

EFFECT OF PULSED ULTRASOUND ON THE FREQUENCY OF SCE AND ON CELL PROLIFERATION

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ABSTRACT

The effects of chronic and acute ultrasound doses on lymphocytes obtained from three males and three females were studied in terms of sister chromatid exchange and cell proliferation index during the various phases of the cell cycle. A decrease in the frequency of M₂ cells in 48-hour cultures (control = 1.01; G₁ = 0.48; S = 0.29; 1% < p < 5%) treated with chronic doses of ultrasound and the absence of these cells in cultures treated with acute doses recommends caution in the use of ultrasound waves for therapeutic purposes. Culture time (42 and 72 hours) had no effect on the frequency of sister chromatid exchange either in the ultrasound-treated group or the control.

INTRODUCTION

Ultrasound (US) can be employed both at high and low intensities for medical purposes. Low intensity applications (5 to 30 mW/cm²) are used for diagnoses, and high intensities (250 to 3000 mW/cm²) for therapy in this case the application can be used from 3 to 50 minutes during several days. The waves can be continuous or pulsed (milisecond (ms) pulses, repeated each 100 ms).

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Improvement of methods for the detection of sister chromatid exchange (SCE) has facilitated its quantification in chromosomes of cells exposed to mutagenic, carcinogenic and/or clastogenic agents (Korenberg and Freedlander, 1974). Even though the exact mechanism of SCE formation is unknown, a wide variety of chemical and physical agents are known to increase their frequency (Latt *et al.*, 1979; Abe and Sasaki, 1982). While alkylating agents and ultraviolet (UV) light are efficient SCE inducers, ionizing radiation and radiomimetic chemicals, which produce molecular damage leading to chromosome aberrations, are inefficient in producing DNA changes leading to SCE (Littlefield, 1982). This suggests that different lesions lead to different effects (Perry and Evans, 1975) and demonstrates a certain specificity in the interaction between genetic material and the environment.

As is the case for X-rays, US seems to be ineffective in increasing SCE frequency (Lundberg *et al.*, 1982; Au *et al.*, 1982; Barnet *et al.*, 1982; Millier *et al.*, 1983; Becher *et al.*, 1983), although a small increase in SCE in human lymphocytes has been reported by some investigators (Licheskind *et al.*, 1979; Haupt *et al.*, 1981; Barrass *et al.*, 1982).

The technique of 5-bromodeoxyuridine (BrdU) incorporation into DNA also permits an accurate and simple study of the kinetics of replication in cell systems. Ionizing X and radiation is known to delay mitosis in mammalian cells *in vitro*, the extent of delay varying with the cell cycle phase (Sinclair, 1968; Woods and Lowenthal, 1984). Maximum sensitivity seems to occur at G₂ in relation to S and G₁ (Dewey and Highfield, 1976). The delay in proliferation can be demonstrated by the accumulation of cells in first division (Purrot *et al.*, 1980; Obe and Beek, 1981). Conflicting results have been obtained in studies of cell proliferation delay induced by US. Liebeskind *et al.* (1979), Siegel *et al.* (1979) and Barnet *et al.* (1982) detected low cell growth, whereas Bleaney and Oliver (1972), Toombs *et al.* (1979) and Becher *et al.* (1983) did not observe this phenomenon.

The objective of the present investigation was to study SCE frequency, cell kinetics and their interrelationships in cultured human lymphocytes submitted to the action of pulsed US used for therapeutic purposes.

MATERIAL AND METHODS

The study was conducted on peripheral lymphocytes in temporary culture obtained from 3 healthy males and 3 healthy females aged 14 to 27 years. Twenty-five drops of whole blood were obtained from each subject and cultured in 10 ml RPMI 1640 medium (Gibco) with phytohemagglutinin added (Ferrari, 1968) and enriched with AB human serum, for 48 and 72 hours.

For SCE detection, cells were exposed to BrdU at a final concentration of 10 µg/ml throughout the incubation period. A hypotonic 0.075 M KCl solution was

used, and 3:1 methanol:acetic acid were used for fixation. Differential staining was obtained by the technique of Korenberg and Freedlander (1974), slightly modified.

SCE analysis

SCE frequency was determined in 48- and 72-hour cultures of cells in second division showing the 46 chromosomes of the complement. Exchanges at the centromere level were excluded; only terminal exchanges (counted as one) and interstitial exchanges (counted as two) were considered.

