Original Article

The Combined Effect of Ultrasound Exposure and Cisplatin on Human Ovarian Carcinoma Cells A2780

(cisplatin / cytotoxicity / ovarian carcinoma cells A2780 / ultrasound)

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Abstract. This article deals with an *in vitro* study of the effect of cisplatin and low intensity ultrasound exposure on the viability of human ovarial carcinoma cells A2780. The effect on the viability of 10^3 cell experimental group samples under the influence of separate and combined cisplatin and ultrasound far field exposure was studied. Viable cells in individual experimental groups were followed at time intervals of 0, 24, 48 and 72 hours following treatment. Another objective of the study was to investigate the effect of the experimental protocol on the combined effect of cisplatin and ultrasound exposure. The effect of the concurrent application of cisplatin and ultrasound exposure was compared with that in which cisplatin was added to the cell suspension after ultrasound exposure. The results of this work confirmed the cytotoxicity of cisplatin and possible stimulation of cancer cells by low intensity ultrasound. However, it was found that ultrasound exposure enhances the action of cisplatin on the viability of A2780 cells and that the effect is dependent on the experimental protocol. In this case the concurrent application of cisplatin and ultrasound was found to be more effective ($P < 0.05$).

Introduction

Tumour processes are a major threat to a considerable part of the population. The use of anticancer drugs in chemotherapy is an important component of therapeutic efforts in this field. However, chemotherapy by means of anticancer drugs has presented some difficulties, i.e., non-selective efficacy, acquired resistance and a significant load of the patient's organism caused by the administration of high doses. Thus, the effort of many research laboratories to develop new anticancer drugs or to introduce therapeutic procedures which would eliminate or at least reduce to acceptable levels the disadvantages of chemotherapy are important. The combined effect of chemotherapy and ultrasound seems to be one of the possible directions in anticancer therapy (Aio et al., 2006). In this case, ultrasound is a physical factor influencing the biological system which enhances the efficacy of substances having anticancer properties. The effects of high intensity ultrasound on living organisms are mainly cavitational and thermal (Miller et al., 1996). Even at intensities below the cavitation threshold ultrasound has an influence on cytoskeletal structures (Hrazdira et al., 1998) and cell surfaces (Korosoglou et al., 2006). Such effects would in turn impact the amount of active substance entering the intracellular space and hence reduce the resistance to the administered anticancer drugs (Stewart, 2007). Studies about the use of ultrasound in targeted drug delivery into the intracellular space have already been published (Schlicher et al., 2006; Yu et al., 2004). In these studies, it was confirmed that it is possible to increase the delivery of active substances to biological objects such as carcinoma cells via ultrasound exposure. It is therefore recognised that the combined biological effect of ultrasound and cytostatic drugs is a promising method in increasing the effectiveness of these drugs in clinical practice. However, no studies have been found in the literature concerning the use of ultrasound with cisplatin and A2780 carcinoma cells. Despite the ever increasing number of anticancer drugs available, cisplatin (cis-diamminedichloroplatinum(II)) is still used intensively, as a monotherapy or in combination with other drugs. Outstanding positive results for cisplatin therapy are found mainly in the treatment of tumours of the testicles, ovaries, urinary bladder and neck (Kelland, 1993; Desoize et al., 2001; Garattini et al., 2001). The main action mechanism of cisplatin is its binding ability as a monofunctional and bifunctional adduct in the N7 position of the DNA purine base (Brabec, 2000). The cisplatin adducts lead to changes in the DNA secondary structure, which consequently influ-

Received December 10, 2007. Accepted June 6, 2008.

This study was supported by grant 305/05/2030/S from the Grant Agency of the Czech Republic.

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Abbreviations: cisplatin – cis-diamminedichloroplatinum(II), PBS – phosphate-buffered saline.

ence the metabolism of the cell (Bose et al., 2000; Lippert, 1999).

Material and Methods

Cell culture and chemicals

Human ovarian carcinoma cells A2780 from Sigma-Aldrich (Prague, Czech Republic) were chosen for this experimental study. The cells were grown in RPMI 1640 medium containing glutamine (BioTech, Ltd., Prague, Czech Republic), 10% foetal calf serum (BioTech) and 100 μg/ml streptomycin/penicillin (BioTech). The cell line A2780 was routinely cultivated in cell culture flasks at 37 °C in 5% atmosphere of CO_2. They were transferred twice a week to a fresh medium. To release the adherent cells from the flask bottom, we used trypsin (BioTech). A stock solution of cisplatin in phosphate-buffered saline (PBS) at a concentration of 130 μM was prepared from crystalline cisplatin (Sigma).

