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REVIEW

BIOEFFECTS OF LOW INTENSITY ULTRASOUND: A SOUND IDEA POTENTIALLY USEFUL FOR THERAPY

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Ultrasound has been in nature long before science discovered it. Defined as sound waves having frequencies too high (above 20 kHz) to be heard by human ear, ultrasound is being used by some animals like dolphins and bats in navigation and hunting for preys; the concept utilized for SONAR (Sound Navigating and Ra nging)－discovered by Pierre Curie in the 1880s. In medicine, the ultrasonic era began with ultrasound technology used for therapy rather than diagnosis. In the 1940s, ultrasound was employed in cancer therapy and later also used in physical therapy. Progress in radiation therapy and chemotherapy eventually phased out ultrasound technology as a treatment option for a number of medical conditions. Initial work on ultrasound imaging started in the late 1940s. Advances in piezoelectric materials improved the ultrasound from gray-scale imaging to real-time imaging. While the recent advances in diagnostic ultrasound is not only rapid and finding a widening use in all medical fields, other uses of ultrasound is continuously being unraveled in various scientific fields; producing outcomes useful to industries, households and even to individuals.

The expanding use of ultrasound led to more and wider research works on the mechanism by which ultrasound interacts with matter, especially on living cells and tissues[1-9]. Equipped with modern technologies and more improved methodologies, medical researchers are now looking back to its potential for therapy. Studies on bioeffects, chemical effects and thermal effects of ultrasound have revealed promising results[4-6]. To explore further the ultrasound's potential for therapy, our group aims to attain, a.) to find an optimal condition to induce cell killing at a certain desired mode of cell death, b.) to find ways to enhance or modulate the bioeffects based on the identified endpoints, c.) to elucidate mechanism of the bioeffects, and d.) to help design a protocol for possible clinical application, especially for cancer therapy.

Materials and Methods

Cells and cell culture

Different types of cancer cell lines were grown in vitro. In culturing the cells, some cell types are suspended in a liquid culture medium (e.g. U937 cells), some types are attached in a plasma treated dish (e.g. HeLa cells), while some cell lines require passaging (inoculating into mice then collect back after sometime) (e. g. Meth A cells).

Ultrasound Apparatus

Different ultrasound machines with different frequencies (48.0 kHz-2.0 MHz) intensities (0.1-5.0 W／cm^2), duty factors (5-100%), pulse repetition frequencies (0.5-100 Hz), and with varying setups were used.

Methods to determine cell killing

Cell killings were assayed by several methods. Cell lysis and loss of viability were determined by direct microscopy with Trypan blue dye exclusion test. Trypan blue is a dye that stains the nonviable cells to blue but not the viable ones. To measure apoptosis and necrosis, cells were stained with fluorescent dyes: Annexin V-FITC and propidium iodide (PI), before doing flow cytometry. The flow cytometer will count cells and detect those stained with annexin V-FITC only (apoptotic cells) and those stained with both Annexin V-FITC and PI (necrotic cells). Apoptosis is a programmed cell death utilized by human body for homeostasis or to maintain physiologic balance. This form of cell death is efficient and is preferred in most therapy that requires cell killing.

DNA transfection

Luciferase assay was employed to assess ultrasound-mediated DNA transfection of cells. DNA transfection by electroporation and lipid mediated transfection were also done for comparative studies and also on combination treatment experiments.

Evaluation of the mechanism of bioeffects

To investigate the mechanisms by which ultrasound induces bioeffects, both the physical and the molecular aspects were evaluated. Electron microscopy was done to visualize greatly enhanced details of the damage induced. Fluorescent probe Fura2-AM was used to determine the concentration of free calcium ions in the cells. For other cellular ions, ion microscopy (officially known as secondary ion mass spectroscopy or SIMS) was also done. For the molecular component of apoptosis, determination of Caspase-3 and mitochondrial membrane potential by flow cytometry were conducted. Analyses of some proteins involved in apoptosis were done using some methods including western blot analysis.

EPR-spin trapping for the detection of free radical formation

Free radical formation, especially hydroxyl radicals derived from sonolysis of water, is an indicator for the occurrence of inertial cavitation during sonication[8,10]. Electron paramagnetic resonance (EPR)-spin trapping with 5,5-Dimethyl-1-pyrroline N-Oxide (DMPO) or 3,5-dibromo-4, nitrosobenzenesulfonate (DBNBS) to detect US-induced free radical production was employed (Fig. 1).

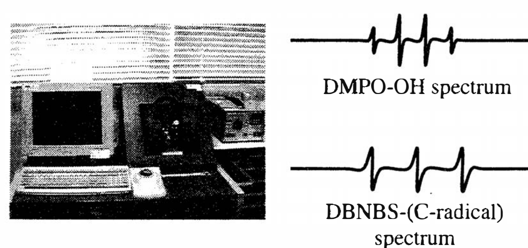

Fig. 1. EPR machine (left). Typical EPR signal showing characteristic spectrum of DMPO-OH (top right) and DBNBS-Carbon radical (below right).

