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Enhancement of ultrasonically induced cell damage by phthalocyanines in vitro

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Abstract

In this work, erythrocytes from carp were used as a nucleated cell model to test the hypothesis that the phthalocyanines (zinc – ZnPc and chloroaluminium –AlClPc) enhance ultrasonically induced damage *in vitro*. In order to confirm and complete our earlier investigation, the influence of ultrasound (US) and phthalocyanines (Pcs) on unresearched cellular components, was studied. Red blood cells were exposed to 1 MHz continuous ultrasound wave (0.61 and/or 2.44 W/cm^2) in the presence or absence of phthalocyanines (3 μM). To identify target cell damage, we studied hemolysis, membrane fluidity and morphology of erythrocytes. To demonstrate the changes in the fluidity of plasma membrane we used the spectrofluorimetric methods using two fluorescence probes: 1-[4-(trimethylamino)phenyl]-6-phenyl-1,3,5,-hexatriene (TMA-DPH) and 1,6-diphenyl-1,3,5-hexatriene (DPH). The effect of US and Pcs on nucleated erythrocytes morphology was estimated on the basis of microscopic observation.

The enhancement of ultrasonically induced membrane damage by both phthalocyanines was observed in case of hemolysis, and membrane surface fluidity, in comparison to ultrasound. The authors also observed changes in the morphology of erythrocytes. The obtained results support the hypothesis that the Pcs enhance ultrasonically induced cell damage *in vitro*.

Furthermore, the influence of ultrasound on phthalocyanines (Pcs) in medium and in cells was tested. The authors observed changes in the phthalocyanines absorption spectra in the medium and the increase in the intensity of phthalocyanines fluorescence in the cells. These data can suggest changes in the structure of phthalocyanines after ultrasound action.

Keywords: Ultrasound; Phthalocyanines; Nucleated red blood cells; Synergistic effect; *In vitro*

1. Introduction

Sonodynamic therapy (SDT) is a new promising method of killing tumor cells, based on the synergistic effect of ultrasound (US) and certain compounds called "sonosensitizers" [1,2]. Compared to the laser bundle that has been applied for years in photodynamic therapy, the ultrasound showed a higher ability to focus on a small region of cancer, penetrating deeply within the tissue, activating the local sonosensitizer and minimizing damage to neighbouring healthy cells [3].

Recently, SDT has been widely examined and many reports demonstrated the synergistic effect of US and sonosensitizers in studies on cells *in vitro* [4,5], as well as in tumor-bearing animals [3,6,7]. These studies have mainly concentrated on the mechanism by which ultrasound increases drug cytotoxicity and different sonosensitizers. Although SDT has been relatively well investigated, the mechanism of killing effects is still unclear and needs additional investigation.

A selection of appropriate compounds, which show sonodynamic properties, is also very important. These compounds, which are used in photodynamic or sonodynamic therapy should penetrate effectively to cancer cells and not show cytotoxic properties in relation to healthy cells. The most widely used sonosensitizers are hematopor-

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phyrine or its derivatives (HpD), but there are also other compounds: anticancer drugs, such as, pheophorbide a [8], dimethylformamide [9], merocyanine [10], erythrosine B, and rose bengal [11], which are reported as sonosensitizers. In this study, ultasonically induced effects of Pcs on nucleated red blood cells are investigated.

Phthalocyanine is a symmetrical, aromatic macrocycle with different metal ions in its central cavity. Physicochemical properties of phthalocyanines depend on the kind of metal in the centre as well as the substituted differential peripheral groups. Sensitizers, including paramagnetic metals in the centre, show weak activity, whereas, dyes with diamagnetic metals (e.g.: zinc, aluminium) are characterized by high photobiological activity [12]. Groups that are substituted also decide about the activities of Pcs, because they influence their solubility. Pcs localized in cells depend on polarity and solubility. More polar Pcs are localized in the cytoplasm and less is found in the cellular membrane [13]. Although phthalocyanines have been reported to localize selectively in tumor tissue and have been proposed as candidates for medical application in view of their photodynamic properties [14,15], the synergistic effect of Pcs and US has been relatively less studied. Yumita and Umemura [16], have noticed a synergistic effect of Pcs and US. They have evaluated chloroaluminum phthalocyanine tetrasulfonate for sonochemical activation in mice *in vivo*. The results showed a significant antitumor effect as evaluated by the decrease in the tumor size.

Our previous results also showed that the effect of US can be enhanced through the addition of phthalocyanines, which results in the increase in hemolysis and lipid peroxidation products, as well as, a decrease in the osmotic resistance of nucleated erythrocytes [17]. However, the presence of Pcs did not enhance ultrasonically induced DNA damage [18]. The authors used Pcs with a concentration of 3 μM and the sample was exposed to the only one intensity of ultrasound, 2.44 W/cm^2.

