Study of cellular response induced by low intensity ultrasound frequency sweep pattern on myelomonocytic lymphoma U937 cells

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Abstract

Purpose This study will analyze the mechanical effects (immediate lysis) and biological effects (cell survival, apoptosis, cell cycle) on U937 cells subjected to different sonication conditions with increasing and decreasing frequencies and burst rate (number of burst of a repeating signal in a specific time unit), in order to determine the best conditions of sonication to produce high mortality, apoptosis and inhibition of hyperproliferation.

Method Cells are been stressed by pulse wave ultrasounds with increasing and decreasing frequencies between 400 and 620 kHz, at burst rates of 0.5, 10, 50 Hz and 50 % duty cycle (percentage of one period in which a signal is active), ultrasound intensities (spatial average-temporal peak) 0.045 and 0.09 W/cm². The sonication durations were 90 and 180 s.

Results The decreasing mode was found to be better than the increasing mode for 10 and 50 Hz burst rates, while at 0.5 Hz the increasing mode gave better results for the time of 180 s. For 10 Hz burst rate, decreasing frequency, 180 s, 0.09 W/cm², 20 % survival rate was found; after 6-h incubation, cells showed 13 % of early apoptosis and 11 % of late apoptosis. For these conditions of sonication, the hyperproliferation of cells was inhibited.

Conclusion Survival rate decreases for increasing intensity and duration with each burst rate. The best performance is decreasing mode in a range between 620 and

400 kHz, duty cycle 50 %, burst rate 10 Hz. In these conditions after 180 s duration, the average survival rate is 20 %, the survived cells manifest apoptosis after 6-h incubation and hyperproliferation is prevented. The results seem to lead toward a non-invasive and effective purging of leukemic cells.

Keywords Low intensity ultrasound · Cell lysis · Hyperproliferation apoptosis · Cell cycle

Sommario

Scopo Questo studio analizzerà gli effetti meccanici (lisi valutata immediatamente dopo la sonicazione) e gli effetti biologici (sopravvivenza delle cellule, apoptosis e lisi cellulare) su cellule U937 soggette a differenti condizioni di sonicazione con frequenze crescenti e decrescenti, a burst rate (numero di impulsi di un segnale che si ripete in una specifica unità di tempo) variabili, al fine di determinare le migliori condizioni di sonicazione per produrre alta mortalità, apoptosi e inibizione dell'iperproliferazione cellulare.

Metodi Le cellule sono state sollecitate con ultrasuoni ad onda pulsata con frequenze crescenti e decrescenti fra 400 e 620 kHz a burst rate di 0.5 Hz, 10 Hz, 50 Hz, con un ciclo di lavoro (frazione di tempo in cui il segnale è attivo in proporzione al tempo totale) del 50 %, intensità (picco spaziale medio-temporale) 0.045 W/cm² and 0.09 W/cm². I tempi di sonicazione erano di 90 s e 180 s.

Risultati La modalità a frequenza decrescente risultava essere migliore di quella crescente per burst rate 10 Hz e 50 Hz, mentre a 0.5 Hz la modalità a frequenza crescente risultava migliore per un tempo di 180 s. Con un burst rate di 10 Hz, a frequenza decrescente fra 620 e 400 kHz, esposizione per 180 s, intensità 0.09 W/cm², fu rilevato un tasso di sopravvivenza cellulare del 20 %; dopo sei ore di

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incubazione le cellule mostravano il 13 % di apoptosi precoce e 11 % di tarda apoptosi. Nelle predette condizioni di sonicazione l'iperproliferazione cellulare era inibita.

Conclusione Il tasso di sopravvivenza delle cellule si riduce se l'intensità e il tempo di esposizione aumentano con ogni burst rate. La migliore performance si ha in modalità a frequenza decrescente in un intervallo fra 620 kHz e 400 kHz, ciclo di lavoro 50 %, burst rate 10 Hz. In queste condizioni dopo un'esposizione di 180 secondi il tasso medio di sopravvivenza è del 20 %, le cellule manifestano apoptosi e l'iperproliferazione cellulare è ridotta. I risultati sembrano condurre verso un'efficiente e non invasiva purificazione delle cellule leucemiche.

