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BIOLOGICAL EFFECTS OF ULTRASOUND ON CULTURED MAMMALIAN CELLS

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INTRODUCTION

Ultrasonic waves are sonic vibrations of frequencies above 16 to 20 KHz which is the upper limit of audibility for the human ear. Above 15 MHz, ultrasonic waves are referred to as microwave ultrasound because at these high frequencies ultrasound tends to act like electromagnetic radiation even though particle vibration is still being produced. Due to practical limitations set by ultrasonic generators the upper limit of ultrasonic frequencies is around 500 MHz. The frequencies of biological interest are in the range of a few KHz to a few MHz.

The lethal effects of ultrasound were first observed by Langevin around 1920 while he was investigating the use of ultrasound for submarine location (Ref. 1). He noticed that small marine organisms that strayed in the path of the ultrasonic beam were killed instantly. This observation gave rise to the idea of using ultrasound as death rays although the idea was not seriously explored.

Ultrasound has since been used for diverse purposes in industry, biology, medicine and dentistry (Ref. 2). Various biological effects of ultrasound have been observed in viruses, microorganisms, plants and animals. These have been reviewed by Elpiner (Ref. 3). Biological effects of ultrasound on cultured mammalian cells have been reported by Clarke and Hill (Refs. 4, 5).

We report here on some of our observations on the effects of ultrasonic waves on the reproductive ability of cultured mammalian cells. Specifically we studied the effect of certain physical factors on survival, the sensitivity of different cell lines and the effectiveness of different frequencies.

MATERIALS AND METHODS

Ultrasonic Equipment: Most of the work

reported here was done on a 1.0 MHz ultrasonic generator (Tomac Model 1700 made by the American Hospital Supply Company for diathermic treatment) after the electronic circuitry was modified to provide independent control of the dose rate (see below) by varying the plate voltage. Later another ultrasonic generator (Fig. 1) was built to provide 0.1, 0.5, 1.0, 2.0 or 3.3 MHz frequencies. The transducers used in all cases were 10 cm² X-cut quartz crystals except at 0.1 MHz in which case a 80 cm² ceramic crystal was used.

Ultrasonic dosimetry: Measurement of actual absorbed dose is complex especially in our experimental arrangement which did not provide free field conditions. However, the intensity (or exposure rate) of ultrasonic energy (I W/cm²) was estimated from the following relation $I = 4 V^2 e^2 / L^2 \rho c$ which gives the output of ultrasonic intensity from a resonant piezoelectric transducer backed by air and radiating into an infinitely thick medium of acoustic impedance ρc (Ref. 6, 7). V = rms voltage applied L = crystal thickness, e = piezoelectric stress constant which for quartz = 0.17. The exposure is the exposure rate multiplied by the time of exposure.

Ultrasonic Exposure: About 5 drops of glycerine were put on the transducer, then a 35-mm plastic petri dish with cells in 2 ml of medium was placed on the transducer and pressed lightly to squeeze out excess glycerine. The oscillator was then turned to the resonant frequency of the crystal and the voltage set to give the desired exposure rate. A schematic of the exposure set up is shown in Fig. 2.

Cell Culture: Conventional cell culture techniques (Ref. 8, 9) were used to obtain survival curves with the following mammalian cell lines: a) M3-1: Chinese hamster bone marrow; b) V79 Chinese hamster lung; c) T-1: human kidney and d) Chang's: human liver.

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RESULTS AND DISCUSSION

Reproducibility of Data: The acoustic intensity measurements we have made refer only to the monitored exposure rather than to the actual intensity in the culture medium dose. It was felt that the data might not be reproducible because of the nature of the experimental setup where there is a possibility of variable loss of sonic energy between the crystal and the cells.

To check the reproducibility of data, survival curves were obtained using M3-1 cells exposed to 1.0 MHz at 1.0 W/cm². Four dishes were used for each dose point and the experiment was repeated four times.

The results are shown in Fig. 3. The data are highly consistent indicating that the plate voltage is a reproducible measure of the survival data in our experimental setup and is therefore related simply to the acoustic exposure and absorbed dose at a given ultrasound frequency.

Physical Parameters: A series of experiments was done to determine how variable experimental conditions may influence the effects of sonication.

i. To determine whether attachment of cells to the petri dish had any influence on survival M3-1 cells were exposed to 1.0 MHz at 1.0 W/cm², either immediately after plating when the cells were rounded and in suspension or 4 hours later when the cells had flattened out and were attached to the bottom of the petri dish. As can be seen from Fig. 4, there is no significant difference in the survival of M3-1 cells when exposed either in suspension or attached to the bottom of a petri dish. This observation is confirmed by the results of an experiment with asynchronous V79 cells in which it is seen that the survival does not change significantly with time after plating (Ref. 10).

