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Studies of the cavitational effects of clinical ultrasound by sonoluminescence: 4. The effect of therapeutic ultrasound on cells in monolayer culture in a standing wave field

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Abstract. In previous work the phenomenon of sonoluminescence (SL) has been used to find the conditions in which transient cavitation during exposure to ultrasound is likely to be maximum. This paper reports the effect of therapeutic ultrasound on growth of mouse tumour cells in monolayer culture when the cells are insonated either at a pressure antinode or at a pressure node in a standing wave ultrasound field that is known to produce strong bands of SL at the pressure antinodes. Reduced cell numbers 72 h after insonation were recorded when the cells were insonated at an antinode but not when they were at a node. The possibility that this effect might be an artefact of the experimental system, and further experiments that could elucidate the nature of the damage, are discussed.

1. Introduction

There are several methods by which ultrasound might cause biological damage (see, for example, Williams 1983) and although a recent review of the safety of diagnostic ultrasound (Wells 1987) has concluded that extensive surveys have failed to reveal any evidence that present ultrasonic diagnostic practice carries any hazard due to ultrasound, the possibility remains that the particular combination of experimental conditions that would demonstrate significant harm has never been selected.

One damage mechanism that still receives considerable attention is transient cavitation, defined here as the growth of a bubble to many times its normal size followed by rapid collapse. The collapse is adiabatic, very high temperatures (of the order of 6000 K) are reached and free radicals have been identified as a result of this process. Thus bubbles transform the relatively low-energy density of the sound field into a very high-energy density at the moment of collapse and there are clear similarities to the method of energy deposition by ionising radiation.

There are several ways in which transient cavitation may be studied, one of which is sonoluminescence (SL)—light emission associated with the high temperatures and free radicals in the collapsing bubbles. In earlier work, we have used the phenomenon of sonoluminescence to investigate and optimise the conditions under which transient cavitation is most likely to occur. Results may be summarised as follows.

(i) There is a threshold for SL and intensities of 1 W m^{-2} or greater should be used for a strong signal (Pickworth *et al* 1988).

(ii) A well aerated, aqueous medium gives a strong light output.

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(iii) A reasonably long pulse length should be used—below 50 cycles there is a marked increase in the threshold ultrasonic intensity for the onset of SL (Pickworth *et al* 1989).

(iv) SL is brightest when there are strong standing waves. Furthermore, if both the ultrasound intensity and standing wave ratio are high, the light appears in well defined bands at the pressure antinodes. There is very little light at the pressure nodes (Leighton *et al* 1988).

These observations were used to devise an experiment that would maximise the chance of observing biological damage at the cellular level.

2. Materials and methods

The cells used were of the established mouse tumour line EMT6/Ca/VJAC. These grow as an attached monolayer on tissue culture plastic with a doubling time during exponential growth of around 12 h. The medium used was Eagles minimal essential medium with Earles salts and supplemented with penicillin and streptomycin and 20% new-born calf serum (all supplied by Gibco Biocult Ltd). To detach cells from the plastic surface a 0.075% solution of trypsin in phosphate buffered saline was used. Following removal of medium, the monolayer was rinsed twice with 2 ml of trypsin solution and the solution aspirated each time. The flask was then incubated at 37 °C for 15 min at which time the cells were suspended in medium and pipetted several times to obtain a single cell suspension.

To insonate a monolayer of cells in a standing wave field, the arrangement shown in figure 1 was used. The cells were seeded onto the lower surface of a polystyrene culture flask (Falcon 3013E, Becton-Dickinson, UK, Ltd) which had been sterilised by gamma irradiation. When orientated as shown in figure 1, the flask had a rectangular base, 75 mm × 35 mm, and was approximately 26 mm deep. Its overall volume was 50 ml. The screw top neck, of circular cross section, was well away from the ultrasound beam. The flask was supported at its edges by a stand, the height of which could be adjusted to within 0.01 mm using three screws. The stand rested on a 6 mm thick brass plate to establish good standing waves and a Therasonic 1030 transducer with an effective radiating area of 440 mm^2 (for details see Pickworth *et al* 1988) was used to insonate this arrangement from the top of the tank with 1.09 MHz continuous wave

