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Survival of Cultured Mouse L Cells Exposed to 1.2 MHz Ultrasound

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1.2MHz 超音波のマウス L 細胞の生残率に及ぼす影響

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培養マウス L 細胞に 1.2MHz 連続波超音波を照射し、コロニー形成能を指標として、生物学的効果を調べた。超音波エネルギー (J/cm^2) と超音波強度 (W/cm^2) を変えて超音波の影響を検討した結果、生物学的効果は超音波強度に強く依存することを認めた。0.3W/cm² 以下の強度では生残率に何らの影響も認められなかった。0.7W/cm² 及び 1.3W/cm² の強度では生残率はエネルギーに対してほぼ指数関数的に減少するが、1.9W/cm² 以上の強度では二相性を呈する。生残率は強く超音波強度に依存するので、超音波の生物効果を論ずる場合、エネルギーのみならず、超音波強度も

重要な指標であると思われる。

さらに、5-ブロムデオキシウリジン (BUdR) を含む培養液で培養した細胞について超音波の影響を調べた。0.7W/cm² および 1.3W/cm² の超音波強度で BUdR の修飾作用が認められ、BUdR 前処理細胞群は対照群に比して、各強度で、2.0 倍及び 1.3 倍の致死効果を示した。しかしながら、0.3W/cm² の強度では対照と同じ値を示し、しきい値を変えるまでには至らなかった。以上より、上記の強度では、細胞内 DNA も超音波の影響を受けることが示唆される。

Introduction

Ultrasound, a form of non-ionizing radiation, has become increasingly popular as a therapeutic and diagnostic tool in medicine and biology. Recent studies have provided some evidence for the biological effects of ultrasound¹⁾²⁾. From studies on the growth curves of L5178Y cells sonicated at 1 MHz of ultrasound in suspension, Clarke and Hill³⁾ reported that the mechanical effect of sonication contributed exclusively to the damage of mammalian cells. Kaufman et al.⁴⁾ reported that the threshold for cell lysis by 1 MHz ultrasound was 1 W/cm² with the maximum effect at 10 W/cm², from studies on trypan blue exclusion and colony-forming ability of cultured cells. Martins et al.⁵⁾ suggested, from the combined effect of 1 MHz ultrasound and X-rays on the colony-forming ability

of the cultured cells, that the damage to the cell membrane caused by ultrasound was responsible for the cell death.

In order to see the relationship between the sound intensity and the colony-forming ability, the cultured mouse L cells were exposed to 1.2 MHz ultrasonic wave of different intensities (0.3, 0.7, 1.3, 1.9, and 2.4 W/cm²). Then to evaluate whether or not DNA takes part in the cell death, BUdR incorporated mouse L cells were used in the present series of colony-forming experiments.

Methods and Materials

Apparatus and dosimetry: The ultrasonic generator used (Kaijo Electric Co., Model 1150) was equipped with a barium titanate transducer with a diameter of 8 cm. This apparatus was improved to change the sound intensity (W/cm²=J/sec.cm²) by varying the applied voltage, and provided a 1.2 MHz ultrasonic continuous wave. The frequency was determined by a syncroscope. The ultrasonic energy output of the transducer, the sound intensity I in W/cm², was estimated from the applied input voltage.

Temperature measurement: The temperature measurement was carried out in a 400-ml water container set on a transducer surrounded by styrol and glasswool, using copper-constantin thermocouple with thermojunctions (Fig. 1). The temperature increase was in proportion to the sound intensity in a 5-min period (Fig. 2).

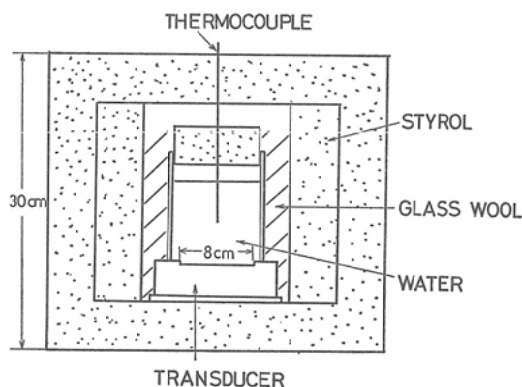


Fig. 1. Exposure system for temperature measurement. Water volume is 400 ml as optimum for determining temperature changes.

