Cytotoxic and anti-proliferative effects of high-energy pulsed ultrasound (HEPUS) on human squamous cell carcinoma cells as compared to connective tissue fibroblasts

Heinrich Iro, B. A. Völklein, Frank Waldfahrer, Thomas Schneider, R. E. Riedlinger, Johannes Zenk

Angaben zur Veröffentlichung / Publication details:

Iro, Heinrich, B. A. Völklein, Frank Waldfahrer, Thomas Schneider, R. E. Riedlinger, and Johannes Zenk. 1998. "Cytotoxic and anti-proliferative effects of high-energy pulsed ultrasound (HEPUS) on human squamous cell carcinoma cells as compared to connective tissue fibroblasts." *European Archives of Oto-Rhino-Laryngology* 255 (8): 420–26. https://doi.org/10.1007/s004050050090.

H. Iro · B. A. Völklein · F. Waldfahrer · T Schneider
R. E. Riedlinger · J. Zenk

Cytotoxic and anti-proliferative effects of high-energy pulsed ultrasound (HEPUS) on human squamous cell carcinoma cells as compared to connective tissue fibroblasts

Abstract The cytotoxic and anti-proliferative effects of high-energy pulsed ultrasound (HEPUS) on human squamous cell carcinoma cells cloned from the hypopharynx (FaDu) and benign connective tissue cells (fibroblasts) were investigated in vitro. Sonication was carried out using an experimental piezoelectric, self-focusing burst-signal transducer. To increase the induction of cavitation, the transducer used was specifically designed to produce multiple oscillations with a high negative pressure amplitude. In both cell lines tested, the application of 100, 800 and 2000 pulses resulted in a high reduction of vital cells. After 2000 pulses, 4.0 ± 1.1% of the fibroblasts but only 2.0 ± 0.4% of the FaDu cells survived HEPUS exposure. A postexposure inhibiting effect of HEPUS for 10 days on the proliferation of surviving cells was noted for the FaDu cells exposed to 2000 pulses, but not as much for the fibroblasts. These findings support the hypothesis that human squamous cell carcinoma cells of the hypopharynx might be more sensitive to HEPUS than fibroblasts and that total tumor cell ablation might be possible in vitro given a sufficient number of HEPUS pulses.

Key words Hypopharyngeal malignancy · Cancer therapy · High-energy pulsed ultrasound · Cell destruction · Cavitation

H. Iro (✉) · F. Waldfahrer · J. Zenk
Department of Otorhinolaryngology, Head and Neck Surgery, Saarland University, D-66421 Homburg/Saar, Germany

B. A. Völklein
Department of Otorhinolaryngology, Head and Neck Surgery, University of Regensburg, Regensburg, Germany

T. Schneider
Department of Medicine I, University of Erlangen-Nuremberg, Erlangen, Germany

R. E. Riedlinger
Department of Acoustics, Institut für Höchstfrequenztechnik, University of Karlsruhe, Karlsruhe, Germany

Introduction

The application of extracorporeal shock waves in the treatment of renal, biliary and salivary stones may induce tissue damage in perifocal areas [5, 6, 8]. It has already been determined during clinical lithotripsy that the negative part of the shock wave is primarily responsible for the induction of cavitation and unintentional tissue damage [4, 15]. Therefore, modification of the pulse characteristics by enhancing the negative peak-pressure amplitude should result in a substantial increase of localized tissue damage.

The physical principles of piezoelectric shockwave generation allow the characteristic waveform of a piezoelectrically generated pulse to be modified. For this reason a specially designed, piezoelectric high-energy ultrasound transducer was shaped to produce multiple oscillations with a high negative pressure amplitude (high-energy pulsed ultrasound or "HEPUS"). Recent data now indicate that a nearly complete destruction of human tumor cells can be achieved in vitro using piezoelectrically generated high-energy pulsed ultrasound [7, 9, 17]. To date little is known about the acute and chronic effects of HEPUS, since its use produces mainly negative pressure amplitudes and causes cavitation on normal and malignant cells. In a first study we examined the acute effects of HEPUS on carcinoma cells of the hypopharynx (FaDu) and oral cavity (SCC4) by varying pulse frequency, cell density, number of pulses and pulse energy. We then found that the cell destruction rate increased with the number of HEPUS pulses and energy. When varying the pulse frequency, the effect was maximal at a repetition rate of 1.0 Hz. The cell density did not show any influence on the cell survival rate [9].

When considering the use of HEPUS in clinical cancer therapy two important questions have to be answered. Thus far no study concerning cell proliferation after HEPUS application has dealt with the long-term effects of our method. It is also essential to know whether there are differences in acute and long-term be-

havior between malignant and benign cells after HEPUS application.

The aim of the present experiments was to investigate the acute effects of HEPUS on normal connective tissue cells and human squamous cell carcinoma cells and also to compare the potential long-term anti-proliferative effects in malignant and benign cell lines in vitro.