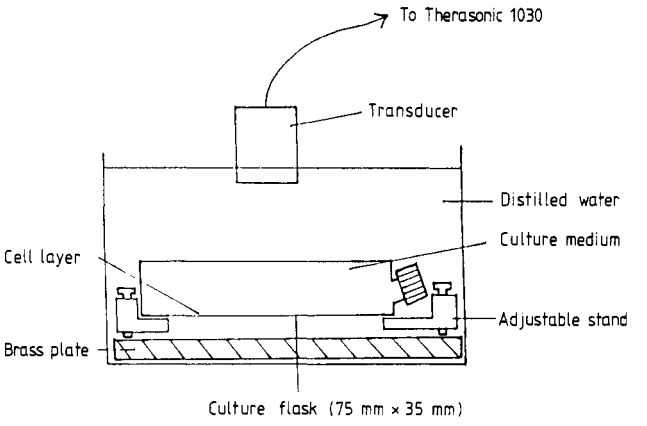

Figure 1. Schematic diagram of the arrangement used to insonate cells.

ultrasound. The distance from the transducer to the cells and the brass plate was about 5 cm. Cells were insonated at room temperature.

To test for standing waves, a culture flask was filled with water and an image intensifier was used to view the arrangement from the side. At 1 W cm^{-2}, bands of sonoluminescence (see Leighton *et al* 1988)) could be seen both between the brass reflector and the base of the culture bottle and within the bottle. The experiment relies upon the ability to place the cells at either the nodes or the antinodes accurately. To do this the positions of these acoustic features had to be calculated. The cells were placed as close as possible to the reflecting surface, since any error in calculating λ is cumulative when used to express the distance between the cells and the reflecting surface as a multiple of the acoustic wavelength. An unavoidable separation of 3.23 mm was caused by the lip of the stand (1.65 ± 0.1 mm), the thickness of the base of the culture flask (1.00 ± 0.01 mm) and a lip on the rim of the culture flask (0.58 ± 0.01 mm).

The speed of sound in water at 20 °C was taken to be 1.48×10^3 ms^{-1} (Bamber 1986), and from this the acoustic wavelength in water at this frequency is 1.36 mm (λ_w). The exact composition of the 'special polystyrene' used for the culture flask is a commercial secret, so the value quoted by Miller (1986) was used for the speed of sound in polystyrene. Using this figure (2.35×10^3 ms^{-1}) 1 mm of polystyrene was equivalent to 0.48 λ_p, whilst 1 mm of water was equivalent to 0.73 λ_w. Hence introducing the polystyrene effectively removed a quarter of a wavelength from the ultrasound pathlength.

To check on the effect of the culture flask, a needle hydrophone (Dapco NP 10-3) was placed between the transducer and the brass plate and used to map nodal and antinodal positions. A culture flask with a sawn off top was then interposed between the hydrophone and reflector, and the node/antinode positions were observed to shift by 0.35 ± 0.05 mm, or about 0.26 λ_w, in good agreement with the above.

The spacing of the bright bands of sonoluminescence (as observed through the image intensifier) could be used to check the above calculations (although the accuracy of these direct measurements was no better than 0.1 mm, so they were not in themselves good enough to determine nodal/antinodal positions to the required precision). These direct observations did agree with the more accurate calculated values.

It should be noted that it is not necessary to know the value of λ_c (the acoustic wavelength in the culture medium) to perform this experiment, although measurements made on the luminescent bands showed that it did not differ from that in water within the limit of accuracy of the measurement.

Since the top side of the brass plate is a displacement node, the surface of the flask will be at a pressure antinode if it is an exact number of half wavelengths away from the plate. In the final experimental arrangement the cell-reflector spacing was 3.74 mm at an antinode (1.00 mm in the flask = 0.48 λ_p and 2.74 mm in water = 2.02 λ_w) and 4.08 mm at a node (1.00 mm in the flask = 0.48 λ_p and 3.08 mm in water = 2.27 λ_w). The error in positioning the cell monolayer at either a node or an antinode is about 0.05 mm, equivalent to 1/7 the distance between them.

Two methods were used to seed a monolayer of cells in the bottles. Either 10^5 cells in 5 ml of medium were seeded uniformly or 10^4 cells in a volume of 0.1 ml were placed near the middle of the culture bottle and allowed to attach to the plastic surface before addition of a further 4.9 ml of medium. The second approach was adopted in an attempt to concentrate the cells near the centre of the ultrasound field.

