

PULSED HIGH-POWER-SONICATION OF CONCREMENTS, CANCER CELLS AND RODENT- TUMORS IN VIVO

R.E.Riedlinger¹, F.Brümmer², D.F. Hülser²

¹ Fachgebiet Akustik, Universität D 7500 Karlsruhe

² Biologisches Institut, Universität D 7000 Stuttgart

Extracorporeal lithotripsy has been successfully established, based on different principles of generating and focusing the shock waves. Lithotripters have also been used to investigate the influence of shocks to cancer cells and solid tumors. With two different trans-mitters (spark-gap type XL-1 and piezo-resonance type MW 2) we applied shock waves and short high power US-pulses to suspended and immobilized tumor cells and multicell spheroids. With MW 2 significant local damage on cell spheroids in gelatine was achieved, caused by locally controlled cavitation. The results are compared to each other.

INTRODUCTION

On Feb 7th 1980 the first extracorporeal lithotripsy was performed on man. Since then over 1 million such patients have been treated. From 1980 to 1985 narcosis and anesthesia were necessary. After 1985 new developed machines made treatment without anesthesia and sedation possible. Since 1985 gallstone patients are also being treated. The lithotripters (about 20) work with different sound sources:

Point sources: spark gap, focussed laser, micro-explosion. Extended sources: piezo-electric transmitter, eddy current transmitter. Focussing is done by reflection on ellipsoids or paraboloids¹, by acoustic lenses or by direct focussing. A survey is given e.g. in /1/.¹rf. /2/. The focussing can be described by the solid angle of the sound field and by the aperture A of the pulser. The machines working without causing pain have a wide solid angle and show a relatively low intensity at the entry region of the body. The essential pulse steepening only takes place in the body.

The time pressure profiles differ: They are characterized by the positive and the negative peak of the pressure amplitudes p^+ and p^- , by the rise time t_r , the pulse width t_w^+ of the primary overpressure, the duration t_w^- of the following underpressure and also by the degree of the nonlinear steepening. Concerning the majority of installed lithotripters here a view of the range of characteristic values:

aperture a :	100 ... 480 mm	solid angle :	50 ... 85° dgr
positive peak of focal pressure p^+ :			10 ... 120 Mpa
negative peak of focal pressure p^- :			6 ... 10 Mpa
rise time t_r from 0 to p^+ :	< 25 ... 600 ns	energy of a single pulse:	2 ... 100 mJ
duration of positive pressure t_w^+ :			150 ... 600 ns
duration of negative pressure t_w^- :			200 ... 2000 ns
average radiated intensity I_{SPTA} :			0,1 ... 10 mW/cm ²
estimated focal peak intensity I_{SPPA} :			6 ... 100 kW/cm ²
nonlinearity-parameter $s_n = 2 * \pi (1/4 - t_r/3 t_w^+)$:			0.3 ... 1.5
axial length of focus ($p^+ -6dB$):	8 ... 150 mm,	radial width of focus ($p^+ -6dB$):	3 ... 40 mm
pulses / treatment :	800 ... 10000,	focussed / radiated energy :	90 ... 99 %

All lithotripters have the effect in common that the positive pressure amplitude clearly exceeds the negative ($p^+/p^- > 4$) in consequence of the steepening. During the development of a lithotripter we learned that a very efficient fragmentation can be achieved with proportions $p^+/p^- = 1$ above $p^+ = 20 \text{ MPa}$ /3/. This is traced back to the effect of cavitation which is being treated more and more in literature about lithotripsy. /4,5/. High pressure values can damage tissue. With focussing sound transmission this effect could possibly be used for the treatment of tumors. Many experiments on tissue with ultrasound up to about 1 kW/cm^2 are known, e.g. /6,7/. More experiments with shockwaves ($p^+/p^- > 3$) on cancer tissue are made possible by the large number of lithotripters installed. They were used for shock treatment of cells and tumors in vitro and in vivo. The following is a summary of results.

in vitro experiments:

Depending on the dosation a damage could be shown on suspended cells treated with differently produced shock waves (spark gap /8,9/, piezo /10/, eddy current /11/). Dunning R3327AT-3 carcinoma and SKMel-28 melanoma cells, treated in vitro with up to 1500 shocks showed reduced cancerogenic activity, when injected in living tissue /8/. The application of up to 2000 shocks on RcPa and NHEK cells in suspension delivered a dosis dependent reduction of living cells and of growth rates /12/.