Cells in suspension might be expected to have a lower survival than attached cells for a given dose, since suspended particles act as inhomogeneous objects that cause scattering, thereby increasing absorption of ultrasonic energy. However, our results show the same survival for a given monitored dose in both cases, indicating that cells in suspension have a greater resistance than attached cells. The increased resistance of cells in suspension may be due to their spherical shape in suspension and the fact that they have a smaller membrane-per-unit volume compared to attached cells.

ii. To see if different amounts of medium in the petri dish may affect survival, M3-1 cells were exposed to 1.0 MHz at an indicated 1.0 W/cm² for 5 and 10 sec with 0.5, 1.9, 2.1, 2.2, 2.5 and 3.0 ml of medium. No significant difference in survival was observed under any of these conditions.

iii. To test the possibility that ultrasound may affect the medium and thus damage the cells only indirectly, 2 ml of cell medium were exposed to 1.0 MHz at 6.0 W/cm² for 10 min and an aliquot of M3-1 cells added within 5 sec of exposure. There was no difference in the fractional survival as compared with controls although cells exposed to that dose would have a survival of less than 10⁻³. Sonicating the medium by itself apparently does not produce appreciable amounts of stable toxic material. In a separate experiment no appreciable amount of free radicals were detected using electron spin resonance (Ref. 10).

iv. To further evaluate the role of medium M3-1 cells were plated in petri dishes and 4 hours later, when the cells had attached firmly to the dishes: a) the medium was sucked and the cells exposed; or b) the dishes were placed in a refrigerator at 0 to -5°C for 10-15 min and the cells exposed when the medium had frozen. In both cases the exposures were to 1.0 MHz at 6.0 W/cm² for 10 sec and in either case the survival was the same as for controls. Liquid medium is needed for ultrasound to be effective.

v. To study how the temperature of the medium at the time of exposure may influence survival, M3-1 cells were exposed to 1.0 MHz at 1.0 W/cm² for various times with the cells maintained at the following initial temperatures: 0 to 0.5°C, 10-15°C and 20-25°C. The results are shown in Fig. 5, and it is concluded that within the limits studied, the temperature of the medium does not affect survival. This is probably because in none of the cases the ambient temperature exceeded 37.5°C.

Cell Line Sensitivity: Cells derived from four different cell lines were exposed to 1.0 MHz at 0.5 or 1.0 W/cm². T-1 and Chang cells are of human origin, have a doubling time of approximately 24 hours and 60-80 chromosomes, V79 and M3-1 cells are derived from Chinese hamster, have a doubling time of 9-12 hours and about 22 chromosomes. The cell size distribution of these cells obtained from a multichannel analyzer connected to a Coulter counter is shown in Fig. 6.

The survival curves for the different cell lines are shown in Figs. 7a and 7b. It is seen that the survival curves are similar and the sensitivity of a cell line does not seem to be related to the origin of the cell line, the chromosome number, the doubling time or the cell size distribution, but is probably related to some structural unit that has similar properties for different cell lines.

Frequency Effect: M3-1 cells were exposed to 0.1, 0.5, 1.0, 2.0 or 3.3 MHz at two different dose rates. Experimental conditions did not permit exposures to be made at the same dose rate for the different frequencies.

The results are presented in Figs. 8a, 8b, 8c, 8d and 8e. By comparing the dose required

for a given survival level it can be seen that 0.5 MHz is the most effective of the frequencies tested and it appears that the effectiveness may increase further at still lower frequencies. For 0.1 MHz a ceramic instead of a quartz transducer was used and the survival curves for that frequency are presented only to show the generality of the dose rate effect and the similarity of the survival curves.

The effectiveness of a given frequency is probably due to the resonance phenomenon discussed by Lependin and Ustinova (Ref. 11), but it is possible that the variation in the effectiveness of different frequencies may be an artifact of dosimetry i. e., it may be due to variations in the standing wave pattern in the culture medium as a function of frequency. However, we have found that under similar exposure conditions, 1.0 MHz is the most effective frequency for inhibition of colony formation in *Saccharomyces cerevisiae* when exposed to the same set of frequencies (Ref. 12).