Results and Discussion

We have confirmed previous findings that low intensity ultrasound can induce cell killing even without significant temperature rise and even at very low intensities. Some factors that enhance these effects and factors that inhibit them were identified and characterized.

Nonthermal ultrasound enhances hyperthermia induced apoptosis

Hyperthermia (40-44℃) is known to induce apoptosis and is being used in medicine to treat tumors. Our study shows that the hyperthermia-induced apoptosis can be enhanced by ultrasound at intensities even below threshold for cell killing[4,9]. Since ultrasound is currently being used to generate heat for hyperthermia therapy, our data help explain why ultrasound was shown to be more effective than other technology used to generate

hyperthermia (e.g. microwave).

Echo-contrast agents enhance ultrasound-induced cell killing and DNA transfection

Echo-contrast agents are usually composed of engineered microbubbles that are injected to the patient before sonographic evaluation to augment echo signals. Considering that these microbubbles are potential nuclei for cavitations, we hypothesized that it can potentiate ultrasound-induced bioeffects. We used different microbubbles (Levovist, Optison and YM454) in our study. Our data showed that Levovist is effective in enhancing ultrasound-mediated gene transfection[11], while in a different study we have shown that Optison and YM454 are effective in enhancing cell killing[3]. The mechanism should include transient pore formation, especially in gene transfection and enhanced cell membrane damage in the cell killing aspect. The main factors involved are the stability and size of the microbubbles as these are responsible in creating inertial cavitation (acoustically generated oscillation and collapse of bubbles).

Ultrasound-induced cell killing enhanced by some agent

Synergistic effect between ultrasound and some agents, especially anticancer drugs, were observed. In one of our studies we used a temperature dependent free radical generator, AAPH, in combination with ultrasound[12]. Cell killing was enhanced and free radical generated by AAPH was increased by sonication. Data also indicated that increased uptake of the agent rather than the increased free radical production was responsible in the enhancement.

Hypotonia enhances ultrasound-induced cell killing

Hypotonia (146 mOsm) can induce nonlethal swelling of cells. This osmotic cell swelling was found to enhance ultrasound-induced cell killing[1]. Although low viscosity can modify acoustic cavitation formation, our data showed that it did not play an important role in the enhancement but rather the mechanical effect of radiation force on the swollen cells. As described by Nyborg (1968) that a force F towards the direction x, acting on a particle of volume v in a liquid medium where radiation pressure (as in acoustic streaming and microstreaming) is applied, F is given by

$$F = v\,(1-\beta)\,\delta T(\delta x)^{-1}$$

where T is the time-averaged volume density of kinetic energy, while $\beta = \rho_o/\rho$, is the density ratio. Here, ρ_o is the density of the medium, and ρ is the density of the particle (cell)[13].

Addition of hypotonic medium into the cells will result to a decrease in β value, thus increasing the value F. This is followed by cell volume increase which will result to a directly proportional increase of the value F. This would mean that a 3 x increase in cell volume (at about 5 min of hypotonia), would result to more than 3 x increase in magnitude for F when combined with the density effect.

It has also been known that cells swell in response to hypotonia, with the cell membrane as the major player of this event. Nyborg extensively described tension on a cell membrane under the influence of internal and external pressures. For a membrane of thickness h the quantity of tension σ is defined by,

$$s = S_o h,$$

where S_o is the stress in the function of pressure (P) acting on the membrane. Furthermore, if membrane thickness h is much less than the cell radius R ($h << R$), tension can be expressed by,

$$2s = R\,(P - P_{ext}),$$

where P is the pressure on the interior of the cell and P_{ext} the pressure outside, while $P-P_{ext}$ is denoted by ΔP. In the case of hypotonic cell swelling, R increases as ΔP increases due to increase in P, thus increasing tension on the membrane. In addition, in the strained membrane, an increased spacing between particles along the axis parallel to the membrane is expected.

Changes in cell shape and size may occur as ΔP increases; the cell may burst at some critical value of ΔP.

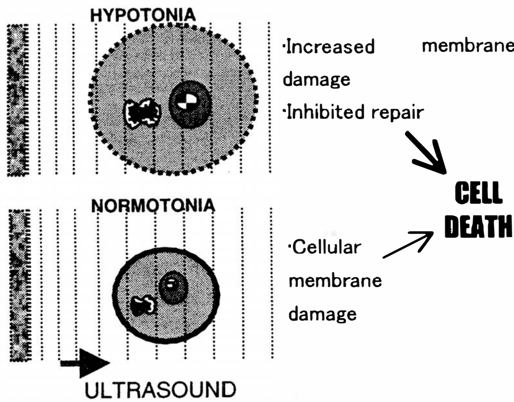

Fig. 2. Mechanism of enhanced ultrasound-induced cell killing by hypotonia.