Ultrasound exposure

A therapeutic ultrasound generator BTL-07 (Beauty-line Ltd., Prague, Czech Republic) working at a frequency of 1 MHz and equipped with a 4 cm^2 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 the intensity of 1 W/cm^2 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 setup ensured a uniform exposure of cells through the entire volume of the cell suspension. The ultrasound intensity was controlled by means of a calibrated PVDF hydrophone, type MH28-6 (Force Institute, Copenhagen, Denmark).

Experimental groups and procedure

The following experimental groups were studied:

– cells incubated following the addition of cisplatin only - *cisPt*,
– cells incubated following 10 min exposure to ultrasound only – *us*,
– cells incubated following the addition of cisplatin and subsequent immediate 10 min exposure to ultrasound - *cisPt+us,*
– cells incubated following 10 min exposure to ultrasound and subsequent addition of cisplatin - *us+-cisPt,*

A control group was also set up for which cells were incubated without the addition of cisplatin and without ultrasound exposure - *contr*.

A calculated volume of the stock solution of cisplatin was added to cells (in RPMI medium) in 96-well plates (10^3 cells per well) to achieve a resulting 1 μM concentration of cisplatin in each well. An equal volume of PBS but without cisplatin was also added to the control samples. Before any experiment, the cells adhering to the flask bottom were trypsinised. No trypsin was added to the wells. The first part of the experiment involved a comparison of the viability of the *cisPt*, *us*, *us+cisPt* and *contr* groups. After incubation in the 96-well plate (0, 24, 48 and 72 h), the cells were washed in PBS and subjected to a standard MTT test of viability (Mosmann, 1983). In the second part of the experiment the viability of *cisPt, cisPt+us, us+cisPt* and *contr* groups were compared. In this case the MTT test was performed only at time interval of 72 h as the results of the first part of the experiment indicated that viability ranges are narrower following this incubation time. The absorbance of the blue product in individual wells was obtained using a microplate reader EL800 (Bio-Tek, VT) at a wavelength 570 nm. The amount of measured blue product is directly proportional to the number of living cells in a sample.

Statistical analysis

The measured values of absorbance were converted into percentage of controls to represent cell viability relative to the control group and the results plotted. Because of the non-normal distribution of the values from individual groups, the non-parametric Mann-Whitney *t*-test at a significance level of $P = 0.05$ was used. The statistical software STATISTICA 7.1 was used to calculate the median and upper and lower quartiles.

Results

The data obtained in the first part of the study permit a comparison of the viability of A2780 cells in the experimental groups *cisPt*, *us* and *us+cisPt* with respect to the *contr* group following incubation times of 0 h (Fig. 1a), 24 h (Fig. 1b), 48 h (Fig. 1c), and 72 h (Fig. 1d).

The graphs clearly show a dependence of both median and inter-quartile range values of relative cell viability on the incubation time. Median values decreased monotonically and minimum values were achieved after 72 h of incubation for all three experimental groups. It is evident that after 48-h incubation the *cisPt* and *us+cisPt* experimental groups exhibited considerably lower median values that the *contr* and *us* groups. After 72-h incubation, the inter-quartile range values decreased to a point which made definite conclusions regarding reduced viability possible. After 72-h incubation, the differences between the control group and *cisPt* and the control group and *us+cisPt* were statistically significant ($P < 0.05$). A statistically significant difference was also found between the groups *us*, *cisPt* and *us+cisPt*. The expected effect of cell stimulation by ultrasound exposure was also confirmed as there was a difference in the percentage of living cells between the control group and the *us* group ($P < 0.05$). In the *us* group, the number of viable cells was higher compared to that in the control group ($P < 0.05$) following short incubations times (up to 48 h) but lower at 72 h. Hence in the second part of the experiment the cells were incubated for 72 h.