The present study is a continuation of the study on the effect of US and phthalocyanines on nucleated erythrocytes *in vitro*. The hypothesis that Pcs enhances ultrasonically induced cell damage has been tested. Hemolysis (lower intensity of US) and membrane fluidity have been determined to confirm that effects on the cellular membrane. The influence of Pcs and US on morphology of nucleated erythrocytes has also been studied, because the authors wanted to test whether Pcs also enhances the cell-damaging effects of US.

Carp erythrocytes have been applied as a useful model to investigate the aspect of toxicology *in vitro*, as their membranes are rich in long-chain polyunsaturated fatty acids, which could be oxidized under oxidative stress conditions induced by chemical or physical factors. These erythrocytes are nucleated, flattened, and ellipsoidal, and they possess, besides hemoglobin (Hb), mitochondria, endoplasmatic reticulum, and other organelles, typical of somatic cells. Their cytoskeletal system consists principally of a membrane skeleton, containing actin and spectrin-family proteins, and intermediate filaments of the desmin class [19]. The important advantage of this model is the fact that many cellular functions and experimental approaches have been well described.

Furthermore, we tested the effect of US on phthalocyanines in medium and in cells because this information can be useful to explain the mechanism of synergistic effect of US and Pcs.

2. Materials and methods

2.1. Chemicals

Fluorescence label: 1-[4-(trimethylamino)phenyl]-6-phenyl-1,3,5,-hexatriene (TMA–DPH), 1,6-diphenyl-1,3,5-hexatriene (DPH), and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). Zinc (ZnPc) and chloroaluminium (AlClPc) phthalocyanines were obtained from Acros Organics (New Jersey, USA). All the other chemicals came from Polish Chemical Reagents (Gliwice, Poland) and were of analytical grade.

2.2. Cell preparations

Healthy fish (*Cyprinus carpio* L) of both sexes, weighing 1–2 kg, were collected from the local fish farm and were acclimated for a few days in aquarium water (temperature 14–16 °C). The whole blood from fish (*C. carpio* L.) was withdrawn by caudal puncture with heparinized syringes. Erythrocytes were isolated immediately after the collection by centrifugation for 5 min with 1500 × *g*, at 4 °C. After the removal of plasma, the erythrocytes were washed thrice with isotonic for carp erythrocytes buffer (0.6%) NaCl solution. After washing, red blood cells were diluted in the incubation medium (90.5 mM NaCl, 3 KCl, 1.3 mM $CaCl_2$, 0.5 mM $MgSO_4$, 6 mM glucose, 1 mM pyruvate, 1 mM Tris–HCl, pH 7.4) to 5% hematocrit for each trial.

The procedures of fish treatment were approved by the Local Ethics Committee in Lodz in compliance with the Polish law.

2.3. Ultrasound and exposure system

Continuous-wave US was generated by an unfocused apparatus for ultrasonic therapy, BTL-07p, produced by Medical Technologies s.r.o. (Prague, Czech Republic). The 1 MHz, 12 mm diameter transducer was immersed in a container with distilled water. To minimize the reflected US, an acoustic reflector (Plexiglas wedge) was placed at the end of the tank opposite the transducer. The reflection coefficient was calculated for pressure amplitude. For the 45° wedge, the reflection coefficient did not exceed 5%, even for a slightly divergent incident wave, and the standing wave could be neglected. Dosimetry was performed with the acoustic absorber in place, using the PVDF bilaminar shielded membrane hydrophone (Sonic Technologies, serial no. 804043; Hatboro, PA). The spatial peak intensi-

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ties in these experiments were in the range of 0.61–2.44 W/cm^2, corresponding to the measured peak positive and peak negative pressures of $p^+ = 0.17$ MPa, $p^- = 0.18$ MPa and $p^+ = 0.34$ MPa, $p^- = 0.33$ MPa, respectively.

The experimental set-up, the plot of reflection coefficient versus angle, lateral ultrasonic pressure distribution and characteristic output values for the ultrasonic unfocused transducer have been described in detail and shown previously [20].

2.4. Chemical treatment of erythrocytes

ZnPc and AlClPc were added to the suspension of erythrocytes to give a final concentration of 3 μM. The cells were incubated with phthalocyanine for 20 min at 22 °C in the dark, to eliminate the influence of light on Pcs and probes. After being exposed to Pcs the erythrocytes were washed and the samples (except the control) were immediately sonicated.

2.5. Sonication

The samples were put into small foil bags 1 cm × 1 cm (width and height) for each trial. The bag was made of thin polyethylene foil, 0.05 mm thickness. Each time the same volume (0.2 ml) of erythrocytes, diluted in incubated medium to 5% hematocrit, was used and they were evenly distributed inside the bag. The samples had not been degasified and rotated. During the exposure, the suspension did not fall to the bottom and the whole sample was sonicated to the same degree, so the cells did not need rotation. Each sample of erythrocytes (except the control) was exposed to 1 MHz of continuous US wave at intensities of 0.61 and/or 2.44 /cm^2 for 5 min.

