THE DEPENDENCE BETWEEN ULTRASONIC EXPOSURE TIME AND BIOLOGICAL REACTIONS IN THE PARAMECIUM

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ABSTRACT

The effects of ultrasonic irradiation at different frequencies, i.e. 0.25, 0.5, 1, and 5 MHz, on the activation of the single cell creature biological reaction have been investigated. The ability shown by ultrasound in promoting and/or accelerating many reactions has been shown to be a useful field. The resonance frequency of Paramecium by using the ultrasound irradiation is an important parameter in this study. All other parameters being keep constant, it has been ascertained that an appropriate frequency value of ultrasound can be selected, capable of driving a biological reaction to its suitable yield. The oscillation of the cells in response to the ultrasound radiation is simulated using Rayleigh-Plesset's bubble activation theory. The resonance frequency of the unicellular creature is then calculated. In our experiment, the resonance (0.5 and 1 MHz) and non-resonance (0.25 and 5 MHz) frequencies were employed.

The resonance frequency of the Paramecium vacuole is among 0.5013 ~ 1.2703 MHz. When the 0.5 and 1 MHz frequencies of ultrasound was irradiated in the samples, the relative growth rate was about 30% higher than that of unexposed sample. It is obviously that the inhibition or enhancement growth conditions did not appear during irradiation the non-resonance frequency ultrasound. In addition, experimental evidence suggests that the sustaining growth effect can be expected, when the irradiation time is divided into parts.

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1. INTRODUCTION

The use of ultrasound within the biological or medical application has been a subject of research and development for many years. In 1927, Wood and

Loomis [1] was the first one using the ultrasound to affect living tissues, they found that ultrasound could produce harmful effects such as the injury and cell death of the small frogs or fish, and the disruption of plant cells. Since then, various interactions between megahertz ultrasound and mammalian tissues have been reported on the investigation of growth and structural alterations. Recently, ultrasound has had extensive application for diagnostic purpose, becoming the modality of choice for many soft tissue examinations. Although the clinical use of ultrasound

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as a diagnostic tool has been widespread, it is increasing interest in regarding the ultrasound induced biological effects, which have demonstrated the potential both to damage and/or stimulate tissues. It must be realized that by careful regulation of the acoustic parameters, the damaged or beneficial results could be available. The acoustic parameters are including the frequency, intensity, waveform or exposure time and so on. This paper is to seek the correlation between ultrasound exposure and the Paramecium biological reaction, as an indication of fundamental interactions between the acoustic parameters and cells in suspensions.

Much interest has arisen around the point of whether or not the bubble involved in the cell can stimulate the cellular structure alterations. The observations of Coakley and Hampton [2] on the suspension of amoeba, which possesses many features typical of higher-order animal cells, was irradiated with 1 MHz ultrasound. The ultrasound-produced cavitations were found the correlation between the number of discrete cavitations occurring and the decrease in cell numbers, on irradiating ultrasound at 515 W/cm² for 10 minutes. Kaufman and Miller [3] used the continuous ultrasound of 1 MHz to expose the suspensions of Chinese hamster V-79 cells for 1 minute at the axial intensity of 2.5 W/cm². They found that there was little growth, as determined by changes in the number of attached cells, for 24 hours after sonication, then the cells will return to normal growth approximately 36 hours after sonication. The lack of growth was appeared due to an approximate balance between proliferation of viable cells and loss of non-viable cells into the medium during the first 24 hours.

On studies of suspensions of cells, a completely different mechanism can be used. Such as the production of destructive bubble activity, called cavitation, it would occur in a liquid medium when the ultrasonic intensity is sufficiently high. In Miller's work [4], the main factors of ultrasound, which induced the biological effects, are sound frequency and intensity. Most effects, including the cell death and the growth decrease, are more strongly dependent on the incident intensity than on other parameters such as exposure duration or total incident energy. The effects of ultrasonic irradiation have been ascertained that an appropriate frequency of ultrasound can induce a sonochemical reaction [5]. A direct explanation of the fundamental role played by ultrasound frequency shows that such an effect will occur when bubbles are at the suitable size or the radius are at the center of a statistical distribution curve. It is also made possible by driving the bubbles' field from its equilibrium distribution state to a different one suitable for transient cavitation to take place.

