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In vitro effects of high-energy pulsed ultrasound on human squamous cell carcinoma cells

Abstract Human squamous cell carcinoma cells cloned from the hypopharynx (FaDu) and oral cavity (SCC-4) were exposed to high-energy pulsed ultrasound (HEPUS) in vitro to evaluate the effects of various physical parameters on cell viability. Such included the number of pulses, voltage applied, pulse repetition rate and cell density. The experimental piezoelectric ultrasound transducer used in the experiments generated pulses with a high negative pressure amplitude. By varying the repetition frequency from 0.6 to 8 Hz, cell viability was found to be least when pulse repetition was approximately 1 Hz. An increase in transducer voltage resulted in a linear decrease in cell viability. The cell survival rate dropped exponentially as a function of the number of pulses applied, reaching 4.2% after 2000 pulses. The cell survival rate exhibited no significant dependence on cell density when cells ranged from 1 to $3.5 \cdot 10^6$ cells ml^{-1}. Data obtained with trypan blue dye exclusion were confirmed by measurements of intracellular lactate dehydrogenase released into an extracellular fluid supernatant. By applying HEPUS to tumor cells, almost complete destruction of the cells could be achieved in vitro. The degree of cell destruction achieved depended significantly on the number of pulses administered, the pulse repetition rate and the transducer voltage applied.

Key words High-energy pulsed ultrasound · Cell viability · Squamous cell carcinoma cells · Treatment

Introduction

When implementing extracorporeal shock wave lithotripsy to treat kidney, gall bladder and salivary disorders non-invasively, the acoustic shock waves used can cause hemorrhage or other trauma to surrounding tissue, especially if shock wave energy is misapplied [5, 8].

Recent investigations with conventional lithotripters have shown that the growth and viability of tumor cells can be affected by insonation with high-energy ultrasound [11, 14]. Electrohydraulic transducers do not permit variation of the pulse wave form, while electromagnetic transducers do so only to a limited extent. In contrast, piezoelectric modalities enable enhancement of the negative pressure amplitude of the shock wave. This occurrence is the most probable cause of tissue injury. During the negative pressure phase liquid will develop gas-filled bubbles (cavitation). The implosion of these microbubbles can induce extreme physical conditions [13], including secondary shock waves, temperatures up to several thousand degrees and pressures up to several thousand bar, with resultant tissue trauma.

Based on the principles of piezoelectric shock wave generation, the present study was devised to determine the effect of high-energy pulsed ultrasound (HEPUS) and the influence of various physical parameters on the viability of human squamous cell carcinoma cells.

Materials and methods

Cell cultures and cell preparation

Human squamous cell carcinoma cells from the hypopharynx (FaDu, HTB 43) and oral cavity (SCC-4, CRL 1624) were obtained from the American Type Culture Collection, Rockville, Maryland, USA. Cells were cultivated in tissue culture flasks (Greiner, Germany) with an EMEM (Ham's F12/DMEM) cell culture medium enriched with 10% or 20% fetal calf serum, 1% penicillin-streptomycin, 1% L-glutamine and 1% sodium pyruvate (all obtained from Gibco/BRL, Germany). The doubling time of the cells grown as a monolayer in an incubator under standard conditions (relative air humidity, 97%; atmospheric CO_2 content, 8%; pH 7.4; 37°C) was approximately 2 days.

Cells were first rinsed twice with physiological saline and subsequently suspended for a short time in a phosphate-buffered saline solution containing low-dose trypsin (0.25%) and 0.1% EDTA. Cells were next resuspended in fresh EMEM cell culture medium enriched with the solutions described above and then concentrated. Immediately after this procedure, 2 ml aliquots of the cell suspen-

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sion were transferred to polyethylene tubes (2 ml volume, 1 mm wall thickness; Nunc, Germany). Tubes were shortened to 44 mm lengths and could be sealed with a lid. These were stored at room temperature until ultrasound experiments were carried out.