In addition to increased membrane damage, in hypotonia, cells were shown to have poor membrane repair ability (Fig. 2). This was supported by ion images[1].

Ultrasound-induced cell killing is inhibited by carbon dioxide

Dose dependent inhibition of ultrasound effects (both bioeffects and chemical effects such as free radical production) was observed when we used equal doses of HCl and H_2CO_3 to generated measurable concentration of CO_2 in the medium used[14]. It is known that CO_2 lowers the final temperature of collapsing bubbles since it has a lower value of $\delta = C_p / C_v$. For the adiabatic collapse of a cavitation bubble, the final intracavity temperature at the end of the collapse, T_f is given by

$$T_f = T_i (R_{max} / R_{min})^{3(\delta - 1)}$$

Where T_i is the initial temperature, ? is the specific heat ratio (C_p / C_v) of the gas inside the bubble; R_{max} is the initial radius of a bubble which collapses to a final radius of R_{min}. Figure 3 below illustrates this concept.

This finding implies how handling of cell samples is important in experiments related to ultrasound effects, while also guides researches to consider CO_2 concentration in the living body when doing in vivo studies.

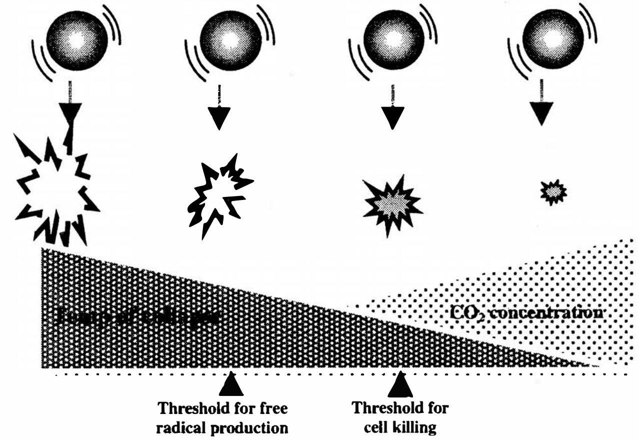

Fig. 3. Dose dependent inhibition of ultrasound-induced cell killing and free radical production due to the decreasing energy of cavitation collapse associated with increasing CO_2 concentration.

Related studies in progress

Based on the above hypothesis on the mechanism of cell killing induced by ultrasound[15], we believe that certain conditions would optimize killing on a desired mode of cell death, e.g. apoptosis. Initial trials have indicated some promising results. We have succeeded killing 80% of cells without significant cell lysis; which means limiting the killing to apoptosis or necrosis. As to ultrasound-induced apoptosis, we have gathered considerable amount of data on its molecular mechanism. In addition, optimal condition for ultrasound-mediated DNA transfection[16] is also providing us data that can apparently compete with other methods currently being used in transfection for gene therapy. In another study we have shown that incubating cells for sometime would inhibit cell killing most likely by accumulation of CO_2 within the medium associated with consumption of other dissolved gasses; and at very high cell density, cell killing is also inhibited[17]. However, these two inhibitory factors would not prevent cell killing if microbubble is added before sonication.

In summary, our data provide information on the potential of ultrasound in therapy (Fig. 4), alone or in combination with other modalities.

The factors that enhance the effects are may

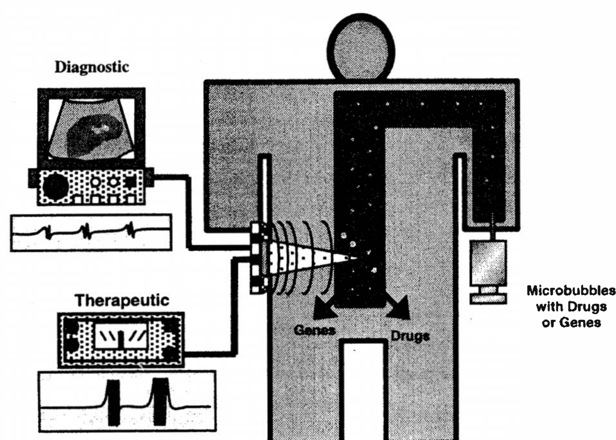

Fig. 4. A schematic diagram of a simultaneous use of therapeutic and diagnostic ultrasound clinically.

be useful in cases where effects of ultrasound are limited; while inhibitory factors may find their use as modulator in the therapeutic process. These informations also gave us better understanding of the mechanism of how ultrasound works and the possible problems that may be encountered when applied in vivo or clinically.

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