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Evaluation of cytotoxic effect of photodynamic therapy in combination with electroporation in vitro

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Abstract

Under the influence of electric pulses cells undergo membrane electroporation (EP), which results in increased permeability of the membrane to exogenous compounds. EP is applied in oncology as a method to enhance delivery of anticancer drugs.

For that reason it was essential to combine photodynamic tumor therapy (PDT)—the cancer treatment method based on the use of photosensitizers that localize selectively in malignant tumors and become cytotoxic when exposed to light, and EP, with the aim to enhance the delivery of photosensitizers into the tumor and therefore to increase the efficacy of PDT.

Thus, the aim of study was to evaluate the cytotoxic effect of PDT in combination with EP. A Chinese hamster lung fibroblast cell line (DC-3F) was used. The cells were affected by photosensitizers chlorin e_6 (C e_6) at the dose of 10 μg/ml and aluminium phthalocyanine tetrasulfonate (AlPcS4) at the dose of 50 μg/ml. Immediately after adding of photosensitizers the cells were electroporated with 8 electric pulses at 1200 V/cm intensity, 0.1 ms duration, 1 Hz frequency. Then, after 20 min of incubation the cells were irradiated using a light source—a visible light passing through a filter (KC 14, emitted light from 660 nm). The fluence rate at the level of the cells was 3 mW/m^2. Cytotoxic effect on cells viability was evaluated using MTT assay.

Our in vitro data showed that the cytotoxicity of PDT in combination with EP increases fourfold on the average. Based on the results we suggest that EP could enhance the effect of PDT.

Keywords: Electroporation; Photodynamic tumor therapy; Photosensitizers; MTT assay

1. Introduction

Electroporation (EP) is a membrane phenomenon that involves the fundamental behaviour of cell membranes [1]. When a cell is exposed to short external electric pulses of high power, the anode-facing side becomes hyper-polarized and the cathode-facing side becomes depolarized depending on the size and the shape of the cell [2,3]. The most frequent application of EP is the introduction of DNA into cells and introduction of some anticancer drugs [4].

Some chemotherapeutic agents used in cancer therapy have limited access to the tumor cells. EP of the cell membrane offers an approach for increased drug delivery into the cells and enhances antitumor effectiveness [5,6]. Electrochemotherapy (ECT) combines administration of non-permeant or poorly permeant chemotherapeutic drugs with application of electric pulses to the tumors in order to facilitate the drug delivery into the cells [5,8].

In vitro studies tested several anticancer drugs for potential application in combination with EP [4,7]. The results of these studies were that only two drugs have been identified as potential drugs for ECT: bleomycin and cisplatin. Bleomycin is hydrophilic and therefore has very restricted capacity of transport through the cell membrane. Its cytotoxicity could be potentiated several times with EP of cells [7]. The transport of cisplatin through the cell membrane is also hampered under usual conditions and therefore EP of cells demonstrated increased cisplatin cytotoxicity [8,9].

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It was shown that in vitro cytotoxicity of some chemotherapeutic drugs could be potentiated several times by exposing cells to short intense electric pulses. When it is essential to introduce photosensitizers into tumor cells, one can expect EP to help photosensitizers penetrate through the cell membrane barrier and thus increase the efficacy of PDT.

Thus, the influence of EP combining with PDT on DC-3F cells viability was studied with the aim to increase the cytotoxic effect of PDT.

2. Materials and methods

2.1. Chemicals

Aluminium phthalocyanine tetrasulfonate (AlPcS4) and chlorin e_6 (C e_6) were purchased from Porphyrin Products, USA. The stock solution of AlPcS4 was prepared in Dulbecco's phosphate buffered saline (DPBS) (5 mg/ml), the stock solution of C e_6 was prepared in ethanol (10 mg/ml) and both were stored at −20 °C in darkness. All experiments were performed diluting the stock solutions to get the appropriate concentration. C e_6 from stock solution of 1 mg/ml at a dose of 5 μg/ml and AlPcS4 from stock solution of 5 mg/ml at a dose of 50 μg/ml were prepared.

2.2. Cell line

The DC-3F, a Chinese hamster lung fibroblast cell line, originates from the Institut Gustave-Roussy and was kindly provided by Dr. Julie Gehl, Copenhagen University, Herlev Hospital, Denmark. Cells were maintained in MEM culture medium (Gibco, Grand Island, NY) with 10% fetal calf serum (FCS) and penicillin-streptomycin.

