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Combined effect of silver nanoparticles and therapeutical ultrasound on ovarian carcinoma cells A2780

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ABSTRACT

Antibacterial activity of silver nanoparticles is widely known and used. The effect of application of therapeutical ultrasonic field in the presence of silver nanoparticles <100 nm was examined on human ovarian carcinoma cell A2780. The cell's viability was examined by MTT assay and by life-time microscopy. The presence of nanoparticles in the cell was examined by using electron transmission microscopy. Experimental results indicate a significant decrease of viability of cell, which was affected by the combined action of ultrasound field and silver nanoparticles, compared to the separate exposure of silver nanoparticles or ultrasonic field. The experiments showed a significant effect of succession of application of these two factors. The presence of nanoparticles inside cells after incubation was showed. The results show the possibility of using of ultrasonic field as a factor that can significantly affect cell viability in the presence of silver nanoparticles.

Introduction

Nanomaterials, including metallic nanoparticles, are at present the subject of study of many research teams and they are increasingly used in a whole spectrum of medical fields (Fukumori and Ichikawa, 2006). Silver nanoparticles (nanoAg) are amongst the most frequently discussed nanomaterials in medicine. The study of metallic nanoparticles is particularly related to their oligodynamic effect which is manifested, amongst others, antibacterially (Rtimi et al., 2013). The antibacterial effect of silver nanoparticles is especially strong, and thanks to this fact they have been successfully employed in medicine and otherwise (Cheng et al., 2004). However, it is also a well-known fact that metallic nanoparticles, including nanoAg, may have an adverse impact on the viability of tissue cells as well as whole organisms (Lanone et al., 2009; Ahamed et al., 2010). Products containing silver are nowadays commonly encountered, for instance in cosmetics, clothing, and cleaning

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agents (Zhang and Sun, 2007; Lee et al., 2007). There are a number of studies proving the possibility of nanoAg permeating into the cells of the human organism, either from textiles, dermatological products, or sticking plaster for wounds (Kulthong et al., 2010; Paddle-Ledinek et al., 2006; Lam et al., 2004).

Ultrasound is one of the most used methods for soft tissue visualisation. Therapeutic ultrasound is another application of ultrasound employed worldwide, particularly in physiotherapy. It is accompanied by the absorption of mechanical energy of tissues in the form of non-thermal and thermal effects, i.e. an increase in the temperature of the environment. To date, the non-thermal effects have only been used experimentally in oncologic treatment – sonodynamic therapy (Rosenthal et al., 2004). One of the characteristic effects of sonodynamic therapy is the loosening of cell membranes, thus causing their increased porosity (Mehier-Humbert et al., 2005). This effect, referred to as sonoporation (Pan et al., 2005) manifests itself by an increased intracellular uptake of drugs and various molecules. An increase in the intracellular concentration of a drug using ultrasound is desirable particularly in connection with targeted therapy of oncologic pathologies, in which the strain put on healthy tissue is decreased thanks to the possibly lower systemic concentrations of the respective drug.

Taking into account the amount of silver in the nanoAg form which is potentially present in the human living environment, it remains a matter of debate whether the simultaneous application of ultrasound and nanoAg may result in an increased toxicity of the nanomaterial in case of its permeation into the human organism and, also, whether this effect can be used in targeted therapy. The question is whether the presence of ultrasound in suitable intensities can cause an increase in the toxicity of nanoAg by using a mechanism similar to the effect of sonodynamic therapy for example? Is it possible to affect the viability of cancer tissue by applying a suitable ultrasonic field in combination with nanoAg? Will be nanoparticles occur inside of cell in connection with presence of ultrasound field?

Materials and methods

Cell cultures and chemicals

Human ovarian carcinoma cell line A2780 was used, obtained from the European Cell Culture Collection, (Sigma–Aldrich, Prague, Czech Republic). 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 line was grown in cell culture flasks in an atmosphere of 95% air and 5% CO_2 at 37 °C. The cells were detached from flask surface by trypsin addition (Bio Tech).

The stock solution of silver nanoparticles <100 nm – nanoAg – in PBS in concentration of 1 mg ml^{-1} was prepared from commercial nanoAg (Sigma–Aldrich, Prague, Czech Republic) by mechanical agitation due to ultrasonic field. This procedure was repeated before each use of this nanoAg solution.

Ultrasound exposure

BTL-07 therapeutic ultrasound generator (Beautyline Ltd., Prague, Czech Republic) working at a frequency of 1 MHz with a 4 cm^2 probe was used as the source of ultrasound. The cells were exposed for 10 min to the far field (20 cm from the probe) and near field (3 cm from the probe) of a horizontal beam of continuous-wave ultrasound at set output intensities of 0.5, 1 and 2 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 set-up provided uniform exposure of the entire volume of cell suspension. Ultrasound intensity and acoustic pressure were measured by means of a calibrated PVDF hydrophone in the space whole ultrasound field, type MH28-6 (Force Institute Copenhagen, Denmark) by using the calibration protocol.

