How does cell culture media with additional lipids and vitamin E affects electropermeabilization, cell-cell fusion and survival of CHO-K1 cell line?

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Abstract. *Cell membrane electroporation is a multipurpose technique suitable for biomedical and biotechnological applications. Lipids are essential components of all cell membranes and play an important role in electroporation and electrofusion. The present study determines the effects of the two physiologically crucial lipids cholesterol, and arachidonic acid and α-tocopherol major component of the lipo-soluble vitamin E on electropermeabilization, cell-cell electrofusion and cell survival. Non-malignant CHO-K1 cell line was used. Before electroporation cell-contacts were established by modified adherence method. Permeabilization was determined by propidium iodide. Propidium iodide positive cells were detected by flow cytometry or microscopy. Cell survival was obtained by spectrometric measurements of crystal violet. The percentage of cell fusion was obtained by manual cell counting of microscopic images. The results indicate that pre-treatment of CHO-K1 cells with cholesterol, arachidonic acid and α-tocopherol does not affect cell membrane permeabilization, cell survival or cell fusion. A slight increase in spontaneous cell fusion was observed in pre-treated cells.*

1 Introduction

Cell membrane electroporation is a multipurpose technique suitable for biomedical and biotechnological applications. The cell membrane exposed to external electric field becomes temporarily permeable allowing the exchange of the molecules between the cell cytoplasm and the surroundings. The molecular transport takes place during the pulse until the recovery of the cell membrane function [1]. The cell membrane recovery, known as resealing depends on electric pulse parameters and characteristics of treated cell. Both, cell membrane permeabilization and resealing depend on biochemical and biophysical characteristics of the cell membrane, anchoring to underlying cytoskeleton and physiological status of the cells [2]–[4]. The incorporation of copolymers or detergents into the cell membrane alters cell membrane resealing and viability [5], [6].

Lipids are essential components of all cell membranes and they play an important role in electroporation [7] and cell signaling [8]. Membrane lipid composition varies across cell types, tissues, and organelles, suggesting their role on cellular functions and membrane plasticity [8]. Cells incorporate the lipids and lipo-soluble vitamins obtained from the diet into their cell membranes. Lipids in the cell membrane have distinct functions. Cholesterol is one of the most important regulators of the lipid organization while phospholipids, are essential building blocks [8]. Membrane lipid composition determines cell membrane fluidity. While cholesterol stabilizes lipid bilayer and increases membrane order parameter, polyunsaturated fatty acids in the lipid tails of phospholipids decrease order parameter in the hydrophobic core of the membrane. Under stress conditions polyunsaturated fatty acids are very susceptible for lipid peroxidation [8]–[11]. The arachidonic acid is an omega-6 polyunsaturated fatty acid and like cholesterol is abundant in the diet [9]. The healthy diet contains also lipo-soluble vitamins [12]. The α-tocopherol is potent antioxidant and the most common form of lipo-soluble vitamin E. When α-tocopherol is present in the cell medium it incorporates into the cell membrane and affect cell membrane fluidity [13]. Like cholesterol also α-tocopherol decrease membrane fluidity. Vitamin E has also anti oxidative role. In mouse melanoma cell line, pre-treatment with α-tocopherol improves viability after electroporation [14]. Harmful reactive oxygen species are formed during electroporation and resulting lipid peroxidation could contribute to cell membrane permeabilization [7]. Vitamin E in the cell membrane acts as a potent free radical scavenger [13] and protects cell membrane lipids from peroxidation damage.

The biochemical composition of the cell membrane affects electroporation related cell-cell electrofusion [15]. Lipid curvature is crucial for fusion pore formation and determines the propensity of lipid bilayers to fuse. A fusion pore comprises a connection between the two merging membranes [16]. This pore is a crucial intermediate of cell fusion in naturally occurring processes needed for syncytia formation during muscle differentiation and in artificially induced electrofusion.

The aim of the present study was to determine the effects of cholesterol, arachidonic acid and α-tocopherol on cell membrane electropermeabilization, cell-cell fusion and cell survival. The cell membrane lipid composition was modified by supplementation of cell culture media with physiologic concentrations of cholesterol, arachidonic acid and α-tocopherol. A non-malignant Chinese hamster ovary cell line CHO-K1 was used and results were compared to different controls; cells not treated with additives in the culture medium and cells not treated with electric pulses.

2 Materials and methods

2.1 Cell culture

CHO-K1, Chinese hamster ovary cells, purchased from European Collection of Cell Cultures, were grown as a monolayer culture in a growth medium F12 HAM nutrient mixture (Gibco) supplemented with 2 mM glutamine, 10% fetal bovine serum (Sigma-Aldrich) and antibiotics. Cell cultures were maintained in the incubator (Kambič, Slovenia) at 37°C in a humidified 5% CO2 atmosphere.