Introduction

It is known that low intensity ultrasounds induce cancer cell death, apoptosis in vitro [1, 2] and in vivo [3, 4] and stiffening of some cancer cells [5], while the healthy cells are more resistant than cancer cells to ultrasonic irradiation [6]. Since cancer cells with high metastatic potential are softer than healthy cells [7–9], cancer cell proliferation should be inhibited by low intensity ultrasounds. Ultrasound bio-effects are due to mechanical, thermal and cavitation contributions. The balance between above contributions changes with frequency: at low frequency mechanical and cavitation effects prevail, while thermal effect prevails at high frequency [10]. Thermal effects are related to the absorption of energy while ultrasounds propagate in a medium. Cavitation is the formation of vapor cavities in the cell solution. Cavitation may be inertial or stable [11–15]. The former occurs at lower intensity: the bubbles oscillate about some equilibrium size for many acoustic cycles. Inertial/transient cavitation is instead characterized by bubble collapse with consequent implosion, energy release and attainment of high pressures and temperatures. The process is characterized by threshold levels of acoustic field amplitude, frequency and bubble size [16]. Modifications of cell membrane can be induced even with levels of ultrasound intensity below the threshold of inertial cavitation. The space between two hydrophobic lipid monolayers inflates and deflates when it is exposed to ultrasound, stimulating cycles of stretch and release in membrane and cytoskeleton, activating mechanosensitive proteins [17].

Low acoustic pressures, less than 0.1 MPa, may already overcome attraction forces between the two lipid layers [18]. Optimal frequencies to produce membrane damage, apoptosis and inhibition of proliferation depend on geometry and mechanical properties of membrane and cells. Variable frequency may increase the probability of

drastically interfering with mechanical and dimensional characteristics of the cell. In connection with this, the aim of the study was to determine the best conditions of sonication to produce high mortality, apoptosis and inhibition of hyperproliferation of cancer cell line, by varying frequency.

Materials and methods

Ultrasound apparatus setup

The ultrasound generating apparatus used consists of 4 SonoPore KTAC-4000, (Nepa-Gene, Chiba, Japan). The KTAC equipment can generate frequencies from 200 to 5000 kHz with a fine adjustment of 1 kHz. The US generator can operate at fixed frequency in the continuous wave mode and in the pulsed wave mode, with a wide range of burst rate from 0.5 up to 100 Hz. Burst rate is the frequency with which the pulses are repeated.

The equipment has also several operating modes including “Increasing frequency”, indicated in the graphs as “sweep 1”, and “Decreasing frequency”, indicated as “sweep 2”, where the frequency gradually ranges, respectively, increasing and decreasing, during each ultrasound pulse output as shown in Fig. 1. The instrument allows to select voltage.

Fig. 1 Sweep 1 and sweep 2

The power reading is displayed during operating. At the same voltage during changing frequency, the power output varies in real time. At 60 V spatial average-temporal peak (SATP) is 90 mW/cm² while at 35 V it is 45 mW/cm². Spatial peak temporal average intensity is the maximum intensity occurring in an ultrasound beam averaged over the pulse repetition period (for pulsed ultrasound, the time from the beginning of one pulse to the beginning of the next).

The probes used in the experiments are the diameter 20 mm KP-S20 (Nepa-Gene, Chiba, Japan).

A short frequency range was selected to have a stable electrical signal and obtain the same balance between cavitation, mechanical and thermal effects at all frequencies, considering also that inertial cavitation threshold varies with frequency. The frequency range between 400 and 620 kHz, with a central frequency 0.510 MHz and sweep width 22 %, was judged the best for the above-mentioned purpose.

Sweep frequency width is the percentage of baseline frequency, which defines the range in which the frequency varies. In this case, frequency changes between 0.400 and 0.620 MHz.