We have also observed a difference in the microscopic appearance of M3-1 cells exposed to 0.5, 1.0 or 2.0 MHz compared to cells exposed to 0.1 or 3.3 MHz (Ref. 10).

Audiofrequencies (20, 50, 60, 80, 100, 500 Hz, 1, 5, 7, 9, 10, 12, 15 and 20 KHz) at intensities as high as 10^{-2} W/cm² (which is the threshold for pain) and for as long as two minutes had very little effect on the survival of M3-1 cells. Because of practical limitations the doses used were much lower than for ultrasonic frequencies.

Growth Curve: Single cell suspensions of M3-1 cells were exposed to 1.0 MHz at 1.0 W/cm² for 60 seconds and the cell multiplicity of the exposed cells as well as those of unexposed cells was determined at various times thereafter.

From Fig. 9 it is concluded that ultrasound extends the lag phase of exposed cells by about 8 hours but the doubling time is not affected. Burns (Ref. 13) has shown that in yeast, sonication selectively changes cell membrane permeability and it is possible that important nutrients may leak out during sonication; the extension in the lag phase may be the period needed to resynthesize and accumulate those nutrients and possibly to repair any sublethal damage.

SUMMARY AND CONCLUSIONS

The colony-forming ability of cultured mammalian cells exposed to monochromatic ultrasonic vibrations of 0.1, 0.5, 1.0, 2.0 or 3.3 MHz was studied.

The measurement of absorbed ultrasonic dose is complex but the approximate ultrasonic energy imparted to the cells was monitored by measuring the plate voltage, and

highly reproducible results were obtained.

Certain factors such as amount of medium, temperature at the time of sonication, and the state of attachment of suspension of cells which might be expected to vary from experiment to experiment did not significantly affect survival. For ultrasound to be effective cells have to be in a liquid medium. Sonication does not produce stable toxic products and no free radicals were detected at the doses used.

Differences in the sensitivities of different cells do not seem to be related to origin of cell line, cell size, distribution, doubling time or chromosome number.

0.5 MHz was found to be the most effective frequency in causing cell death in M3-1 cells exposed to 0.5, 1.0, 2.0 or 3.3 MHz.

Cells that survive ultrasonication show an extended lag phase but the doubling time is not affected.

ACKNOWLEDGMENTS

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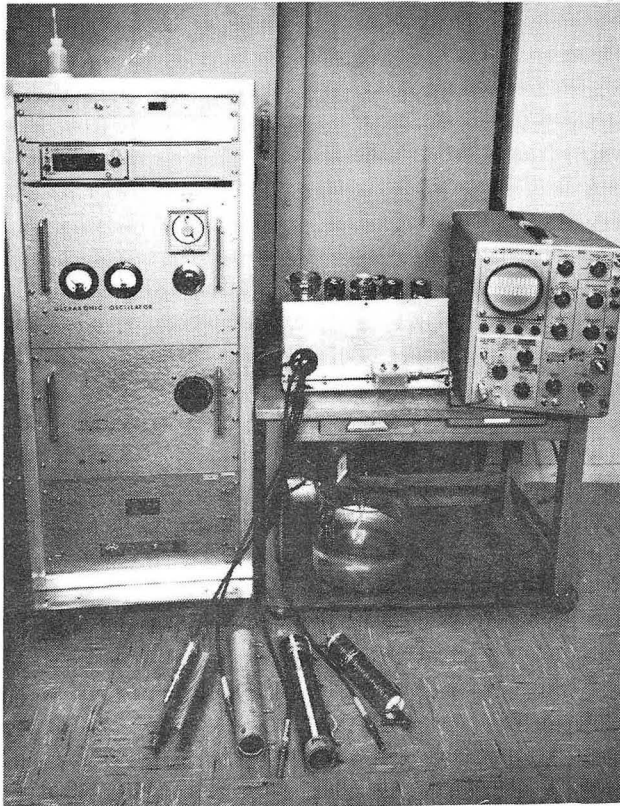


Fig. 1. Ultrasonic equipment for producing 0.1, 0.5, 1.0, 2.0, and 3.3 MHz frequencies.