Experimental design

The cells of A2780 line were incubated for up to 72 h after the following modes of treatment:

- addition of nanoAg only (*Ag*)
- 10-min exposure to ultrasound only (*us*)
- addition of nanoAg and subsequent 10-min exposure to ultrasound (*Ag + us*)
- 10-min exposure to ultrasound followed by addition of nanoAg (*us + Ag*).
- without addition of nanoAg and exposure to ultrasound (*cont*).

Viability test

The following procedure was used to compare the viability of *us*, *Ag*, *Ag + us*, *us + Ag* and control cells: a cell suspension was obtained by trypsination of cells adhering to the flask bottom. To each well of a 96-well plate containing 5×10^4 cells in RPMI medium, a calculated volume of nanoAg stock solution was added to achieve a final concentration of 3.5 μg ml^{-1}. An equal volume of PBS free of nanoAg 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. 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 of mitochondria (i.e. viability) in living cells.

Statistical analysis

The absorbance value for each group was converted into cell viability as follows: the median absorbance value of the control group *contr* was taken as 100%; the absorbance of each experimental group was expressed as a percentage of control group value. Because of a non-normal distribution of the values in the individual groups, the non-parametric Mann–Whitney *U*-test at a significance level of $2\alpha = 0.05$ was used. The statistical software STATISTICA 9 was used to calculate the median and the upper and lower quartiles. The data of

viability of each experimental group shown in graphs are obtained from 5 repeated independent experiments, each one experiment involving analysis of samples on 96-well plate.

Real-time light microscopy

All of the experimental groups were observed by real-time light microscopic technique. The cultivation conditions and experimental groups were the same as in the viability experiment. Microscopic observations were performed with an Olympus Cell^R imaging station (Olympus C&S Ltd., Prague, Czech Republic). Relief phase contrast method was used as a mode of light microscopy to examine specimens. Images were obtained by Hamamatsu ORCA-R2 digital camera. Each sample was scanned with time-lapse interval of 20 min. The number of cells and their growth was assessed.

Transmission electron microscopy

The cells from all experimental groups were observed by transmission electron microscope MORGAGNI 268D (FEI Company, Brno, Czech Republic) by using non-contrasted ultrathin section (60 nm). The cultivation conditions and experimental groups were the same as in the viability experiment. Fixation process was done in 300 mmol/l glutaraldehyde in 100 mmol/l cacodylate buffer and postfixation in 40 mmol/l osmium tetroxide in 100 mmol/l cacodylate buffer. Samples were dehydrated by increasing concentration of ethanol (30–100%) and embedded in LR White embedding medium.

Results

A dose dependent response was observed and LD_{50} concentration value in time 48 h was calculated as 3.5 $\mu g\ ml^{-1}$ for viability assays. The obtained data in the first part of the study permit a comparison of the viability of A2780 cells in the experimental groups *us*, *Ag*, *Ag + us* and *us + Ag* with respect to the control cells *cont* following incubation times 24, 48 and 72 h. All of these experiments were done in two modes of ultrasound exposure – far field (Fig. 1) and near field (Fig. 2) – at nominal intensities of 0.5, 1 and 2 W cm^{-2}. The graphs show a dependence of both median and interquartile range value of relative cell viability on incubation time and on experimental design for each ultrasound intensity and exposure mode separately.

The results of experiments performed so far show clear differences in the value of viability between individual incubation periods of the respective experimental group affected by silver nanoparticles. All the experimental groups (*Ag*, *us + Ag*, and *Ag + us*) show a gradual decrease in viability in time. The lowest values of viability are observed in cells cultivated in a silver environment at 72 h for each group, and in the case of ultrasound action disregarding its intensity; the lowest viability value is again recorded at 72 h. On the other hand, the viability of cells affected solely by the ultrasonic field remains almost unchanged throughout the observed time period, with an increase in the viability being possible at 72 h. From the overall evaluation of all the experiments, it is evident that a there is a difference between the cells affected by silver nanoparticles only, and the groups of cells that were affected by a combination of silver nanoparticles and the ultrasonic field. This effect of decreasing viability by the combined effect of the ultrasonic field and nanoparticles can be clearly observed at the intensities of 1 and 2 W cm^{-2}. From the graphical representation of the results, it is evident that this is not a plain summation of a decrease in viability. Evaluating the effect of the ultrasonic field alone, the conclusion may be drawn that the measured data show a greater decrease in viability in the experimental groups resonated in the near field. Statistically significant differences (significance level $2\alpha = 0.05$) in the value of viability are observed in the *us + Ag* and *Ag + us* experimental groups (Table 1). In all the experiments, either depending on the intensity and type of the ultrasonic field, or depending on the time of the incubation period, the *Ag + us* experimental group manifests a decreased viability value as opposed to the *us + Ag* experimental group. A maximum suppression of viability was reached in the *Ag + us* experimental group resonated in the near field. The resulting value of viability that was obtained in this group at 72 h equals 20% of the viability of the *cont* (control) group.

