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Biological effects of combined ultrasound and cisplatin treatment on ovarian carcinoma cells

Vladan Bernard [a,*], Jiřina Škorpíková [a], Vojtěch Mornstein [a], Iva Slaninová [b]

[a] Masaryk University, Faculty of Medicine, Department of Biophysics, Brno, Czech Republic
[b] Masaryk University, Faculty of Medicine, Department of Biology, Brno, Czech Republic

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ABSTRACT

The effects of low-power ultrasound, the anti-cancer drug cisplatin, and their combined application were studied in two lines of human ovarian carcinoma cells, A2780 and A2780cis. Four modes of treatment were used: exposure to ultrasonic field, application of cisplatin, exposure to ultrasound followed by cisplatin, and presence of cisplatin followed by exposure to application ultrasound. Ultrasound was used at intensities of 0.5 W/cm^2 and 1.0 W/cm^2 for 10 min, cisplatin was applied at concentrations of 1 μM and 6 μM per cell suspension treated in A2780 and cisplatin-resistant A2780cis cells, respectively. The results of each experimental treatment were assessed by the resultant cell viability related to the viability of control cells, using a standard MTT test. It was shown that a combined effect of ultrasound and cisplatin was more effective than that of ultrasound or cisplatin alone. It also appeared that the order of application played a role, with the cisplatin-ultrasound treatment lowering cell viability more than the ultrasound-cisplatin treatment. It can be assumed that the exposure of cells to a low-power ultrasonic field has an immediate effect on the structure of cell surfaces and, consequently, on entry of cisplatin into the cell.

The study also included observations on changes in the cell cycle associated with the treatments used in both cell lines and their evaluation by flow cytometry.

1. Introduction

Ultrasound has a wide range of applications in current medicine and is commonly used in both diagnosis and therapy. Therefore, as broad as possible knowledge of its effects on biological systems is necessary and this fact warrants ongoing investigations. Exposure of biological systems to a low-power ultrasonic field produces the mechanical stress, known as non-thermal effects, and an increase in temperature. This thermal effect is assumed to be due to absorption of ultrasonic energy that increases vibrational and rotational energy of molecules in the system [1].

At the cellular level, ultrasound produces changes in inner structures, particularly on the membranes of mitochondria or endoplasmic reticulum or in microtubular and microfilamental components of the cytoskeleton [2]. It has been reported that exposure to ultrasound can affect cell structures and surfaces in terms of their porosity [3,4]. Under certain conditions, the changed porosity influences the amount of active substances entering the cell and is one of the factors responsible for resistance to anti-cancer drugs [5].

The incidence of cancer and related pathological conditions in the population is increasing. Chemotherapy, an important anti-cancer treatment, is using agents based on heavy metal complexes that are the effective components of treatment [6,7]. However, in addition to advantages, their use also brings about some disadvantages, such as non-selective efficacy, acquired resistance and serious side effects for the patient. Therefore, research into new active anti-cancer agents or the development of such therapeutic procedures that would reduce or eliminate the disadvantages of chemotherapy are very important.

A promising trend in anti-cancer therapy appears to be the use of a combined effect of chemotherapy and low-power ultrasound. Studies have been published on application of ultrasound during a targeted intracellular delivery of drugs and macromolecules [8,9]. Other findings have shown that the effect of cytostatic drugs or in vivo chemosensitivity of tumours is enhanced by the presence of an ultrasonic field [10,11]. In this case, ultrasound as a physico-mechanical factor is co-acting with anti-cancer agents. The advantages of this co-acting approach include a lower dose of the cytostatic drug applied and its targeted effect within the area defined by an ultrasonic field.

* Corresponding author. Address: Department of Biophysics, Faculty of Medicine, Masaryk University, Kamenice 3, 625 00 Brno, Czech Republic. Tel.: +420 549 49 1320.
E-mail address: vbernard@med.muni.cz (V. Bernard).

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Table 1
Significance of differences in viability values, as assessed by the Mann–Whitney test.

	contr	*cisPt*	*cisPt + us*	*us + cisPt*	*us*
A2780 0.5 W/cm²					
contr	–	•	•	•	•
cisPt	•	–	•	•	•
cisPt + us	•	•	–	•	•
us + cisPt	•	•	•	–	•
us	•	•	•	•	–
A2780 1W/cm²					
contr	–	•	•	•	•
cisPt	•	–	•	•	•
cisPt + us	•	•	–	•	•
us + cisPt	•	•	•	–	•
us	•	•	•	•	–
A2780cis 0.5W/cm²					
contr	–	•	•	•	•
cisPt	•	–	•	•	
cisPt + us	•	•	–		•
us + cisPt	•	•		–	•
us	•		•	•	–
A2780cis 1W/cm²					
contr	–	•	•	•	•
cisPt + us	•	–	•	•	
cisPt + us	•	•	–		•
us + cisPt	•	•		–	•
us	•		•	•	–

Empty box, no significance; •, statistical significance at $p < 0.05$; cell lines exposed to ultrasound as follows: A2780 0.5 W/cm², A2780 1.0 W/cm², A2780cis 0.5 W/cm², A2780cis 1.0 W/cm²; *cisPt*, cells incubated with cisplatin; *cisPt + us*, cells exposed to ultrasound in the presence of cisplatin; *us + cisPt*, cells exposed to ultrasound followed by incubation with cisplatin; *us*, cells exposed to ultrasound only.