2.6. Hemolysis

Erythrocyte suspensions (Ht = 5%) in incubated medium (pH 7.4), after exposure to Pcs, were sonicated at 0.61 W/cm^2 for 5 min. After centrifugation the degree of hemolysis (%) was determined by measuring the hemoglobin content in the supernatant at 540 nm. Hemolysis in each sample was expressed as a percentage of the absorbance in distilled water.

2.7. Fluorescence anisotrophy

To demonstrate the changes in the fluidity of the plasma membrane, the authors used the spectrofluorimetrical method and two fluorescence probes: TMA–DPH and DPH. The lipid probe DPH is located relatively deeply in the hydrocarbon interior of the lipid bilayer. TMA–DPH, in contrast to DPH, is known to be located in the polar head-group region of the plasma membrane [21]. Measurements of fluorescence anisotropy of TMA–DPH and DPH were performed at room temperature with a Perkin-Elmer luminescence spectrometer (Model LS50B, United Kingdom), using the excitation wavelength of 358 nm, the emission wavelength of 428 nm for TMA-DPH, and 348 and 426 nm for DPH, respectively. Prior to anisotropy measurement, samples of red blood cells were diluted in hematocrit of 0.05%. The final concentration of the fluorescence probe in the samples was 1 μM.

The degree of fluorescence anisotropy (r) was calculated according to the equation:

$$r = (I_{vv} - I_{vh}xG)/(I_{vv} + I_{vh}xG)$$

where I_{vv} and I_{vh} are the intensities measured with the polarization plane parallel (I_{vv}) and perpendicular (I_{vh}) to that of the excitation beam. G is a factor used to correct the instrument's polarization and is given by the ratio of vertically to horizontally polarized emission components when the excitation light is polarized in the horizontal direction.

2.8. Morphological studies

Smears of erythrocyte suspension were stained using the Unny–Pappenheim method and fixed by alcohol–ethyl ether (1:1) solution. Observations were made using Optiphot-2 microscope (Nikon, Warszawa, Poland). Images of RBCs were registered (recorded) by coupling DXM 1200 CCD (Nikon) camera with the microscope.

2.9. Absorption spectra

To test the influence of US on Pcs, absorption spectra of these compounds were made. This information could be useful to explain the mechanism of their synergistic effect. Pcs (stock solution in DMSO) were diluted in the incubation medium to the concentration of 3 μM, and a Cary 1 (Varian, Australia) UV–visible spectrophotometer was used to measure the absorption spectra of the Pcs solutions before and after sonication (2.44 W/cm^2).

2.10. Fluorescence intensity

Pcs strongly absorb red light at the range of 600–700 nm and show fluorescence. To study the effect of US on Pcs located in RBCs, the phthalocyanines fluorescence in cells before and after US exposure was measured with the help of a flow cytometer Becton Dickinson (LSR II), with excitation of 633 nm (red laser) and emission filter 650–670 nm.

2.11. Statistical analysis

The results are presented as mean ± S.D. from six individual experiments, for all methods, with the exception of fluorescence of Pcs in cells (four treatments). Each experiment was performed on a fresh set of blood and repeated thrice. Statistical evaluation of the difference between sonicated erythrocytes without Pcs and sonicated erythrocytes with Pcs was performed by using the Student's t-test. $P < 0.05$ and below was accepted as statistically significant.

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3. Results

Phthalocyanines at the concentration used and US at intensity 0.61 W/cm^2 did not show a significant effect on hemolysis of nucleated erythrocytes in comparison to the control. However, sonication of red blood cell suspension (5 min, I = 0.61, 1 MHz) in the presence of zinc and chloroaluminium phthalocyanines (3 μM) caused an increase in hemolysis. The percentage of hemolysis was significantly higher after the combined US and Pcs action (Fig. 1).

Fluorescence anisotropy of probes TMA-DPH and DPH, incorporated in the cell membrane is reversely related to membrane fluidity. The results of this study show (Fig. 2) that the values of TMA-DPH fluorescence anisotropy (r) in erythrocytes incubated with phthalocyanines significantly decrease ($p < 0.001$) in comparison to the control, and they are on the same level as those in the result with US action. These data demonstrate that phthalocyanines as well as US lead to an increase in erythrocyte membrane fluidity. The joint action of US and phthalocyanines enhances this effect. ZnPc and US action is more effective than the action of AlClPc and US ($p < 0.001$).

No significant changes in DPH fluorescence anisotropy (r) were observed in erythrocytes exposed to Pcs and US, when compared to control and sonicated erythrocytes (Fig. 3). US and phthalocyanines together led to an increase in r values in comparison with US exposure alone, but these changes were not statistically significant.