The effect of silver nanoparticles and the ultrasonic field on the cells of ovarian carcinoma was also evaluated using the real-time microscopy technique. Using a combination of pictures (examples of selected experimental groups can be seen in Fig. 3), a video sequence was made which allowed observation of the cell growth dynamics and their final confluence. The final value of confluence in the *cont* group was evaluated subjectively as being 100%. The *Ag* experimental group in which the final silver concentration was 3.5 $\mu g\ ml^{-1}$ was evaluated as having 60% confluence. When the concentration of silver nanoparticles was doubled to 7 $\mu g\ ml^{-1}$, it was found that the number of cells in the cultivation vessel did not grow in time and the confluence during the cultivation did not change. An evaluation of the effect of the ultrasonic field alone, either in the near or in the far field shows the same behaviour of the *us* experimental groups as that of the control group. The coverage was determined as being 100%. In case of the combined effect of nanoparticles and ultrasound (*Ag + us* and *us + Ag*), the behaviour of the cells and their confluence was evaluated in a similar way as in the *Ag* 3.5 $\mu g\ ml^{-1}$ experimental group. In the experimental groups affected by silver nanoparticles, non-adhering and apoptotic cells were observed in greater numbers compared with the control group and the group of cells that were affected by ultrasonic field only, either near or far.

When observing the cells using transmission electron microscopy (TEM), it was found that silver nanoparticles are found inside A2780 cells (Fig. 4). The distribution of particles in the intracellular space of the cells was assessed as being accidental, with some exceptions. In the intracellular space of some of the cells, objects corresponding to lysosomal structures were found (Fig. 5), inside of which objects there was an increased concentration of the studied silver nanoparticles. The pictures obtained using TEM also showed the presence of apoptotic cells in the experimental groups affected by silver nanoparticles (*Ag*, *Ag + us* and *us + Ag*). Marked differences between the numbers of nanoparticles in the