Pulse generation and HEPUS application

Figure 1 shows the characteristic differences of a shock wave used in lithotripsy and the pulsed ultrasound used in this study. The electroacoustic ultrasound transducer used was a specially designed experimental piezoceramic burst-signal test generator (GMW2, Department of Acoustics IHE, University of Karlsruhe, Germany), as schematized in Fig. 2. The pressure-versus-time wave form of the exponentially decreasing bipolar oscillations generated by the test generator was characterized by a short rise time (< 170 ns) and a high negative pressure amplitude with an p^+/p^- ratio of 0.5–1. In the present study pressures up to 30 MPa were applied.

For subsequent experiments the transducer was filled with partially degassed water (< 2.3 mg O_2/l; 37°C ± 1°C).

The polyethylene tubes containing tumor cell suspensions were positioned by means of a platform adjustable in three dimensions (x, y, z). Two laser beams served to define precisely the focus position of the transducer into the tubes. The base of the tube was located 14 mm below the focus and the surface of the water 30 mm above the focus.

Extensive in vitro pressure measurements were conducted beforehand with a needle probe hydrophone (Imotec 300/005/04; Imotec, Germany) and a polyvinylidene difluoride coaxial hydrophone (Department of Acoustics IHE; University of Karlsruhe) yielded a focus area of 3 × 3 × 11 mm (50% isobar = –6dB line) when the maximum transducer voltage was applied. Individual high-energy pulses had a good degree of reproducibility. Further pressure measurements were carried out using small plastic tubes made of polyethylene, polypropylene and polystyrene (all obtained from Nunc) placed in the focal zone of the transducer. The polyethylene containers were best suited for the HEPUS experiments due to their minimum pressure reduction (approx. 7%).

Parameters investigated

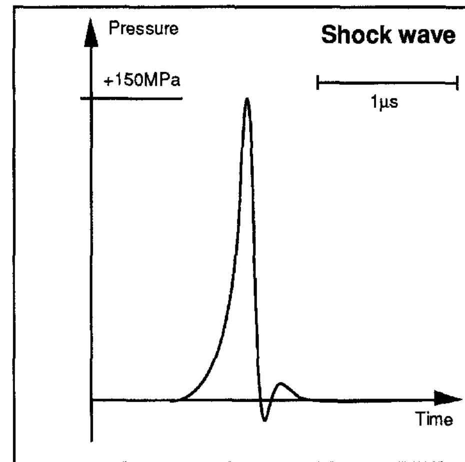
Before and after exposure of each tumor cell suspension to ultrasound the fraction of living cells present was determined quantitatively by trypan blue dye exclusion [7]. Three samples were examined per parameter setting.

Intact cells were counted in a hemocytometer in quadruplicate. Identically prepared control cell samples were positioned at the edge of the water bath outside the ultrasonic field and were subsequently handled by the same procedures as the tumor cells.

Assayable enzymes found in the supernatant of the fluid after centrifuging suspended cells were lactate dehydrogenase (LDH), aspartate aminotransferase, alanine aminotransferase, γ-glutamyltransferase, α-amylase, lipase and total serum protein. However, LDH proved to be indicative of cell damage induced and revealed the highest absolute values with small variations of enzyme levels also detected. Enzyme levels measured in individual samples were normalized to LDH values of corresponding cell suspensions subjected to insonation by an ultrasonic oscillator (Branson, USA), providing evidence for complete cell destruction.

Variable treatment parameters

On the basis of a standard value of f = 1 Hz for pulse repetition rate, a standard transducer voltage of $U = U_{max}$, and a standard cell density of $\rho_{cell} \approx 2 \cdot 10^6$ cells ml^{-1}, individual parameters of ultrasound application varied as follows: number of pulses (n) = 100–2000; generator voltage (U) = 54–100% U_{max}; pulse repetition rate (f) = 0.6–8 Hz; density of suspended cells (ρ_{cell}) = 1.3–3.5 · 10^6 cells ml^{-1}.