Analysis of cell proliferation

The cell proliferation index (PI) was calculated by determining the relative frequency of cells in first, second and third division (M_1 , M_2 and M_3), and of interphase cells. The following ratio was used for 48-hour cultures:

$$P_{ij} = \frac{\text{No. of cells in } j\text{th division}}{\text{Total No. of cells}} \times 100$$

$$j = 1,2$$

For 72-hour cultures, 100 metaphases were examined and classified as M_1 , M_2 and M_3 , and the respective proportions were computed by the following ratio:

$$P_{ij} = \frac{\text{No. of cells in } j\text{th division}}{100}$$

$$j = 1,2,3$$

Ultrasound treatment

Cultures harvested at 48 and 72 h were exposed to US for 60 minutes in a fractionated (15 minutes, chronic dose - CD) and in a continuous (acute dose, AD) manner at the beginning and after 24 h of incubation, corresponding to phases G_1 and S respectively.

Ultrasound was applied as follows: 1.5 MHz frequency, 57 mW/cm² intensity; 200 μ s pulse width; 1000 pulses per second (pps); and 27 V amplitude. These US parameters are used therapeutically to accelerate the formation of bone callus.

RESULTS

SCE frequency was unchanged in 48 and 72-hour cultures at all exposures when the treated experimental groups (G_1 and S) were compared with the control by the Friedman statistical parameter (S) as in Table I. Culture time was not a limiting factor for SCE frequency. The variation of mean SCE values per cell in the experimental groups was random.

Table I - SCE (Mean \pm SEM) per cell from 48 and 72 hour cultures treated with chronic (CD) and acute doses (AD) of ultrasound during phases G_1 and S of the cell cycle.

Culture time and dose	Experimental group		
	Control	G_1	S
48 hour			
CD	8.88 \pm 0.43	9.80 \pm 0.51	10.67 \pm 0.56
AD		0	0
72 hour			
CD	10.48 \pm 0.31	12.50 \pm 0.35	11.66 \pm 0.40
AD		11.73 \pm 0.48	11.20 \pm 0.38

Table II shows the calculated PI_j for 48 and 72-hour cultures exposed to US under CD and AD conditions. No significant differences in M_1 cells treated under CD conditions in 48 hour cultures were observed between the experimental groups ($K = 3$, $N = 6$, $S = 4.33$; $P > 10\%$). M_2 cells showed a decrease when the weighted means for the experimental groups were compared (control = 1.01; $G_1 = 0.48$ and $S = 0.29$) ($K = 3$; $N = 6$; $S = 8.72$; $1\% < P < 5\%$). Differences between the experimental groups C and S were detected by the multiple comparison test. The distribution of calculated PI_j in culture treated under AD conditions for M_2 cells were absent in all groups except the control. The Friedman statistical parameter did not detect significant differences for M_1 cells ($K = 4$, $N = 6$, $S = 5.60$; $P > 10\%$).

The frequencies of M_1 , M_2 and M_3 cells calculated for 72-hour cultures exposed to US under CD and AD conditions, showed no delay in cell proliferation either for CD conditions ($K = 4$, $N = 6$; $S = 2.00$; $P > 10\%$), or under AD conditions ($K = 4$, $N = 6$; $S = 0.20$; $P > 10\%$), since there was no accumulation of M_1 cells and consequent reduction of M_3 cells.

Table II - Proliferation indices determined for M₁, M₂ and M₃ cells from 48 and 72 hour cultures treated with chronic (CD) and acute doses (AD) of ultrasound during phases G₁ and S of the cell cycle.

Culture time and dose	Experimental Group								
	G ₁			Control			S		
	M ₁	M ₂	M ₃	M ₁	M ₂	M ₃	M ₁	M ₂	M ₃
48 hour									
CD	4.47	0.48	0	6.18	1.01	0	3.54	0.29	0
AD	2.85	0	0				3.38	0	0
72 hour									
CD	34	37	29	39	36	25	42	35	23
AD	40	34	26				41	32	27

Friedman's multiple comparison test (M₂).

C G₁ S

DISCUSSION

Comparative studies of the effect of ultrasound radiation on mammalian cell cultures should be conducted not only to elucidate the mechanism of action of this radiation, but also to establish a radiobiological basis for therapeutic and diagnostic purposes. Studies of cell proliferation kinetics have shown that most cells exposed to moderate X-ray doses die after at least one division and that abnormalities and prolongation of division time frequently precede cell death (Sasaki, 1984). Thus there is a correlation between cell death, chromosome aberrations and SCE, which share some common damage to DNA (Natarajan *et al.*, 1984; Singh *et al.*, 1984).