2. Cell cultures and chemicals

Human ovarian carcinoma cell lines, A2780 and its cisplatin-resistant form A2780cis were used. They were obtained from the European Cell Culture Collection. RPMI-1640 medium with L-glutamine (Bio Tech, Ltd., Prague, Czech Republic) supplemented with 10% foetal calf serum (Bio Tech) and 100 μg/ml streptomycin/penicillin (Bio Tech) was used. The cell lines were grown in cell culture flasks in an atmosphere of 95% air and 5% CO_2 at 37 °C, with cisplatin added to medium for A2780cis cultivation. The cells were detached from glass by trypsin addition (Bio Tech).

The stock solution of cis-Dichlorodiammine Platinum (II) (*cisPt*) in PBS was prepared from crystalline *cisPt* (Sigma). The stock solution of propidium iodine at a concentration of 1 mg/ml was prepared from crystalline propidium iodine (Sigma).

3. Ultrasound exposure

A BTL-07 therapeutic ultrasound generator (Beautyline Ltd., Prague, Czech Republic) working at a frequency of 1 MHz and equipped with a 4 cm² probe was used as the source of ultrasound. The cells were exposed for 10 min to the far field of a horizontal beam of continuous-wave ultrasound at intensities of 0.5 W/cm² or 1.0 W/cm² in a thermostated 37 °C water bath. The exposure was carried out in a polyethylene tube fastened to a rotating holder (3 rpm). This experimental set-up provided uniform exposure of the entire volume of cell suspension. Ultrasound intensity was controlled by means of a calibrated PVDF hydrophone, type MH28-6 (Force Institute Copenhagen, Denmark).

4. Experimental design

The cells of both A2780 and A2780cis lines were incubated for 72 h after the following modes of treatment:

- addition of cisplatin only (*cisPt*);
- 10-min exposure to ultrasound only (*us*);
- addition of cisplatin and subsequent 10-min exposure to ultrasound (*cisPt + us*);
- 10-min exposure to ultrasound followed by addition of cisplatin (*us + cisPt*);
- neither addition of cisplatin nor exposure to ultrasound (*control*).

The treatment of each experimental group was carried out in several replicates.

5. Viability test

The following procedure was employed to compare the viability of *us*, *cisPt*, *cisPt + us*, *us + cisPt* and control cells: a cell suspension was obtained by tryptinisation of cells adhering to the flask bottom. To each well of a 96-well plate containing 10^3 cells in RPMI medium, a calculated volume of *cisPt* stock solution was added to achieve a final *cisPt* concentration of 1 μM (A2780) and 6 μM (A2780cis) per well. An equal volume of PBS free of cisplatin was added to the control cells. No trypsin was added. After incubation for 72 h, the cells were washed in PBS and evaluated by a standard MTT test of viability [12]. Using an EL800 microplate reader (Bio-Tek, USA) the absorbance of a colour product in each well was recorded at 570 nm. The amount of the colour product is directly proportional to the metabolic activity (i.e., viability) of living cells.

6. Cell cycle analysis

Before harvesting the cells, culture medium with floating cells was transferred into a test tube; then adhering cells were harvested by trypsinisation into the same tube, washed twice with ice-cold PBS and fixed in 70% ethanol for 30 min at room temperature. Subsequently, the cells were washed with PBS, incubated with 17 μg/ml RNase A at 37 °C for 30 min, and stained with 3 μg/ml propidium iodide solution for 10 min (in darkness, at room temperature).

Data on cell cycle status were obtained with a Cytomics FC 500 (Beckman Coulter, Inc.) flow cytometer system, using the FL3 channel (emission at 620 nm), with reading set to linear acquisition. Between 10,000 and 20,000 events were evaluated. Data from each sample were saved as separate flow cytometry standard files using CXP analysis software (Beckman Coulter, Inc.), and were analysed for cell cycle phases using Multicycle AV software for Windows (Phoenix Flow system, San Diego, USA). DNA content analysis included determination of the percentages of G1, G2/M, S-phase and sub-G1 (apoptotic) fractions.

7. Fluorescence microscopy

Cells stained with propidium iodide for cell cycle analysis were transferred to Vectashield mounting anti-bleaching medium (Vector Laboratories Inc., USA) and the presence of apoptotic nuclei was evaluated by fluorescence microscopy (Leitz Laborlux S fluorescence microscope).

8. Statistical analysis

The absorbance value for each group was converted into cell viability as follows: the median absorbance value of the control group was taken as 100%; the absorbance of each experimental group was expressed as a percent control value, i.e., its viability relative to that of the control group. Because of a non-normal distribution of the values for individual groups, the non-parametric