Materials and methods

Statistical analysis

All data were subjected to the Wilcoxon rank sum test. Significance was defined as $P < 0.05$.

Pulse generation and HEPUS application

The electro-acoustic ultrasound transducer used for HEPUS generation is a specially designed experimental piezoceramic burst-signal test generator (GMW2; Department of Acoustics IHE, University of Karlsruhe, Karlsruhe, Germany; Fig. 1). The transducer is bowl-shaped and self-focusing (aperture = 362 mm, f = 200 mm, C = 230 nF). In contrast to conventional shockwaves (Fig. 2A), the pressure-versus-time waveform of the exponentially decreasing bipolar oscillations generated by the GMW2 (p+/p– ratio of 0.5–1.0 and rise time of 170 ns) is characterized by a high negative pressure amplitude that produces transient cavitation (Fig. 2B).

For pressure measurements two different hydrophones (Imotec Hydrophone, no. 300/005/04; Imotec, Würselen, Germany; and Marconi Coplanar Hydrophone IP035; Gec-Maconi, Cnelmsford, UK) and an oscilloscope (Gould Instrument, no. 145000043; Seligenstadt, Germany) were used. Since individual high-energy pulses demonstrated a high degree of reproducibility, pressures up to 30 MPa were applied with a constant transducer voltage of 3.2 kV. For propagation of the pulse energy, the transducer was filled with partially degassed water (2.3 mg O_2/l at 37 °C). The spatial pressure profile yielded a focal volume of 11 mm • 3 mm • 3 mm (z, y, x; 50% isobar = –6 dB line) when the maximum transducer voltage was applied [7].

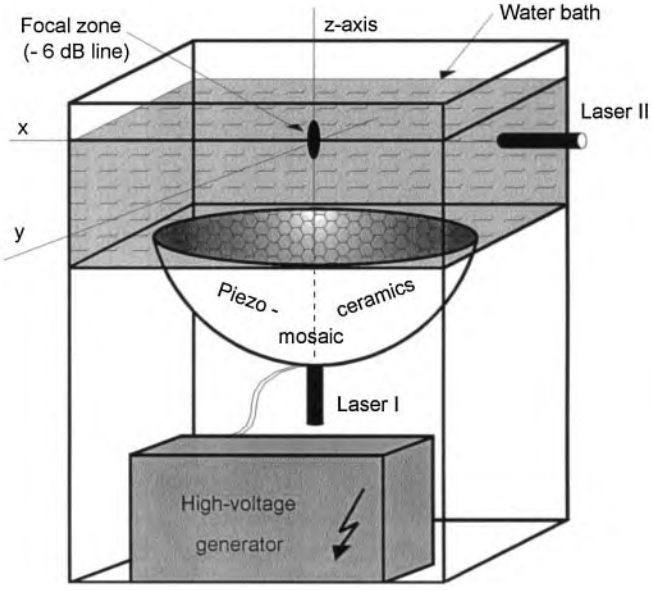

Fig. 1 Diagram of the GMW2 piezoelectric test generator. The sample tubes containing the cell suspensions are positioned in focus by means of two lasers

Polyethylene tubes (2 ml volume; 1 mm wall thickness; Nunc, Germany) containing tumor or non-neoplastic cell suspensions were positioned in the focus of the transducer by means of a platform which could be adjusted in three dimensions (x, y, z). Two laser beams served to define precisely the acoustic focus position of the transducer used. The base of each tube was located 14 mm below the focus while the surface of the water was 30 mm above the focus.

A pulse repetition frequency of 1 Hz, a constant transducer voltage $U = U_{max} = 3.2$ kV and a cell density of 1–3 • 10^6 cells/ml were employed as standard parameter settings [9]. The number of the pulses applied was varied as later described.

Cell cultures and preparation

Human squamous cell carcinoma cells (FaDu; American Type Culture Collection-ATCC, Rockville, Md., USA) and normal connective tissue cells (using fibroblasts isolated from a normal skin biopsy after informed consent) were cultivated in Eagle's Minimum Essential Medium (EMEM) and Dulbecco's modification of EMEM (DMEM) enriched with 10% fetal calf serum (FCS), 1% penicillin-streptomycin, 1% L-glutamine and 1% sodium pyruvate (all obtained from Gibco/BRL Company, Germany) as monolayers in tissue culture flasks (Greiner Company, Germany). Standard conditions for cell growth were 37 °C temperature, 97% relative air humidity, 8% atmospheric CO_2 and pH 7.4.

Before HEPUS treatment, cells were rinsed twice with 0.9% NaCl solution and then suspended for a short time in phosphate-buffered saline (PBS) containing low-dose trypsin (0.25%) and 0.1% EDTA. Cells were resuspended in fresh medium, following which cell viability was evaluated in a hemocytometer by the trypan blue dye exclusion test [11]. Immediately after this procedure, 30 aliquots of 2 ml each of each cell suspension were transferred to sterile polyethylene tubes that had been shortened to a length of 44 mm and were sealed with a plug. Thirty tubes of each cell type were treated in each of four different groups: 0 pulses (controls), 100 pulses, 800 pulses and 2000 Pulses.

