Effect of near field ultrasound on carboplatin treatment of ovarian carcinoma cells

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It has long been shown that therapeutic ultrasound has the potential to affect cells surfaces and membranes. In this study, the effects of ultrasound in near field mode, the anti-cancer drug carboplatin and their combined application were studied on human carcinoma cells A2780. Four modes of treatment were used: exposure to ultrasonic field, application of carboplatin, exposure to ultrasound followed by carboplatin and carboplatin treatment followed by exposure to near field ultrasound. The value of viability was measured by standard MTT test. The value of ultrasound intensity was set 1 W·cm⁻² and 1 MHz frequency was used. The real value of acoustic pressure during in vitro experiments was assessed by hydrophone. The results showed that a combined effect of ultrasound and carboplatin influenced the viability of human carcinoma cells A2780 more than the application of ultrasound or carboplatin alone. It could be assumed that exposure of cells to ultrasonic field had an immediate effect on the structure of cell surface and consequently on the entry of carboplatin into the cell. The results of our experiments demonstrated possibility of using therapeutic ultrasound in potentiating the cytostatic treatment of human carcinoma cells.

Keywords: Carboplatin, Ovarian carcinoma cells, Sonodynamic action, Ultrasound, Viability

Cancer is the second most frequent cause of human mortality, regardless of sex or age. Ovarian cancer is the second most frequent malignancy of the female genital tract and is first among all the other gynecologic cancers in mortality rate. At present, available option for treatment is platinum chemotherapy. Besides standard cisplatin, several platinum derivatives are currently used for the treatment of ovarian carcinoma. For effective therapy, various combinations of chemotherapeutic agents are used. Cytostatic effects of a platinum derivative carboplatin in combination with other drugs are also currently studied. However, major problem with chemotherapy is toxicity. Especially the commonly used generation of platinum derivatives exhibit high toxicity as compared to the newly synthesized one, for example oxaliplatin or tetraplatin, This presents a risk for the patient and is responsible for complications in the following treatment stages. Although dose reduction is one option for lowering toxicity, but it negatively affects positive effects of the treatment. Another approach includes enhancing the effects of cytostatic drugs by other factors. This approach also includes the use of ultrasonic energy. Such use of ultrasound is generally referred to as sonodynamic therapy.

Sonodynamic therapy is defined as action of ultrasound on biological objects, leading to increase in activity of cytotoxic agents applied simultaneously with ultrasonic field. The original concept of sonodynamic therapy is focused on non-specific, mainly thermal effects of ultrasonic fields that appear to be synergic with the effect of applied drugs or other substances. Current (not yet quite concise) understanding of the sonodynamic mechanism is based on the non-thermal effects, in particular those associated with cavitation phenomena and hydrodynamic stress (acoustic microstreaming, shear forces). The general principle of sonodynamic therapy can be explained as the process of sonoporation in some cases. According to Postema et al., sonoporation process is the mechanism how the mechanical effects of ultrasound are manifested. This action can be described particularly as a change in consistency of cell membrane and an increase in its porosity. Such changes of cell membrane have been observed even in the case of low intensity ultrasound.
A considerable number of drugs exhibit a greater effect in the target cell under such conditions.

In this study, we have attempted to address the following: i) whether the resulting effect of cytostatic drug, assessed as the factor influencing the cell viability, is dependent on the distribution and intensity of ultrasound field and the concentration of cytostatic drug, and ii) whether it is possible to potentiate the cytotoxic effect of a platinum drug using an appropriate ultrasonic field.

Materials and Methods

The viability expression of carcinoma cells A2780 in presence of drug carboplatin, ultrasound and combination of ultrasound and carboplatin was studied. The parameters of ultrasound field were also studied.

Human ovarian carcinoma cell line A2780 used for experiments was obtained from the European Cell Culture Collection. RPMI-1640 culture medium with L-glutamine (Bio Tech, Ltd., Prague, Czech Republic) supplemented with 10% fetal calf serum (Bio Tech Ltd., Prague, Czech Republic) and 100 µg/ml streptomycin/penicillin (Bio Tech Ltd., Prague, Czech Republic) was used. The cells were grown in cell culture flasks in an atmosphere of 5% CO₂ in air at 37°C and then detached from growing flask by trypsin addition (Bio Tech Ltd., Prague, Czech Republic). The stock solution of carboplatin (cis-Diamine[1,1-cyclobutanedicarboxylato] platinum[II]) in PBS was prepared from a lyophilized commercially available drug cycloplatin (Lachema, Czech Republic). Cycloplatin consisted of 200 mg carboplatin, 100 mg citric acid and NaOH in one vial.

Ultrasound exposure

The 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 near field (4 cm distance from probe) of a horizontal beam of continuous wave ultrasound at nominal intensities of 1 W·cm⁻² in a 37°C tempered water bath. The exposure was carried out in polyethylene tubes fastened to a rotating holder (3 rpm). This experimental set-up provided a uniform exposure of the entire volume of cell suspension. Ultrasound intensity was checked using a calibrated PVDF hydrophone, type MH28-6 (Force Institute Copenhagen, Denmark).