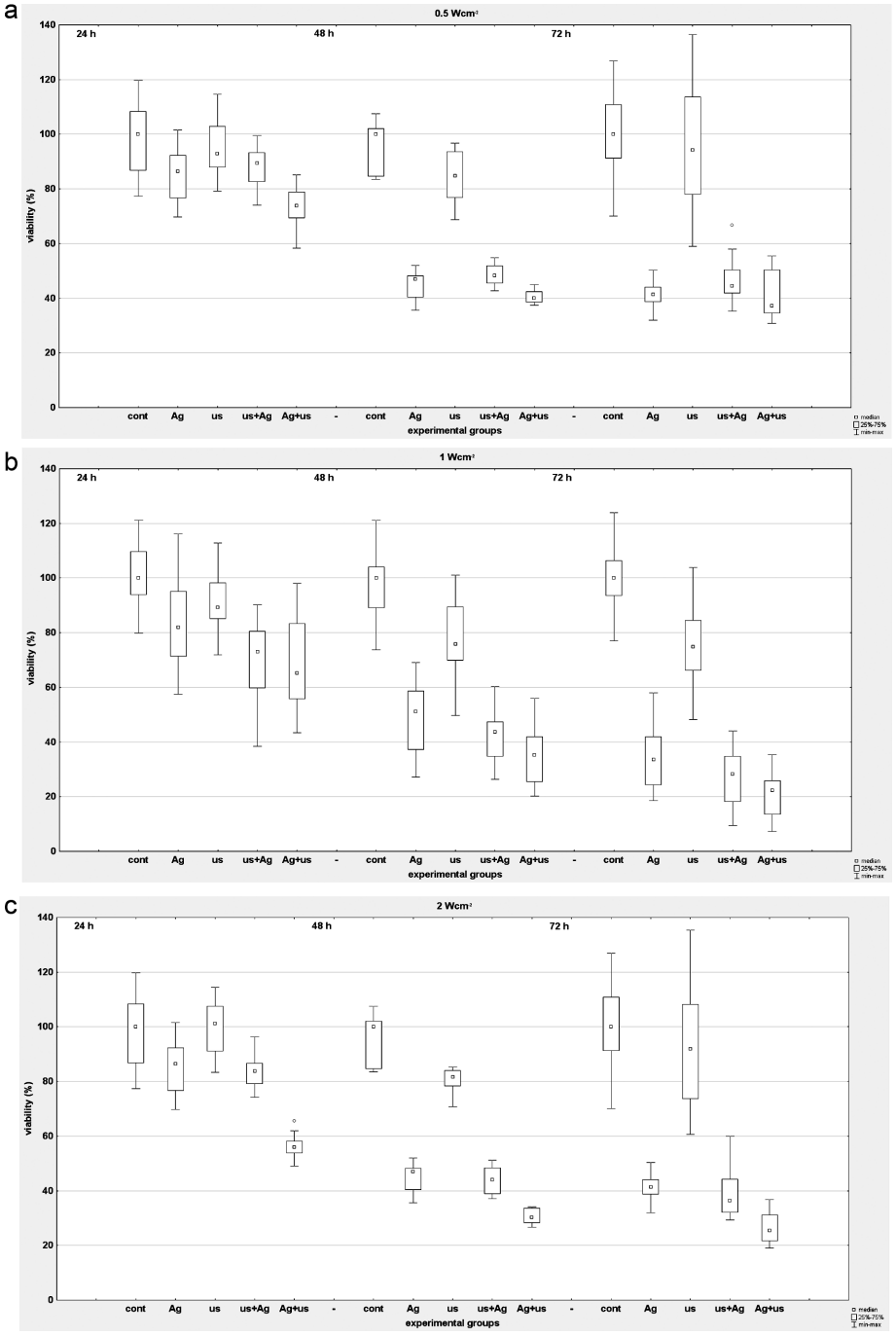

Fig. 1 – (a)–(c) Cell viability of the cell line A2780 for incubation time 24, 48 and 72 h under the ultrasound exposure at intensities of 0.5, 1 and 2 W cm^{-2} in far field mode. Experimental groups are following: *cont* = control group; *Ag* = cells incubated with nanoAg only; *us* = cells exposed to ultrasound only; *us + Ag* = cells exposed to ultrasound followed by incubation with nanoAg; *Ag + us* = cells exposed to ultrasound in the presence of nanoAg.

intracellular space in the individual experimental groups were not found.

Discussion

The use of ultrasound as a supporting factor in drug treatment is well-known and frequent, for example in the form of the so-called sonoporation with the effect of a transitional change of cellular structures (Wu et al., 2006). This change of the natural structure of cellular surfaces allows an increased permeation of even large molecules into the cell. Some experimental studies even suggest an ultrasound-conditioned induction of pores larger than 100 nm in the cellular membrane (Zhou et al., 2009; Qiu et al., 2012). Such a size of cellular pores appears to be sufficient for the permeation of various nanomaterials.