After HEPUS exposure, vital tumor cells of identically treated samples of each cell type ($n = 30$) were counted in a hemocytometer. The remaining cell suspensions of each respective group and cell line were mixed in a 100 ml tube. Following this, 2 ml aliquots of each stock suspension were transferred to tissue flasks ($n = 30$/group and cell type; N = 240) in order to attain identical concentrations of treated tumor cells in corresponding tissue flasks prior to recultivation. To obtain the cell proliferation rate after HEPUS, three corresponding tissue flasks of each cell line and sonication modality were incubated with 0.25% trypsin for 10 min and the number of intact cells determined at scheduled time intervals (every 24 h up to 240 h).

Parameters investigated

The number of applied pulses was modified to compare the destructive effect of varying numbers of HEPUS pulses on normal and malignant cells. In order to investigate any potential long-term anti-proliferative effect, the cell proliferation rates after sonication were determined as described previously.

The fraction of living cells was evaluated by trypan blue dye exclusion before and after exposure of each tumor/normal cell suspension to HEPUS and every 24 h over a total time interval of up to 240 h. Additionally, lactate dehydrogenase (LDH) was measured in the supernatant after initial HEPUS exposure, since LDH proved to be indicative of the induced cell damage [9]. The absolute values of LDH were referred to the LDH values of corresponding cell suspensions ($n = 3$) subjected to sonication by an ultrasound oscillator providing 100% cell destruction. Identically treated samples for each cell line which were positioned at the edge of the waterbath outside the HEPUS focus served as controls