The pulse type parameter used was sine wave.

The cells U937 were sonicated in multi-wells (Lumox Multiwell 24 TC Quality, Greiner-Bio). The plate bottom consists of a transparent film for ultrasounds. The plate was placed on top of the US unfocused transducer (diameter, 20 mm) after applying coupling gel (Pharmaceutical Innovations Inc., NJ, USA) between the transducer surface and the bottom of the plate. The distance from the transducer was less than 1 mm. In particular, four wells were simultaneously sonicated in the same sonication condition, placing the plate so that the four interested wells were above the four transducers and perfectly centered.

Cell culture and US treatment

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 °C in humidified air with 5 % CO₂. 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 10⁶ cells/mL and 2 mL were aliquoted in each well of Lumox Multiwell 24 TC Quality (Greiner-Bio). Dishes were placed in the sonication setup as mentioned before and tested at chosen parameters for 90 or 180 s. Following US treatment, cells were either subject to

viability testing or returned to incubator for 6 h before detection of apoptosis and cell cycle was done.

Cancer cells were submitted to mechanical stress produced by increasing and decreasing ultrasound frequency between 400 and 620 kHz. For this purpose, different burst rates (i.e., 0.5, 10 and 50 Hz) were used. The low values of power recorded during the experiment and intensity recorded in probe calibration phase seem to keep the phenomenon well below inertial cavitation threshold. The cells were subjected to sonication in pulsed wave of duration 180 and 90 s and intensity 90 and 45 mW/cm².

Thermal effects may be rated not significant as temperatures read by a digital meter connected to one of the four probes were always below 29 °C.

Measurement of cell viability

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 was pipetted in a counting slide and introduced in TC20TM Automated Cell Counter (Bio-rad, USA). The number of total and alive cells was displayed immediately on the monitor. Figure 2 shows typical cell patterns for different experimental conditions.

Detection of apoptosis and cell cycle

Apoptosis and cell cycle assays were detected with a TaliTM Image-Based Cytometer (Invitrogen by Life Technologies). Annexin V Alexa Fluor 488 and Propidium Iodide (Tali apoptosis Kit by Life Technologies) were used for apoptosis. Annexin V labeled with a fluorophore or biotin can identify apoptotic cells by binding to phosphatidyl serine exposed on the outer leaflet. Propidium iodide (PI) is a cell-impermeant and fluorogenic DNA binding dye used for identifying necrotic cells. PI is impermeant to live cells, but easily enters dead cells where it binds to nucleic acids and becomes fluorescent. Viable cells are both annexin V and PI negative; cells in early apoptosis are annexin V positive and propidium iodide negative; cells in late apoptosis or already dead are both annexin V and propidium iodide positive and only propidium iodide positive cells are dead cells.

After 6-h incubation in humidified air with 5 % CO₂ at 37 °C, cells were collected, centrifuged and deprived of supernatant. Cells were suspended in the 100 µL annexin binding buffer and 5 µL annexin V Alexa Fluor[®] 488 was added. After gently mixing, the annexin V Alexa Fluor[®] 488 mixture was incubated for 20 min, at room temperature, in the dark, then centrifuged and resuspended in

Fig. 2 TC 20 images. Sonication conditions: frequency ranging between 400 and 620 kHz and vice versa, duty cycle 50 %, burst rate 10 Hz, sweep interval 0.2 ms, duration 180 s. The red cells are died, the green cells are alive. The slide chamber (10 μ L) keeps less than

0.5 % of the cell samples from a total of more than 2 million cancer cells. The TC 20 counts less than 0.02 % of the cells in the well (volume counted in each chamber is 0.4 μ L)

100 μ L Annexin binding buffer. 1 μ L TaliTM propidium iodide was added to each 100 μ L sample and properly mixed. The samples were incubated at room temperature in the dark for 5 min and then analyzed with TaliTM Image-Based Cytometer. Apoptotic cells showed green fluorescence, dead cells showed red and green fluorescence (observed as yellow), and live cells showed low or no fluorescence.