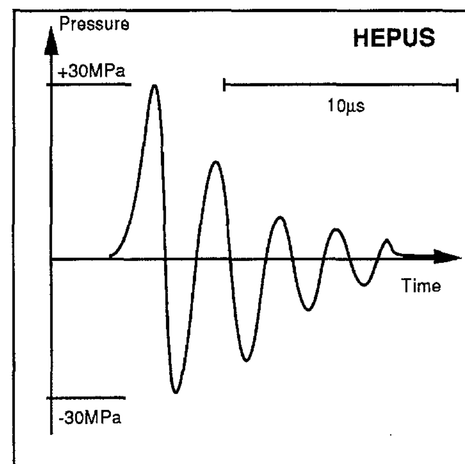


Fig. 1 Pressure-versus-time profile of a piezoelectrically generated shock wave (*above*) for stone disintegration and HEPUS wave form (*below*). Multiple oscillations and high negative pressure phases are illustrated

Results

At settings for standard parameters (SP) of the generator voltage and pulse repetition rate an exponential decrease was found in the number of cells surviving HEPUS applications. This was observed in samples having an initial cell density of 2 · 10^6 cells ml^{-1} as the number of pulses applied increased (Fig. 3a). Approximately 50% of the cells were already destroyed after 300 pulses. After 800 pulses 14% of the cells had survived. A further increase in the number of pulses applied resulted in virtually complete destruction of cells. Survival rate was 4.2% after 2000 pulses.

An increase in transducer voltage in reference to SP resulted in a linear decrease of cell survival rate (Fig. 4a). At 54% of the maximum generator voltage output, 24% of the cells were destroyed. When applying maximum energy at a pulse repetition rate of 1 Hz to samples with a cell density of 2 · 10^6 cells ml^{-1}, trypan dye exclusion

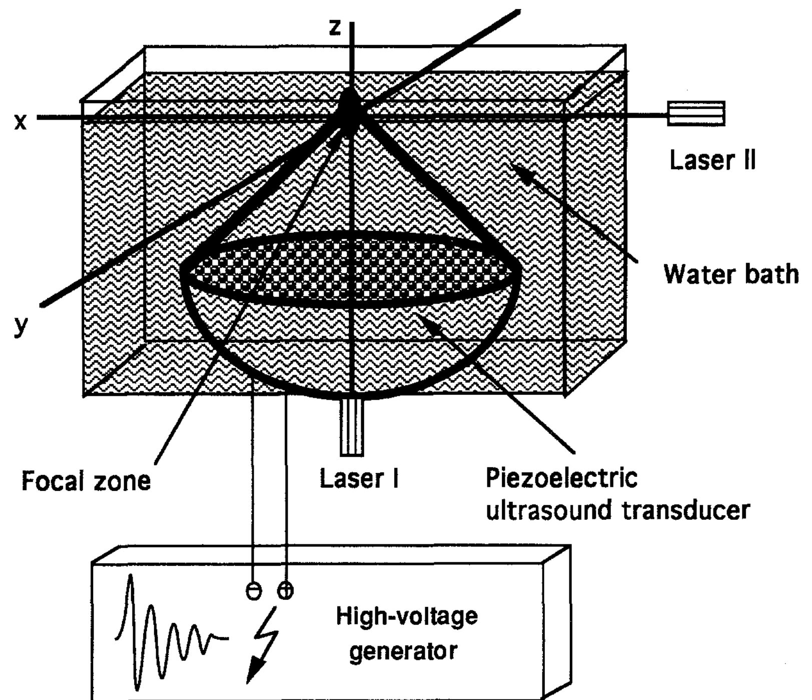


Fig. 2 Diagram of the piezoelectric test generator GMW2. The sample tubes containing the cell suspension were positioned in the focus by means of the two laser beams and then exposed to HEPUS

showed that 16% of tumor cells were intact after 400 pulses as compared to control samples.

Insonation of cell suspensions with varying pulse repetition rates in repeated measurement cycles showed maximum cell destruction at a frequency of 1 Hz (Fig. 5a). Any slight deviation from this value towards a higher or lower frequency range led to a distinct increase in cell viability. However, no correlation was observed between cell destruction and the density of cells suspended in the experimental medium (Fig. 6). At constant values for 400 pulses a pulse repetition rate of 1 Hz and maximum generator voltage, U_{max} cell survival rates reached 34 ± 4.5% of the corresponding figures noted for control samples.