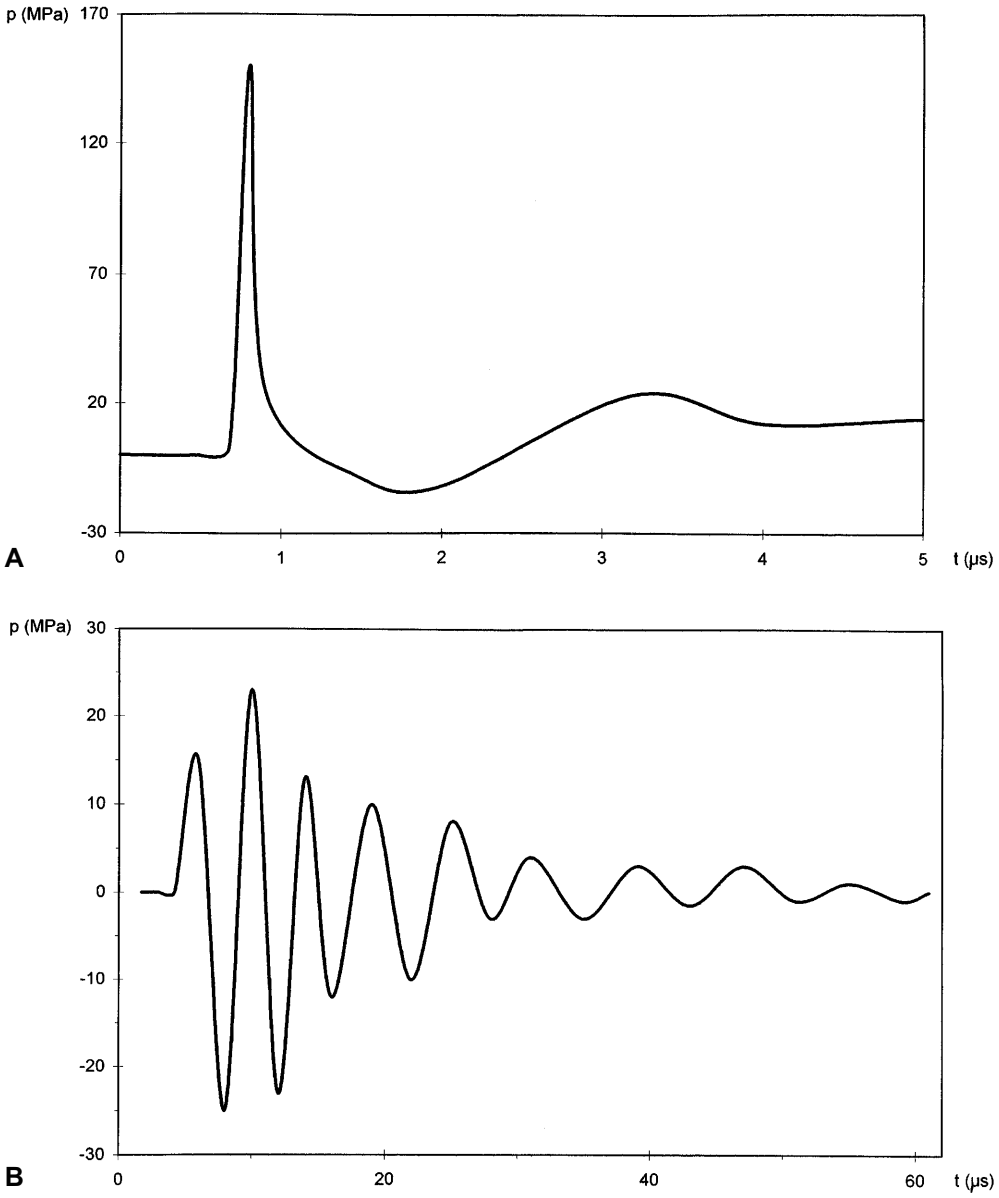

Fig. 2 **A** Pressure-vs-time waveform of a high-energy shockwave used in extracorporeal shockwave lithotripsy. **B** Pressure-vs-time waveform of piezoelectrically generated high-energy pulsed ultrasound (HEPUS). Exponentially decreasing bipolar oscillations with a high negative peak pressure amplitude are measured in the focal zone

(n = 30). The rate of surviving cells after initial sonication represented the mean of 30 independent samples exposed to HEPUS.

Results

Acute HEPUS effects

Compared with the controls (Fig. 3A), the application of 100 pulses resulted in a significant ($P < 0.05$) reduction of vital fibroblasts and FaDu cells (Fig. 3B) This incidence was 79 ± 6% and 74 ± 9% surviving cells, respectively.

After 800 pulses, 10 ± 2% of the fibroblasts and 11 ± 0.5% of the respective FaDu cells were determined to be vital by trypan blue dye exclusion (Fig. 3C).

After treatment with 2000 pulses, 4 ± 1.1% of the fibroblasts and 2 ± 0.4% of the malignant FaDu cells had survived treatment (Fig. 3D). In this case the difference between both cell lines was significant ($P < 0.05$).

As shown in Fig. 3E–H, LDH measurements confirmed the cell count data, with higher cell destruction rates correlating with higher LDH levels in the supernatant.

Tumor cell proliferation after HEPUS

Fibroblasts

An initial decrease in the number of vital cells in controls and in the fibroblast cell line exposed to 100 and 800 pulses was seen during the first 24 h after HEPUS application and recultivation. The number of fibroblasts treated with 2000 pulses decreased until 48 h after HEPUS exposure (Figs. 4, 5A) After this delay of proliferation, an exponential increase in the number of vital cells was observed in the fibroblast cell populations exposed to 100, 800 and 2000 HEPUS pulses and was similar in the control group.