The survival rate of L1210 cells, Lewis-Lung and A-Mel3 cells in suspension decreases with increasing number of applied shocks. The cancerogenic potential in tissue is reduced /13/. With equivalent amounts of total energy cytotoxic effects on L1210 cells are stronger when applying a higher energy/pulse than increasing the number of applied shocks of lower energy /14/. In some cases up to 5000 electromagnetically generated shocks reduced the cell viability in AT-4 cell suspensions and multiplied 3 times the capability of colony inhibition caused by Interferon-gamma and Velbe /11/.

in vivo experiments:

High energy shock waves (heSW) and high energy pulsed ultrasound (hepUS) have also been reported to produce a significant cytoreduction in vivo. The effects of shock waves on tissue are : vasoconstriction, stasis within the capillaries, micro-hemorrhages, ruptures of endothelial cells, defects in vascular basal membranes, leakage of venules for makromolecules, creation of amorphous conglomerates in venules, micro- bleedings and hematomas and extravasation of red blood cells /15/.

In vivo experiments on R3327AT-3 Tumor nodules showed a lower growth for five days following exposure to 1500 shocks /8/. 1400 electrohydraulic shocks on FANFT tumors, induced on mice, led to significant reduction of growth rate. A synergistic effect in combination with Doxorubicin was observed /12/. Shockwaves reduced the micro-circulation in vivo, leading to stasis. Cytoreduction and complete disappearance of A-Mel3-hamster- melanome was achieved in vivo by repetitive shock treatment /16/. 1500 piezoelectrically generated shock pulses applied to R3327-MatLyLu tumor-bearing animals did not lead to increasing metastasis, but split lung metastasis rate into half /10/.

From this actual position the question becomes evident, if the described effects are mainly provoked by the shocks or if they are mostly caused by cavitation.

The in vivo experiments with short high power ultrasound signals /17,18/ give some arguments: Applying up to 900 hepUS-pulses on Dunning prostate tumor nodules in vivo caused hemostasis . Local necrosis was present at 24 hrs. L3 tumors in nude mice received 1000 signals. The tumor growth was delayed by six days, whereby

inoculated Meth-A fibrosarcomas was delayed by 6 to 8 days. In 5 of 5 animals a single treatment with only 100 hepUS pulses induced permanent local tumor control of intradermally growing Meth-A sarcomas and complete removal after 6 to 14 days. However only partial results exist to these experiments, so no general statement can be made /17,18/.

Total tumor reduction was also achieved by multiple heSW treatment with appropriate delay /16/. In comparison it is remarkable that hepUS needs much lower pressure amplitudes p^+ , however the negative pressure amplitude p^- is higher than with lithotripter shock waves. But the variety of in vivo experiments as well as the methods of the pulse treatment are difficult to compare up to now. In this consequence we intend treatment of cells and cell-aggregations (multicell-spheroids) in suspension as well as in a tissuelike environment, to compare the effects of heSW and hepUS.

MATERIAL AND METHODS

Generation of the hepUS-Pulses :

The US-pulses used for this research were produced by a silicon-backed piezoceramic mosaic transmitter MW2 built in co-production with the Richard Wolf GmbH. It is shaped like a spherical bowl of 32 cm aperture, 20 cm radius and a solid angle of 130° . We used the same adjustments which were chosen for the in vivo experiments with hepUS on rodent tumors /17,18/.