LDH levels measured in the supernatant fluid of the FaDu cell suspensions following HEPUS applications agreed quantitatively and qualitatively with cell count results. Thus, increasing the number of pulses also led to an exponential increase in enzyme levels (Fig. 3b). The linear decrease in cell viability with increasing generator voltage was verified experimentally by a corresponding increase in LDH concentrations (Fig. 4b). The results of pulse repetition rates were also paralleled by the data derived from cytometry. The highest enzyme levels in the supernatant fluid following insonation of the tumor cell suspensions were also measured at 1 Hz (Fig. 5b).

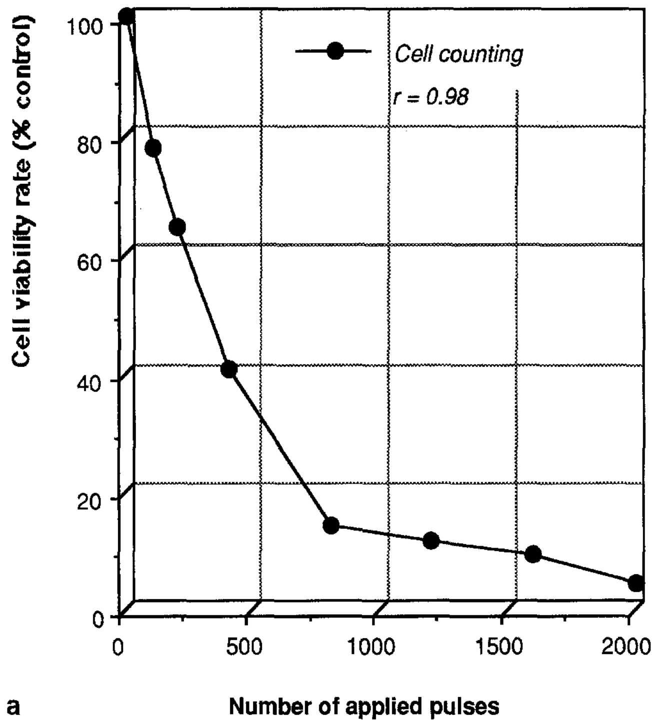
Similar results were obtained with SCC-4 cells (Fig. 7).