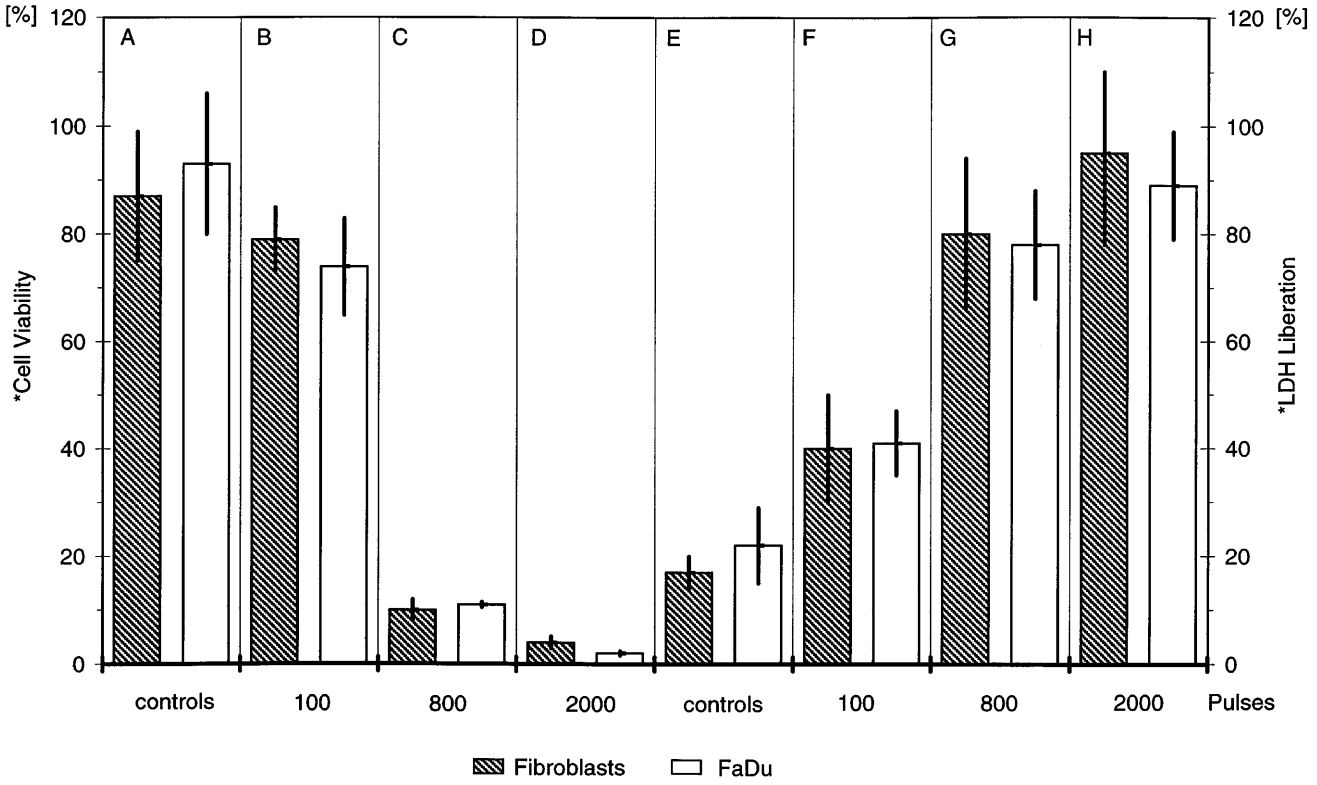

Fig. 3 Acute HEPUS effects. Viability rate (% of vital cells before HEPUS treatment) of fibroblasts and FaDu cells immediately after HEPUS exposure with no (**A**), 100 (**B**), 800 (**C**) and 2000 (**D**) pulses. Corresponding lactate dehydrogenase (LDH) liberation in the supernatant (**E–H**)

FaDu cells

The number of vital FaDu cells sonicated with 100 pulses decreased until 24 h after initial HEPUS treatment, whereas the FaDu cells exposed to 800 pulses decreased until 96 h after initial sonication. The number of vital cells exposed to 2000 pulses showed a constant decrease during the entire observation period. After 240 h and treatment with 2000 pulses cell viability was 0.1% compared to the cell number before HEPUS treatment (Fig. 4) and under 5% compared to the number of vital cells directly after treatment (Fig. 5B).

After 24–96 h, post-exposure cell proliferation of the malignant FaDu cells showed an exponential increase in cell numbers only for those cells treated with 100 and 800 pulses and was very similar to the control group.

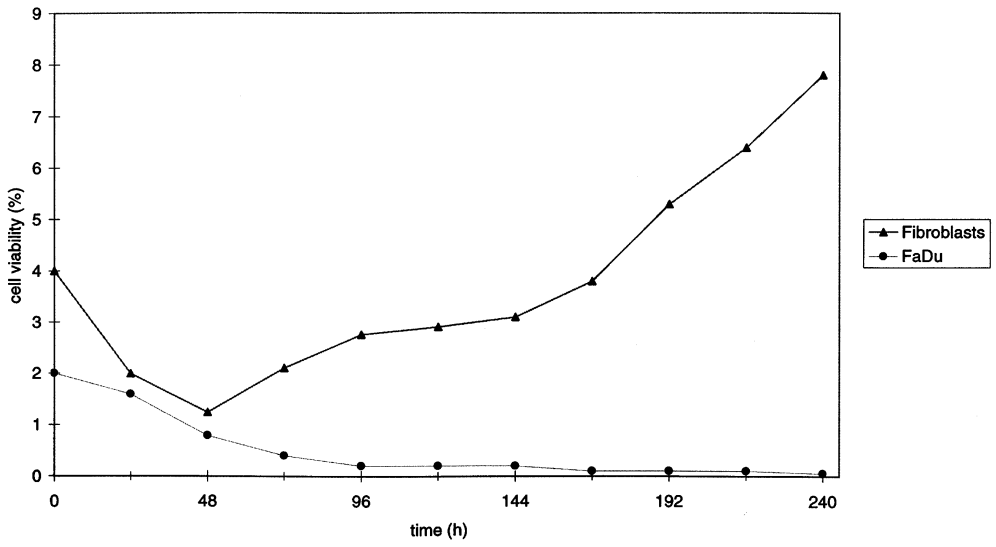

Fig. 4 Cell proliferation after HEPUS treatment of FaDu cells and fibroblasts after 2000 pulses. The rate of vital cells is referred to the number of vital cells before HEPUS exposure (100%; time = 0 h: cell viability immediately after HEPUS treatment)