The results of the second part of the experiment with experimental groups *cont, cisPt, cisPt+us* and *us+cisPt*

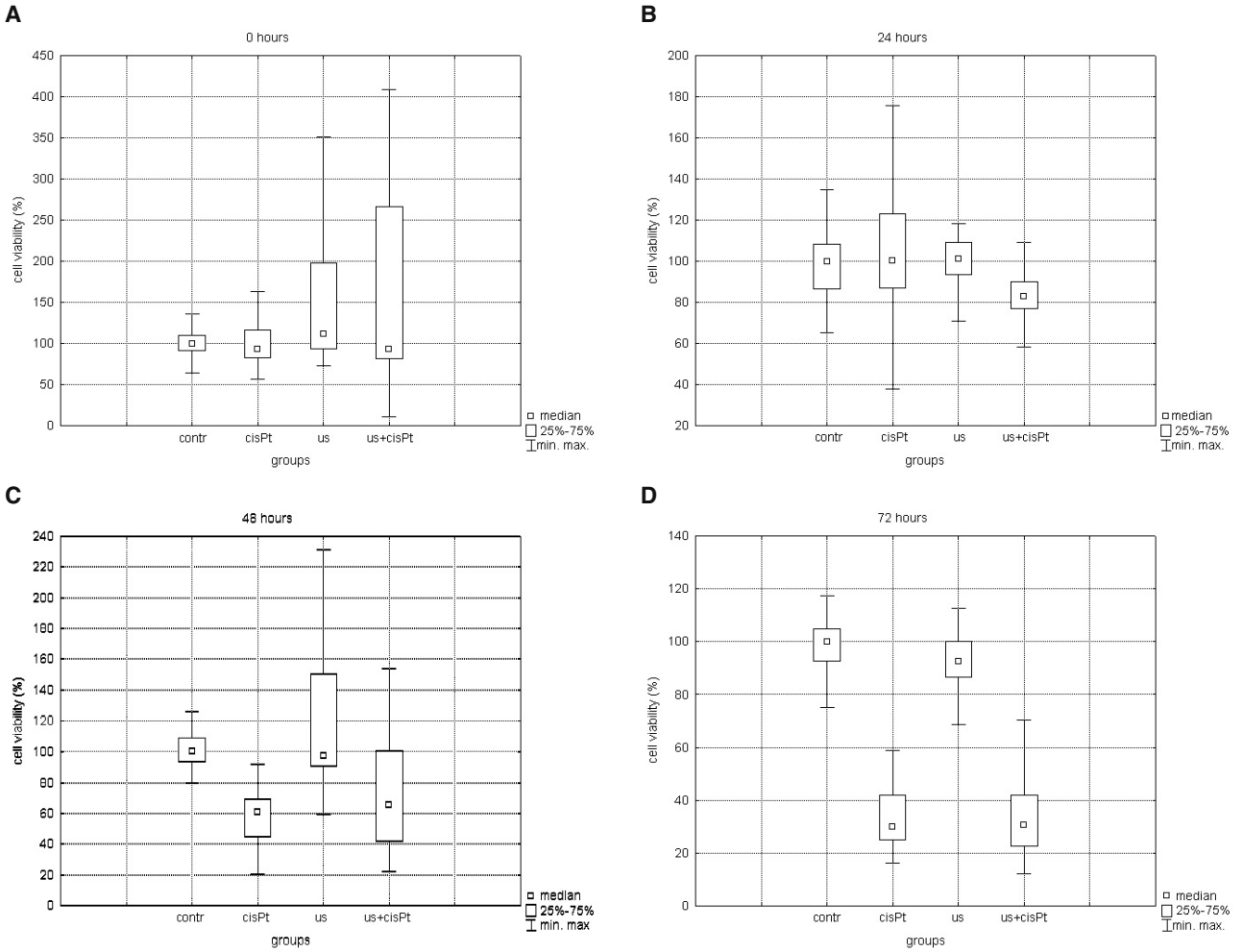

Fig. 1. Graphs showing the viability in individual experimental groups as the percentage of living cells compared with the control group for incubation times 0 h (a), 24 h (b), 48 h (c), and 72 h (d). Following groups are involved: *contr* – control group of A2780 cells, *cisPt* – cells incubated with cisplatin (1 μM in each well), *us* – cells exposed to ultrasound in the far field (1 W/cm^2, 1 MHz, 10 min), *us*+*cisPt* – cells exposed to ultrasound (1 W/cm^2, 1MHz, 10 min) and then incubated with cisplatin (1 μM in each well).

at an incubation time of 72 h are shown in Fig. 2. This additional experiment was done in order to find out whether the decrease in viability of cells treated with cisplatin following ultrasound pre-treatment would be the same as in the case of cells undergoing concurrent ultrasound-exposure and cisplatin treatment (identical ultrasound exposure and cisplatin concentration).

Statistically significant differences in the percentage of viable cells between the groups *cisPt* and *cisPt*+*us* ($P < 0.05$), and *cisPt* and *us*+*cisPt* ($P < 0.05$) were found as well as the *cisPt*+*us* and *us*+*cisPt* ($P < 0.05$).