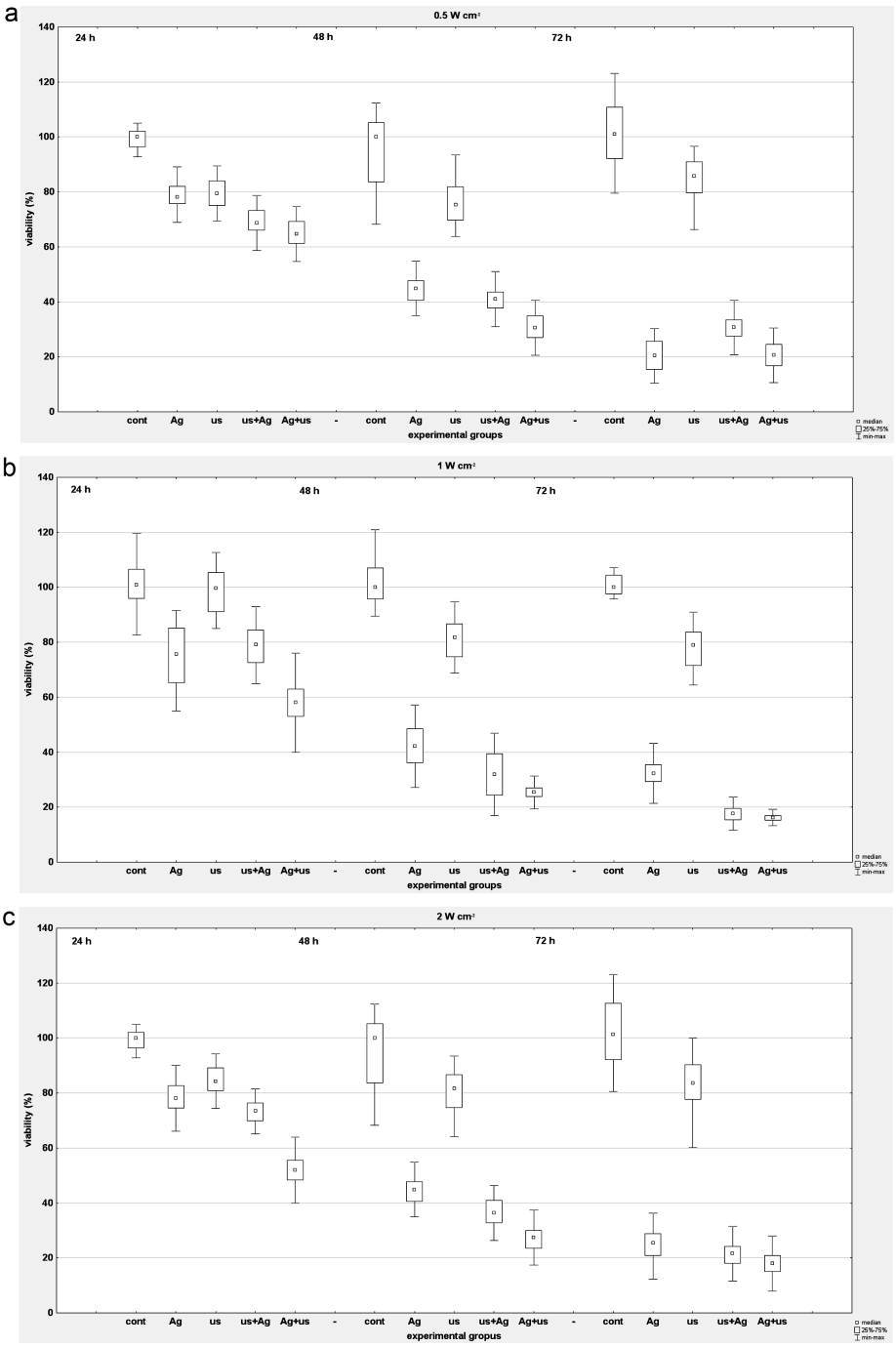

Fig. 2 – (a)–(c) Cell viability of the cell line A2780 for incubation time 24, 48 and 72 h under the ultrasound exposure at intensities of 0.5, 1 and 2 W cm^{-2} in near field mode. Experimental groups are following: *cont* = control group; *Ag* = cells incubated with nanoAg only; *us* = cells exposed to ultrasound only; *us + Ag* = cells exposed to ultrasound followed by incubation with nanoAg; *Ag + us* = cells exposed to ultrasound in the presence of nanoAg.

The effects of the ultrasonic field alone at the level of cells have been noted by a number of researchers, their form being evaluated in relation to the used intensities of the ultrasonic field. Significant changes in cell morphology and viability are obtained when different levels of cavitation intensity of the ultrasonic field are used (Schlicher et al., 2010). The authors of the present study have previously also experimented with the effects of the ultrasonic field on various types of cell cultures, however, at low cavitation intensities and thus with no cavitation effects present (Bernard et al., 2010, 2012). The results of these experiments reveal a limited effect on cellular viability by the ultrasonic field alone.

In the introductory part of this article several questions concerning the effects of a combined action of the ultrasonic field and silver nanoparticles at the level of cells were asked; can the presence of the ultrasonic field of a suitable intensity

Table 1 – Significant differences in viability values for the experimental groups *us + Ag* and *Ag + us*, as assessed by Mann–Whitney test.

Far ultrasound field			
Intensity/time	24 h	48 h	72 h
0.5 W cm^{-2}	•	•	•
1 W cm^{-2}	□	•	•
2 W cm^{-2}	•	•	•
Near ultrasound field			
Intensity/time	24 h	48 h	72 h
0.5 W cm^{-2}	•	•	•
1 W cm^{-2}	•	•	•
2 W cm^{-2}	•	•	•

• Statistically significant at level $2\alpha = 0.05$, □ no statistically significant between experimental groups *us + Ag* and *Ag + us*; cells exposed to ultrasound intensity 0.5, 1 and 2 W cm^{-2} for far and near field, incubation time 24, 48 and 72 h.

cause an increase in the toxicity of nanoAg by a mechanism similar to the effect of enhancing treatment during a sonodynamic therapy by cytostatics? Is it possible to moderate the viability of cancerous tissue by the action of a suitable ultrasonic field and nanoAg? In order to answer these questions it was necessary to design experiments the outcome of which would be at least a partial description of the simultaneous action of the ultrasonic field and the oligodynamic effect of metallic nanoparticles on tissue cultures.

One of the performed experiments was the evaluation of cellular viability using the observation of mitochondrial activity by the MTT test. In these tests, the effect of ultrasound in the near as well as far field and its combination with the application of silver nanoparticles was observed. The effect of silver nanoparticles alone was also tested. The results of the experiments show a significant effect on cellular viability by the action of nanoAg (in the *Ag* experimental group). The value of viability in the *Ag* experimental group decreases in time. This downward tendency of the viability value of the cells, proportional to the period of cultivation, was expected and it corresponds with the known antibacterial and oligodynamic effects of silver. The found concentration 'LD_{50}' does not contradict the LD_{50} concentrations of silver nanoparticles that have been published by other researchers (Ahamed et al., 2010; Mukherjee et al., 2012). The published results of other researchers reveal that the toxically effective concentration of nanoAg depends on the size of the nanoparticles, and in particular, on the type of the cells used in the experiments.