2.3. Electroporation

Cells were harvested using trypsin-EDTA, washed once in SMEM medium (Gibco, Grand Island, NY) and counted. Viability was estimated using Nucleo Counter (ChemoMetec, Denmark) exclusion test. Cells were chilled on ice and 400 μl of cell suspension (2.2×10^6 cells/400 μl) was put into each of 4-mm-wide cuvettes (CLPDirect, USA). Then 40 μl of the photosensitizer solution or in the case of controls, the same amount of isotonic saline was added. After that some of the cuvettes were exposed to eight electric pulses at an electric field intensity of 1.2 kV/cm, with pulse duration of 0.1 ms, 1 Hz frequency using a BTX T820 square wave electroporator (BTX, San Diego, USA). Then, pulsed and unpulsed cuvettes covered with lids to maintain the pH and sterility were placed in a heat block at 37°C for 20 min.

2.4. PDT procedure

While handling the samples containing photosensitizers, precautions were taken to avoid irradiating the samples with room light. This was done by reducing the sources of illumination to a minimum and by protecting the samples from light with aluminium foil. After EP, the cells were replaced in the glass test tubes and incubated for 20 min to let cells membrane recover after EP. After that selected specimens of cell suspension were exposed to light. For irradiation a visible light source (lamp) which passing through the KC 14 filter (emitted light from 660 nm) was used. The fluence rate at the level of the cells was 3 mW/m^2. After 20 min, exposure cells were placed into the incubator for 20 min. Then cells in suspensions were diluted by a factor of 100 with RPMI 1640 culture medium (Gibco, Grand Island, NY) with 10% FCS and penicillin–streptomycin and seeded in 96-well plates.

2.5. MTT assay

The 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test was used for the assessment of cell viability. After culturing for 96 h, 10 μl of 5 mg/ml MTT stock solution (dimethylthiazol-diphenyltetrazoliumbromide thiazolil blue; Sigma, USA) was added to each well. Four hours later, the reaction was stopped by addition of 10% sodium dodecylsulfate in 0.01 M HCl. After 20 h of incubation at 37 °C, optical density (OD) was measured in a Multiscan MS ELISA reader (Labsystems, Finland), with a 540-nm filter.

2.6. In vitro experimental design

In each experiment, there were seven experimental groups: I, Control, untreated and sham exposed; II, EP (1.2 kV/cm, 99 μs, 8 el. pulses); III, Photosensitizers, C e_6 (10 μg/ml) or AlPcS4 (50 μg/ml); IV, Light exposure (3 mW/cm^2); V, Combination of EP and light illumination; VI, PDT only, cells with C e_6 or

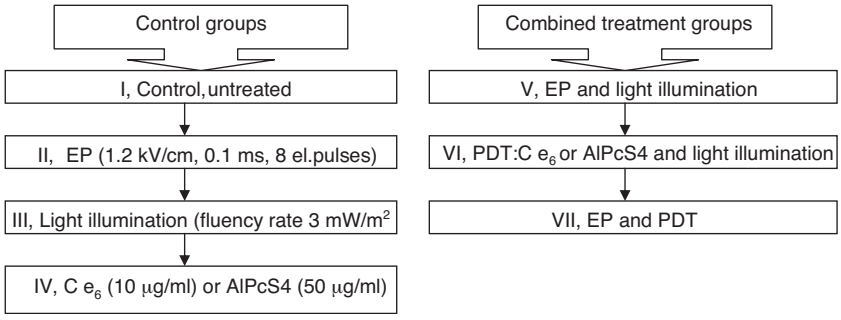

Fig. 1. Design of the experiments.

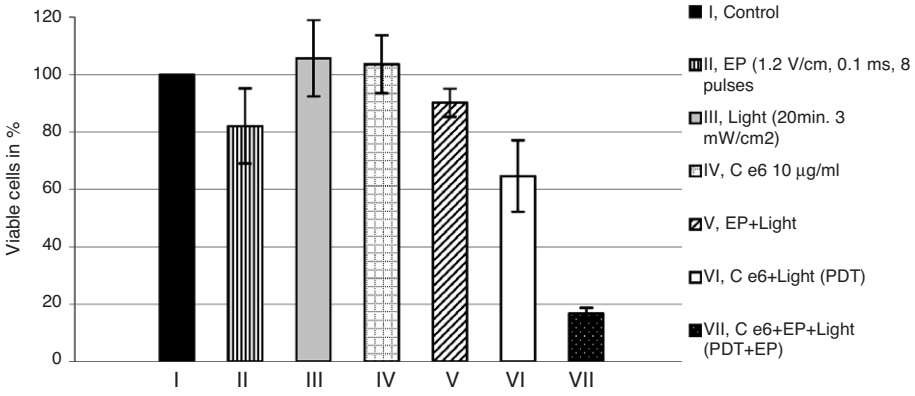

Fig. 2. Combined effect of chlorin e_6 mediated-PDT and electroporation on viability of DC-3F cells. EP—electroporation, C e_6—chlorin e_6.