The cell cycle analysis was performed using the same TaliTM Image-Based Cytometer. The number of cells in each phase of cell cycle (G1, S, and G2/M) was determined using an optimized solution of propidium iodide (Tali[®] Cell Cycle Assay by Life Technologies). Propidium iodide produced a fluorescence signal proportional to DNA mass that directly indicates which cycle phase cells are going through. Immediately after sonication and after 6-h incubation, cells were collected, washed, fixed with ice-cold 70 % ethanol in distilled water, placed at -20°C overnight, washed, stained with the Tali[®] Cell Cycle Solution, and analyzed using the Tali[®] Image-Based Cytometer.

Statistical analysis

The 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. The survival rate is the complement to 100 of this value. In all experiments, the cells with diameter smaller than 6 μm or larger than 18 μm were not considered; this allowed cell fragments to be excluded.

Experimental data gathered in this study will be presented as the mean $\pm$ standard deviation (SD).

Statistical analysis was carried out using Student's *t* test and *P* values less than 0.05 were regarded as significant.

Results

It was observed that sonicating cells at 90 mW/cm^2 , duration 180 s, sweep 2 (decreasing mode) led to kill more cells than sweep 1 (increasing mode) for burst rates 10 and 50 Hz. However, for burst rate 0.5 Hz, increasing mode seems to be more effective than decreasing mode. In particular, at burst rate 10 Hz, average survival rate in decreasing mode was 20.2 % while in increasing mode it raised to 39.4 %. The same behavior was seen at 50 Hz: for sweep 2, survival rate is 23.2 % while for sweep 1 survival rate is 48.6 %. At 0.5 Hz burst rate, increasing mode became more effective allowing 10 % more cell killing than decreasing mode: the corresponding survival rates are 33.1 % (decreasing mode) and 24.4 % (increasing mode). Experimental data are summarized in Fig. 3.

Increasing the burst rate from 10 to 50 Hz leads to kill less cells for intensity 90 mW/cm^2 and 180 s duration.

Fig. 3 Cell survival rate ultrasound intensity SATP 90 mW/cm^2 at different burst rate. Frequencies ranging between 400 and 620 kHz and vice versa, Duty cycle 50 %, burst rate 0.5 Hz (dashed), 10 Hz (dotted), 50 Hz (continuous), sweep interval 0.2 ms, duration 180 s

Fig. 4 Cell survival rate burst rate 10 Hz. Frequencies ranging between 400 and 620 kHz and vice versa, Duty cycle 50 %, sweep interval 0.2 ms, duration 90 s. Ultrasound intensity SATP 90 mW/cm² (continuous), 45 mW/cm² (dotted)

Fig. 6 Cell survival rate 0.5 Hz. Frequencies ranging between 400 and 620 kHz and vice versa, Duty cycle 50 %, sweep interval 0.2 ms, duration 90 s. Ultrasound intensity SATP 90 mW/cm² (continuous), 45 mW/cm² (dotted)

Fig. 5 Cell survival rate burst rate 50 Hz. Frequencies ranging between 400 and 620 kHz and vice versa, Duty cycle 50 %, sweep interval 0.2 ms, duration 90 s. Ultrasound intensity SATP 90 mW/cm² (continuous), 45 mW/cm² (dotted)

Fig. 7 Apoptosis assay burst rate 10 Hz. Annexin V Alexa Fluor 488 and propidium iodide assay for frequencies ranging between 400 and 620 kHz and vice versa, duty cycle 50 %, sweep interval 0.2 ms, duration 180 s, ultrasound intensity SATP 90 mW/cm². Alive cells (first bar), dead cells (second bar), early apoptosis (third bar), late apoptosis (fourth bar)

However, the 0.5 Hz burst rate kills more cells than 10 and 50 Hz burst rates in sweep 1.