Erythrocytes damage after US action as well as US and phthalocyanines exposure was confirmed by the changes in morphology. Fig. 4 (magnitude 1000) shows the possible changes in nucleated erythrocytes exposed to these factors. Erythrocytes of a changed shape and vacuolization of cytoplasm as well as erythrocytes, at the stage before or during hemolysis were observed. The action of Pcs alone at the concentration used did not cause changes in the morphology of erythrocytes.

Knowing that sonication with phthalocyanines led to enhanced cell damage, the authors tested the influence of US on Pcs. This information could help to explain the mechanism of the sonodynamic effect. On the basis of the obtained spectra of Pcs in medium it was observed that there was maximum absorbance decrease and changes in absorption spectra shape, for both Pcs after sonication (Fig. 5a and b). These results could suggest changes in the phthalocyanines structure.

The intensity of phthalocyanines fluorescence was also checked when Pcs were located in cells. The data are shown as a ratio of the sample fluorescence after sonication (F) to the control sample fluorescence – before sonication (Fo). As seen in Fig. 6, sonication led to an increase in the phthalocyanines dye fluorescence intensity.

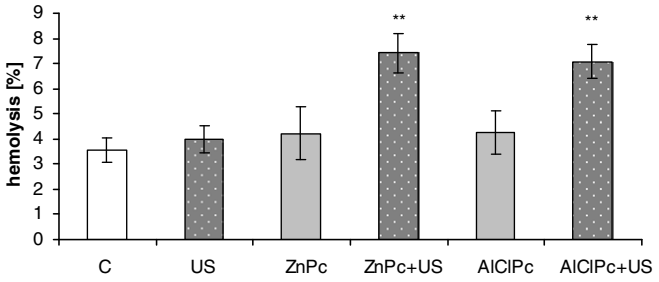

Fig. 1. Effect of US, Pcs and Pcs together with US on hemolysis of carp erythrocytes. US was used at the intensity 0.61 and Pcs at concentration 3 μM. The data were obtained from six individual treatments. Error bars denote SD. ** denote $p < 0.001$ compared with the cells treated with US.

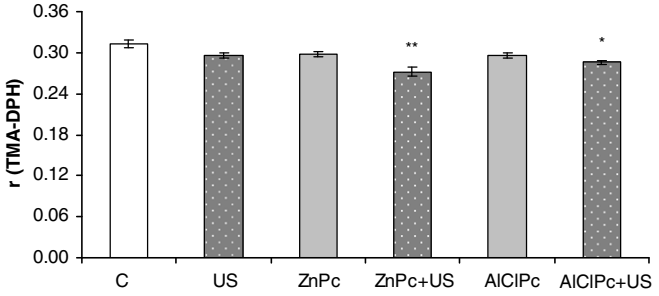

Fig. 2. Fluorescence anisotropy TMA–DPH in nucleated erythrocytes membrane exposed to US, Pcs and Pcs + US. US was used at the intensity 2.44 W/cm^2 and Pcs at the concentration 3 μM. The data were obtained from six individual treatments. Error bars denote SD. * denotes $p < 0.01$ and **$p < 0.001$ compared with the cells treated with US. Significant effect relative to control was also obtained for cells treated Pcs or US alone ($p < 0.001$).

4. Discussion

The aim of this *in vitro* study was to test the hypothesis that Pcs enhance damage of nucleated erythrocytes caused by US. This study is a continuation of an earlier study [17,18]. The investigation was completed with lower intensity (hemolysis) of US wave as well as other parameters of cells (membrane fluidity and morphology of nucleated erythrocytes). Erythrocytes after incubation (20 min) with zinc or chloroaluminium pthalocyanines (3 μM) were sonicated ($I = 0.61$ and/or 2.44 W/cm^2). Previously, it was seen that a joint action of US (2.44 W/cm^2) and Pcs led

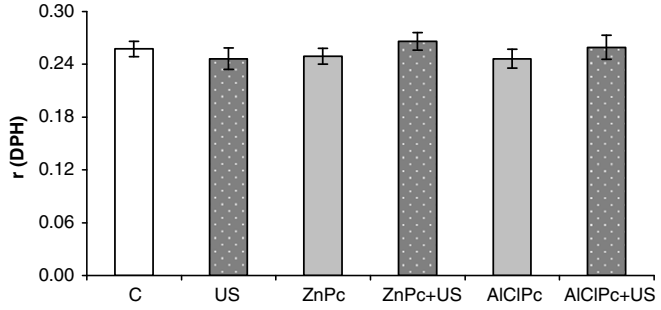

Fig. 3. Fluorescence anisotropy DPH in nucleated erythrocytes membrane exposed to US, Pcs and Pcs + US. US was used at the intensity 2.44 W/cm^2 and Pcs at the concentration 3 μM. The data were obtained from six individual treatments. Error bars denote SD.