The purpose of this paper is then to explore the

relationship between the ultrasound frequency and the activation of the Paramecium at its growth condition. Many studies have suggested that ultrasound can active the soft connective tissue or bone, accelerate and augment the healing of the fracture part. In vitro study, the authors reported increased prostaglandin E₂ production with ultrasound exposure in mouse osteoblasts and suggested this as a possible mechanism in fracture healing [6]. Similarly, Kouji and associates [7] were interested in ST2 cells of bone marrow stromal origin by using the low intensity of pulsed ultrasound. Their results supported that the clinically used equipment to accelerate the healing processes of fractured bone, induces a direct anabolic reaction of osteogenic cells, leading to bone matrix formation. Furthermore, Jamie and colleagues [8] examined the effects of a single 5-min CW exposure of 3 MHz ultrasound on the growth and functional activity of a human osteoblast-like cell line (MG63 cells) and human periodontal ligament (PDL) cells in vitro. Their results showed that ultrasound has potentially important effects on the functional activities of connective tissue cells in vitro, which could markedly influence tissue repair and regeneration processes in vivo. As mentioned above, the acceleration of the normal fracture repair process or the activation of the damaged tissue cells was focused on the multi-cell creature. When the multi-cell creature is exposed to ultrasound, the reaction will lead the biological effect becomes complex. It would be difficult to examine the precise effects of ultrasound on fundamental cellular and molecular processes involved in the repair and regeneration of either hard or soft tissues. Thereby, a single cell creature was an appropriate choice for this study. The Paramecium, which possesses many features typical of higher-order animal cells, is an appropriate choice for this study. Moreover, reporting on the effects of ultrasound on Elodea at 1 MHz, Miller [9] noted that the cells near the gas body of the leaves had a considerably lower cell death threshold than any other cells. In 1979, Miller investigated the biological effects of the Elodea leaves in ultrasound fields for 100 sec in the frequency range 0.45 to 10 MHz, and found that the intensity thresholds were strongly dependent on frequency [10]. It can be explained that the gas bodies near these cells are more strongly activated than the others, because these gas bodies are resonant at the resonance frequencies. One would expect strong activation of the cells in suspensions only when the sonication frequency is at or near the resonance frequency of the cells. In this paper, the physical theory for the oscillation of the bubble mode in response to the ultrasound radiation is adapted from Rayleigh-Plesset's bubble activation theory. There are four different ultrasonic transducers were employed in the experiment, which were operated

at central frequencies of 0.25, 0.5, 1, and 5 MHz, respectively. All insonated samples were exposed to ultrasound with a spatial peak temporal peak intensity (SPTP) of 0.127 mW/cm². Cell proliferation assay was used as a model to discuss the biological effect of ultrasound. The growth rate for each of the populations examined, are described and compared to predictions based on the resonance theory.

2. THEORY

The response of a bubble to a sound field is calculated evaluating mainly the steady-state solutions. Frequency response curves are given for different pressure amplitudes of the sound field as well as for different bubble radii. For sufficiently high-pressure amplitude, no steady-state solutions may be obtained within a reasonable time because of the violent change of the oscillation. Thus the bubble behavior at higher sound pressure level cannot be given in terms of response curves based on steady-state solutions.

The low amplitude oscillations of bubbles were able to characterization by linear resonance theory of simplified model system. This is the same theory that can describe the oscillations and resonance of a mass on a spring with damping. The model describes the low amplitude pulsation of free spherical bubbles as discussed by Coakley and Nyborg [11]. Since the bubble activation is typically studied under low power conditions, the relatively simple linear theory is adequate for analyzing most experimental results.

A reasonable definition of bubble dynamics is: the induction and maintenance of oscillation by the interaction of an ultrasonic field with stabilized volumes in a liquid. The oscillations are often associated more with the structure than with the pulsation of the bubbles. This relationship needs to study carefully what effects would appear in the surroundings of the activated bubbles, including the quantitative comparisons between theory and experimental results. In this study, the effects of ultrasonic irradiation at different frequencies on the activation of bubbles are an important factor to induce a change of inside the cells. The basic dynamical problem of acoustic cavitation is to determine the fluid medium system, together with the motion of bubble wall (the bubbles' field), when under the influence of an acoustic pressure (the acoustic field) [7].

The liquid medium is only one phase present and the liquid can be characterized by physical parameters, such as temperature (T), viscosity (η), density (ρ) and surface tension (σ), etc. The acoustic field comprised of acoustic waves traveling through the liquid medium, and it can be characterized by the frequency $f = \omega/2\pi$ and by the pressure $P = P_A \sin \omega t$

where P_A is the peak acoustic pressure amplitude value. The bubbles' field constitutes the population of gas filled cavities. A physical description of such cavities can be achieved by taking into account their volume, and the parameters of their fluid content; i.e., the pressure, density and the politropic ratio γ of the gas.