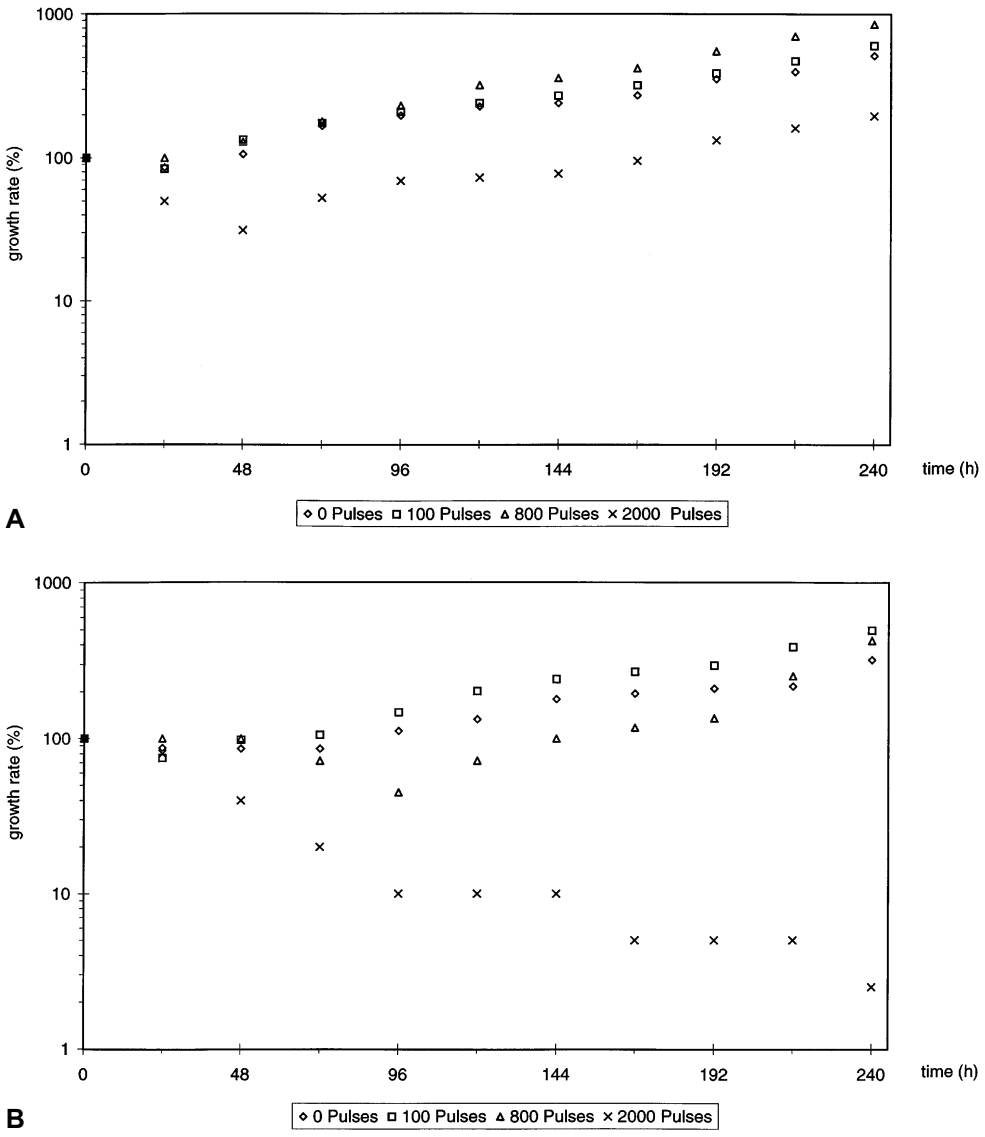

Fig. 5 A, B Proliferation kinetics of sonicated cells after HEPUS application in a logarithmic scale. The number of vital cells directly after treatment (time = 0 h) is set at 100% for both cell types and all numbers of pulses to compare cell proliferation. The individual data points (> 24 h) represent the mean of three tissue flasks each. **A** Changes in fibroblasts. The controls and treated cells show a decreasing number in the first 24–48 h after treatment and afterwards have recovered again with a constant proliferation. **B** Results in FaDu cells. The decrease of numbers lasted 96 h after treatment with 800 pulses. However, application of 2000 pulses resulted in a decrease up to 240 h and no recovery of proliferation

Discussion

To date a number of studies on the effect of shock waves and HEPUS on malignant cells under in vitro conditions have been published [1, 3, 7, 9, 10, 12, 14, 16, 20]. Our's is the first study dealing with cell proliferation after HEPUS application and comparison of HEPUS effects on malignant and benign cell-lines of the head and neck. One major problem in contrasting all studies is the wide range of physical and analytical differences in experimental design, especially in the characteristics of the applied ultrasound or shockwaves. In contrast to the conventional mode of extracorporeal shockwave application used in most of the various studies, the experimental piezoelectric pulse transducer of our investigation was specially shaped to produce an exponentially decreasing bipolar oscillation with a high negative pressure amplitude giving rise to increased cavitation [7, 9, 17]. The induction of cavitation achieved with high negative peak pressures is considered to be an important mechanism of cell destruction by high-energy ultrasound sources [4, 15, 18].