The same trend can be observed for the duration of 90 s (Figs. 4, 5). In particular, at 90 mW/cm² and 10 Hz burst rate, survival rate for increasing mode was 64.5 % but dropped to 48 % for decreasing mode. At burst rate 50 Hz, increasing mode yield 73.5 % survival rate versus only 38 % of decreasing mode. At 0.5 Hz burst rate, intensity 90 mW/cm², and 90 s duration, decreasing mode returns more effective than increasing mode: in fact, sweep 1 yields a survival rate of 67.8 % while sweep 2 yields a survival rate of 45 % (Fig. 6).

At 45 mW/cm², there was never a significant difference in cell killing between sweep 1 and sweep 2. The response of cells to sonication at 90 mW/cm² is more significant than that at 45 mW/cm². Cell killing rate increases with duration and intensity. From power output detection at increasing or decreasing frequency in a range between 400

and 620 kHz, duty cycle 50 %, 10 Hz burst rate, sweep interval 0.2 ms (sweep interval is defined as the resolution of frequency sweep change), duration 180 s, 90 mW/cm² intensity we detected a maximum power output of 0.7 W, which leads to assume to be below the threshold of inertial cavitation. This is confirmed by the fact that temperatures were always below 26 °C (detected in sweep 1 with a room temperature 24.6 °C).

Microscopic observations of the culture after 6-h incubation in humidified air with 5 % CO₂ at 37 °C revealed that cells do not show autofluorescence. Sonication conditions were as follows: frequency ranging from 400 to 620 kHz and vice versa, duty cycle 50 %, burst rate 10 Hz, sweep interval 0.2 ms, duration 180 s, intensity 90 mW/cm².

Experimental data are summarized in Figs. 7 and 8.

Fig. 8 Cell cycle. After sonication and after 6-h incubation for frequencies ranging between 400 and 620 kHz and vice versa, duty cycle 50 %, Burst rate 10 Hz, sweep interval 0.2 ms, duration 180 s, ultrasound intensity SATP 90 mW/cm²

Fig. 9 Apoptosis assays burst rate 0.5 Hz. Annexin V Alexa Fluor 488 and propidium iodide assay for frequencies ranging between 400 and 620 kHz and vice versa, Duty cycle 50 %, sweep interval 0.2 ms, duration 180 s, intensity 45 mW/cm², alive cells (first bar), dead cells (second bar), early apoptosis (third bar), late apoptosis (fourth bar)

Early and late apoptosis was detected in cells exposed to ultrasounds in both increasing and decreasing modes in greater extent than in the control. Again decreasing modes was much more effective than increasing mode. For 10 Hz burst rate, decreasing frequency, 180 s, 0.09 W/cm², after 6-h incubation, cells showed 13 % of early apoptosis and 11 % of late apoptosis, against 9 % of early apoptosis and 5 % of late apoptosis in decreasing mode and about 3 % of early apoptosis and 1 % late apoptosis in the controls.

At burst rate 0.5 Hz, intensity 45 mW/cm², duration 180 s, with sweep 2, survival rate was 87.7 %. Interestingly, no significant differences between increasing mode, decreasing mode and control apoptosis were observed (see Fig. 9).

Discussion

Mechanical, morphological and functional changes in the cell may be correlated with changes in ultrasound frequency and intensity.

The influence of burst rate on the effect of 1 MHz fixed frequency sonication was analyzed in previous works [19]. At low burst rates (<5 Hz), sound streaming (convection, flow of matter due to the acoustic radiation) prevails while at higher burst rate standing waves prevail. Buldakov et al. [19] observed that gap-time length increasing between pulses (off-time) by low value of burst rate determines stirring of the solution and interrupts the surface: this reduces the possibility of standing wave formation. In this case, cells more likely will be in contact with oscillating cavities and the effects are more random and less investigable.

By decreasing off-times, stirring of the solution tends to disappear. The solution seems to be continuously irradiated allowing standing waves to form. For high values of burst rate (10 and 50 Hz), effects related to the ultrasound streaming can be disregarded as standing waves are well established.