The acoustic wave traveling through the medium will induce the bubbles that will pulsate with a radial motion of their walls. The bubble's wall motion can be described by the Rayleigh-Plesset equation [5]:

$$R\ddot{R} + \frac{3}{2}\dot{R}^2 = \frac{1}{\rho}[(P_0 + \frac{2\rho}{R_0})(\frac{R_0}{R})^{3\gamma} - \frac{2\sigma}{R} - \frac{4\eta\dot{R}}{R} + P_\infty]. \quad (1)$$

Where R_0 is the bubbles' radius at equilibrium, P_0 is the gas pressure above the surface of the liquid and the P_∞ is the pressure in the liquid far from the bubble. By using the suitable approximations, for example, substituting $R = R_0 + r$, where $r/R_0 > 1$, expanding in powers of $1/R_0$ and retaining only the first-order terms, Equation (1) can reduce to the more concise form:

$$\ddot{r} + \omega_r^2 r = \frac{P_A}{\rho R_0} \sin \omega t. \quad (2)$$

The eigen-frequency of this simple equation is similar to that referring to a linear oscillation system under an impressed sinusoidal pressure:

$$(\omega_r)^2 = \frac{1}{\rho R_0^2} [3\gamma(P_0 + \frac{2\sigma}{R_0}) - \frac{2\sigma}{R_0}]. \quad (3)$$

If a bubble of equilibrium radius R_0 is submitted to an acoustic field, its wall will pulsate steadily with the same frequency of the driving field. As this frequency approaches the eigen-frequency ω_r , resonance effects would occur. In this research, the physical parameters are measured such as the temperature $T = 20.0^\circ\text{C}$, the politropic ratio $\gamma = 1.4$, the surface tension $\sigma = 72.75 \text{ dyn/cm}$, the density of the liquid $\rho = 1.013 \text{ g/cm}^3$, the hydrostatic pressure $P_0 = 10^6 \text{ dyne/cm}^2$, and the viscosity factor $\eta = 1.002 \times 10^{-2} \text{ g/cm}\cdot\text{s}$. By using the values of above parameters, the resonance frequency of Paramecium can be obtained. The resonance frequency may be calculated and falls into the range of 0.5013 ~ 1.2703 MHz for the vacuole radius R_0 of the Paramecium in the range of 3 ~ 7 μm , as shown in Fig. 1. Of course, the results could be obtained by allowing the ultrasonic frequency to become the determining variable in the sonicated system. For the theoretical results, as this phenomenon can be considered as a form of biological catalysis, it can be used to accelerate the cell division and induce a activation reaction, and to ensure assuring maximum efficiency. This work was to demonstrate

Fig 1. A transmitted-light photomicrograph of a Paramecium cell. A vacuole indicated by the arrow.

the relationship between the theoretical vacuole oscillation system and the activation reaction of Paramecium cell growth.

3. METHOD

3.1. Cell Culture and Proliferation

The cell types used in the experiments were Paramecium samples. Most of the cells consist of one or more vacuoles in the cytoplasm. The cells were grown in suspension using distilled water supplemented with minimum essential medium. They were water bath at $26 \pm 1^\circ\text{C}$ under a room temperature and were harvested for experimental use. The cell suspensions were diluted by the addition of growth medium to yield cell densities of 0.15×10^3 cells/ml. From this moment, the samples would transfer to the exposure chamber with 5 ml volumes. The exposure chamber consisted of a cylindrical shape hole in the center. A 5 mm thick plastic plate sealed the front and the back of the chamber. Following the transfer of the samples to the exposure chamber, these suspensions were then used directly in the experiments.

A light microscope fitted with a graticule eyepiece was used to measure the cell proliferation. Growth rates were calculated by counting cell numbers each 12 hr within the ten randomly selected regions of each chamber. Each region was obtained to an volume of approximately $3 \mu\text{l}$. The chambers incubated at 26°C for approximately 96 hr. Counts of the numbers of the Paramecium cells were made during each 12 hr for each chamber. Relative growth rate is the ratio of the number of irradiated cells to the number of control cells. The growth curve of the control cells was illustrated in Fig. 2. In this figure, the growth of the Paramecium cells were apparently elevated at hour 36

to 72 and was nonsignificant proliferation at the period from 84 to 96 hr. The treated cells were exposed to ultrasound on the period range from 36 to 48 hours. There was a good reason to choose this period: After this moment, the cell proliferation assays showed an obvious increase. When the samples were exposed or sham exposed to ultrasonic irradiation, the cells with their affected proliferation were visible.

Fig 2. The growth curve of control cells were recorded in the range of 0 ~ 96 hr.