In contrast to high-intensity focused ultrasound (HIFU), no thermal effects are involved in cell and tissue damage induced by HEPUS [7]. Direct and indirect cavitational and cytochemical effects of high-energy ultrasound and shockwaves include rupture of membranes, damage of cell organelles, formation of free radicals and strand breaks in DNA and can influence proliferation of cells surviving initial sonication [3, 10, 14, 16, 19]. When evaluating vital cells, no avital cells that had remained morphologically intact were detected. We conclude from our findings that rupture of the cell membrane is the main reason for cell death. Cell-cell and cell-tube interactions can be excluded as indirect contributions to cell destruction because cell destruction is independent of cell density [9].

The application of HEPUS resulted in a significant reduction of vital squamous cell carcinoma cells. Two thousand pulses proved to be sufficient for the destruction of

more than 95% of the malignant cells treated. Therefore, a nearly 100% destruction of all malignant cells might be possible with a further increase in the number of pulses. Compared with other studies using standard lithotripters or experimental systems equipped with electrohydraulic or electromagnetic generators, the destructive potential of HEPUS on malignant cells appears to be higher [7, 9, 17]. After 100 and 800 pulses, cell destruction rates of fibroblasts were similar to those for malignant FaDu cells. After sonication with 2000 pulses, the rate of tumor cell destruction was significantly higher than that for the respective fibroblasts.

The initial growth delay (24–96 h) after sonication was not only observed in sonicated cells, but to a lesser extent in controls, and could be a side-effect of the recultivation procedure.

In our study a post-exposure long-term (up to 240 h) growth-inhibiting effect of HEPUS was seen in FaDu cells treated with 2000 pulses. In this group 95% of the malignant cells surviving initial treatment were found to be non-vital by the end of the observation period. Although the increase in the number of viable fibroblasts after 2000 pulses detected at daily measurements was clearly smaller than for control cells or cells treated with fewer pulses, the growth of fibroblasts surviving initial sonication with 2000 pulses was not inhibited completely. This might be a plausible indication that connective tissue cells like fibroblasts could have a higher resistance or capacity of regeneration after HEPUS exposure than malignant FaDu cells treated with an equal number of pulses.

Brümmer et al. [2] compared the different effects of shockwaves on normal and malignant cells using an experimental electrohydraulic lithotripter. However, they were unable to detect a specific difference between normal and malignant cells concerning their sensitivity to shockwaves. The question arises whether the tissues used (i.e. embryonic chicken cells of the thigh muscle and kidney) truly represent fully differentiated cells as concerns the mechanisms of cell repair and proliferative capacity.

In contrast, Wada et al. [20] examined the effects of electrohydraulic shockwaves produced by a Dornier kidney lithotripter on malignant cell lines and fibroblasts. By measuring the intracellular RNA synthesizing activity and intracellular ATP levels before and after treatment, they concluded that the sensitivity of malignant tumor cells to shockwaves was greater than that of fibroblasts. These latter findings correspond to our results.

As both of our cell lines grew in similar suspension media and cells were all treated under identical conditions, the influence of these parameters on the results of our experiments could be minimized accordingly. It is also rather unlikely that the differences in size between FaDu cells and fibroblasts could be the cause of the variability of cell viability after HEPUS treatment, as Brümmer et al. [2] have proven for shockwaves.

Russo et al. [16] examined the long-term anti-proliferative effects of shockwaves in Dunning R3327AT-3 rat prostatic carcinoma cells and the human melanoma cell line SK-Mel-28 by using a clonogenic assay after the application of electrohydraulically generated shockwaves. Both cell lines demostrated a marked decrease in the ability of the exposed cells to form colonies. After application of 1500 shockwaves cell viability was reduced to 68% and the clonogenic survivers of the Dunning tumor cells amounted to 22% of the controls. The SK-Mel-28 cells appeared more vulnerable, exhibiting a decrease of 69% in cell viability after 200 shockwaves and no clonogenic survivors compared to controls.

Oosterhof et al. [12] have demonstrated a decrease in the clonogenic potential of Dunning R-3327 derived PAT-2 prostatic cancer cells that was dependent on the number of electromagnetically generated shockwaves applied to the cells using a Siemens Lithostar lithotripter. After application of a maximum of 3000 shockwaves, cell viability measured by trypan blue dye exclusion was 65% and the cells no longer exhibited any clonogenic potential in soft agar. However, there exist striking differences to our results obtained with our specially designed HEPUS generator for cell destruction. Cell proliferation was stopped only in the case of Fadu cells, with an initial cell viability falling below 5%. The fibroblasts still showed slight proliferation after applying the same procedure.

Our findings are inconsistent with the clonogenic potential of the Dunning tumor cells and SK-Mel-28 cells [12, 16]. In contrast, our results indicated that cell proliferation was completely inhibited only when the initial destruction rate was nearly 100%. This difference might be attributable to different techniques employed for the recultivation.