Generation of shock waves :

The shock waves used in these experiments were produced in an experimental lithotripter XL1 (Dornier Medizintechnik). They are generated by spark discharge in the geometric focal point of a half ellipsoid in brass of 14 cm aperture and 68° solid angle. The XL1 contains the same SW-generator as the lithotripter HM3 (80 nF) in clinical service. For the installation of the sample and other details refer to /19/. Degassed water of 21°C was used as coupling. (XL-1: $< 3\text{ mg O}_2/\text{l}$; MW2: $< 1,5\text{ mg O}_2/\text{l}$). As precise as possible the preparation and treatment of the samples was realized in the same way. In both cases 250 and 750 events were applied.

Cell cultures :

To determinate the effects of heSW and hepUS we used 3 cell systems:

1.) cell line L 1210 : L1210 cells, a lymphotic mouse leukemia cell line were maintained as suspensions at 37°C under an atmosphere of 5% CO_2 in air at pH 7,4. They were grown in tissue culture flasks filled with RPMI 1640 medium with NaHCO_3 and supplemented with 15% fetal calf serum. Prior to use 100 μl mecaptoethanol (0.4 mg/l) were added to 100 ml RPMI 1640 medium. Cell cultures were routinely tested for mycoplasma contamination. For treatment with shock waves the cells were pelleted by centrifugation (10s, 1000g), resuspended in culture medium to a final cell concentration of about 2×10^5 cells/ml.

2.) human erythrocytes: Using concentrates of erythrocytes from overaged blood conserves (Katharinenhospital Stuttgart) we made suspensions with a concentration of $3\text{--}5 \times 10^7$ cells/ml. For this purpose the erythrocytes were washed twice in a phosphate buffered solution (without Mg and Ca), centrifugated (1min, 600g) resuspended in PBS and transferred into pipettes.

3.) multicell spheroids: In order to estimate the sonication of tissuelike material, we used cell aggregations, so called multicell- spheroids. BICR/M1R_k cells, a strain originating from a transplantable mammary tumor of the Marshall rat were cultured at 37 ° C in Dulbecco's modified Eagle's medium supplemented with 3,7 g/l NaHCO₃ , 100 mg/l streptomycine sulfate, 150 mg/l penicilin G and 10% calf serum. Spheroid cultures in glass spinner flasks on magnetic stirrers were agitated by 120 rpm. Culture medium was renewed daily. The samples of cells and - aggregates were filled into PE-pipettes of 3.5 ml volume 4 cm length and 1.3 cm diameter.

Before and after heSW or hepUS treatment the concentrations of intact L1210 cells were measured using a Coulter Counter. The release of hemoglobine was the measure of damage of the human erythrocytes. After treatment these cells were centrifugated. The content of hemoglobine was determined by the Cyanmethemoglobine-method. For light microscopical histology cells were fixed in Bouin-solution for 2h at room temperature, followed by dehydration in 70% ethanol for 2-3h , 80% and 90% isopropanol (replaced 3 x) for 1,5h at 45 ° C. Cells were then impregnated overnight at 57 ° C in a mixture of equal parts isopropanol and the embedding medium Paraplast Plus (PP), infiltrated for 2x7h at 57 ° C with pure PP, followed by embedding in PP. All specimen were cut to 5 µ m sections and stained with hematoxylin/erythrosin.

RESULTS

Both methods of sonication led to a dose dependent damage of cells in suspension. The number of damaged L1210 cells (Fig.3 a, 3b) as well as the released hemoglobine (Fig.3a, 3b) increased with the charging voltage, the pressure amplitude and the number of pulses. The L1210 damage is highly correlated with hemolysis in the heSW- (Fig.3a) as well as in the hepUS - treatment (Fig.3b) . Both treatments of the cell aggregations in suspension led to destruction of spheroids (not shown here).