The results describing the development of viability in time for the *us* experimental group show almost identical values for all the studied periods of cultivation in the individual intensities used. A comparison of resonations in the near and far field reveals that the effect of the near ultrasonic field causes a slight decrease in the manifestations of the viability of the resonated cells. This effect can be attributed to the manifestations of the ultrasonic field in the near Fresnel region of the ultrasonic beam where the ultrasonic field is more homogenous as compared to the far field, and where higher values of acoustic pressure, or intensities, are obtained (Gutiérrez et al., 2012).

Significant results were obtained in the experimental groups when the effect of nanoAg and the ultrasonic field at different time sequences of *Ag + us* and *us + Ag* exposition were combined. In both of these experimental groups a decrease in viability in time (24, 48 and 72 h) was observed. This effect is apparent in the used 1 and 2 W cm^{-2} intensities of the ultrasonic field. A statistically significant difference in the viability values between the *Ag + us* and *us + Ag* experimental groups allows a possible hypothesis that in this case it is not a plain summation of nanoAg effects and the ultrasonic field. If it were not so and the cause would be the summation only, it would be also possible to suppose that the sequence of nanoAg application and the ultrasonic field has no effect on the resulting value of viability. A statistic evaluation reveals a significantly lower value of viability manifestations in the *Ag + us* group. The effect of sequential application of the ultrasonic field and the presence of nanoAg on cellular viability can be explained in many ways. On of the possible mechanisms which no doubt plays an important role here is the mechanical effect of the ultrasonic field on the cells which leads to a change of the structure and porosity of cell membranes, including a translocation of membrane proteins (Nejad et al., 2011; Tachibana et al., 1999). In this case the entry of nanoparticles into the intracellular environment would be facilitated, or the previously blocked binding sites of cellular compartments for nanoAg would be made accessible. This theory can explain that the maximum suppression of viability was achieved at the moment of the ultrasonic effect on the cell cultures during their cultivation with silver nanoparticles, not conversely.

The principle of drug and nanoparticle delivery by ultrasound is not unambiguously explained even in the publications by other researchers; however, it can certainly differ according to the character of the studied substance. One of the suggested theories agreeing with the results of the present study is the ultrasound-conditioned 'acoustic radiation force induced displacement' (O'Neill et al., 2009). A similar hypothesis involving the existence of hydrodynamic stress that allows sonoporation is described in a review article by Rosenthal et al. (2004). In both cases the precondition is a change of cellular structures that can only be temporary and reversible. This mechanism of a temporary change of the structures of cell membranes is also attested by experimentally obtained data.

It appears that a relevant physical explanation of the observed effects could be also the fact that the density of silver particles is very different from the density of the aqueous environment and thus, under the effect of inertia forces, the silver particles will follow the longitudinal oscillations of the aqueous environment with phase displacement. In this way the nanoparticles can easily 'shoot through' into the inside of cells where they become stuck in the inner, more viscous environment. The so-called vibration potentials, well-known but rarely mentioned, have long had been described to work in a similar way (Raoul and Ernest, 1982).

Also, it is necessary to consider the possibility of an effect of the ultrasonic field on the silver nanoparticles themselves. The

