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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 $1500 \times g$, 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-