Spheroids, were no longer affected by 250 shocks of the XL-1 when immobilized in 12%-gelatine (Fig.4 a), but were damaged by 250 pulses from the MW2 as follows : Because of the small focal region of the MW2 (ca 2mm radius, ca 5 mm length) it was possible to position the focus in the middle part of the sample pipettes. In consequence of it, the pipettes were cut in three parts. The contents were separately examined by histology. The histological results for the cell spheroids in front (Fig.4 d) and beyond the focal region correspond to the untreated controls (Fig.4 b). The spheroids in the middle part of the pipette however were significantly damaged (Fig. 4 c). The cell aggregations then showed destruction of the surface contour up to total disintegration of the aggregation. Nevertheless aggregates were still present unaffected by the pulse treatment. During pulse treatment of pure 12%-gelatine we observed ruptures of ca 3mm length and directed parallel to the MW2 axis in the fokal region. Identical numbers of pulses (250) lead to a lower damage rate in suspension cells with heSW (18 kV, 50%) than with hepUS (6 kV, 40%). In spite of this hepUS still shows obvious damage of embedded cell aggregations.

DISCUSSION

The shapes of the hepUS and shock waves differ seriously in the ratio p^+/p^- . The dosis dependent damage of both types of sonication of single suspension cells could be shown by the destroyed L 1210 cells as well as the hemolysis of human erythrocytes under comparable experimental conditions.

Shock waves damage cells and cell aggregations in suspension but not in gelatine. hepUS damages cells and cell aggregations in suspension and also damages cell aggregations in gelatine. The small focal range of this experimental setup made aiming on cells and cell aggregations possible without hurting neighbour cell aggregations. (Fig. 4 c,d) Proceeding on the assumption of the same degree of damage of single suspension cells and using the instrument and treatment parameters (50%, U=18 kV, n=250 heSW, C=80 nF) (50%, U=4 kV, n=750 hepUS, C=230 nF) for comparison of the total energy ($0,5 CU^2 n$) the SW treatment has an energy of ca 3000 J, the US sonication ca 1400 J. Using hepUS waves with less energy we achieve the same damage rate of cells with a much smaller focussing zone. An explanation for this is the high focussing gain of the hepUS machine and the high p+/p- ratio of its generated pulses.

This enables locally controlled cavitation, which is shown by the rupture of the gelatine and the destruction of the cell spheroids it contains. The same effects cannot be seen by application of heSW in the same biological system. We suppose that damage of cells with heSW and hepUS basically depend on the effects of cavitation.

From this point of view the generation of high overpressure amplitudes basically does not take part in the damage of cells.

Acknowledgement: We greatly acknowledge Mrs. B. Rehkopf, Mrs. S. Pilkuhn and Mr. M. Keller for technical assistance. This work was supported in part by Bundesministerium für Forschung und Technologie, Dornier Medizintechnik, and Richard Wolf GmbH.

REFERENCES

- 1 Rassweiler, J.; Westhauser, A.; Bub, P.; Eisenberger, F.: Second-Generation Lithotripters: A comparative Study. J. of Endourology 2 : 2: 193-204, 1988
- 2 Wess, O.; Marlinghaus, E.H.; Katona, J.: LARS -eine großaperturige Leistungsschallquelle für medizinische Anwendungen. Proc.DAGA'89 Duisburg 1989
- 3 Riedlinger, R.; Ueberle, F.: Berührungsfreie piezoelektrische Nierensteinzertrümmerung. Proc.DAGA 86, 829-832, 1986
- 4 Church, C.C.: A theoretical Study of Cavitation generated by an Extracorporeal Shock Wave Lithotripter. JASA Suppl1: 83: 88, 1988
- 5 Coleman, A.J.; Saunders, J.E.; Crum, L.A.; Dyson, M.: Acoustic Cavitation generated by an extracorporeal shockwave lithotripter. Ultrasound in Med. and Biol. 13: 2: 69-76, 1987
- 6 Lele, P.P.: A simple Method for Production of trackless focal lesions with focused ultrasound: physical factors. J. Physiol. 160: 494-512, 1962
- 7 Fry, F.J. (Ed.): Ultrasound . Its applications in Medicine and Biology Part I, Part II , Elsevier, Amsterdam 1978
- 8 Russo, P.; Stephenson, A.; Mies, C. et al.: High Energy Shock waves suppress Tumor Growth in vitro and in vivo. The J. of Urology vol 135: 626-628, 1986
- 9 Brümmer, F.; Brenner, J.; Bräuner, Th.; Hülser, D.F.: Effect of shock waves on suspended and immobilized L1210 cells. Ultrasound in Med. and Biol. 15:3:229-239, 1989
- 10 Geldorf, A.A.; de Voogt, H.J.; Rao, B.R.: High energy shock waves do not affect either primary tumor growth or metastasis of prostate carcinoma R3327-MatLyLu. Urol. Res 17:9-12, 1989