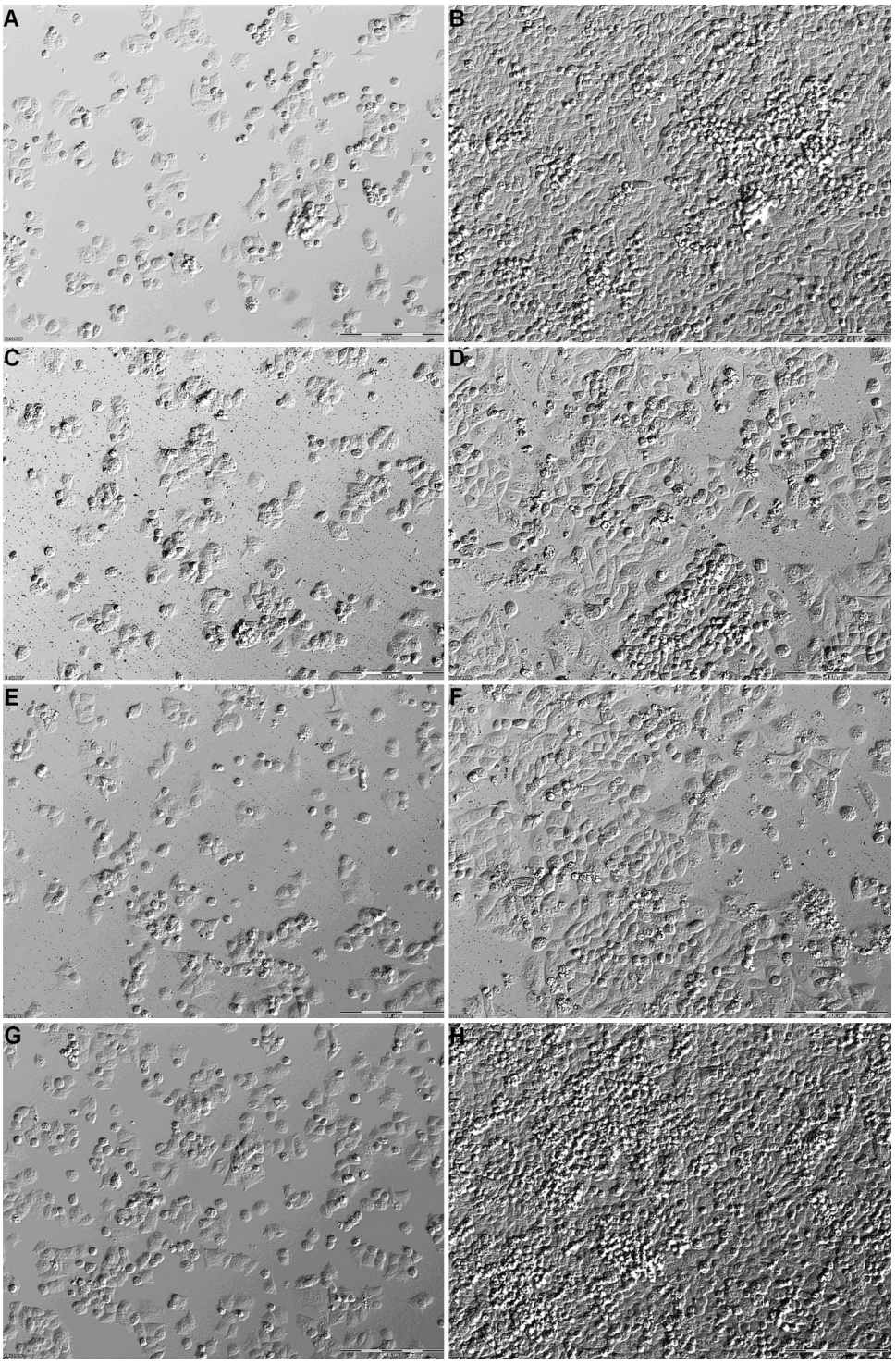

Fig. 3 – Life-time microscopy, scale bars = 200 μm. (A) Control group *cont* – 1 h, (B) control group *cont* – 72 h, (C) cells incubated with nanoAg Ag – 1 h, (D) cells incubated with nanoAg *Ag* – 72 h, (E) cells exposed to ultrasound 1 W cm^{-2} in presence of nanoAg *Ag* + *us* – 1 h, (F) cells exposed to ultrasound 1 W cm^{-2} in presence of nanoAg *Ag* + *us* – 72 h, (G) cells exposed to ultrasound 1 W cm^{-2} only *us* – 1 h and (H) cells exposed to ultrasound 1 W cm^{-2} only *us* – 72 h.

presence of the ultrasonic field can doubtless affect their mutual aggregation with the resulting effect of deaggregation, which leads to the diminution of the particles and their potentially easier permeation into cells and, occurring at the same time, an increase in the free surface area of the silver.

Microscopy studies reveal a marked difference between the cells incubated after the application of ultrasound alone, and the cells that have been affected by the presence of silver nanoparticles. In a sequence of pictures (see Fig. 3 for illustrative representatives), an increased presence of apoptotic cells in the

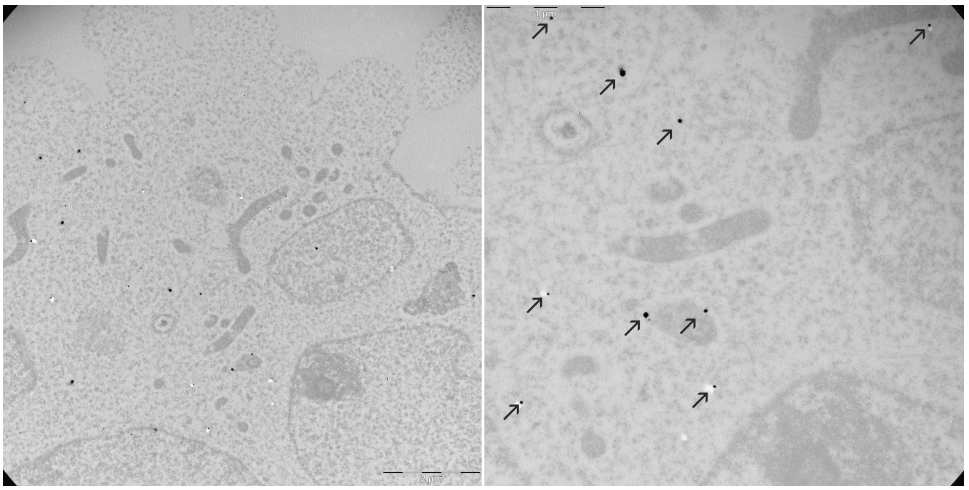

Fig. 4 – Transmission electrone microscopy image of silver nanoparticle inside of cell (→), experimental group *Ag + us*.