Twenty-four hours after cell seeding, flasks were completely filled with medium (to eliminate impedance discontinuities) and insonated for 10 min at either 1, 2 or

3 W cm^{-2} at either a pressure node or pressure antinode. Control flasks were handled in precisely the same manner except for exposure to ultrasound. Immediately after treatment the culture medium was removed and replaced by the usual volume, i.e. 5 ml.

Removal of the cells from the polystyrene surface as a result of mechanical damage by the ultrasound is a distinct possibility. Therefore several small fields of view each 1 mm^2 in area were selected and prior to insonation these areas were inspected down the microscope and diagrams were drawn showing the location within the areas of individual cells. An attempt was made to re-identify the cells immediately after insonation and twice a day thereafter these cells or their daughters were scored until the cells became too crowded for positive identification.

Seventy-two hours after insonation the cells in each flask were harvested using trypsin, resuspended in an appropriate volume of medium, and counted under a haemocytometer. In each experiment either four or five flasks received the same treatment.

As a basis for comparison of any effect, a set of 14 flasks was seeded with 10^5 cells each, 4 flasks were reserved as controls and the remainder were exposed to a range of doses of 250 kVp x-rays 24 h later at a dose rate of 0.6 Gy min^{-1}. Again the cells were harvested and counted after a further 72 h.

3. Results

Table 1 shows several examples of the number of cells in selected 1 mm^2 areas just prior to and after insonation. It is clear that no cells were lost at either 1 or 2 W cm^{-2} at either the antinodes (table 1(*a*)) or the nodes (table 1(*b*)). However, at 3 W cm^{-2} behaviour is very erratic with none, some or all the cells dislodged. There is no obvious difference in behaviour at nodes and antinodes but any attempt to monitor overall growth of cells after 3 W cm^{-2} would clearly be unreliable.

At 1 and 2 W cm^{-2} there is an increase in cell numbers during the 24 h after insonation. The precise increase depends on the phase in the cell cycle when the cells were first mapped and it is not possible to decide from these observations whether or not the insonated cells are growing less rapidly than the controls.

Table 2(*a*) shows a typical set of results for the number of cells per flask counted 72 h after treatment at antinodes. Table 2(*b*) shows a set of results after treatment at nodes. The difference in cell numbers in control flasks between experiments is due to a number of factors, especially variable delay in resuming growth after plating out. Since final results are quoted relative to controls, this variation is unimportant. The consistency in number of cells per control flask within an experiment is important.

Pooled results of all experiments are shown in table 3 with the average number of cells in control flasks normalised to 100 for each experiment. Figures shown at 1 and 2 W cm^{-2} are weighted means to allow for the fact that the number of replicate flasks in different experiments was variable (for technical reasons, e.g. an occasional infection, the number of flasks counted was sometimes as low as three). The error figure quoted is the standard error of the mean within one group of experiments. When cells were insonated at an antinode the number per flask was reduced. There was no reduction in cell numbers when the monolayer was placed at a node. Neither was there any difference, within the statistical accuracy of the results, between the two seeding methods.

Figure 2 shows the results of experiments in which cells were exposed to 250 kVp x-rays and harvested for counting 72 h later. From this curve the dose of x-rays required to cause equivalent inhibition of growth may be obtained.

Table 1. Number of cells present in a 1 mm^2 area at various stages just before, and after, treatment at antinodes (*a*) and treatment at nodes (*b*).

Intensity	Just before insonation	Just after insonation	24 h later	48 h later	72 h later
(*a*) *Treatment at antinodes*					
control	22	—	40	58	81
	16	—	65	150	—
	12	—	36	120	—
	20	—	23	47	110
1 W cm^{-2}	38	38	60	70	110
	37	37	72	200	—
	9	9	32	90	300
	16	16	33	75	120
2 W cm^{-2}	31	31	44	72	100
	27	27	65	132	300
	10	10	17	60	180
	41	41	100	300	—
3 W cm^{-2}	51	0	0	0	0
	26	20	31	59	117
	13	13	31	43	101
	40	0	0	0	0