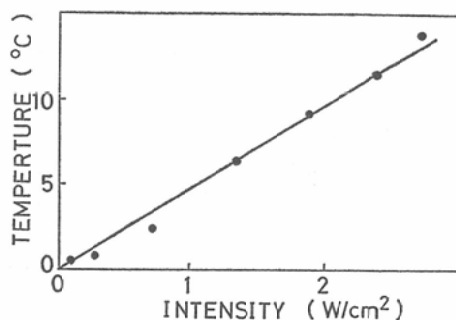


Fig. 2. Temperature increase of water, exposed to 1.2 MHz ultrasound with various intensities for 5 min. Temperature increased linearly with intensity.

Chemical effect: The chemical effect of ultrasound was examined in a cell exposure system using two types of chemical dosimeters; the ferrous to ferric (Fricke) dosimeter and the liberation of iodine from KI-starch solution in the presence of chloral hydrate⁶⁾. Changes in optical density of the two chemical systems were determined with a spectrophotometer (Hitachi Ltd., Model 340) at wave lengths of 304 and 555 nm, respectively. Changes in optical density apparently increased at an intensity of over 1.3 W/cm². In KI-starch system, pH changes far below the threshold of the intensity (1.3 W/cm²) (Fig. 3).

Cells and exposure condition: The mouse L cells were grown in a standard monolayer culture in

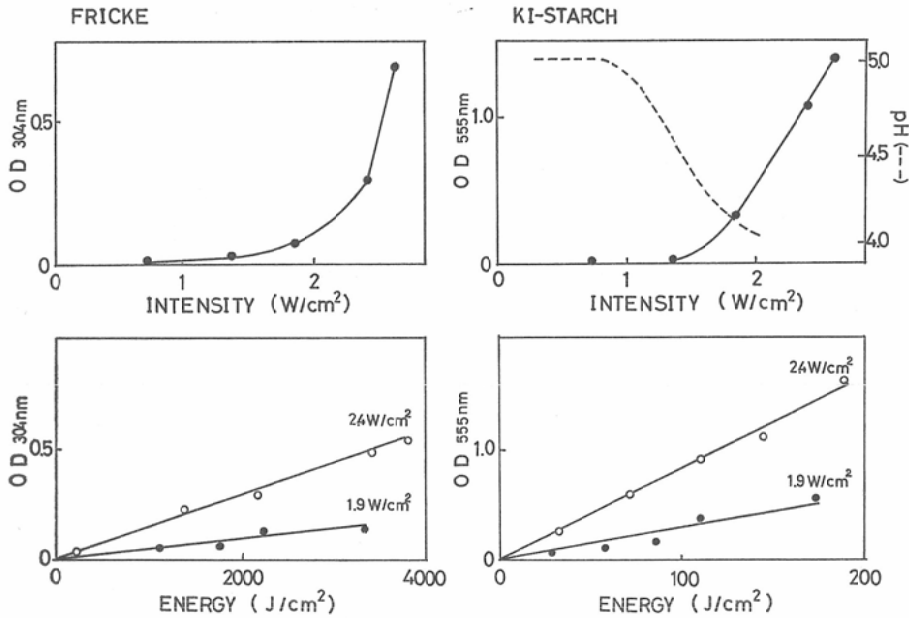


Fig. 3. Change in optical density of chemical dosimeters. Fricke system and KI-starch system were exposed at various energy and intensities. Exposure time of Fricke and KI-starch was 15 and 1 min, respectively.