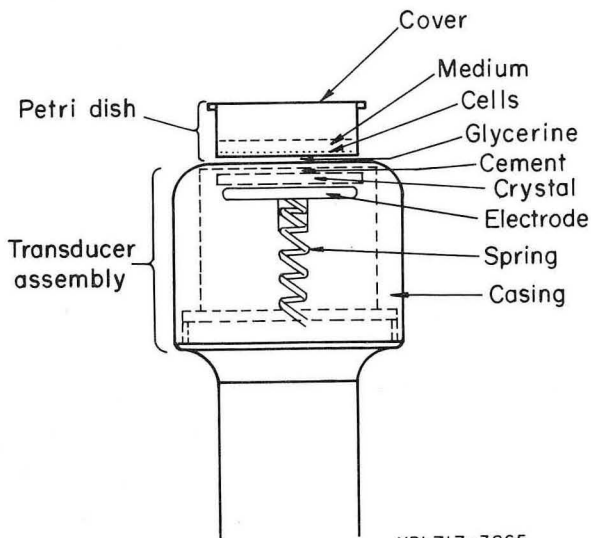


Fig. 2. Schematic representation of the exposure setup.

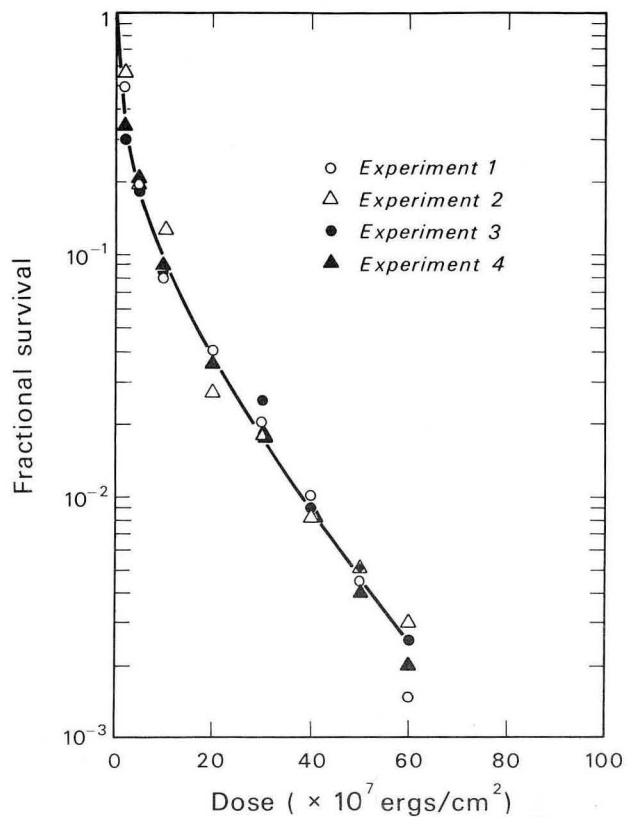


Fig. 3. Fractional survival, in 4 different experiments, of M3-1 cells exposed to 1.0 MHz at 1.0 W/cm².

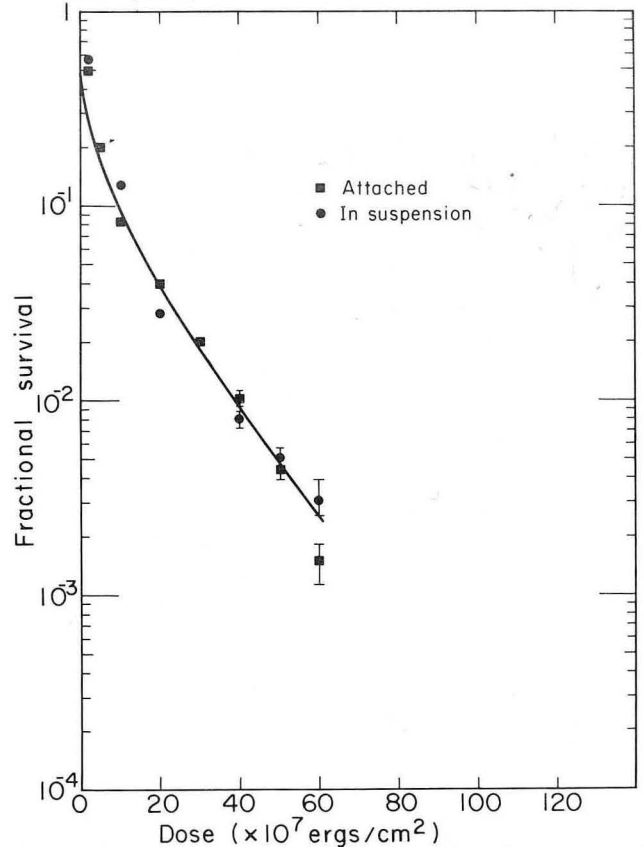


Fig. 4. Survival curve of M3-1 cells exposed to 1.0 MHz at 1.0 W/cm² in suspension or attached to the bottom of the petri dish.

