ACTION OF TWO FREQUENCY ULTRASONIC FIELD ON IN VITRO CANCER CELLS

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The main aim of the present work was to investigate the possibility of enhancing the anticancer effect of low intensity ultrasound (US) thanks to an interacting ultrasonic field characterized by highly different frequencies. The occurrence of sonoluminescence and cytotoxicity of cancer cells was studied in vitro under the influence of a low frequency (LF) and high frequency (HF) ultrasonic field.

The experimental chamber was a stainless steel cylinder of 90 mm in diameter and 120 mm in height. Piezoceramic transducer with a titanium wave – guide (sonotrode) was used to generate low frequency (LF) US field with a resonance frequency 21.7 kHz. Diameter of the radiating surface of the sonotrode was 12 mm. The sonotrode was driven by the LF generator USG 5–22 (BSUIR, Minsk). US intensity (I) was estimated by the calorimetric method and was varied in the range 0.1–3 W/cm2.

The high frequency (HF) flat piezoceramic transducer of 25 mm in diameter with a resonance frequency F0 = 1.5 MHz was mounted at the cell bottom. It was driven by the HF generator USGH 8–3M (BSUIR, Minsk). The region of the chamber between LF and HF radiators was viewed through a 25–mm light–guide by a photomultiplier Philips XP 1110, whose output was labeled by L. It was driven by Philips high voltage supply PW 4025. The chamber containing cell suspensions 30 mm in diameter and 70 mm in height was mounted on the HF transducer by epoxy resin. HF field utrasound intensity in this inner chamber was 0.55 W/cm2. The photomultiplier output was connected to a Hewlett Packard 54601 multichannel memory oscilloscope. The liquid medium for the in vitro ultrasound experiments was physiological saline solution (NaCL 9 mg/mL).

The HT–29 human colorectal adenocarcinoma cell line (ATCC, Rockville, USA) has been cultured in Minimum Essential Medium Eagle supplemented with 2 mM L–glutamine, 100 UI/mL penicillin, 100 µg/mL streptomycin and 10% fetal bovine serum. After US exposure, 1.5 × 103 HT–29 cells have been seeded in 100 µL of growth medium in replicates (n = 8) in a 96–well culture plate and the effect on HT–29 cell growth has been evaluated using a WST–1 cell proliferation assay (Roche Applied Science, Penzberg, Germany). Cytotoxicity was expressed as a percentage with respect to control cells, i.e., untreated cells.

Figure 1a shows SL intensity versus US intensity (I) for the LF field alone.

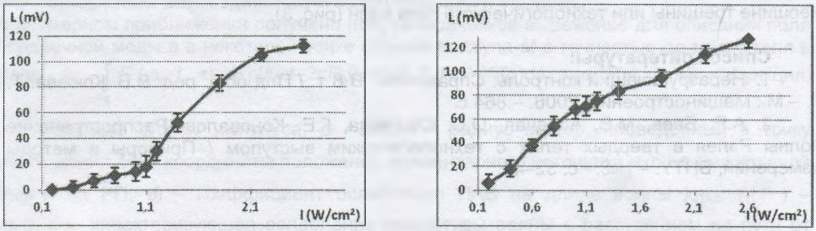


Figure 1 – Photomultiplier output (L) versus LF field US intensity (I). LF field alone (a) and HF+LF fields (b) with HF intensity 0.45 W/cm2

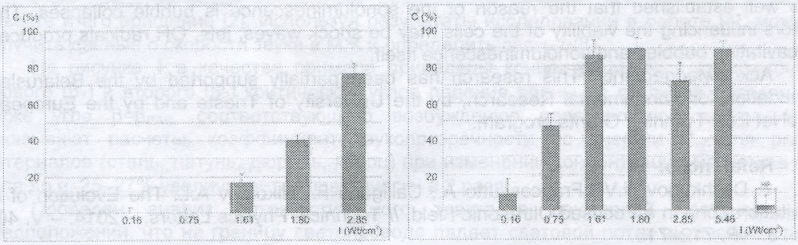
The SL intensity threshold (i.e., appearance of SL pulses) was around 0.3 W/cm2 and the SL intensity increased along with the US intensity (I) even if slowly up to 1.1 W/cm2

In accordance with a previous work [1], in this stage bubbles generating SL emission can be classified as stable. This means that they do not collapse intensively and SL emission

is of low level. The same is true for shocks generated by bubbles and OH radical production. At the second threshold of SL appearance (approximately 1.1 W/cm2) the slope of L(t) was changed in a sudden manner, and the SL intensity (L) increased significantly. In this stage of cavitation zone development sudden increase of L was accompanied by wider distribution of the recorded maximal values of the hydrophone output. Apparently, it can be accounted for the stronger bubble collapses and for the onset of an avalanche–like multiplication of cavitation bubbles causing a sharp increase in SL intensity. At low US intensity only one peak at F0 could be found in the cavitation noise spectra (not shown here). At low US intensities only one peak at F0 could be found in the spectra (not shown here). 2F0 peak appears at the first stage. After SL appearance at US intensity below 1.1 W/cm2 higher harmonics can be seen in the spectra. The stronger is the SL intensity during this stage of the cavitation zone development, the higher harmonics appear in the spectra. During this stage low level broadband signals sometimes appear in the cavitation noise spectrum as well. The harmonics are superimposed to the broadband component. After quick increase in SL intensity (I ≥ 1.1 W/cm2) wide–band is seen in acoustic spectra and its intensity increases with increasing the SL intensity. The intensity of the fundamental component and of the low harmonics tend to decrease after achieving maximum of the SL intensity.