AlPcS4 exposed to the light; VII, Combined treatment, EP and PDT: after adding photosensitizers C e_6 or AlPcS4 cells were electroporated and exposed to light (Fig. 1).

3. Results and discussion

The aim of this in vitro study was to determine whether EP could potentiate the cytotoxicity of PDT. Our previous preliminary data indicated that EP enhances accumulation of photosensitizers C e_6 and AlPcS4 inside the tumor and determines its more even distribution in cancerous tissue [10,11].

The C e_6 is a second-generation photosensitizer, which belongs to the group of chlorines. The long-wavelength absorptions naturally led to the studies of their potential usage as photosensitizers in PDT. The other photosensitizer used–AlPcS4 belongs to phthalocyanines–tetrapyrrolic macrocycles that, unlike the porphyrins and chlorins, have nitrogen atoms linking the individual pyrrole units instead of methine bridges. The periphery of the macrocycle is extended by benzene rings, which strengthens the absorption at longer wavelengths as compared to porphyrins. An incorporated metal ion-aluminium enhances photosensitizer's triplet yield and lifetime, which is important in order to increase the photodynamic activity [12].

To determine the cytotoxicity of C e_6-mediated PDT in combination with EP DC-3F cells were exposed to PDT as well as to PDT in combination with EP and cells viability was evaluated (Fig. 2).

The difference of cells viability in control groups–untreated cells (I), affected by a pulsed electric field (II), exposed to light (III), treated by photosensitizer (IV)–was statistically insignificant. The influence of EP and light (V) and PDT (VI) on viability of DC-3F cells was noticed but the effect was insignificant. Statistically significant ($p<0.03$) suppression of cells viability was obtained combining PDT and EP (VII). Cells viability suppression was 3.8-fold greater than that of PDT alone. For comparison at the PDT group cells viability was suppressed 1.5 times from the control while at PDT-EP group it was 6 times from the control.

In order to determine DC-3F cells' response to AlPcS4-mediated PDT and its combination with EP the similar experiment as in the case of C e_6-mediated PDT was performed (Fig. 3).

Statistically significant changes in cells viability were obtained in both groups—cells treated with AlPcS4-mediated PDT (VI) ($p<0.05$) and treated with PDT in combination with EP (VII) ($p<0.045$) in comparison with cells viability in the remaining groups (I–V). The viability of the cells in the group (VII) was suppressed 3.7-fold greater than that in PDT alone

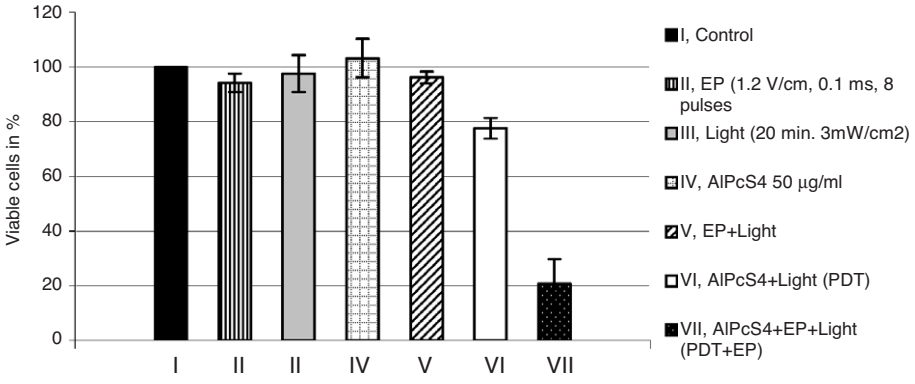

Fig. 3. Combined effect of aluminium phthalocyanine tetrasulphonate—mediated PDT and electroporation on viability of DC-3F cells. EP—electroporation, AlPcS4—aluminium phthalocyanine.

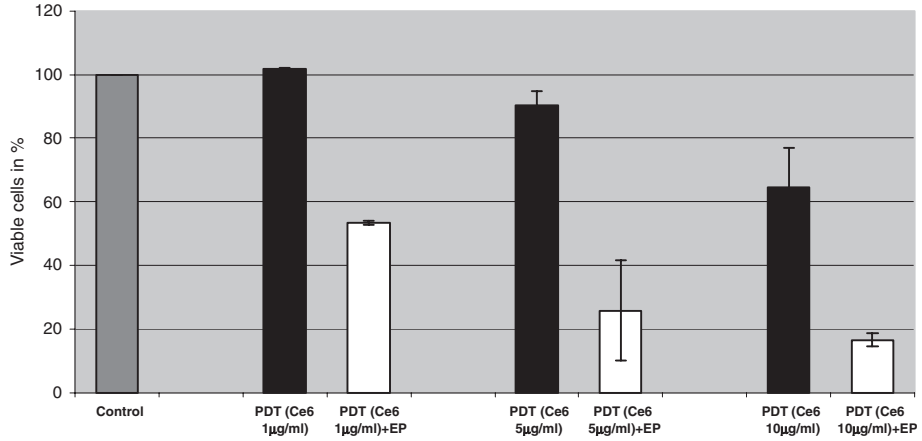

Fig. 4. Dependence of DC-3F cells viability on chlorin e_6 dose (1, 5 and 10 μg/ml) in combination of PDT with electroporation. PDT—photodynamic tumor therapy, EP—electroporation, C e_6—chlorin e_6.