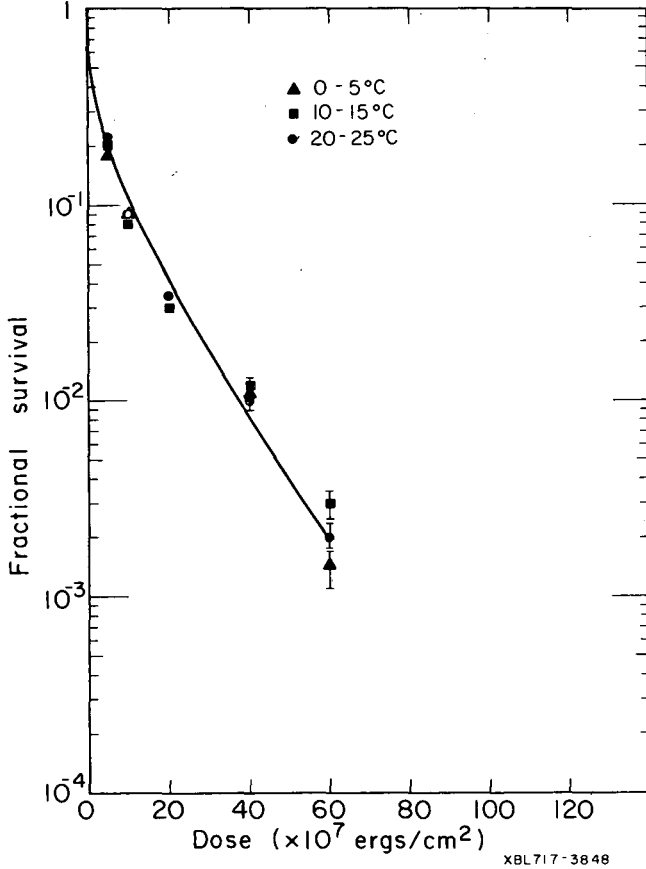


Fig. 5. Survival curve of M3-1 cells exposed to 1.0 MHz at 1.0 W/cm² at 0-5°C, 10-15°C, and 20-25°C.

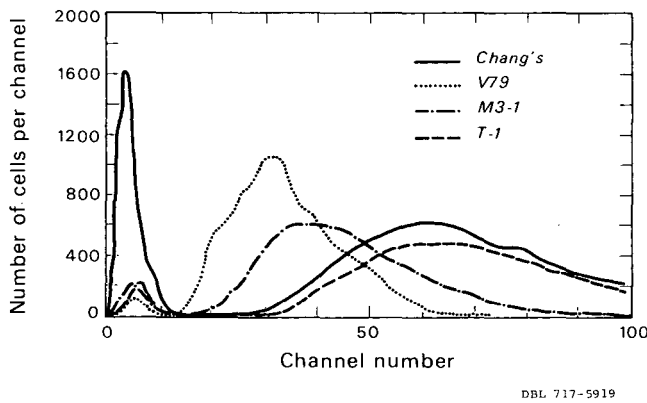


Fig. 6. Cell size distribution, in isotone, of cells derived from different mammalian cell lines.