Eagle's MEM containing 10% fetal bovine serum. One day before the experiment, the medium was refed and cells in the log-phase were prepared for the ultrasonic exposure, using 5-cm polystyrene petri dishes as exposure vessels. Transducer-dish distance was 3 cm and the surrounding space was filled with 18°C water. The mouse L cells were harvested by trypsinization and inoculated in petri dishes at a concentration of 200 cells/dish. Samples were exposed to ultrasound at different intensities. After incubation for 10 days, the number of colonies was scored. The plating efficiency was about 85%. Under this condition the temperature and pH in the medium were nearly constant during the exposure (Fig. 4).

Drugs: 5-bromodeoxyuridine (BUdR) was purchased from Wako Pure Chemical Industries, Ltd., Tokyo. BUdR toxicity was examined by determining the colony-forming efficiency after exposure to

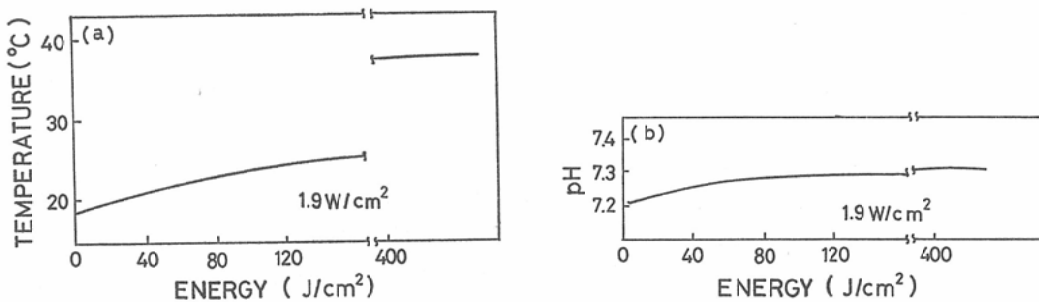


Fig. 4. (a) Temperature increase in the condition of cell exposure.
(b) pH change in the condition of cell exposure.

medium containing BUdR at various concentrations for more than 48 hr. After treatment at a concentration of $10 \mu\text{g/ml}$, which gave no toxicity to the cells, the cells were exposed as scheduled.

Results

There was a possibility that some chemical changes induced in the culture medium by ultrasonic exposure would lead to cell death. To examine the possibility of this kind of indirect effect, untreated cells were added to dishes immediately after exposure of only the culture medium and were incubated for 10 days. The result is shown in Fig. 5. Although longer exposure seriously reduced the ability of the medium to support the cell growth, the indirect chemical effect did not play a major role in cell death for a short time such as killing 99% of the cells in this system.

The mouse L cells were exposed to 1.2 MHz ultrasound at intensities of 0.3, 0.7, 1.3, 1.9, and 2.4 W/cm^2 . The survival fractions were plotted against the total energy given, and show in Fig. 6.

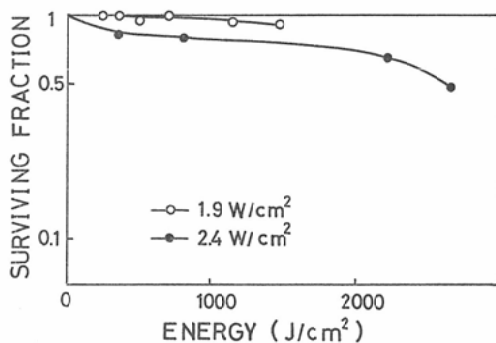


Fig. 5. Effect of exposed medium on survival of mouse L cells.