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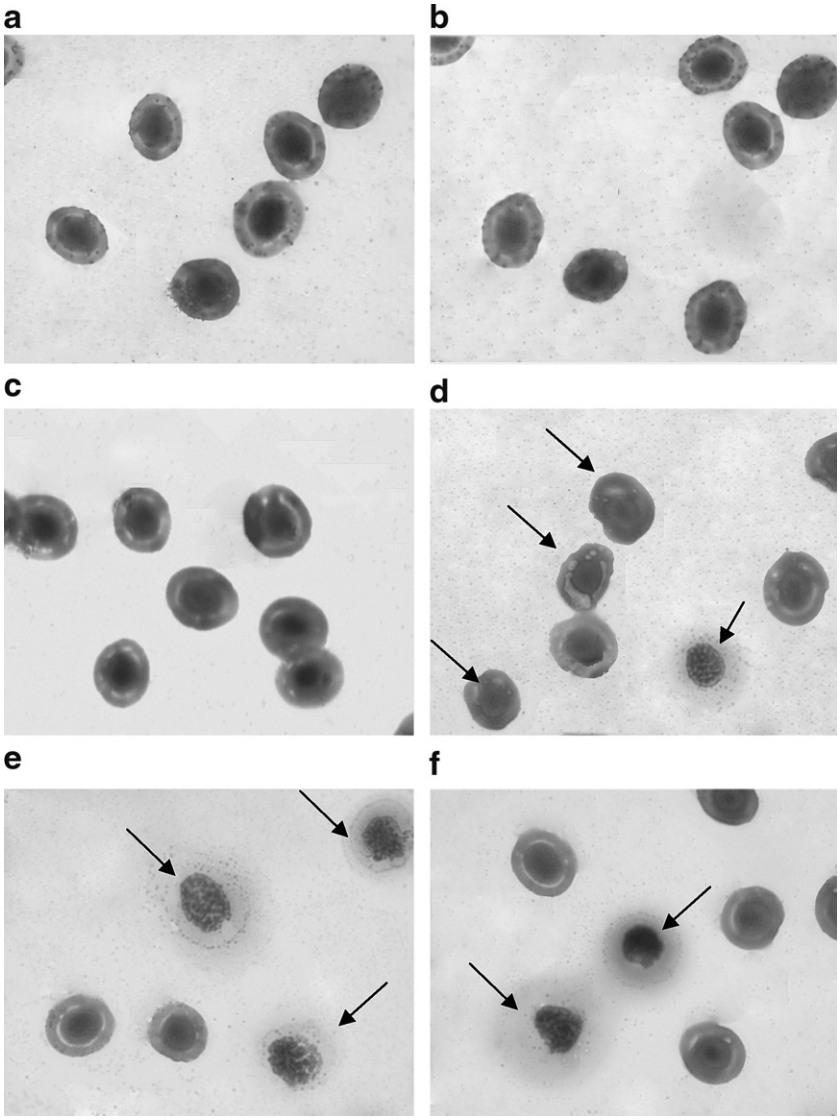

Fig. 4. Images of nucleated erythrocytes: (a) control and exposed to: (b) ZnPc, (c) US (I = 0.61 W/cm^2), (d) US (I = 2.44 W/cm^2), (e) ZnPc + US (I = 2.44 W/cm^2) and (f) AlClPc + US (I = 2.44 W/cm^2), (magnitude 1000). Pcs was used at concentration 3 μM. Possible changes were marked by arrow.

to a higher increase in cellular membrane damage in comparison with sonication without Pcs. In the current investigation, the enhancement of ultrasonically induced cell membrane damage was also observed, which resulted in an increase in hemolysis (lower intensity of US – 0.61 W/cm^2) and fluidity in the plasma membrane surface.

Sonosensitizers located inside a cell depend on its polarity and solubility as well as the type of cell [22,23]. Phthalocyanines, used here are hydrophobic and placed in cellular membrane. The cellular membranes are rich in lipids and are potential targets of oxidative damage. Lipid peroxidation can induce modification in structure, fluidity, and permeability of an erythrocyte membrane. Changes and modification of membrane can decrease stability and increase sensitivity of the cells. An increase in lipid peroxidation after sonication with Pcs has been reported in the previous study [17]. Many authors suggest that lipid peroxidation decreases membrane fluidity [24,25]. However, the results of the investigation on this matter are divergent [26]. A significant increase in membrane surface fluidity is observed, but no changes in the fluidity of the internal region. These results mean that the hydrophobic region of the lipid bilayer is less susceptible to fluidity changes, after sonication with and without Pcs, than the hydrophilic region. Benderitter et al. [27], observed an increase in the fluidity of polar region, and this effect is connected to the lipid oxidation process. The current results support the previous study [17] and the hypothesis that Pcs enhance ultrasonically induced damage of cellular membrane.