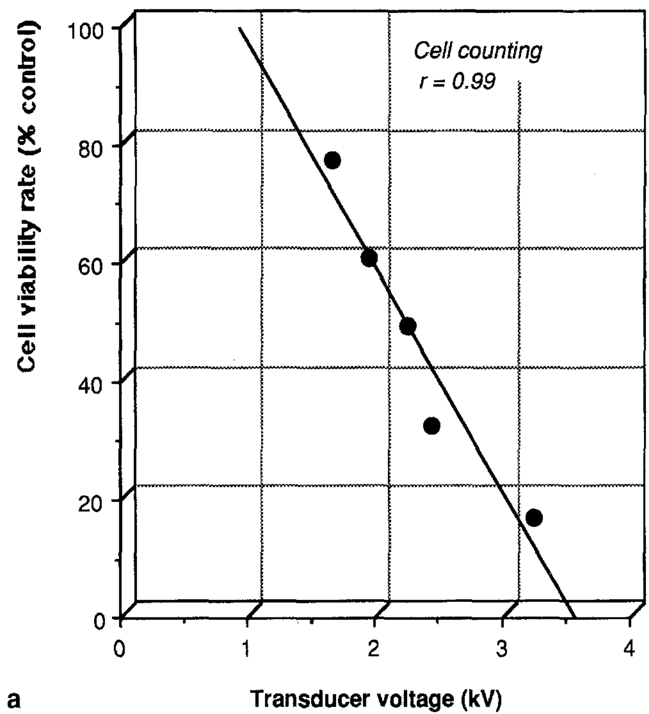
Discussion

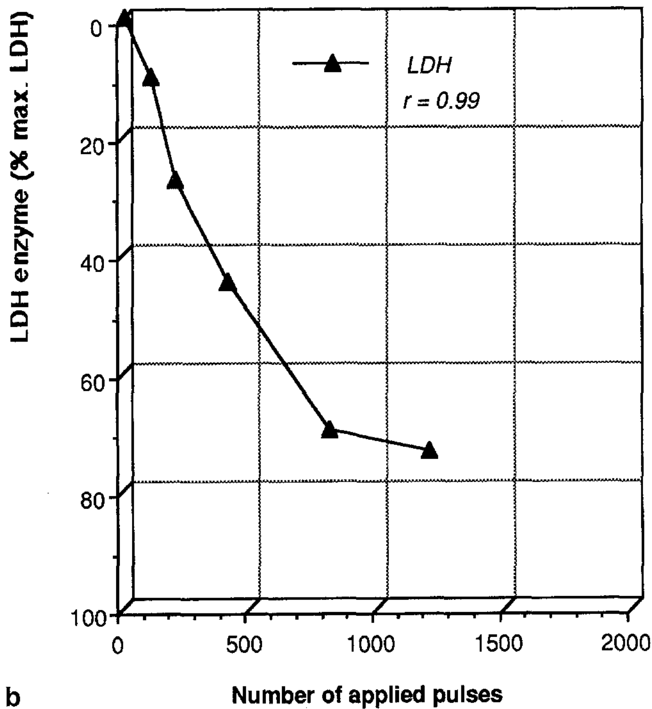
Within the scope of extracorporeal shock wave lithotripsy, the destructive effects of shock waves on tumor cells have been investigated in a number of studies [2, 3, 9, 10, 12, 14–16]. These studies have demonstrated that the effects observed were dependent on the shock wave energy applied. For various series of human and animal tumor cells, cell survival rates < 10% have been attained, even after administration of 1000 pulses [9, 11]. All these investigations were conducted with standard lithotripters or experimental systems equipped with electrohydraulic or electromagnetic generators. However, these systems offered only a limited scope for modifying the physical features of the wave form. In contrast, the structural principles of piezoelectric generators and the characteristic wave form of a piezoelectrically generated pulse can be shaped to produce multiple oscillations with a high negative pressure amplitude.

It is already known from clinically established lithotripsy that the negative pressure component of a shock wave is primarily responsible for undesired tissue damage on this occasion [4, 10]. The geometric dimensions of the piezoelectric generator allow the pulse energy to be focused on an extremely small target area. This proves to be an essential prerequisite for precision in in vivo HEPUS treatment of tumors. For this reason, the size of our polyethylene sample tubes was reduced to more closely match the geometry of the focal zone within the spatial limits imposed by our experimental set-up.

The rate of cell destruction achieved in human squamous cell carcinoma cells with the GMW2 prototype piezoelectric generator exceeded in vitro results obtained in studies on tumor cells using other experimental systems or conventional shock wave generators available to date. Cell survival following the application of 1000 pulses was of the order of several per cent with the GMW2 system. The rate of cell destruction showed a direct exponential dependence on the number of pulses administered.







