BIOPHYSICAL INVESTIGATIONS OF THE IN VITRO EFFECTS OF SHOCK WAVES AND ULTRASOUND

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Abstract: To investigate the interactions of ultrasonic waves with biological tissues, we developed and standardized several in vitro models. Using these systems - artificial stones, human erythrocytes, L1210 mouse leukemia cells, multicellular spheroids, cavitation assay - we are able to elucidate the mechanisms of interaction as well as the cause of clinically observed side effects.

I. INTRODUCTION

Side effects of ultrasonic waves on organs, tissues, and cell suspensions have been investigated for several decades. Since the advent of extracorporeal shock wave lithotripsy of kidney and gall stones, accompanied by tissue damage and obvious impairment of organ function [3], the importance of quantitative analysis of biological effects increased. Physical characterization of focused high energy shock waves including quantitative measurements of the peak negative pressure needs highly sophisticated technical equipment [5,8]. We, therefore, characterized the acoustic output as well as the biological effects of different shock wave sources in clinical use for the therapy of kidney stones and experimental ultrasound devices for tumour therapy.

II. IN-VITRO-MODELS

Artificial stones, 15 mm in diameter, 1.46 - 1.54 g in weight, made of a mixture of dental cement and glass microspheres were shock wave treated until disintegration into fragments smaller than 2 mm. The number of shock waves needed for this approach is a measure of the fragmentation efficacy.

Human erythrocytes obtained by venous puncture from volunteers were washed and suspended in phosphate buffered saline. Cell suspensions ($3\text{-}5\cdot10^7$ cells/ml) were transferred in polyethylene pipettes and treated with shock waves. After determination of the number of intact cells as well as the amount of free haemoglobin in the supernatant, the resulting dose-response-curves were calculated.

L1210 cells, a lymphocytic mouse leukemia cell line, were concentrated to $2\text{-}3\cdot10^5$ cells/ml and treated with shock waves. The percentage of geometrically destroyed and intact cells was measured, and the latter fraction was investigated for physiologically viable and dead cells using a double staining technique with fluorescent dyes and flow cytometry. A LD_{50} was calculated from the dose-response-curves [4].

Multicellular spheroids, three-dimensional growing cell aggregates with numerous cell-cell and cell-matrix interactions, are a model for tissue *in vivo*. For the initiation of multicellular spheroids, single cell suspensions were seeded into plastic dishes, and within three days small cell aggregates were formed. These aggregates were transferred into spinner flasks and can be cultured up to several 100 µm in diameter. Shock wave treated spheroids were investigated by light and electron microscopical histology.
Spheroids immobilized in 12% gelatine were recovered by liquefying the gel at 37°C, and processed in the same way [1].

The occurrence of acoustic cavitation, accompanied by free radicals and chemical oxidation products, was investigated by our cavitation assay. This assay uses the radical-sensitive fluorescent dyes dihydroethidium and dichlorofluorescin-diacetate which are suitable for extra- and intracellular measurements. After sonication of dye solutions and stained cells, the increase in fluorescence of the oxidation products ethidium and dichlorofluorescein was determined by fluorometry and flow cytometry [9].

III. RESULTS AND DISCUSSION

Using our standardized *in vitro*-models, we demonstrated the influence of various parameters on fragmentation efficacy and on biological as well as on chemical effects.

We examined the fragmentation efficacy of different types of lithotripters with regard to emitted acoustic energy, temperature, gas content, and position of the surface of the waterbath. Under the same experimental conditions, we compared two identical lithotripters (Dornier XL-1 in Stuttgart and Munich) and determined fragmentation efficiencies of 100 ± 1 and 105 ± 1 shock waves. This reveals the high reliability and reproducibility of this stone model.

A simple and well established model for biological effects are human erythrocytes. The occurrence of free haemoglobin in the urine of patients after lithotripsy indicates the clinical relevance of this model. The number of destroyed erythrocytes after sonication of erythrocytes *in vitro* accounts only for half of the released amount of free haemoglobin, indicating a transient increase in membrane permeability in intact erythrocytes. By combining experiments with artificial stones and erythrocytes, we characterized several electrodes for electrohydraulic lithotripters with regard to their efficiency and tissue damage.

After shock wave treatment, viable and dead mouse leukemia L 1210 cells were distinguishable, resulting in different dose-response-curves related to experimental conditions. Degassing (<2 mg O_2/l), heating (41°C vs. 37°C vs. 21°C) and lowering the surface (2 cm vs. 10 cm behind the focal region) of the water in the tank of an electrohydraulic lithotripter resulted in an increase of destroyed and dead cells.
With this experimental approach we investigated several cell lines of different origin for the sensitivity of normal and tumour cells to shock waves. Our results indicate that under carefully controlled and constant experimental conditions, cells have different sensitivities to shock waves, but no general difference between normal and malignant cells can be seen [6].
Long-term investigations using growth curves or the MTT-cell proliferation assay [2] resulted in delayed proliferation of cells after shock wave treatment for a lag-phase of 72 hours.

Shock wave treated multicellular spheroids exhibited severe damage in light- and electron microscopical histology, *e.g.*, fragmentation of spheroids, alterations of organelle ultrastructure, perinuclear cisterns, and transient membrane ruptures. Immobilizing spheroids in 12% gelatine leads to a tissue phantom with acoustic properties known for biological tissues and organs, *e.g.*, sound velocity of 1530 m/s vs. 1520 m/s for blood and 1570 m/s for muscle [7]. With immobilized spheroids, we were able to separate direct shock wave effects from secondary mechanisms, *e.g.*, cavitation, and detected differences in the pattern of damage after treatment with shock waves or high energy pulsed ultrasound.

A closer view on the mechanisms of cellular damage using the cavitation assay revealed the occurrence of extra- and intracellular cavitation-generated free radicals. We found a dose- and repetition frequency-dependent increase in fluorescence of the oxidation products after shock wave treatment. This increase was higher for 8 Hz than for 1 Hz. The contribution of these free radicals to the shock wave induced cell damage was demonstrated using antioxidants.

Our standardized *in vitro* models (artificial stones, flow cytometry of single cell suspensions, histology of suspended and immobilized multicellular spheroids, cavitation assay) are reliable and reproducible test systems for detailed characterization of shock waves and ultrasound and their interactions with biological tissues.

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