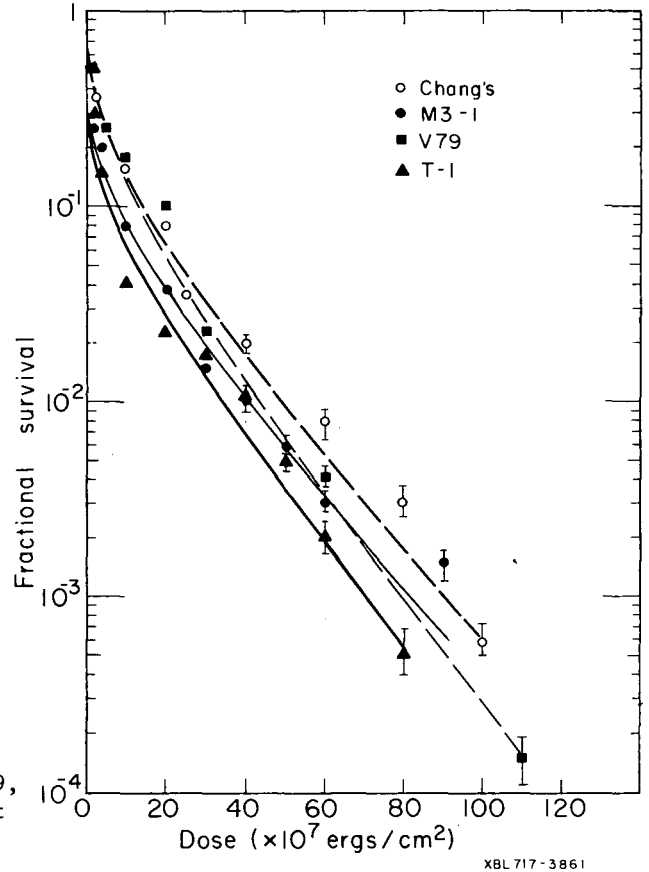
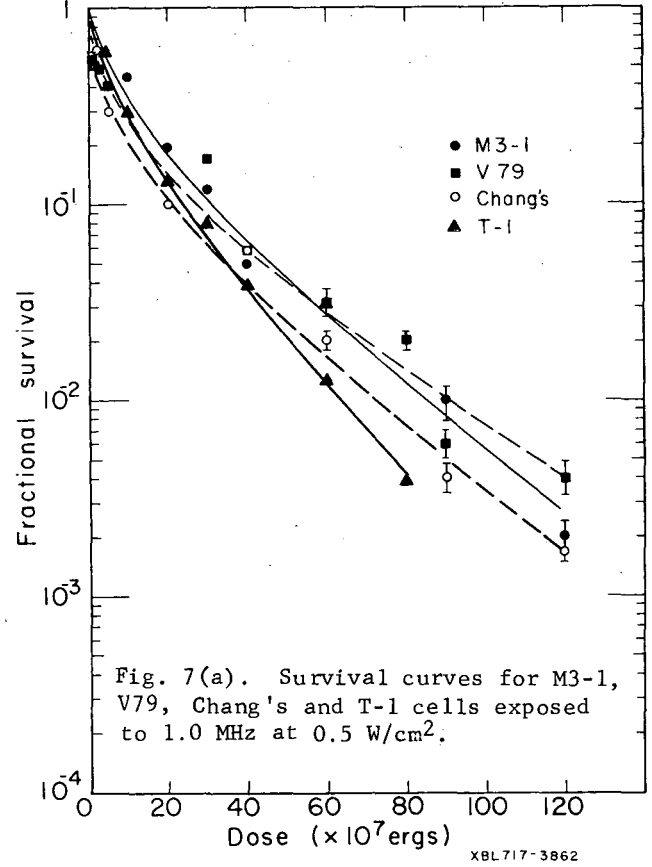


Figure 7(b). Survival curves for M3-1, V79, Chang's and T-1 cells exposed to 1.0 MHz at 1.0 W/cm².

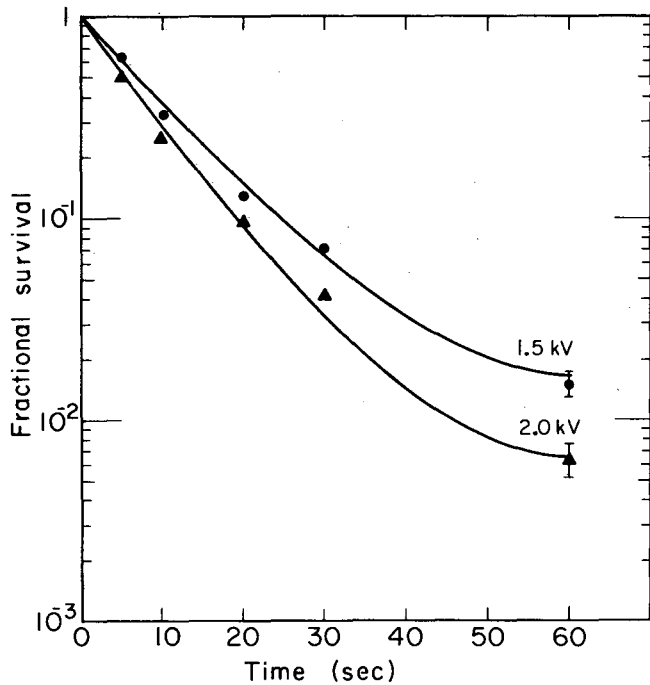


Fig. 8(a). Survival curves for M3-1 cells exposed to 0.1 MHz at 1.5 and 2.0 kV.