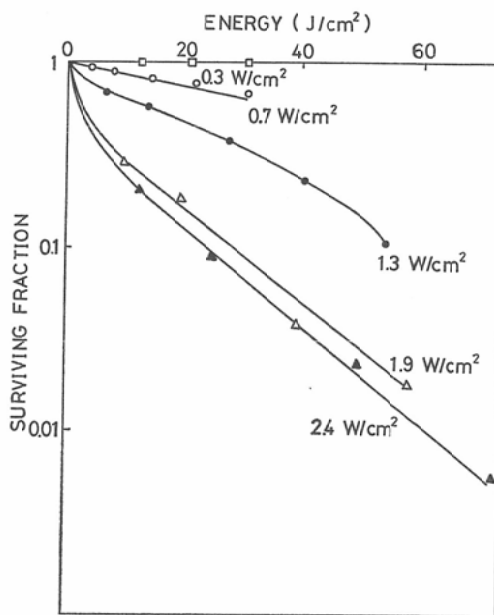


Fig. 6. Survival of mouse L cells as a function of total energy.

This graph shows that the survival rate to the energy given decreased exponentially at intensities of 0.7 and 1.3 W/cm^2 , and decreased biphasically at intensities above 1.9 W/cm^2 , with appearance of an upward curvature followed by a straight portion. No lethal effect on cells due to exposure was observed at intensities less than 0.3 W/cm^2 . The survival fractions in the same experiment were available to show the apparent threshold in intensity effect as shown in Fig. 7, in which survivals were graduated with the intensity. The threshold in intensity effect for the cell killing was 0.3 W/cm^2 . The lethal effect by the total energy given was more remarkable at higher intensities than at lower intensities. However, at intensities of above 1.9 W/cm^2 , no further increment in the lethal effect was observed.

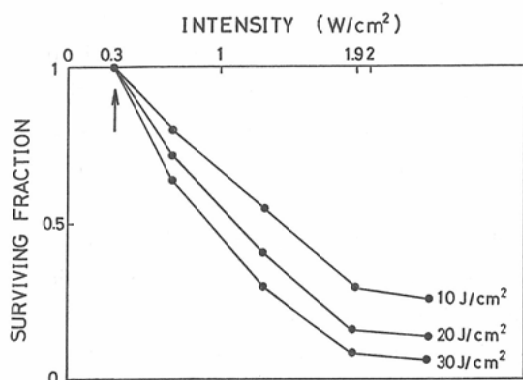


Fig. 7. Intensity effect of ultrasound on mouse L cells. The arrow indicated the threshold of ultrasonic intensity (0.3 W/cm^2).

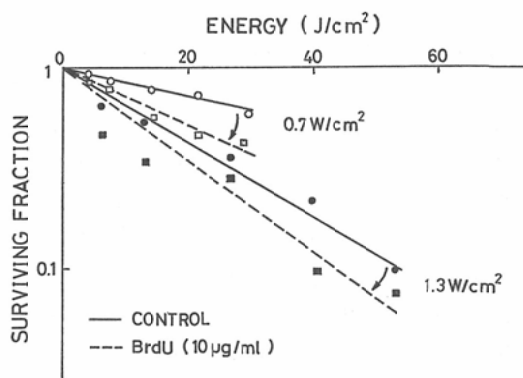


Fig. 8. Survival of BUdR pretreated mouse L cells as a function of total energy. Concentration of BUdR was $10 \mu\text{g/ml}$.

Sensitized effect of ultrasonic exposure by BUdR at a concentration of $10 \mu\text{g/ml}$ was observed at the intensity of 0.7 and 1.3 W/cm^2 (Fig. 8). In order to see the relationship between the total energy and the survival, the curve fitting was carried out by a computerized statistical program. The exponential curve proved to be more fitted than other curves. The regression coefficient ratio of the exposed to the control cells was found to be 2.0 and 1.2 for 0.7 W/cm^2 and 1.3 W/cm^2 , respectively. We take, tentatively, the values of these coefficients as a measure of the sensitization by BUdR. Sensitization by BUdR was not found at intensities less than 0.3 W/cm^2 and at a concentration below $5 \mu\text{g/ml}$.