3.2. Instrumentation

An exposure system comprised of ultrasound transducer operating in alternating pulses was devised, and is illustrated in Fig. 3. In Fig. 3, the exposure chamber was constructed from thin wall plastic containers and the ultrasound transducers were fixed on one surface of the box. A rubber, used as a wave absorber was placed in the tank opposite the transducer to minimize reflections. Four different narrow band transducers were employed in the experiment. They

Fig 3. Schematic diagram of the isonation and measurement apparatus used.

were operated at central frequencies of 0.25, 0.5, 1, and 5 MHz, respectively. Sound fields were measured with a bilaminar polyvinylidene fluoride (PVDF) hydrophone. The exposure chamber was placed near the transition zone between near- and farfield regions of the beam. The function generator was a HP 33120A unit, produced by Hewlett Packard. This apparatus can be set to work with arbitrary waveform. In this experiment, it was set to the tone pulse mode, pulsing 1:1 and 1:9. The pulse repetition frequency of each pulse was 25 kHz. All insonated samples were exposed to ultrasound with a spatial peak temporal peak intensity (SPTP) of 0.127 mW/cm². After irradiation, the number of the Paramecium cells in the treated and control samples were counted.

In each exposure ultrasonic frequency, the transducers were operating in a pulsed mode with two duty cycles of 1:1 and 1:9. The first series of experiments were conducted a continuous exposure duration. The exposure duration of a pulse mode 1:1 and 1:9 was continued and maintained 12 and 60 min, respectively. The total "with signal" time was about 5 min in each trial. The other series of experiments was separated the exposure duration into parts and set to work in the culture range of 36 ~ 48 hr. The irradiation time of the pulse mode of 1:1 was about 2 min in each two hours. It was replicated 6 times in the range of 36 ~ 48 hr. For the pulse mode of 1:9, the irradiation time was 10 min in each two hours and replicated 6 times as the pulse mode of 1:1. The total length of "with signal" time was held the same as the continued exposure duration series. In addition, each of which was accomplished the ultrasound exposure or sham exposure treated cells would be re-incubated up to the 96 hr. After completion of the two series of experiments, the ultrasonic effects could be analyzed by comparing the number of cells in the treated and control samples.

4. RESULT AND DISCUSSION

The relative growth rate for the population of the Paramecium suspensions obtained using the various exposure frequencies with the pulsed mode of 1:1 are illustrated in Fig. 4. In Fig. 4(a), the sample of cell suspension was exposed or sham exposed to 0.25 MHz ultrasound with continued and separated duration time. It should be noted that in the total culture range 0 ~ 96 hr, the change of cell numbers was not significant in the first 24 hr. Thus, the change of the relative growth rate was recorded in the range from 36 to 96 hr. The error bars represent the standard errors of the means. As can be seen in this figure, there was no apparent difference in the exposure setup. The relative growth rate in samples exposed using a separated exposure

duration was essentially <1 at all culture period. The minimum growth rate was about 0.945 compared with the control cells. With continued exposure duration, the relative growth rate was appeared little inhibition of Paramecium proliferation. The growth rate was essentially >1 at all culture period and the maximum was about 1.09. That is, under the exposure frequency of 0.25 MHz, the cells appeared lower activity and the cell reproduction was not significant by ultrasound irradiation. Similarly, from Fig. 4(b), it is clearly to find that the 5 MHz ultrasound exposures also produced a little increased effects of the cell growth. The calculated resonant frequency range of cell vacuole was about 0.5013 ~ 1.2703 MHz. Thus, at the non-resonance frequencies of ultrasound (Figs. 4(a) and 4(b)), it was found to be no apparent difference in the cell proliferation at any exposure duration. In contrast, Figs. 4(c)-(d) demonstrates that the cell numbers were increased substantially at most exposure duration. In Fig. 4(c), the results were obtained from 0.5 MHz ultrasound irradiation. Notably that the frequency 0.5 MHz was approached to the range of theoretical resonance. Although the cell growth was apparently reduced at first 12 hr by using the separated exposure duration. The maximum relative growth rate was about 1.119 at the culture time of 96 hr. Figure

(a)

(b)

Fig 4. Relative growth rate of *Paramecium* cells as a function of the culture time of exposure, by presenting the data for each frequency (duty cycle 1:1) 0.25, 0.5, 1, and 5 MHz, respectively. The exposure duration was divided to the continued and separated parts. Each point represents the mean of $n = 10$ observations and error bars represent the standard errors of the means. Panel (a): irradiating frequency = 0.25 MHz; panel (b): 0.5 MHz; panel (c): 1 MHz and panel (d): 5 MHz.