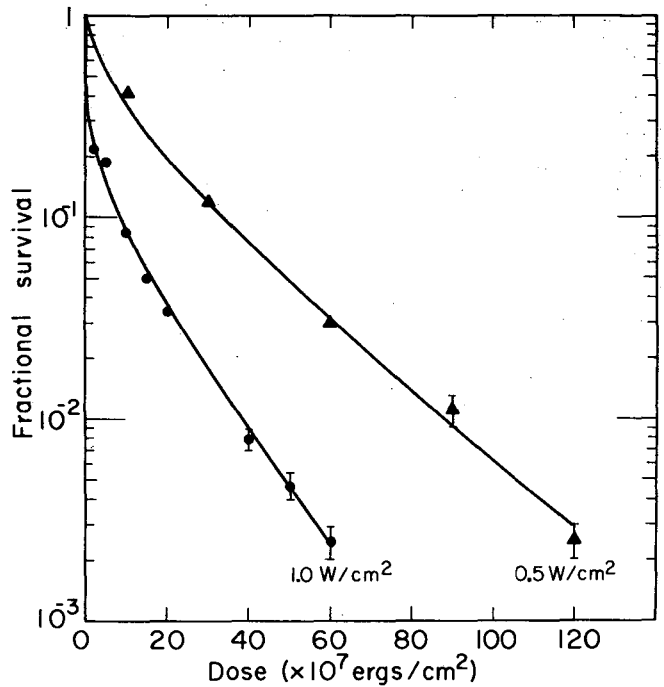


Fig. 8(c). Survival curves for M3-1 cells exposed to 1.0 MHz at 0.5 and 1.0 W/cm².

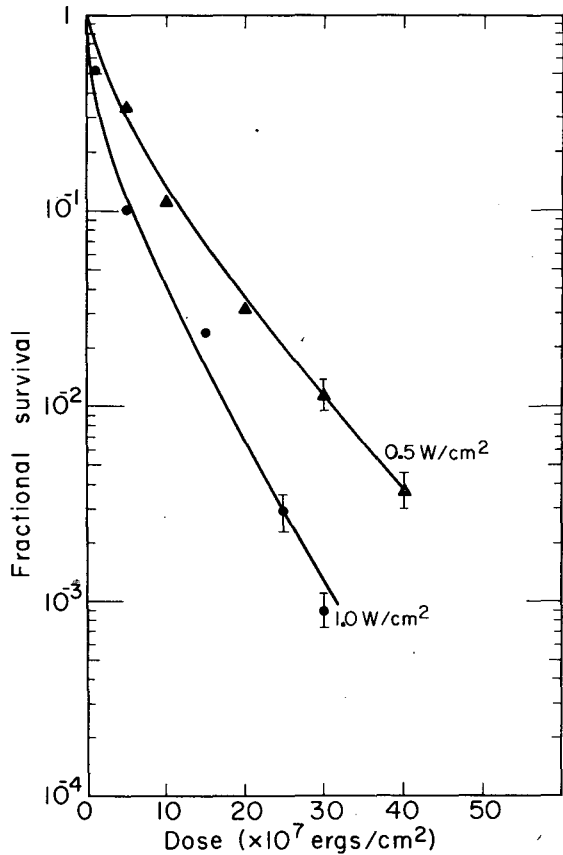


Fig. 8(b). Survival curves for M3-1 cells exposed to 0.5 MHz at 0.5 and 1.0 W/cm².

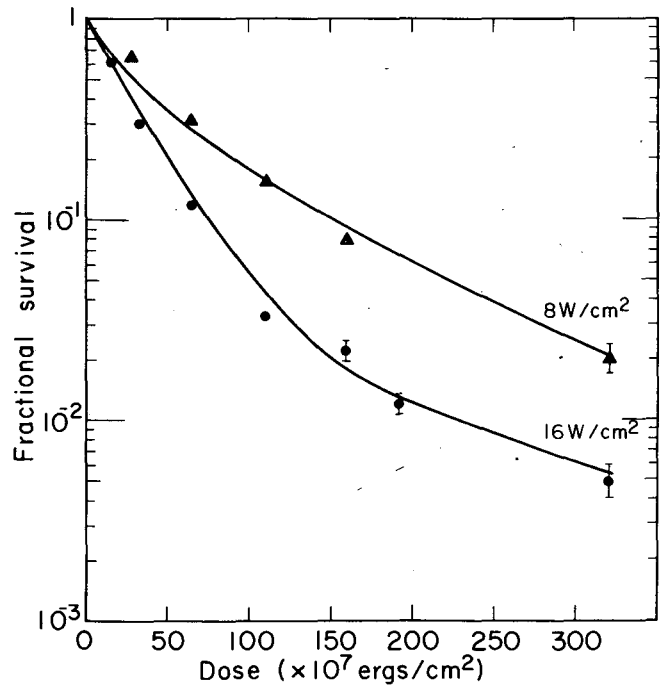


Fig. 8(d). Survival curves for M3-1 cells exposed to 2.0 MHz at 8.0 and 16.0 W/cm².