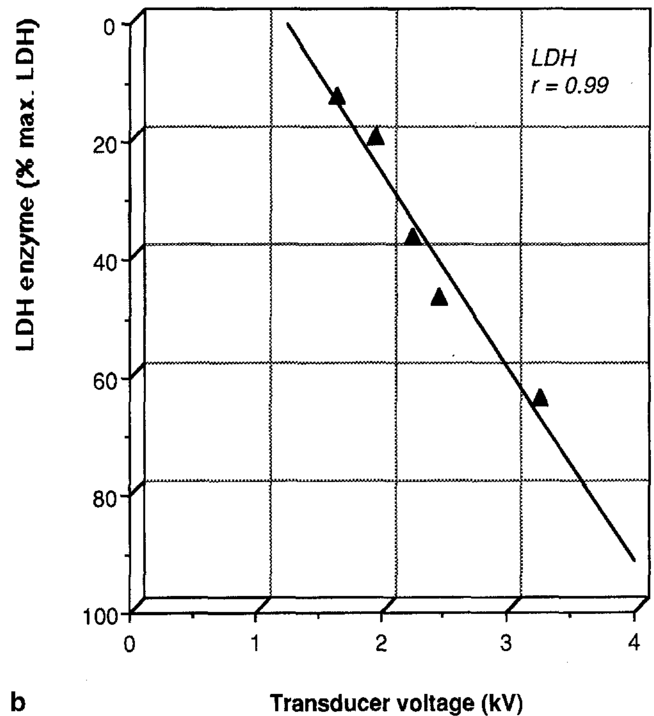
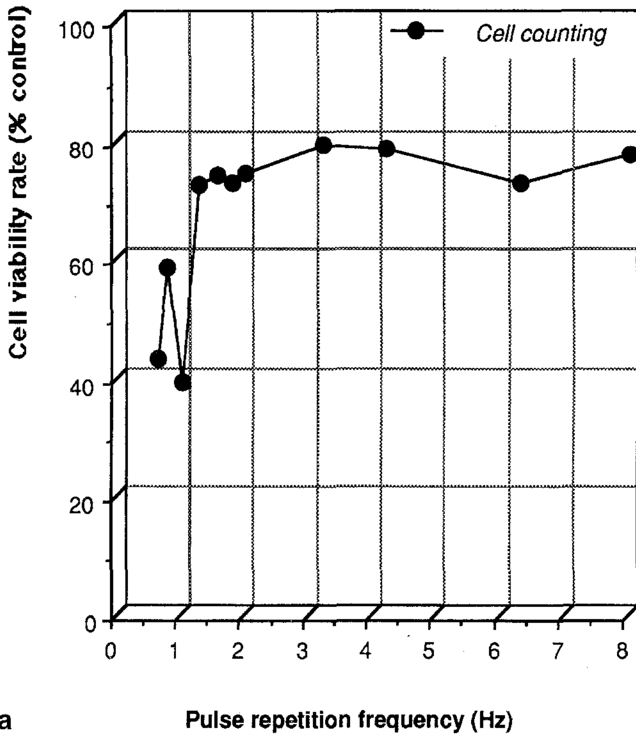


Fig. 3 a Cell viability (% of control) dependent on the number of pulses applied (mean; n = 3). Correlation coefficient (exponential fit): r = 0.98. Standard settings: f = 1 Hz; $U = U_{max}$; $\rho_{cell} \approx 2 \cdot 10^6$ cells ml^{-1}. **b** Lactate dehydrogenase (% of LDH maximum) levels in the supernatant of the cell suspensions after HEPUS applications as a function of the number of pulses applied (mean; n = 3). Correlation coefficient (exponential fit): r = 0.99. Standard settings: f = 1 Hz; $U = U_{max}$; $\rho_{cell} \approx 2.5 \cdot 10^6$ cells ml^{-1}

Fig. 4 a Cell viability (% of control) dependent on the applied transducer voltage (mean; n = 3). Correlation coefficient (linear): r = 0.99. Standard settings: f = 1 Hz; no. of pulses = 400; $\rho_{cell} \approx 2 \cdot 10^6$ cells ml^{-1}. **b** Lactate dehydrogenase (% of LDH maximum) level in the supernatant of cell suspensions after HEPUS applications as a function of the transducer voltage applied (n = 1). Correlation coefficient (linear): r = 0.99. Standard settings: f = 1 Hz, no. of pulses = 400; $\rho_{cell} \approx 2 \cdot 10^6$ cells ml^{-1}