Experimental design

The A2780 line cells were incubated for 48 h after the following modes of treatment: (i) Addition of carboplatin only (carbo) with two concentrations; (ii) 10-min exposure to median doses of ultrasound alone (us); (iii) addition of carboplatin and subsequent 10-min exposure to ultrasound (carbo-us); (iv) 10-min exposure to ultrasound, followed by addition of carboplatin (us-carbo); and (v) no carboplatin, no exposure to ultrasound (control). The treatment for each experimental cohort was performed several times.

Viability test

The following procedure was employed to compare the viability of us, carbo, carbo-us, us-carbo and control cells: A cell suspension was obtained by trypsinization of cells adhering to the flask bottom. To each well of a 96-well plate containing 5 × 10⁴ cells in RPMI medium, a calculated volume of carboplatin stock solution was added to achieve a final carboplatin concentration of 5 × 10⁻⁵ M or 2.5 × 10⁻⁵ M per well. An equal volume of carboplatin-free PBS was added to the control cells. No trypsin was added. After incubation for 48 h, the cells were washed with PBS and evaluated by a standard MTT viability test. Using an EL800 microplate reader (Bio-Tek, USA), the absorbance of a color product in each well was recorded at 570 nm. The amount of the color product was directly proportional to the metabolic activity (i.e., viability) of measured cells.

Measurement of ultrasound pressure

The experiments included determination of ultrasound pressure value in axial axis of ultrasound probe inside of water bath. The acoustic pressure was measured by a MH28-6 hydrophone. The pressure value [Pa] was calculated using the calibration protocol of the used hydrophone from the measured voltage by the following equation:

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-2.5dB = 20 \cdot \log \left( \frac{U}{79.4nPa} \right). 
\]

Pressure of 1 Pa corresponds to the voltage \( U = 59.54 \cdot 10^{-9} \) V. The values were measured along the hydrophone axis each 0.5 cm. The hydrophone was connected to a Goldstar OS-3020 oscilloscope (Goldstar Precision Co., Korea).

Results

For the A2780 cell line affected by both drug concentrations, the viability values of us, carbo,
us-carbo and carbo-us were compared with that of the control cells. The results are presented in Fig. 1. It was apparent that the viability depended on the experimental design, especially on the sequence of application of ultrasound and cytostatic drugs, as well as concentration of cytostatic drug.

The results showed that carboplatin concentrations of $5 \times 10^{-5}$ M and $2.5 \times 10^{-5}$ M corresponds to ID$_{20}$ and ID$_{50}$, respectively. Ultrasound exposure also decreased the viability; the us cells viability was nearly 20% of non-affected control group. The graphs in Fig. 1 clearly showed a dependence of median and inter-quartile range values of relative cell viability on the combined application of ultrasound and the drug. Maximal suppression of viability was observed for the experimental group carbo-us. The experimental group us-carbo showed low levels of viability for both concentrations of carboplatin, as compared to carboplatin or ultrasound exposure alone (Fig. 1A, B).

Statistically significant differences (Table 1) in the percentage of viable cells between experimental groups and control group were found in all cases ($p<0.05$). Despite the fact that the median value of viability in the experimental group carbo-us was lower than in the experimental group us-carbo, these differences were not statistically significant. This result was valid for the last-mentioned experimental groups under conditions of identical median concentrations of carboplatin and the same ultrasound exposure with intensities of 1 W·cm$^{-2}$. The difference between the experimental groups us-carbo and carbo-us with $2.5 \times 10^{-5}$ M and $5 \times 10^{-5}$ M concentrations of carboplatin was statistically significant ($p<0.05$). The experimental group involving combined application of drug carboplatin and ultrasound field showed a low value of variance. The highest value of variance was found in the experimental groups us and control.

The results of acoustic pressure measurements showed a decrease in the value, depending up on the increasing distance from ultrasound probe (Fig. 2). It was evident that in the near distance, the values of acoustic pressure were more variable (Table 2). The difference between pressure values for distance 1 cm and 4 cm was almost 40% of the maximum value. The results from 4 cm distance showed a much less variability in acoustic pressure. Since the absorption of ultrasound by the polyethylene tube wall of

![Fig. 1—A2780 cell line viability for incubation time of 48 h under the 1 W·cm$^{-2}$ ultrasound exposure and application of carboplatin (contr = control group; us = cells exposed to ultrasound only; carbo = cells incubated with carboplatin only; us-carbo = cells first exposed to ultrasound and then incubated in presence of carboplatin; carbo-us = cells exposed to ultrasound in the presence of carboplatin. Fig. 1A and B shows the results for $2.5(\times)10^{-5}$ M and $5 \times 10^{-5}$ M carboplatin concentration, respectively)]

![Fig. 2—Graph of mean value and standard deviation of acoustic pressure dependence on distance from the ultrasound probe in a water bath]
was about 30% as determined by hydrophone measurement at distance of 4 cm from ultrasonic probe, hence the acoustic pressure in the cell suspension was 1.2 MPa inside the polyethylene tube.