Discussion

There are a considerable number of studies on the biological effect of ultrasound. The intensity effect of 1 MHz ultrasound on cultured mammalian cells has never been clear. Clarke and Hill³⁾ reported that the threshold of intensity and optimum intensity for disintegration of L5178Y cells were at about 1 and 5 W/cm^2 , and that the same pattern was found for the liberation of free iodine in solutions of potassium iodine. Kaufman et al.⁴⁾ reported 1 W/cm^2 as the threshold of intensity for cell lysis of HeLa cells by 1 MHz ultrasound. Martins et al.⁵⁾ reported that M3-1 cell killing was not observed at an exposure rate of 0.125 W/cm^2 .

In our experiments, the threshold of intensity for cell death was observed at the intensity of 0.3 W/cm^2 . Although the difference of the thresholds in intensity depends on the cell species and on difficulty of dosimetry, it is quite possible that the intensity plays one of the major roles in the lethal effect of ultrasound. Within the intensity ranges assayed, as shown in Fig. 7, the intensity effect of ultrasound to mouse L cells was divided into three regions; no killing region, "proportional" region, and plateau region (Fig. 7). If the origin of cell disintegration preferentially depends on the mechanical shearing force associated with cavitation, the no killing region would concern the intensity for cavitation threshold and the plateau region would be the "minimal" intensity at which the cavitation nuclei disappear in the medium so far as assayed in the present experiments. Although the mechanism of the

intensity effect is poorly understood, our interpretations are as follows: At the no killing region (at lower intensities), cavitations formed by the energy absorption of sound are sparsely distributed in the vessels and, accordingly, the energy emitted from the cavitation does not transfer to the subcellular components in the cell. At the plateau regions (at very high intensity), on account of the mutual interactions between cavitations, the actual number of the cavitation turns out to be much less than would result from increase in the number of cavitations.

For the chemical effect, Clarke and Hill³⁾ found a close relation between iodine release and cell death, but our experiments did not show any strong correlation between the two respective chemical systems and cell death. Namely, the threshold of intensity for biological effect was 0.3 W/cm^2 and that for chemical effect was 1.3 W/cm^2 . The difference may be due to the influence of chloral hydrate used in the potassium iodine solution or to the measurement of released iodine. Further experiments on the chemical effect are necessary.

Martins et al.⁵⁾ reported that a small amount of synergism between ultrasound and X-rays was observed, and that the extent of synergism might be due to an interaction between nuclear damage caused by X-rays and the damage to the cell membrane caused by ultrasound. To examine the possible effect of ultrasound on DNA damage in cell nuclei, the cells were pretreated with BUdR for partial replacement instead of thymidine. If ultrasound could affect DNA in the cell nuclei and modify the physicochemical properties of DNA, the modifying action would reflect on the intensity effect. It was found that the cells that incorporated BUdR in the cell nuclei increased the effectiveness of the ultrasound by a factor of 2.0 and 1.2 at intensities of 0.7 and 1.3 W/cm^2 , while sensitization by BUdR was not observed at the intensity of 0.3 W/cm^2 . This result shows that DNA also would be damaged by the ultrasonic exposure, the damage of which leads to death of the cell. The cell membrane as well as nuclei could be a structure that might be drastically changed by the occurrence of energy absorption. If damages could be triggered by the resonant energy absorption of the subcellular components followed by cavitation, the membrane damage may have some share in the cell death. We have not as yet sufficient data on the primary target for ultrasound to give an exact evaluation of these two possibilities.

In order to elucidate the mechanism of the biological effect of ultrasonic exposure, investigation on the effect of ultrasound and chemical modifiers or ionizing radiation should be undertaken.

Conclusion

Mouse L cells were exposed to 1.2 MHz ultrasound to investigate the biological effect of ultrasonic exposure. The survival of the cells indicated the intensity effect of ultrasound and the threshold of ultrasonic intensity was 0.3 W/cm^2 . When mouse L cells, pretreated with BUdR, were exposed to ultrasound, the lethal efficiency was higher than in the control cells. Since BUdR is incorporated into DNA in the cell nuclei, this result would suggest that ultrasound also acted on the cell nuclei as well as the cell membrane.

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