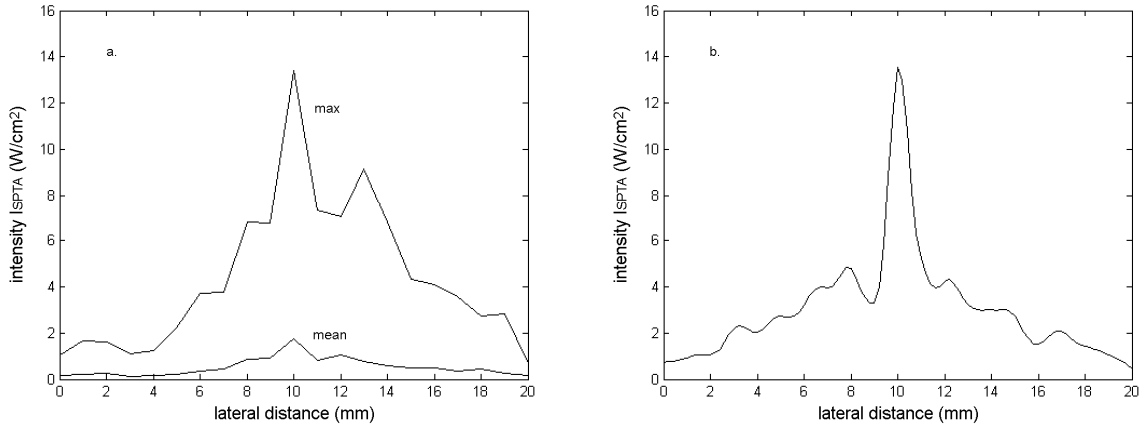

Fig. 4. Lateral acoustical intensities I_{SPTA} measured across diameter of one well of 12 Well Cell Culture Plate. a. Standing wave condition, mean intensity over 3 min sonication and max intensity - maximum intensity value recorded during 3 min sonication at I_{SATA} = 0.3 W/cm^2. b. Free field condition at I_{SATA} = 5.8 W/cm^2.

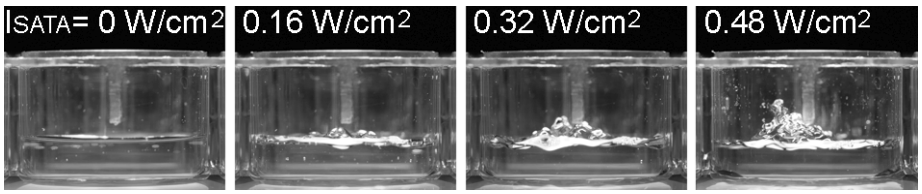

Fig. 5. Pictures of the one well of 12-well plate during sonication at intensities I_{SATA} = 0, 0.16, 0.32 and 0.48 W/cm^2.

3. CONCLUSION

The standing wave conditions effectively increases the intensity of the ultrasonic wave at the level of cells. It allows reducing the sound power radiated by the transmitter. To achieve 50% of cell viability, the intensity I_{SATA}, proportional to the power emitted by the transmitter, decreases from 5.8 W/cm^2 to 0.3 W/cm^2 which is 18 times more than the estimated value. The rationale behind this fact may be unsettled water surface in the well by the acoustic wave and the momentary focus of the ultrasonic beam at the level of the cell (Fig. 5). For free field conditions and the intensity I_{SATA} = 5.8 W/cm^2, the maximum intensity of I_{SPTA} was 13.5 W/cm^2 (Fig. 4b). A similar value of the maximum intensity was measured in the standing wave conditions (Fig. 4a). It should be noted that the average intensity in the same conditions was 7.5 fold lower at 1.8 W/cm^2. So for 50% necrosis of the population, there were enough single pulses with an intensity corresponding to the free field conditions. However for the standing wave conditions 100% efficiency cell destruction is not achieved. The explanation could be a small diameter transducer (20 mm) smaller than the diameter of the orifice of the 12 well plate (22 mm), and the intensity of acoustic waves near the edges of the hole was insufficient to destroy the cells. In the free field, at the intensity of 7 W/cm^2, all cells were destroyed, but the presumed cause was an excessive temperature rise from 37°C to over 60°C [13].

In summary, sonication of the cells from the bottom, in the standing wave conditions, can effectively and reproducibly destroy cells by ensuring the sterility of the multi-well culture plate with cells and without the risk of overheating.

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