Ag, *Ag + us* and *us + Ag* experimental groups can be seen. These results correspond with the results of viability tests, although it is a fact well-known to the authors of this article that manifestations of viability and the quantity of apoptotic cells in a population cannot be compared. From a time lapse study, it is apparent that an increase or decrease in the number of cells does not occur suddenly, but throughout incubation.

It is evident from the results obtained by the transmission electron microscopy technique that the nanoparticles in question have an ability to permeate into the intracellular environment. This finding may play an important role particularly in the security issue of the nanoparticles occurring in the human living environment. A subjective TEM picture evaluation has not shown that the number of freely diffused nanoparticles in relation to the ultrasound sequence and the application of silver nanoparticles in experiments (in the *us + Ag* and *Ag + us* experimental groups) is higher than normal. However, the validity of this assertion must be treated with caution which has to be always used when evaluating microscopy pictures. It is problematic to determine the total number of particles permeating into the inside of a cell, particularly in regard to the lysosomal structures that are found there – these apparently fulfil the function of a defence mechanism that removes the entered nanoparticles from the cell. Thanks to this mechanism, many free particles from the intracellular space are caught and removed during incubation, and without a good knowledge of this process it is impossible to ascertain precisely the initial number of the particles. This assertion is, however, only a conjecture of the authors of this study, and it has to be further analysed.

As the final evaluation of the research that has been carried out, it may be stated, on the basis of the results of the experiments, that there occurs a gradual decrease in the viability of cells affected by silver nanoparticles in time (24, 48, and 72 h). One possible explanation of this fact is that the toxicity of silver nanoparticles which become stuck inside of cells gradually increases. A slightly changing viability value in the experimental group affected solely by the ultrasonic field has also been observed in the studied time period, which fact indicates a one-time and immediate effect on the cells by the

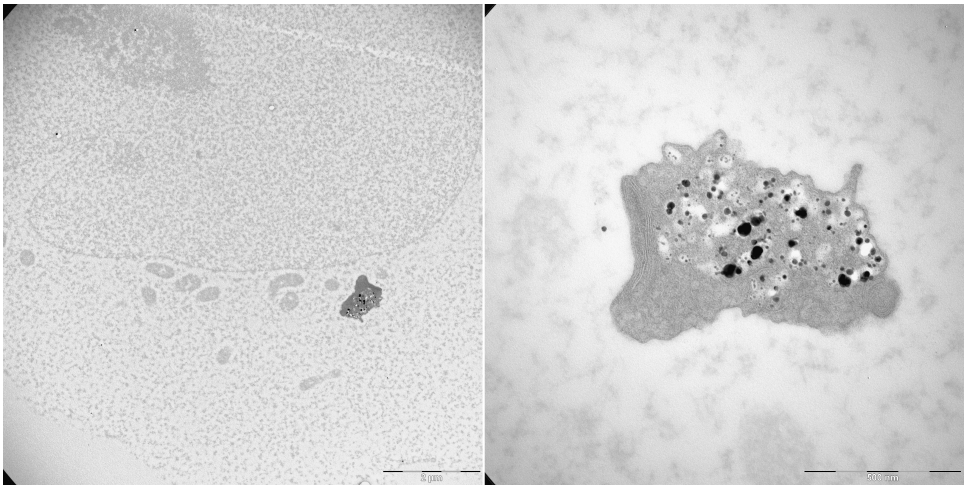

Fig. 5 – Transmission electrone microscopy image of lyposomal structure with incorporated silver nanoparticle in high concentration.

ultrasonic field, with no changes occurring during the following cultivation. A maximum suppression of viability was found in the experimental groups when the action of silver nanoparticles and the ultrasonic field were combined – i. e. the results do not indicate a plain summation of the effects of the two factors. This fact indicates a possible effect of an application sequence of nanoAg and the ultrasonic field.

An important fact is also the evidence of the permeation of silver nanoparticles into the intracellular space and their presence in the cell with no specific distribution.

The results of the experiments show that there exists a possibility of increasing the effects of silver nanoparticles in order to suppress cellular viability by applying the ultrasonic field. It is good to take this fact into consideration when the prevention of possible health risks associated with the occurrence of metallic nanoparticles in the human organism in ultrasonographic therapeutic applications comes into question. On the basis of the obtained data, the authors of the present study also hold that it is possible to use a suitable combination of a local application of the ultrasonic field and silver nanoparticles for the purpose of the so-called target therapy. However, in such case there is a need of more experiments to be performed, particularly in vivo.

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