Addition of the HF field to the LF field (Figure 1b) causes a strong enhancement of SL generation in the range of the LF field US intensities 0.2–1.2 W/cm2. In this range of US intensities L was increased 9 times at 0.4 W/cm2 and 5 times at 1 W/cm2. At higher LF field intensities, the increase of SL intensity after addition of the HF field was not so strong.

Figure 2 shows the results of cytotoxicity (C) measurements for the same experimental conditions showed in Figure 1. Figure 2a shows the action of the LF field alone and Figure 2b shows combined simultaneous action of both fields. The column extreme on the right in Figure 2b shows the cytotoxic effect of HF alone.



a b

Figure 2 – Cytotoxicity of HT–29 48 h post–treatment for different LF field intensities

When LF field was on alone (a) exposure period for all intensities was 2 min, except for 1.8 W/cm2. For the 1.8 W/cm2 it was 5 min. When both fields were on (b), i.e., for the interacting fields, exposition period was 2 min for all intensities excluding 2.85 and 5.46 W/cm2; for these two intensities it was 1 min (note that at these two intensities HF field was switched off, i.e., LF was acting alone).

At US intensities below 1.1 W/cm2 for LF field alone no cytotoxicity of cancer cells was observed (Figure 2a). A low cytotoxic effect was observed up to 1.61 W/cm2, after which the cytotoxic effect significantly increased along with US intensity. This is correlated with SL generation (Figure 1a) as SL intensity was very low at US intensities below 1.1 W/cm2 and significantly high at US intensity higher than 1.1 W/cm2.

In interacting fields (Figure 2b) cytotoxicity was observed at all the intensities studied as well as sonoluminesce (Figure 1b).

For cancer cells cytotoxicity (Figure 2b), a strong synergism was observed when the two US fields (LF + HF) worked simultaneously as well as for SL generation (Figure 1b). For example, for LF alone (Figure 2a) at I = 0.75 W/cm2 cytotoxicity was absent (C = 0) and for HF field alone C was approximately 2; when working simultaneously LF and HF fields at I =

0.75 W/cm2 cytotoxicity was more than 20 times higher than the simple sum of the cytotoxicity generated by each field separately. At higher intensities the cytotoxic synergetic effect decreased (Figure 2b) as well as sonoluminescence (Figure 1b).

In accordance with a previous work [2], the main factor of enhancement of cavitation activity in interacting fields is the generation of new cavitation nuclei upon bubble collapses in the LF field. By collapse the cavitation bubbles break down into fragments. The number of these fragments, i.e. smaller bubbles, can be rather big. Since their sizes are substantially smaller than the size of the initial bubble, these nuclei can be suitable for cavitation in the HF field. These new nuclei contain much less air than the initial bubbles from which these nuclei have been formed; therefore, they are likely to collapse in the HF field at a higher rate than the bubbles drown from the nuclei stable existing in the liquid. Thus, owing to this mechanism both the number of cavitation bubbles and the efficiency of their collapse can increase. The last can entail an increase in the maximum pressures and temperatures attained in the vapor–gas mixture inside bubbles and, as a consequence, an increase of the SL intensity and other cavitation effects as for example cytotoxicity in our case.

The reason for the decrease of the synergetic effect for both SL generation and cytotoxicity at high intensities of US may be that high bubble volume concentrations can be induced by the reasons discussed in a previous work [3]. Briefly, the main causes might be the screening action of cavitation and the inter–bubble impact. The increase of the intensity of bubble interactions will increase the probability of the bubbles deformation and their collapse in a non–spherical way. The latter decreases the efficiency of energy concentration by bubbles thus decreasing the cavitation activity.

In conclusion, a strong cytotoxic effect of US has been observed with the addition of the HF field to the LF field in the range of US intensities not much higher than SL thresholds for both US fields. The degree of correlation between the phenomena studied can be considered as a proof of the association of cytotoxic effect with transient cavitation, as far as it is well established that the reason of the sonoluminescence is bubble collapses. The factors influencing the viability of the cells may be shock waves, jets, OH radicals produced by cavitating bubbles and sonoluminescence itself.

Acknowledgements. This research has been partially supported by the Belarusian Foundation for Fundamental Research, by the University of Trieste and by the European IncoNet EaP Twinning Grants program.

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