Intensity	Just before insonation	Just after insonation	24 h later	56 h later	72 h later
(*b*) *Treatment at nodes*					
control	10	—	26	45	200
	20	—	50	150	—
	26	—	50	150	—
	8	—	19	65	200
1 W cm^{-2}	12	12	29	45	200
	31	31	100	—	—
	15	15	30	110	—
	7	7	27	80	250
2 W cm^{-2}	17	17	47	66	200
	37	37	100	—	—
	17	17	29	60	200
	15	15	35	100	—
3 W cm^{-2}	8	4	8	28	68
	10	0	0	0	1
	17	3	9	22	40
	12	12	40	100	—

4. Discussion

The mechanism of transient cavitation by ultrasound may be capable of causing harmful effects analogous to those of ionising radiation on biological material. A good way to search for such effects is to optimise the likelihood that they will occur. This work has shown that when a cell monolayer is placed at a pressure antinode in a standing wave ultrasound field, where bright bands of sonoluminescence indicate that transient cavitation is high, cellular multiplication is depressed during the subsequent 72 h. If the cell monolayer is moved as little as 0.34 mm to the adjacent pressure node, there is no inhibition of cellular multiplication.

Table 2. Number of cells per flask counted 72 h after treatment at antinodes (*a*) and nodes (*b*). Results are shown for each of four replicate flasks orginally seeded with 10^4 cells.

No of cells in controls	No of cells after 1 W cm^{-2}	No of cells after 2 W cm^{-2}
(*a*) *Antinodes*		
2.8×10^5	2.3×10^5	1.8×10^5
3.0×10^5	2.5×10^5	2.9×10^5
2.8×10^5	2.8×10^5	2.3×10^5
2.6×10^5	2.6×10^5	2.2×10^5
(*b*) *Nodes*		
5.0×10^5	4.8×10^5	4.6×10^5
4.6×10^5	5.0×10^5	4.7×10^5
4.8×10^5	4.6×10^5	5.0×10^5
4.6×10^5	4.8×10^5	4.9×10^5

Table 3. Average number of cells per flask normalised to a control value of 100.

	Control (%)	1 W cm^{-2} (%)	2 W cm^{-2} (%)
Pressure antinodes			
Cells uniformly seeded (4 experiments)	100	75 ± 6	63 ± 8
Cells locally seeded (3 experiments)	100	82 ± 7	70 ± 5
Grand average (7 experiments)	100	78 ± 4	68 ± 4
Pressure nodes			
Cells uniformly seeded (3 experiments)	100	100 ± 8	104 ± 5
Cells locally seeded (3 experiments)	100	95 ± 7	99 ± 6
Grand average (6 experiments)	100	97 ± 5	101 ± 2

Results show that the effect is greater at 2 W cm^{-2} than at 1 W cm^{-2} and a calibration experiment shows that the doses of ionising radiation required to produce a comparable inhibition of cellular multiplication are quite high at about 2 Gy and 1.4 Gy respectively. If the effect of ultrasound is real, there is clearly a substantial microdosimetric effect associated with the banding pattern in the ultrasound field.

As discussed at length by Wells (1987) alternative causes of the observed effect must be considered carefully. For example, observations under the microscope showed clearly that 3 W cm^{-2} could shear cells from the polystyrene suface. The mechanical damage incurred would almost certainly inhibit cell division. Mechanical damage sufficient to prevent cell division but not sufficient to dislodge cells might have occurred at 2 and 1 W cm^{-2}. However, sequential microscopic observations did not suggest that a substantial percentage of the cells were incapable of dividing after 2 W cm^{-2}.

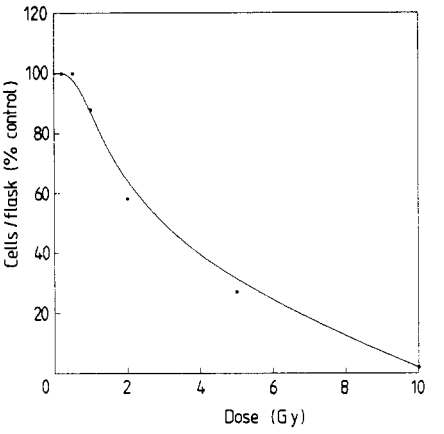

Figure 2. Number of EMT 6 cells per flask, expressed as a percentage of controls, 72 h after various doses of 250 kVp x-rays.