4(d) shows a very notable response between the exposure resonance frequency and the cell proliferation. The relative growth rate was maintained above the value of 1.1 at the period of 48 ~ 84 hr, and the maximum value was 1.324 at the separated exposure duration. It means that under the resonance frequencies irradiated, the cell growth rate was enhanced in excess of about 10%. Comparing both exposure duration, the continued exposure was found to attain the maximum growth rate immediately. However, the effects could not maintain longer period. The separated exposure duration not only appeared the maximum growth rate, but also maintained the increasing effects. In the experimental series, the decreased effects were attained to a maximum value of

9.1% under the 0.5 MHz ultrasound exposures, and the maximum value of the growth rate was approached to 32.4% under the 1 MHz ultrasound irradiation.

Figure 5 presents the relative growth rate obtained using the pulsed ultrasound with the duty cycle of 1:9 at the various exposure durations. Similar procedures of the experiments were undertaken in an attempt to demonstrate the dependence for the different duty cycle in cell growth at various exposure duration. The results of 0.25 and 5 MHz ultrasound exposure are illustrated in Figs. 5(a) and (b). The data show that the cell numbers of the treated or control cells were almost the same at the continued exposure duration. With separated exposure duration, the irradiation frequency 0.25 and 5 MHz produced little inhibition at the 48 hr culture period. The relative growth rate was approximately 0.92 at the 48 hr. Nevertheless, the relative growth rate did not maintain the lower level. The treated cell numbers were increased and approached to the control cell numbers in the next 12 hr. Thus, there is no apparent data appeared that the frequency 0.25 and 5 MHz could produce any apparently inhibited or increased effects at the continued or separated exposure duration.

Figs. 5(c) and (d) present the data of relative growth rate obtained using the 0.5 and 1 MHz ultrasound exposures, respectively. From Figs. 5(c), the maximum relative growth rate was 1.123 by using the 0.5 MHz exposure at the continued duration. The relative growth rate was essentially >1 in samples exposed using both exposure durations at all culture period (there was only one exception in the 36 hr culture time at the separated duration). Figs. 4(c) and 5(c) illustrate the dependence of the cell proliferation on the resonance frequency of ultrasound irradiation. Fig. 5(d) was irradiated the samples using the 1 MHz ultrasonic frequency, it shows the same effects as 0.5 MHz at both exposure duration. It leads to the conclusion that resonance frequency of ultrasound has potentially important effects on the activities of *Paramecium* cells. In addition, pulsed ultrasound at resonance frequency and operating with the 1:1 duty cycle produced greater effects than the duty cycle with 1:9.

The exposure technique applied in this study had the advantage of utilizing the theoretical resonance frequency for the exposure basis. There are still a few unresolved problems remained to predict the experiment results precisely. Two factors, for example, which exist between the theory and the experimental results are; (1) uncertainties in the physical parameters of the vacuoles and cells, and (2) uncertainties in the application of the idealized theoretical model to actual biological material. Such interaction effects may be an important consideration in theoretical models of the response of *Paramecium* cells to ultrasound exposure.

(a)

(b)

(c)

(d)

Fig. 5. Relative growth rate of Paramecium cells as a function of the culture time of exposure, by presenting the data for each frequency (duty cycle 1:9) 0.25, 0.5, 1, and 5 MHz, respectively. Each point represents the mean of $n = 10$ observations and error bars represent the standard errors of the means. Panel (a): irradiating frequency = 0.25 MHz; panel (b): 0.5 MHz; panel (c): 1 MHz and panel (d): 5 MHz.

5. CONCLUSION

In the investigations reported here, some subjects are examined. First, the populations of the Paramecium cells in different exposure duration and duty cycle were chosen for examination the growth effects of ultrasound. Second, the ultrasonic fields for resonance and non- resonance frequencies in the range of 0.25 to 5 MHz are employed. Third, the results, in terms of the relative growth rate for each of the populations examined, are described and compared to predictions based on the resonance theory. The theoretical model of the vacuole was used to calculate the resonance frequency. It is assumed that growth conditions for any frequency represents a constant level of activation, then the cell growth would be enhanced at the resonance frequency range, and increase for frequencies above and below resonance range at which the cell growth is less active. This theoretical prediction can be compared with experiment, and the functional form of the data is generally similar to the theoretically expected form. The maximum cell growth rate correspond to the expected resonance frequencies of the vacuole, and the growth rate appeared no significant difference outside the range. The theory for the oscillation of the cell vacuoles is therefore a useful guide in predicting the most efficiency sonication frequencies, and presumably for other unicellular creature. Quantitative comparison between theory and experiment is difficult and should examine repeatedly. However, the general concepts of the biological effects appear to be correct in our experiment. It appeared the possibility to elucidate the complex etiology of the effects of ultrasound on Paramecium.

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