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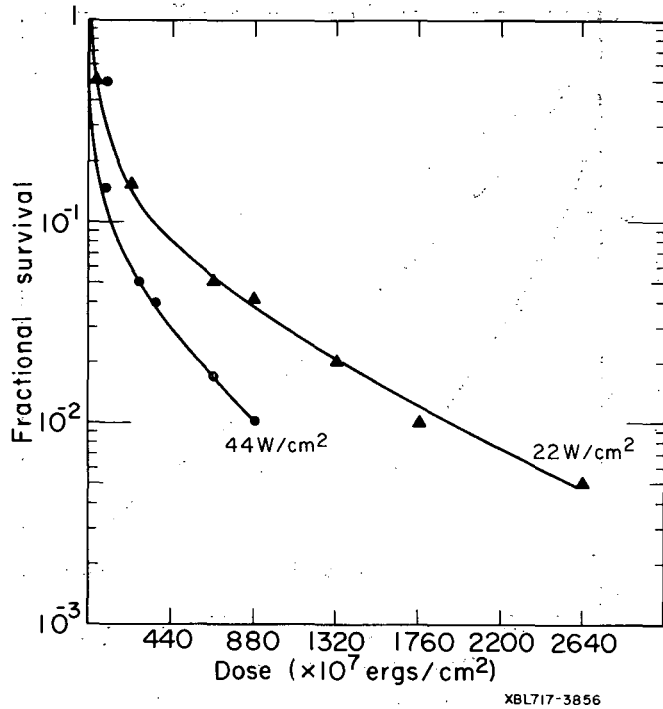


Fig. 8(e). Survival curves for M3-1 cells exposed to 3.3 MHz at 22.0 and 44.0 W/cm 2 .

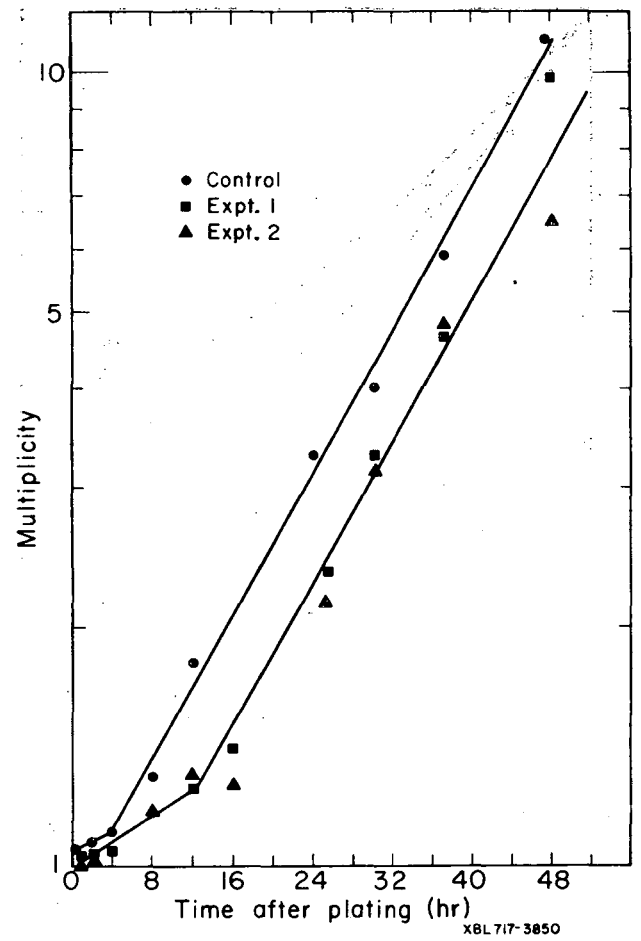


Fig. 9. Effect of 1.0 MHz frequency ultrasound on the growth curve of M3-1 cells exposed to 1.0 W/cm 2 for 60 sec.

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