Sonication with and without Pcs caused changes in the morphology of erythrocytes. Erythrocytes of a changed shape, vacuolization of cytoplasm, as well as erythrocytes at the stage before or during hemolysis were observed. The results obtained with the microscopic observations were in agreement with the earlier investigation data. Deformation of whole cells was the result of membrane

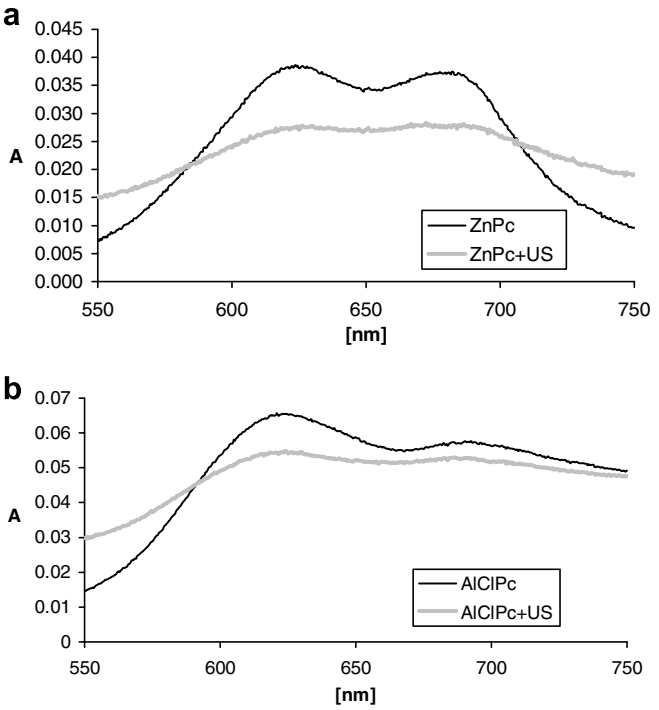

Fig. 5. Absorption spectra of Pcs in medium before and after sonication ($I = 2.44$ W/cm^2) (a) ZnPc and (b) AlClPc.

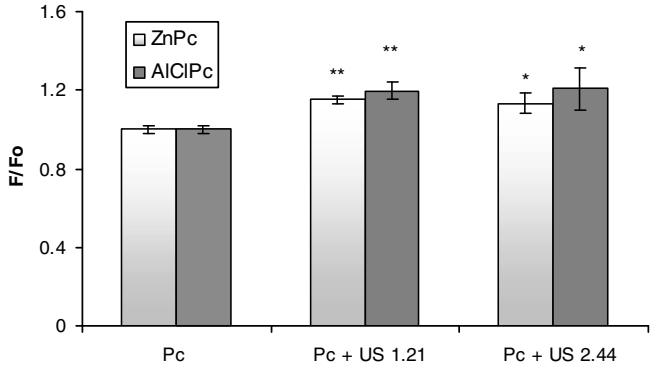

Fig. 6. Fluorescence of Pcs in cells. The data (four individual treatments) are shown as a ratio of the samples fluorescence after sonication (F) to the control sample fluorescence – before sonication (Fo). Error bars denote SD. $*$ denotes $p < 0.01$ and $**$ $p < 0.001$.

damage, namely lipid peroxidation or changes in membrane fluidity. The presence of erythrocytes at the stage of hemolysis confirmed the earlier data, which were obtained with the help of the spectophotometric method. Mehier-Humbert et al. [28], observed pores in the membrane induced by US cavitation. Plasma membrane poration could be useful because it could facilitate drug transport through the membrane, however, it could also damage or kill the cells if the pores were too large or if the cell membrane could not reseal quickly. The morphological changes in cells included: cell shrinkage, membrane blebbing, chromatin condensation, nuclear fragmentation, and apoptotic body fragmentation, after US exposure [29–31]. However, all changes leading to the formation of apoptotic cells were observed a few hours after sonication. In this study, similar morphological changes were not observed, because erythrocytes were analyzed immediately after sonication.

Another point of this report was to test the studying hypothesis that US could contribute to changes in the phthalocyanines structure. The authors assessed the influence of US on Pcs absorption spectra before and after sonication and studied the level of Pcs fluorescence in cells. On the basis of the spectra it was confirmed that US caused changes in the Pcs structure because the shape of the spectra was changed and absorbance was lower. These changes could have been caused by hydroxyl radical reactions with phthalocyanines leading to a formation of their radical products. Other researchers also observed a decrease in the concentration of phthalocyanine derivatives after sonication. Gonze et al. [32], described an indirect product generation because of sonication, on account of an attack of radicals or pyrolysis of different organic compounds. Tu et al. [33], demonstrated destruction of metallophthalocyanines (copper and nickel) in aqueous medium by US (20 kHz, 37–59 W/cm^2). They explained the mechanism of this phenomenon with an intermediate radical generation, which could release metal. However, Banks et al. [34], showed that metal release was significantly slower than phthalocyanines decolorization.