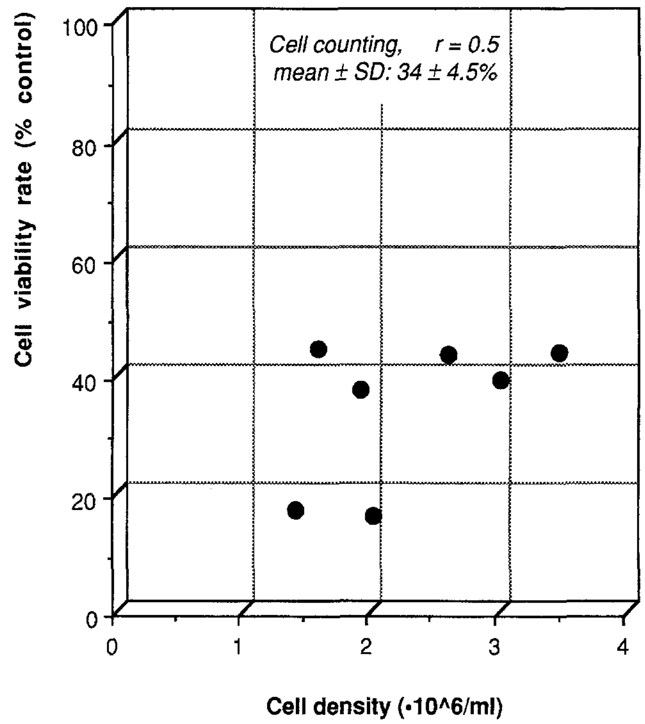


Fig. 6 Cell viability (% of control) dependent on cell density (millions per milliliter; mean; n = 3). Correlation coefficient: r = 0.5. Standard settings: f = 1 Hz; $U = U_{max}$; no. of pulses = 400

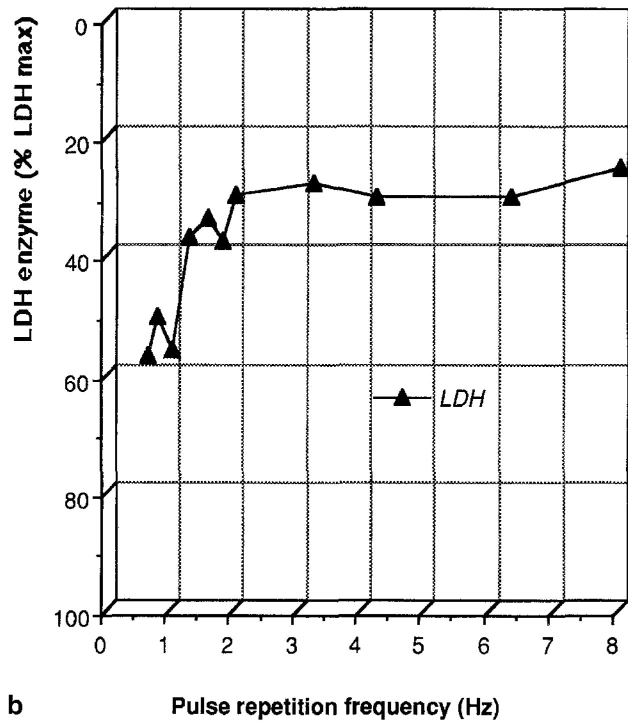


Fig. 5 a Cell viability (% of control) dependent on the pulse repetition rate (mean; n = 3). Standard settings: $U = U_{max}$; no. of pulses = 400; $\rho_{cell} \approx 2 \cdot 10^6$ cells ml^{-1}. **b** Lactate dehydrogenase (% of LDH maximum) levels in the supernatant of cell suspensions after HEPUS dependent on the pulse repetition rate (mean; n = 3). Standard settings: $U = U_{max}$; no. of pulses = 400; $\rho_{cell} \approx 2 \cdot 10^6$ cells ml^{-1}