Discussion and Conclusions

In our study, we investigated the viability of the A2780 cell line of human ovarian carcinoma under the influence of the cytostatically active pharmaceutical cisplatin and the exposure to ultrasound separately and in combination. The cytostatic activity of cisplatin on human ovarian carcinoma cells reported in earlier studies e.g. by Kolfschoten et al. (2002) was also confirmed. In the experiments on the biological effect of ultrasound exposure we also confirmed the enhancement of cell viability as already reported by Arthur et al. (2007*)* and Škorpíková et al. (2001). In the experimental groups exposed solely to ultrasound, the A2780 cells became stimulated after shorter incubation. On the contrary, after the longest incubation time (72 h), a relatively outstanding inhibitory effect of the ultrasound exposure was found compared with the control groups. This explains our choice of incubation time for the second part of the experiment. Based on the evaluation of the experiments with combined effect of ultrasound exposure and cisplatin, we conclude that the viability of the *cisPt*+*us* groups after 72-h incubation was lower than in the group *us*+*cisPt*. A possible explanation is via the effect of ultrasound on membrane permeability. Some authors have reported increased membrane porosity and loosening of structures on cellular surfaces after ultrasound exposure (Tachybana et al., 1999). These changes

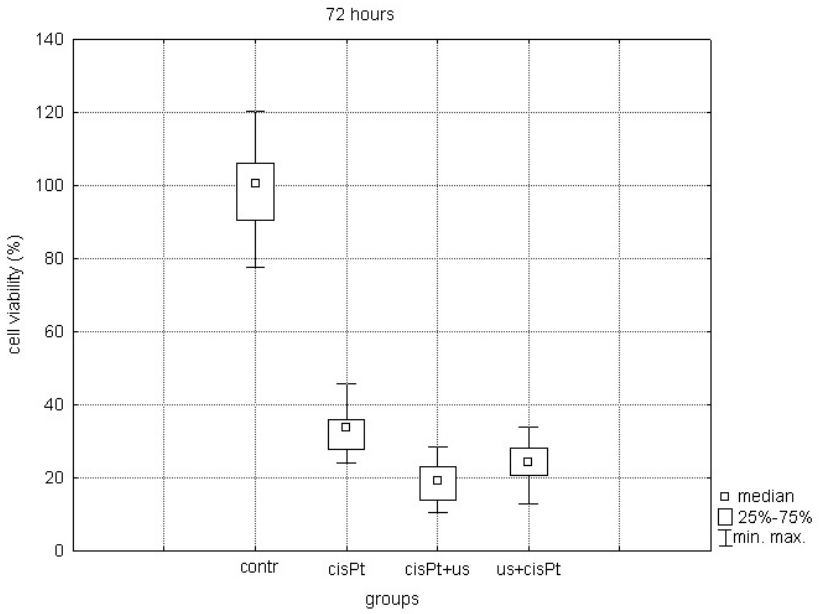

Fig. 2. Viability of the individual groups expressed as percentage of control cell number for the incubation time of 72 h. Following groups are involved: *cont* – control group, *cisPt* – cells incubated with cisplatin (1 μM in each well), *cisPt+us* – cells exposed to ultrasound (1 W/cm^2, 1MHz, 10 min) in the presence of cisplatin (1μM), *us+cisPt* – cells exposed to ultrasound (1 W/cm^2, 1 MHz, 10 min) and then incubated with cisplatin (1 μM).

would facilitate the transport of cytostatic substances into the cells. The higher viability of the experimental group *us+cisPt* in comparison with the group *cisPt+us* indicates that these changes of cellular surfaces are reversible, and that such a reversal takes place after a short time interval following the ultrasound exposure.

The finding that the influence of the combined effect of cisplatin and ultrasound on viability is dependent on experimental protocol opens new research perspectives. Provided that ultrasound exposure of tumours *in situ* would lead to similar effects, i.e. to the increase of membrane permeability for anticancer drugs, the local tumour-targeted application of ultrasound could lead to the amplification of the anticancer therapy or to the reduction of therapeutic doses of anticancer drugs necessary, thereby reducing the deleterious side effects of these remedies. However, it seems that the sole application of low intensity ultrasound may cause stimulation of cancer cells, and therefore should be avoided.

Acknowledgement

The authors thank Mr. C. J. Caruana of the Department of Biomedical Physics, Institute of Health Care, University of Malta for his consulting support and Mrs S. Modrová of the Department of Biophysics, Faculty of Medicine of Masaryk University, Brno, Czech Republic, for her help in the experimental part of the work.

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