In this case, the higher mortality observed at varying frequencies may be caused by frequency. Low intensity values let presume to be below the threshold of inertial cavitation. The space between two hydrophobic lipid monolayers of cell membrane inflates and deflates with a certain frequency. The acoustic pressure has oscillatory behavior between the “negative” pressure phase for which the two layers move away from each other thus overcoming molecular attraction forces, inertial forces of surrounding water and viscous forces, and the “positive” phase where the opposite occurs. These periodic expansions/contractions may stimulate cycles of stretch and release in cell membrane and cytoskeleton that may activate mechanic-sensitive proteins, increasing cell permeability and biological effects.

The membrane exposed to low intensity ultrasounds becomes a mechanical oscillator and source of intracellular cavitation activity, in a way very similar to a gas bubble in an acoustic field.

Optimal frequencies to produce membrane damage, apoptosis and inhibition of proliferation depend on geometry and mechanical properties of membrane and cells.

Each frequency will interact differently with mechanical and geometric properties of the cell and its membrane: frequency changes, regardless of using the decreasing mode or the increasing mode, improve the probability of drastically interfering with mechanical and dimensional characteristics of the cell and cause the reduction of the average diameter of the cells population, destroying cells

of specific diameter after sonication. It was observed that even the sonication at constant frequency induces the shrinking of the cells [20, 21].

The best performance of decreasing mode respect at increasing mode is hardly explicable, since nothing let us assume that the decreasing mode favors constructive interference. The only possibility is that in decreasing mode higher power peak is available.

In previous studies [22], low intensity ultrasound apoptosis was related with membrane damage.

According to this model, the degree of membrane damage and the ability of the cells to repair the damage determine the mode of cell death. Cells able to successfully repair the damage may eventually survive, the others trigger DNA fragmentation which leads to apoptotic death. Sometimes, the cell dies before having fully repaired the damage: hence, it comes to late apoptosis.

From cell cycle analysis, it is noted that the S-phase of DNA replication dominates in both initial and final control assays.

In the decreasing mode, the G1 phase tends to dominate. This may suggest that in the present experiments it was inhibited duplication of the DNA and then cell proliferation. This arrest is related to DNA damage induced by sonication. Consequently, also the number of cells that are replicating DNA is smaller.

Cell cycle and apoptosis assays show a consistent behavior. In fact, in case of DNA damage, the p53 protein is activated. The active form of p53 accumulates and binding to the regulatory region of the p21 gene (protein inhibitor of Cdk) stimulates the transcription of the gene itself. The p21 protein binds to both G1/S-Cdk both S-Cdk, turning them off, so that the cycle stalls in G1 [23]. This allows cell to induce repair processes, if possible. If DNA damage is substantial and irreparable, p53 can induce cell suicide, apoptosis. In the decreasing mode, we detected an increasing in apoptosis and late apoptosis.

Conclusion

Sonication at decreasing frequency in a range between 620 and 400 kHz, duty cycle 50 %, burst rate 10 Hz, sweep interval 0.2 ms, induces 20 % survival rate after 180 s duration. As survival rate exhibited inverse dependence from time, it can be hypothesized that a 100 % killing rate can be achieved with a longer duration.

The same sonication condition inhibits duplication of the DNA and cell proliferation. Furthermore, apoptosis (13 %) and late apoptosis (11 %) were observed after 6-h incubation. Since other study report on optimal apoptosis detected after 12-h incubation [2], it is expected to have better results with a post-treatment longer incubation.

Consistent results were obtained from three completely different biological assay methods (cell survival, apoptosis and cell cycle).

Therefore the mechanical effect (80 % cell killing rate) and biological effect (apoptosis and inhibition of hyperproliferation) observed submitting myelomonocytic lymphoma cells to ultrasound at decreasing frequency from 620 to 400 kHz with 10 Hz burst rate, duration 180 s and intensity 90 mW/cm², may be interesting for a non-invasive and effective purging of leukemic cells.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest.

Human and animal studies The study described in this article does not contain studies with animal and human subjects performed by any of the authors.

Informed consent For this type of study formal consent is not required.

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