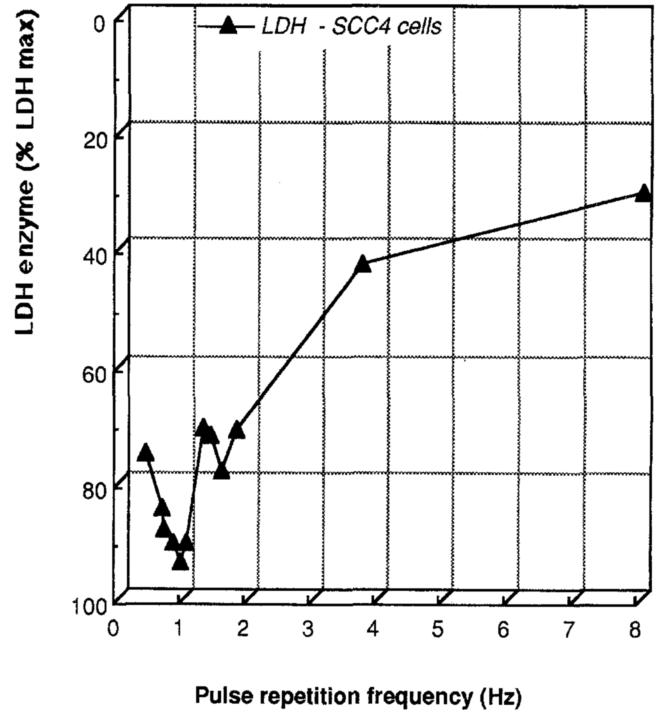


Fig. 7 Lactate dehydrogenase (% of LDH maximum) levels in the supernatant of the cell suspensions (SCC-4) after HEPUS dependent on the pulse repetition rate (mean; n = 3). Standard settings: $U = U_{max}$; no. of pulses = 400; $\rho_{cell} \approx 2 \cdot 10^6$ cells ml^{-1}

A distinct linear dependence was also exhibited by the transducer voltage applied and was proportional to the pressure output. Tumor cell death seemed to depend directly on the positive and/or negative pressure, because the pressure output was proportional to the voltage applied. This contrasted with stone disintegration, where the total energy and the energy per pulse, respectively, is the important parameter [6]. This might imply that the various mechanisms of stone disintegration and cell destruction are not necessarily the same.

Apart from the total energy applied comprising the number of pulses and transducer voltage, the pulse repetition rate also appeared to markedly influence insonation effects. In a number of experimental series a reproducible maximum cell destruction rate was observed at a pulse repetition rate of 1 Hz. Even a slight deviation from this pulse repetition frequency under otherwise constant experimental conditions resulted in distinctly enhanced viability parameters of the tumor cells studied.

The increase in cell survival at pulse repetition rates > 1 Hz could be caused by cavitation effects. As such, the energy transported by a subsequent pulse might be shielded and dissipated by the cavitational cloud produced by the preceding pulse *outside* the test tube. Hence, the size of the cavitational cloud increases with increasing pulse repetition rates.

The following explanation for the observed increase in cell viability at a pulse repetition rate of < 1 Hz appears plausible. The time duration to the subsequent pulse becomes longer than the lifetime of the cavitation bubbles *inside* the test tube and is necessary for cell destruction. Previous measurements of the lifetime of cavitation bubbles and the size of cavitation clouds generated by the GMW2 have shown that such effects must be taken into account [10].

For the reasons given above, the effect of maximum cell destruction observed in vitro at a pulse repetition rate of 1 Hz must therefore not necessarily imply a duplication of results when solid tissue is exposed to HEPUS. With in vivo applications, numerous factors must be taken into consideration, such as cavitation at the surface of the skin and in gas-containing structures in organs. These factors tend to preclude the application of ultrasound pulses at 1 Hz.

The exact mechanism of cell destruction by HEPUS is yet unresolved. Recent investigations, however, indicate that the cell membrane, cellular organelles and the cell nucleus are damaged by the administration of high-energy shock waves [1]. This assumption is strengthened by our observation that counts of suspended cells following trypan blue dye exclusion show no morphologically intact and at the same time avital cells (i.e. blue, trypan-positive cells). LDH levels determined in supernatant fluid in our culture media after insonation correlate with the cytometry results and also point toward the destruction of the cell membrane at an early stage.

The promising effects achieved by applying piezoelectrically generated HEPUS to human squamous cell carcinoma cells in vitro give rise to the expectation that this technique may also be used to selectively destroy tissue in vivo. Initial preliminary experiments with normal and tumor cells have revealed no significant differences in viability after exposure to HEPUS. However, the acoustic energy produced by the transducer can be focused within a small volume deep in tissue (unpublished data). Successful eradication of tumors appears advisable only in cases where tumor cells can be selectively and irreversibly destroyed by transduced energy without risking the simultaneous destruction of adjacent tissue or domains of vital intact tissue.

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