The intensity of dye fluorescence in cells depends on the efficiency of dye incorporation and type of cells. The fluorescence intensity in various cell-lines can be different, because of possible differences in dye location in different types of cells [15]. An increase in phthalocyanines fluorescence intensity (15–20%) in cells is observed after US exposure (Fig. 6). These data indicate that, as a result of Pcs structure changes, products showing higher fluorescence are formed. The alternative possibility is an increase in membrane permeability by sonication [28], and Pcs at a higher level penetrating to cells and showing higher fluorescence.

Summarizing, the obtained results support the hypothesis. The data show that the Pcs enhance action of US in relation to cell membrane *in vitro*. The conclusion is in agreement with what has been suggested in the previous results: Pcs show sonodynamic properties. Our results can also suggest changes in the structure of phthalocyanines after sonication, but the mechanism of this action is not clear yet.

References

[1] N. Miyoshi, J.Z. Sostaric, P. Riesz, Correlation between sonochemistry of surfactant solutions and human leukemia cell killing by ultrasound and porphyrines, Free Radic. Biol. Med. 34 (2003) 710–719.

[2] T. Yu, Z. Wang, T.J. Mason, A review of research into the uses of low level ultrasound in cancer therapy, Ultrason. Sonochem. 11 (2004) 95–103.

[3] Y. He, D. Xing, G. Yan, K. Ueda, FCLA chemiluminescence from sonodynamic action *in vitro* and *in vivo*, Cancer Lett. 182 (2002) 141–145.