- 11 Oosterhof, G.O.N. et al.: The in vitro effect of electromagnetically generated shock waves (Lithostar) on the Dunning R3327 PAT-2 rat prostatic cancer cell-line. *Urol.Res.* 17:13-19, 1989
- 12 Randazzo, R.F.; Chaussy, C.G.; Fuchs, G.J.; Bhuta, S.M.; Lovrekovich, H.; de Kernion, J.B.: The in Vitro and in Vivo Effects of Extracorporeal Shock Waves on Malignant Cells. *Urol.Res* 16:419-426, 1988
- 13 Wilmer, A.; Gambihler, S.; Delius, M.; Brendel, W.: Shock Waves Enhance the Cytotoxicity of Cisplatin but not of Adriamycin. *Eur.surg.Res.* 20:89-90, 1988
- 14 Gambihler, St.: Wirkung von Stoßwellen auf Tumor-Zellen in vitro. Symp.der Körper Stiftung.Hamburg: "Stoßwellenanwendungen in der Medizin". München, 16.2.1988
- 15 Brendel, W.; Delius, M.; Goetz, A.: Effect of Shock Waves on the microvasculature. *Prog.appl.Microcirc.* Karger, Basel. 12:41-50, 1987
- 16 Goetz, A.E.; Königsberger, R.; Conzen, P.; Brendel, W.: Experimentelle Tumorthherapie mit Schockwellen - Erste Ergebnisse. 64.Tagung d.Verein.d.Bayr.Chirurgen Jul1987, Bad Reichenhall(D)110, 1987
- 17 Riedlinger, R.; Ueberle, F.; Zanger, U.: HEPUS (High Energy Pulsed Ultrasound): Apparatus and Physical Aspects of Tumor-Insonification. *Biomed.Technik* 33 : SupVol 2 : 139-140, 1988
- 18 Hahn, E.W. et al.: High Energy Pulsed Ultrasound (HEPUS): Cytotoxic effects on rodent tumors. *Biomed.Technik* 33: SupVol 2: 141-142, 1988
- 19 Bräuner, Th.; Brümmer, F.; Hülser, D.F.: Histopathology of Shock Treated Tumor Cell Suspensions and Multicell Tumor Spheroids. *Ultras.in Med.and Biol.* 1989 in press.

FIGURES

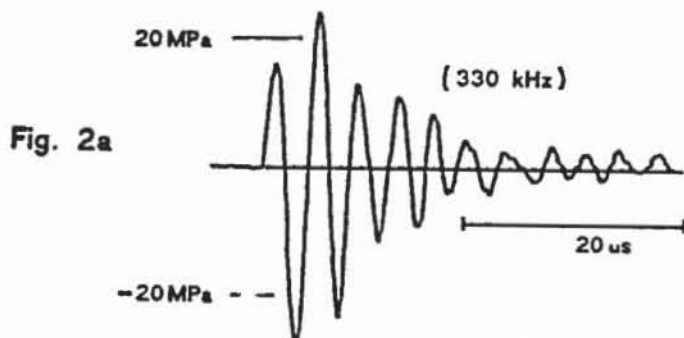
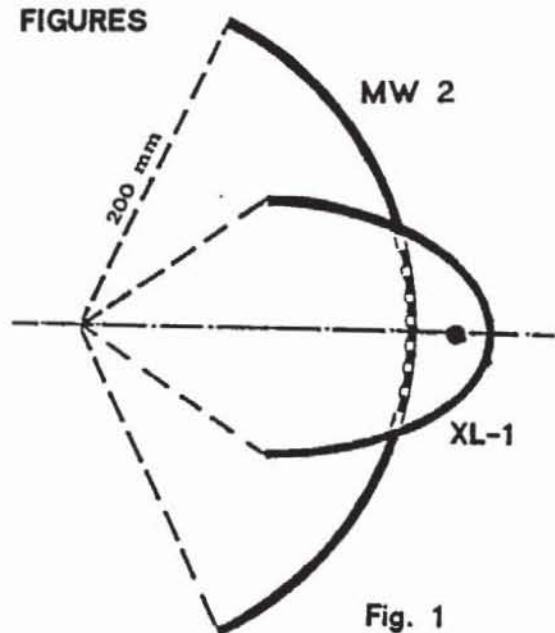