group. For comparison at the PDT group cells viability were suppressed 1.3 times from the control while at PDT-EP group it was 4.8 times from the control samples.

As the aim of the study was to reveal the combination efficacy of PDT with EP, for this purpose the different doses of photosensitizers were chosen to reveal the dependence on the strength of the effect.

In Figs. 4 and 5, the influence of EP on the efficacy of PDT using different doses of photosensitizers was demonstrated. Statistically significant ($p<0.05$) values were calculated from six averaged samples per group. The EP has positive effect on PDT activity even applying from 5 to 10 times smaller doses of photosensitizers (C e_6 applied in three different doses: 1, 5 and 10 μg/ml, AlPcS4: 10, 25 and 50 μg/ml). This suggests that applying electric pulses it is feasible to reduce the dose of photosensitizers used in PDT thus achieving the effect and herewith excluding the possibility of high doses phototoxicity.

Thus, according to our in vitro results combining the AlPcS4 or C e_6-mediated PDT with EP the viability of DC-3F cells considerably decreases as compared to the effect of PDT alone. The higher effectiveness of combined PDT and EP on cells viability could lie in the hydrophilic nature of those photosensitizers since electroporation could facilitate drug transport through cell membrane for those molecules that are poorly or fully non-permeant and the selection of hydrophilic compounds is limited.

There is only some data published on delivery of photosensitizers into tumor cells by EP. According Zhou et al. [13] the photosensitizers–thiopyronine, protoporphyrin, zinc phtalocyanine, copper phthalocyanine–rapidly diffuse into the electroporated cells. Johnson et al. [14] has used EP for delivery of 5-aminolevulinic acid (5-ALA) into the tumors. The authors have shown that 5-ALA can be efficiently delivered transdermally. Such delivery results in a nearly three-fold increase in protoporphyrin IX production in comparison with the passive transdermal delivery. Therefore, the enhanced delivery of photosensitizers by applying EP is subject for the further investigation, comparing different photosensitizers and different experimental models and regimes trying to achieve the most optimal effect.

4. Conclusion

Our results demonstrate that electric pulses used in combination with PDT enhance the photodynamic effectiveness. EP, applied with PDT, increases its cytotoxicity 4-fold on the average greater comparing to cytotoxic effect of PDT alone in appropriate conditions. Based on the results we suggest that

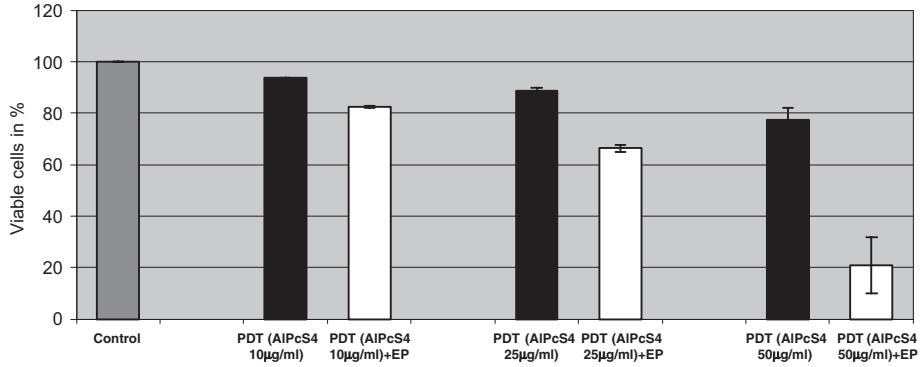

Fig. 5. Dependence of DC-3F cells viability on AlPcS4 dose (10, 25 and 50 μg/ml) in combination of PDT with electroporation. PDT—photodynamic tumor therapy, EP—electroporation, AlPcS4—aluminium phthalocyanine tetrasulphonate.

EP could enhance uptake of certain photosensitizers (AlPcS4 and C e_6) by cells and therefore the cytotoxic effect of PDT on cells viability is increased. Also reducing the doses of photosensitizers in magnitude (from 5 to 10 times) the same effect could be achieved when combined with EP. A very interesting perspective of these experimental data is that with the help of EP it may be possible to avoid the high dose phototoxicity of sensitizers used in PDT.

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