Discussion

The present study was aimed to determine the effect of near field ultrasound application in combination with cytostatic action of carboplatin on viability of cultured carcinoma cells. The near ultrasound field was used since such a field could be applied on both surface and subsurface occurring tumors, depending upon the thickness of skin-tissue. We investigated the effect of ultrasonic field of high acoustic pressure with median value greater than 1.2 MPa, either alone or in combination with cytotoxic chemotherapy. The initial assumption was that the presence of higher intensity ultrasonic field will reduce exposure times. Another assumption was the expected effect of reducing chemotherapy dosage, while retaining the outcome of viability of cells. This was achieved by the higher value of the ultrasonic field near the probe, unlike in the far field arrangement and might be due to cavitation phenomena produced by near field. Cavitation phenomena are one of the possible options to influence the structure of membranes and thus promote drug introduction into the cell. Change in viability was expected due to this effect.

The change of viability was measured in time interval of 48 h after treatment of the cells. This is the time interval in which repair of cell damage caused by ultrasound may be assumed. The results were confirmed by the axial mapping of ultrasonic field near the ultrasound probe. The value of acoustic pressure of ultrasonic field was quite high in the immediate proximity of ultrasonic probe, but it decreased rapidly when the distance from the probe exceeded 2 cm. However, at a distance greater than 4 cm, low axial variability in the measured acoustic pressure was detected, allowing the homogenous application of ultrasound on the sample cells in the rotating tube. The measurement results also confirmed that at the same output power settings on the ultrasonic generator, acoustic pressure values might vary considerably in the whole space of ultrasound field. Measurement of ultrasonic acoustic pressure field was important with regard to the possible occurrence of tumor cells in different tissue depths in vivo. The results showed that close to the ultrasound probe, small changes in the axial direction produced a major change in the acoustic pressure.

It was also necessary to consider the possibility of existence of transient cavitation when evaluating the experiment. The existence of transient cavitation depends on the presence of gas bubbles of specific size under given acoustic pressure. Without optimal conditions, only pseudocavitation effects are present. However, cell surfaces are also shown to be affected by the pseudocavitation and by collapse cavitation as well.

Effect of ultrasound field on the cells was evident as shown Fig. 1. We were able to compare this effect
with previously published experiments. When we used acoustic pressure lower than 1.2 MPa by using ultrasound in far-field mode, the viability values were approximately 70% of values of the experimental group us\textsuperscript{12}. It was evident that the effect of ultrasonic field of common intensities that were used for therapy exhibited a high potential to affect cells. Various effects on cells due to ultrasonic field of variable parameters are known\textsuperscript{13}. The advantage of ultrasound was the possibility of setting of its exact spatial distribution (by using ultrasound probe with different diameter of piezoelectric elements or used ultrasound sonication in far or near field settings) and thus regulating the final effect.

The use of ultrasound for enhancement of drug effects has been reported by some authors, especially in the general context of sonodynamic therapy\textsuperscript{14,15}. These authors have suggested that it is possible to use ultrasound to enhance drug effects and the effect is mainly due to the activation of drugs or (which seems much more probable) ultrasonic disruption of the structure of cell membranes and changes of their permeability. The results of our study demonstrated such pronounced adjuvant effect of the ultrasound during carboplatin treatment of cells. The viability values for our experimental groups carbo-us and us-carbo were lower than the values for experimental group us or carbo. Further, there was no significant difference between the median values at two concentrations of carboplatin.

Our results showed that the same decrease in viability could be achieved with a smaller concentration of the drug. We also demonstrated that viability values for us-carbo and carbo-us were not only a simple summation effect of viability value belong to us ultrasound and cytostatics carbo groups alone. The sum of the decrease of viability caused by ultrasound and carboplatin alone was not the same as the decrease for the combination of carboplatin and ultrasound exposure. The same result on decrease of the viability values of both experimental groups us-carbo and carbo-us at carboplatin concentration of 2.5 \times 10^{-5} M and same acoustic pressure level of 1 W/cm\textsuperscript{2} was achieved. It could be assumed that the effect was produced by ultrasound by affecting cell membrane and surface structures. We also found that the effect was of long-term nature. We observed no significant difference when we compared experimental groups us-carbo and carbo-us. This indicated that the strengthening effect was present even when the drug was applied without previous ultrasonic field application. In earlier case of some our experiments, this effect was not observed with far ultrasound field of low intensity\textsuperscript{12}.

In conclusion, our results suggested that ultrasound field as a physico-mechanical factor could be used as an adjuvant agent in cytostatic treatment. It could allow administration of lower doses of the drugs, while maintaining the same final effect and could also reduce some drug side effects, such as a nephrotoxicity and ototoxicity typical for platinum cytostatic drugs\textsuperscript{16}. Although the adjuvant effect of ultrasound was confirmed for selected cytostatic agent in vitro in the present study using an experimental set-up model of sonodynamic therapy, further studies are warranted to confirm these findings in in vivo studies.

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