Another possibility is that the ultrasound might have released toxins from the polystyrene surface. However, the culture medium was changed after treatment and seemingly no such 'toxins' were released at pressure nodes.

Growth inhibition was not significantly different when an attempt was made to localise the cells near the centre of the ultrasound field, even though the ultrasound intensity should on average be greater there. There are several possible reasons. First, these cells were seeded in a small volume (0.1 ml) so errors in the seeding concentration were greater. Second, since the cells could not be seen during insonation, they may not have been as close to the ultrasound axis as we thought. Third, cell counting was less accurate at the end of the experiment. Finally, when the cells were localised they may have become overcrowded during the growth period. This would inhibit cell division in the controls with an apparent reduction in any effect of the ultrasound.

Clearly, these experiments require to be repeated, either by another experimenter or in another laboratory. Also, more sophisticated methods are required to elucidate the nature of the damage. For example we need to know if chromatid aberrations or single strand breaks in DNA occur after insonation at a pressure antinode. Such changes should be readily detectable if the effect is comparable to that produced by a dose of 2 Gy of x-rays.

If these results are confirmed and the nature of the biological damage can be identified at the cellular level, important new information will become available that will help to establish threshold levels for damage when ultrasound is used clinically for diagnosis and therapy.

5. Conclusion

On the basis of earlier work on the phenomenon of SL *in vitro*, an experimental arrangement was adopted that would maximise the likelihood that cells in monolayer culture would be damaged by mechanisms associated with collapse cavitation. There was a significant inhibition in growth by 72 h after insonation. The possibility that this effect is an artefact of the experimental system has not yet been completely eliminated. However, the fact that a movement of 0.34 mm in the position of the monolayer from a pressure antinode to a pressure node eliminated the growth inhibition

suggests that the effect is real and is strong evidence in support of further work on this experimental system.

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Résumé

Etude des effets de cavitation avec un appareil à ultrasons à usage clinique à l'aide de la sonoluminescence.

Dans un travail précédent, les auteurs ont utilisé le phénomène de sonoluminescence (SL) pour trouver les conditions dans lesquelles une cavitation transitoire apparaît durant l'exposition aux ultrasons avec une intensité maximale. Ce travail rapporte l'effet d'ultrasons à usage thérapeutique sur la croissance de cellules tumorales de souris en culture monocouche quand des cellules sont soumises aux ultrasons, soit au niveau d'un ventre de pression, soit au niveau d'un noeud de pression, dans un champ ultrasonore à ondes stationnaires reconnu capable de produire de fortes bandes de SL aux ventres de pression. Les auteurs ont enregistré une diminution du nombre des cellules, 72 heures après l'exposition aux ultrasons, lorsque les cellules étaient exposées au niveau d'un ventre, mais pas quand elles étaient situées au niveau d'un noeud. Les auteurs discutent la possibilité que cet effet puisse être un artéfact du système expérimental, et des expériences complémentaires qui pourraient élucider la nature des dommages.

Zusammenfassung

Untersuchungen der Hohlraumeffekte von klinischem Ultraschall durch Sonolumineszenz: 4. Der Einfluß von therapeutischem Ultraschall auf einschichtige Zellkulturen in einem Stehwellenfeld.

In vorhergehenden Arbeiten wird das Phänomen der Sonolumineszenz (SL) verwendet zur Bestimmung der Bedingungen unter denen die Ubergangshohlraumbildung während der Beschallung mit Ultraschall ein Maximum erreicht. In der vorliegenden Arbeit wird über den Einfluß von therapeutischem Ultraschall auf das Wachstum von Mäusetumorzellen in einschichtigen Zellkulturen berichtet, wenn man die Zellen entweder an Druck-Antiknoten oder an Druckknoten in einem Stehwellenultraschallfeld beschallt, von dem man weiß, daß es starke Bänder von SL bei Druckantiknoten erzeugt. Eine Reduzierung in der Anzahl der Zellen wird 72 h nach Beschallung beobachtet, wenn die Zellen an einem Antiknoten beschallt worden waren, nicht aber an einem Knoten. Die Möglichkeit, daß dieser Effekt ein Artefakt des experimentellen Systems ist, sowie weitere Experimente, die die Natur dieses Schadens aufklären könnten, werden diskutiert.

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