It is thus possible that with a sufficient number of HEPUS pulses a complete destruction of all tumor cells can be achieved not only in vitro but also in vivo, while damages inflicted on neighboring connective tissue cells remain minimal. To determine whether or not this is true in vivo will require further study.

Acknowledgements This project was supported by the Wilhelm Sander Foundation in Neustadt an der Donau, Germany. The authors wish to thank Prof. Dr. M. Grade, University of Applied Sciences, Cologne, for revising the English manuscript.

References

1. Brümmer W, Brenner J, Brauner T, Hülser D (1989) Effect of shockwaves on suspended and immobilized L1210 cells. Ultrasound Med Biol 15:229–239
2. Brümmer F, Suhr D, Hülser DF (1992) Sensitivity of normal and malignant cells to shockwaves. J Stone Dis 4:243–248
3. Clayman RV, Long S, Marcus M (1991) High-energy shockwaves: in vitro effects. Am J Kidney Dis 17:436–444
4. Coleman AJ, Saunders JE (1987) Comparison of extracorporal shockwave lithotripters. In: Coptcoat MJ, Miller RA, Wickham JEA (eds) Lithotripsy II. BDI Publishing, London pp 121–131
5. Delius M, Gambihler S (1992) Sonographic imaging of extracorporeal shockwave effects in the liver and gallbladder of dogs. Digestion 52:55–60
6. Ell C, Kerzel W, Heyder N, Langer N, Mischke U, Giedl J, Domschke W (1989) Tissue reactions under piezoelectric shockwave application for the fragmentation of biliary calculi. Gut 30:680–685

7. Feigl T, Völklein B, Iro H, Ell C, Schneider T (1996) Biophysical effects of high-energy pulsed ultrasound on human cells. Ultrasound Med Biol 22:1267–1275
8. Iro H, Wessel B, Benzel W, Zenk J, Meier J, Nitsche N, Wirtz P, Ell C (1990) Gewebereaktionen unter Applikation von piezoelektrischen Stoßwellen zur Lithotripsie von Speichelsteinen. Laryngo Rhino Otol 69:102–105
9. Iro H, Feigl T, Zenk J, Waldfahrer F (1996) In vitro effects of high-energy pulsed ultrasound on human squamous cell carcinoma cells. Eur Arch Otorhinolaryngol 253:11–16
10. Kondo T, Kodaira T, Kano E (1993) Free radical formation induced by ultrasound and its effects on strand breaks in DNA of cultured FM3 A cells. Free Radic Res Commun 19 [Suppl 1]:193–200
11. Lindl T, Bauer J (1989) Zell- und Gewebekultur, 2nd edn. Gustav Fischer, Stuttgart, p169
12. Oosterhof GON, Smits GAHJ, Ruyter JE de, Moorselaar RJA van, Schalken JA, Debruyne FMJ (1989) The in vitro effect of electromagnetically generated shockwaves (Lithostar) on the Dunning R3327 PAT-2 rat prostatic cancer cell-line. Urol Res 17:13–19
13. Prat F, Chapelon JY, Chauffert B, Ponchon T, Cathignol D (1991) Cytotoxic effects of acoustic cavitation on ht-29 cells and a rat peritoneal carcinomatosis in vitro. Cancer Res 51: 3024–3029
14. Prat F, Sibille A, Luccioni C, Pansu D, Chapelon JY, Beaumatin J, Ponchon T, Cathignol D (1994) Increased chemotoxicity to colon cancer cells by shockwave-induced cavitation. Gastroenterology 106:937–944
15. Riedlinger RE, Brümmer F, Hülser DF (1989) Pulsed high-power sonication of concrements, cancer cells and rodent tumours in vivo. Ultrason Int Conf Proc, Madrid, Butterworth & Co ltd, ISBN 408054182, pp 305–312
16. Russo P, Stephenson RA, Mies C, Huryk R, Heston WDW, Melamed MR, Fair WR (1986) High energy shock waves suppress tumour growth in vitro and in vivo. J Urol 135:626–628
17. Schneider HT, Feigl T, Löhr M, Riedlinger R, Hahn EG, Ell C (1994) In vitro effects of high-energy pulsed ultrasound (HEPUS) on human tumour cells. Eur J Gastroenterol Hepatol 6:257–262
18. Steinbach P, Hofstädter F, Nicolai H, Rössler W, Wieland W (1992) In vitro investigations on cellular damage induced by high-energy shock waves. Ultrasound in Med Biol 8:691–699
19. Suhr D, Brummer F, Irmer U, Schlachter M, Hülser DF (1994) Reduced cavitation by the antioxidative effect of vitamin E. Ultrasonics 32:301–307
20. Wada S, Kishimoto T, Nishisaka M, Ameno Y, Kamizuru M, Sakamoto W, Ikemoto S, Maekawa M (1990) Effect of high energy shock wave on human malignant urological cells. Japanese Journal of Endourology and ESWL 3:135–138