[4] N. Yumita, K. Sasaki, S. Umemura, R. Nishigaki, Sonodynamically induced antitumor effect of a gallium-porphyrin complex, Jpn. J. Cancer Res. 87 (1996) 310–316.
[5] N. Yumita, K. Kawabata, K. Sasaki, S. Umemura, Sonodynamic effect of erythrosin B on sarcoma 180 cells *in vitro*, Ultrason. Sonochem. 9 (2002) 259–265.
[6] N. Yumita, K. Sasaki, S. Umemura, A. Yukawa, R. Nishigaki, Sonodynamically induced antitumor effect of gallium-porphyrin complex by focused ultrasound on experimental kidney tumor, Cancer Lett. 112 (1997) 79–86.
[7] N. Yumita, R. Nishigaki, S. Umemura, Sonodynamically induced antitumor effect of Photofrin II on colon 26 carcinoma, J. Cancer Res. Clin. Oncol. 126 (2000) 601–606.
[8] K. Umemura, N. Yumita, R. Nishigaki, S. Umemura, Sonodynamically induced antitumor effect of pheophorbide a, Cancer Lett. 102 (1996) 151–157.
[9] R.J. Jeffers, R.Q. Feng, J.B. Fowlkes, J.W. Hunt, D. Kessel, C.A. Cain, Dimethyloformamide as an enhancer of cavitation-induced cell lysis *in vitro*, J. Acoust. Soc. Am. 97 (1995) 669–676.
[10] K. Tachibana, T. Uchida, K. Tamura, H. Eguchi, N. Yamashita, K. Ogawa, Enhanced cytotoxic effect of Ara-C by low intensity ultrasound to HL-60, Cancer Lett. 149 (2000) 189–194.
[11] S. Umemura, N. Yumita, K. Umemura, R. Nishigaki, Sonodynamically induced effect of rose bengal on isolated sarcoma 180 cells, Cancer Chemother. Pharmacol. 43 (1999) 389–393.
[12] D.J. Ball, S.R. Wood, D.I. Vernon, J. Griffiths, T.M.A.R. Dubbelman, S.B. Brown, The characterization of three substituted zinc phthalocyanines of differing charge for use in photodynamic therapy. A comparative study of their aggregation and phothosensitising ability in relation to *m*THPC and polyhaematoporphyrin, J. Photochem. Photobiol. B: Biol. 45 (1998) 28–35.
[13] S.M.T. Nunes, F.S. Sguilla, A.C. Tedesco, Photophysical studies of zinc phthalocyanine and chloroaluminium phthalocyanine incorporated into liposomes in the presence of additives, Braz. J. Med. Biol. Res. 37 (2004) 273–284.
[14] B.W. Pogue, I.D. Pitts, A.M. Mycek, R.D. Sloboda, C.M. Wilmot, J.F. Brandsema, J.A. O'Hara, *In vivo* NADH fluorescence monitoring as an assay for cellular damage in photocynamic therapy, Photochem. Photobiol. 53 (2001) 859–870.
[15] K. Wiktorowicz, J. Cofta, A. Dudkowiak, A. Waszkowiak, D. Frąckowiak, Preliminary studies of phthalocyanines sensitizers incorporated into human leukemia cells from two cell-lines, Acta Biochim. Polon. 51 (2004) 703–710.
[16] N. Yumita, S. Umemura, Ultrasonically induced cell damage and membrane lipid peroxidation by photofrin II: mechanism of sonodynamic activation, J. Med. Ultrason. 31 (2004) 35–40.
[17] K. Milowska, T. Gabryelak, Synergistic effect of ultrasound and phthalocyanines on nucleated erythocytes *in vitro*, Ultrasound Med. Biol. 31 (2005) 1707–1712.
[18] K. Milowska, T. Gabryelak, Reactive oxygen species and DNA damage after ultrasound exposure, Biomol. Eng. 24 (2007) 263–267.
[19] W.D. Cohen, I. Sanchez, N. Rayos, A.-V. Dadacay, Utility of smooth dogfish erythrocytes for studies of cellular morphogenesis and the cytoskeleton, Mar. Mod. Elec. Rec, Aug. 9, 1996 [serial online <http://www.mbl.edu/BiologicalBulletin/MMER/COH/CohTit.html>].
[20] K. Milowska, T. Gabryelak, G. Lypacewicz, R. Tymkiewicz, A. Nowicki, Effect of ultrasound on nucleated erythrocytes, Ultrasound Med. Biol. 31 (2005) 129–134.
[21] M. Shinitzky, Y. Barenholz, Fluidity parameters of lipid regions determined by fluorescence polarization, Biochim. Biophys. Acta 779 (1978) 89–137.
[22] E.B. Chernyaeva, J. Greve, B.G. de Grooth, G. Van Leeuwen, Intracellular phthalocyanine localization: confocal laser scanning microscopy studies, Proc SPIE, Microsc. Hologr. Interfer. Biomed. 2083 (1994) 62–70.
[23] S. Duk, R. Biolo, W.G. Love, G. Jori, P.W. Taylor, Localization of zincII–phthalocyanine within implanted tumors after intravenous administration of a liposomal formulation, Proc. SPIE, 5th Int. Photodyn. Assoc.Biennial Meeting 2371 (1995) 194–197.
[24] K. Kolanjiappan, S. Manoharan, M. Kayalvizhi, Measurement of erythrocyte lipids, lipid peroxidation, antioxidants and osmotic fragility in cervical cancer patients, Clin. Chim. Acta 326 (2002) 143–149.
[25] L. Öztürk, B. Mansour, M. Yüksel, A.S. Yalçin, F. Çelikoğlu, N. Gökhan, Lipid peroxidation and osmotic fragility of red blood cells in sleep-apnea patients, Clin. Chim. Acta 332 (2003) 83–88.
[26] W. Leyko, D. Ertel, G. Bartosz, Effect of hyperthermia and lipid peroxidation on the erythrocyte membrane structure, Int. J. Radiat. Biol. 59 (1991) 1185–1193.
[27] M. Benderitter, L. Vincent-Genod, A. Berroud, S. Muller, M. Donner, P. Voisin, Radio-induced structural membrane modifications a potential bioindicator of ionizing radiation exposure? Int. J. Radiat. Biol. 75 (1999) 1043–1053.
[28] S. Mehier-Humbert, T. Bettinger, F. Yan, R.H. Guy, Plasma membrane poration induced by ultrasound exposure: implication for drug delivery, J. Contr. Rel. 104 (2005) 213–222.
[29] H. Ashush, L.A. Rozenszajn, M. Blass, M. Barda-Saad, D. Azimov, J. Radnay, D. Zipori, U. Rosenschein, Apoptosis induction of human myeloid leukemic cell by ultrasound exposure, Cancer Res. 60 (2000) 1014–1020.
[30] L. Lagneaux, E.C. de Meulenaer, A. Delforge, M. Dejeneffe, M. Massy, C. Moerman, B. Hannecart, Y. Canivet, M-F. Lepeltier, D. Bron, Ultrasonic low-energy treatment: a novel approach to induce apoptosis in human leukemic cells, Exp. Hematol. 30 (2002) 1293–1301.
[31] Z-.M. Tian, M-.X. Wan, M-.Z. Lu, X-.D. Wang, L. Wang, The alteration of protein profile of Walker 256 carcinosarcoma cells during the apoptotic process induced by ultrasound, Ultrasound Med. Biol. 31 (2005) 121–128.
[32] E. Gonze, L. Fourel, Y. Gonthier, P. Baldo, A. Bernis, Wastewater pretreatment with ultrasonic irradiation to reduce toxicity, Chem. Eng. J. 73 (1999) 93–100.
[33] S.P. Tu, D. Kim, T.F. Yen, Decoloriaztion and destruction of metallophthalocyanines in aqueous medium by ultrasound: a feasibility study, J. Environ. Eng. Sci. 1 (2002) 237–246.
[34] C.E. Banks, A.H. Wylie, R.G. Compton, Ultrasonically induced phthalocyanine degradation: decolouration vs metal release, Ultrason. Sonochem. 11 (2004) 327–331.

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