Fig. 2b

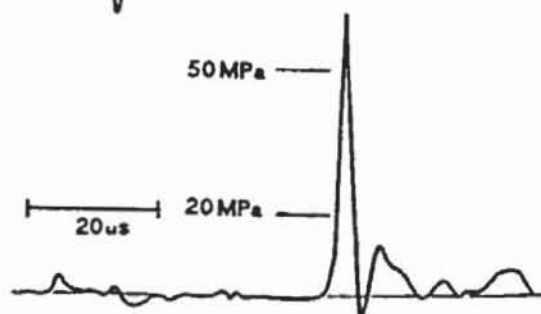


Fig. 1 : Contours of the MW 2 ultrasound pulser and the XL-1 shock wave source . schematic drawing, but in scale.

Fig. 2a : hepUS time pressure profile near focus. generator voltage: 2 kV

Fig. 2b : heSW time pressure profile /15/.

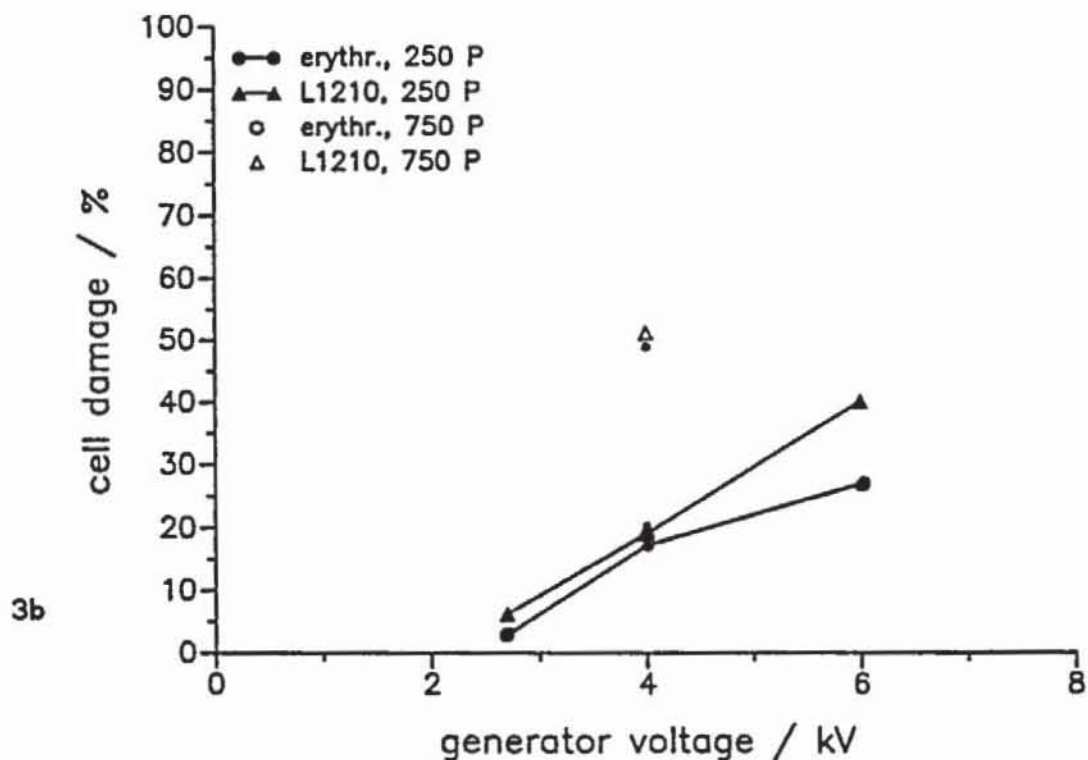
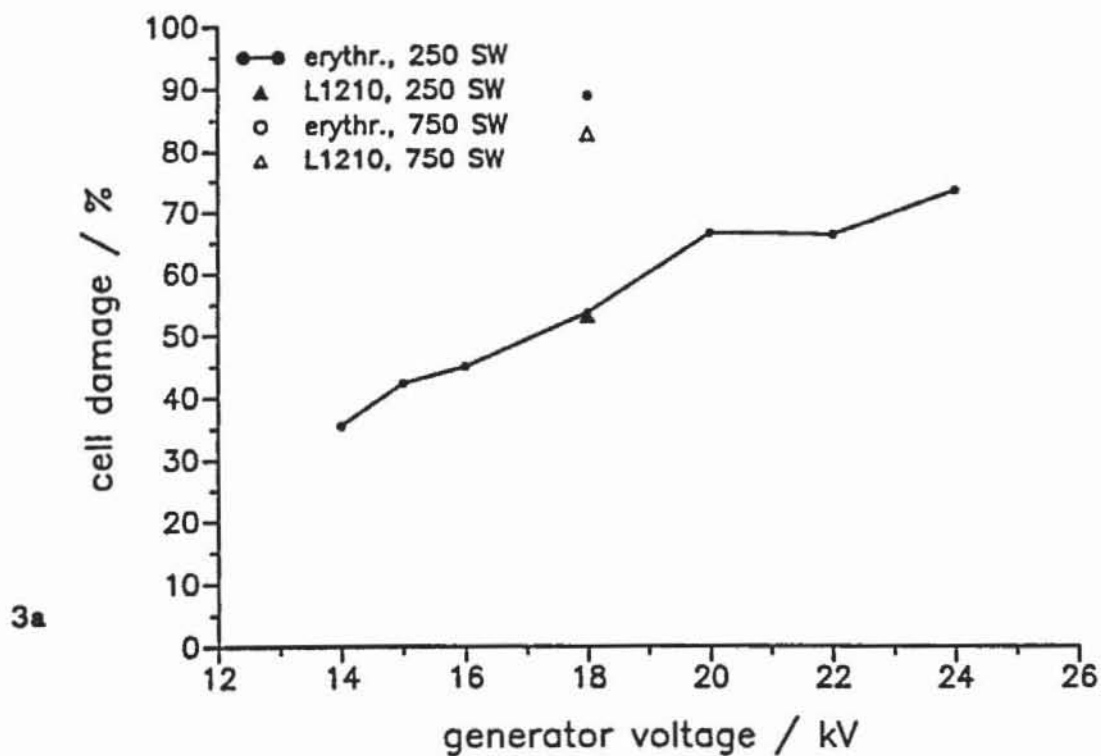


Fig. 3 : Sonication by 250 and 750 events. Effects on human erythrocytes (● ○) and on L1210 mouse leukemia cells in suspension (▲ △) , demonstrated by the increase of damaged cells after treatment with different generator voltages

3a: of the experimental lithotripter XL-1 (Fig. 3 a)

3b: of the ultrasound - pulser MW 2 (Fig. 3 b)

In both cases the coupling water was partially degassed and at 37°C.