The SCE technique has proved to be minimally useful for the detection of damage induced by physical mutagens (Abe and Sasaki, 1982; Littlefield, 1982; Lundberg *et al.*, 1982; Au *et al.*, 1982; Barnet *et al.*, 1982; Becher *et al.*, 1983; Millier *et al.*, 1983). This is definitely due to the fact that events responsible for the chromosome breaks predominating in cells treated with physical agents are not the same that trigger SCE formation (Perry and Evans, 1975). The results in Table I sug-

gest that pulsed-wave ultrasound applied under the conditions and parameters used here is harmless at the SCE level, confirming reports by other authors (Barnet *et al.*, 1982; Au *et al.*, 1982; Becher *et al.*, 1983; Millier *et al.*, 1983).

The absence of effect of culture time on SCE frequency (Crossen, 1982) was also confirmed in the present study, in both the treated and the control groups. (Table I). This demonstrates that, while studies of chromosome aberrations for evaluation of the mutagenicity of various agents should be conducted on 48-hour lymphocyte cultures so that this action may be detected in M_1 cells, the study of SCE frequency in M_2 cells can be conducted at any culture time.

Beek and Obe (1974) found that the two lymphocyte subpopulations in 48-hour cultures differ in sensitivity to chemical agents, with the population that divides first (M_2 cells) being 3 times more sensitive than the second (M_1 cells). There is a strong possibility that unrepaired DNA damage will result in delayed proliferation (Purrot *et al.*, 1980; Popescu *et al.*, 1986) of cells simultaneously treated with chemical and physical agents. Treatment during the cell cycle shows that the G_1 phase is less sensitive than phases S and G_2 . Sensitivity to radiation seems to be highest at G_2 (Dewey and Highfield, 1976), whereas stage G_1 seems to have a protective mechanism. The absence of this stage in mammalian embryos implies that these embryos are more exposed to the action of radiation (Prescott, 1982).

The reduction in M_2 cells in 48-hour cultures (Table II) may permit us to assume, by extrapolation, that exposure to pulsed US at the dosages used here should be avoided during the initial stages of pregnancy, especially for the treatment of fractures in the pelvic and coxofemoral regions.

The results obtained here for 72-hour cultures suggest that cell recovery may occur over this culture time. If this were the case, a lower frequency of M_3 cells and a consequent accumulation of M_1 cells should be detected, but no such occurrence was observed (Table II).

After lymphocyte stimulation with phytohemagglutinin, the cultures contain cells of different generations, leading to the heterogeneity detected in 72-hour cultures (Morimoto *et al.*, 1983). This heterogeneity was probably responsible for the random response of cells treated with both doses of US. The complex nature of lymphocyte cultures may mask the results of studies on cell proliferation and chromosome aberrations for the detection of the mutagenicity of several agents, as was the case in the present study. Thus, this system should be selected with caution for mutagenicity studies (Beek and Obe, 1974, 1976; Morimoto *et al.*, 1983).

Kremkau *et al.* (1976) noted that some chemical agents such as nitrogen mustard potentiate the action of US, and Jacob (1979) and Purrot *et al.*, (1980) reported that BrdU potentiates the action of X-rays, as revealed by cell toxicity and by reduction of cell proliferation.

Table II shows a decrease in M_1 cells in US-treated groups in relation to the control, although the difference was not statistically significant. It is possible that

BrdU incorporation into two of the DNA strands in M_1 chromosomes makes the DNA more unstable, a fact that would cause a reduction in M_1 cells. When incorporation involves 3 strands (M_2 cells), sensitivity to US ($x = 0.38$) is approximately 3 times that observed in the control ($x = 1.01$) in 48-hour cultures.

DNA damage can be detected through a variety of molecular and cytologic parameters (Preston *et al.*, 1981; Latt *et al.*, 1981; Popescu *et al.*, 1986). The induction of chromosome changes may have influenced the proliferative capacity of US-treated cells in 48-hour cultures in the present study, since chromosome alterations characteristic of several forms of cancer seem to be responsible for changes in structure and expression of genes responsible for the regulation of cell growth (Popescu *et al.*, 1986).

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RESUMO

Os efeitos de doses agudas e crônicas - ultra-som pulsado - foram estudados em linfócitos de três indivíduos do sexo masculino e três do sexo feminino, observando-se a frequência de trocas entre cromátides irmãs e o índice de proliferação celular nas várias fases do ciclo celular. A diminuição da frequência de células M_2 em culturas de 48 horas (controle = 1,01; $G_1 = 0,48$; $S = 0,29$; $1\% < p < 5\%$) tratadas com doses crônicas de ultra-som e a ausência total dessas células em culturas tratadas com doses agudas recomendam cautela no uso de ondas ultra-sônicas com finalidades terapêuticas. O tempo de cultura (42 e 72 horas) não modificou a frequência de trocas entre cromátides irmãs tanto no grupo tratado pelo ultra-som como no grupo controle.

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