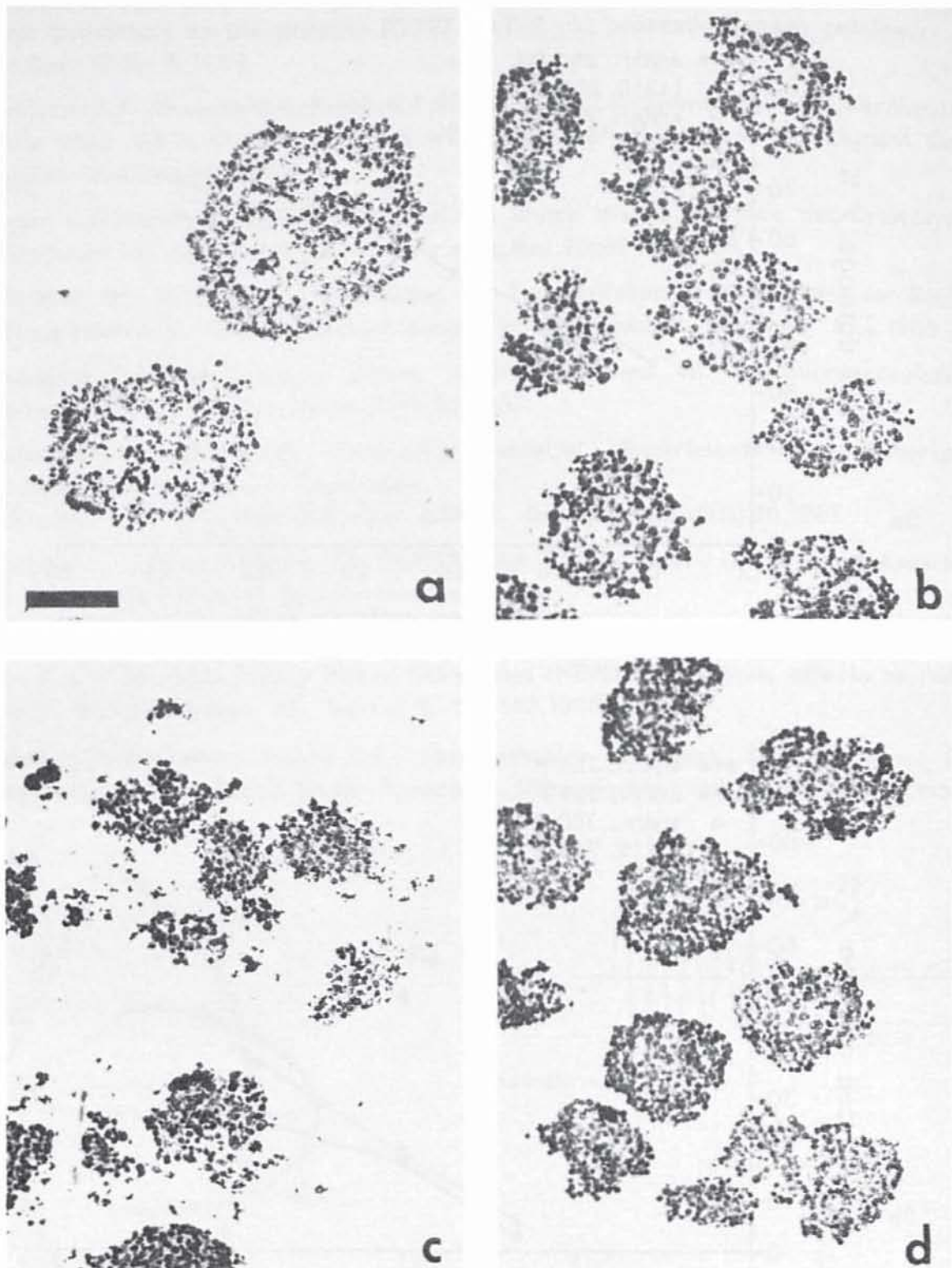


Fig. 4 : Multicell tumor spheroids of the rat mammary tumor cell line BICR/M1R_k after different experimental conditions. bar : 200 μ m

- 4a: Section through speroids immobilized in gelatine treated with 250 shock waves (XL-1 : 18 kV, 1 Hz)
- 4b: Section through untreated control speroids.
- 4c: Section through speroids from the middle part of the test tube (focal zone) immobilized in gelatine and treated with 250 US-pulses (MW2 : 4 kV, 1 Hz)
- 4d: Section through speroids from the lower part (nearer to source) of the test tube immobilized and treated as mentioned in 4c.