2.2 Pre-treatments

Cells were pre-treated with different lipid soluble agents at physiological concentrations: 50 μM cholesterol, 0,5 μM arachidonic acid and 50 μM α-tocopherol. For control treatment, cells were grown in the complete culture medium without addition of lipo-soluble agents. Cells were grown in 6 well plates at the concentration 1.5×10^5 cells/ml for 48h in the incubator at 37°C. At the day of experiment, cells were tripsynised and seeded for electrofusion protocol. Both pre-treated and non-pre-treated cells were exposed to electric pulses and a control, cells not exposed to electric pulses, was prepared in each experiment.

2.3 Electroporation and electrofusion protocol

For electroporation of CHO cells parallel wire electrodes were used. The electric pulse parameters were a train of 8 of 100 μs duration applied at repetition frequency 1 Hz and electric pulse amplitude 1.2 kV/cm.

On the day of the experiment, cell suspension was prepared by 0.25 % trypsin/EDTA solution. Trypsin was removed and 5 ml of culture media was added. Cells were gently rinsed from the bottom of 6 well plate and homogenous cell suspension was prepared. Close cell-cell contacts were established by modified adherence method [17]. A 40 μl drop of cell suspension in concentration 2×10^6 cells/ml was placed per well in a 24-multiwell plate (TPP, Switzerland). Drops were incubated in the incubator with 5 % CO_2 at 37 °C for 25-30 min. When cells were slightly attach to the surface they were washed with 1 ml of isotonic buffer (K_2HPO_4/KH_2PO_4, pH 7,4) . For electroporation 350 μl of isotonic buffer was added and a train of 8×100 μs pulses with the frequency 1 Hz was applied at two parallel wire electrodes (Pl/Ir = 90/10) with four mm gap. Electric field strength was 1.2 k V/cm. After delivery of pulses, the cells were left undisturbed for ten minutes for the cell fusion to take place. For electropermeabilization with propidium iodide (PI) the dye was added to electroporation buffer (final concentration 150 μM) and cells were electroporated and analyzed immediately after pulse delivery by flow cytometry. For in situ permeabilization cells were observed before, between and immediately after electric pulse application. Objective magnification was 40× images were acquired in contrast and fluorescence setup (Leica). Cell survival was determined by crystal violet method. Samples were grown for 24h after electroporation in a 24 well plate. Cells were gently rinsed with physiologic solution 0,9 % NaCl, stained with 0.05 % crystal violet in 30% ethanol for 30 minutes at room temperature. Dye was then removed and cells were washed three times with NaCl. After, lyses with 10% acetic acid samples were measured in microplate reader (Tecan) at 595 nm. For each plate, four blanks without cells were used and the values were subtracted from the obtained results. Cell survival was calculated and expressed as percentage of the control treatment.

Cell electrofusion yield was determined microscopically. Cells were rinsed with NaCl and stained with crystal violet. Stained samples were rinsed three times with NaCl. Washed samples were observed under 40× objective magnification and at least five images per treatment were acquired. Cell were counted manually using Image J software.

Fused cells were determined by their morphological characteristics (Figure 1), characterized by large surface and two or more nuclei in the center of the cytoplasm. Fusion yield was determined as number of multinucleated cells divided by total number of cells. The percentage of polynucleated cells in the control (cells not exposed to electric pulses) was also determined. The percentage obtained in the control was subtracted from electric pulse treated cells to obtain electrofusion percentage.

The results are presented as mean (± standard deviation STD) for a given treatment obtained from three independent experiments.

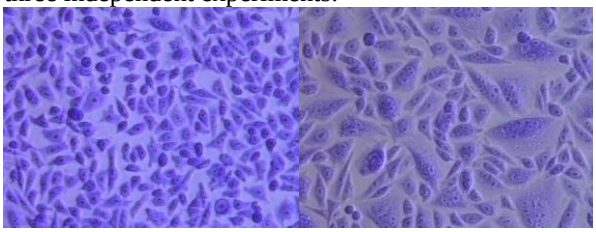

Figure 1: Bright field image. Left panel: cell culture. Right panel: electrofused CHO cells stained by crystal violet. Fused cells were obtained by cell exposure to 8x100μs electric pulses, 1 Hz, 1.2 kV/cm . Images were captured 24 after electroporation. Objective magnification was 40×.

3 Results

3.1 Effect of pre-treatment of CHO cells with cholesterol, arachidonic acid and α-tacopherol on cell membrane permeabilization

Pre-treatment of CHO-K1 cells in the medium with the physiologic concentration of cholesterol, arachidonic acid and α-tocopherol did not affect cell membrane permeabilization. Eight pulses of 100 μs duration and amplitude of 1.2 kV/cm delivered at repetition frequency 1Hz permeabilized all cells in the treated sample. In the control treatment (no electric pulses and no pre-treatment) 12% of permeabilized (PI positive)

cells was observed by flow cytometry (Figure 2). The percentage of permeabilized cells in the control treatment was higher than expected, therefore additional experiments were conducted to confirm the obtained results.

In the additional experiment, permeabilization of CHO cells was observed in situ (Figure 3) and percentage of permeabilized cells was detected by counting of PI positive cells before and after electric pulse application in selected cell population.

The selected methodology excludes any false positive results. In the left panel of the Figure 3 phase contrast of the CHO is presented while on the right panel PI positive cells (red) indicate the grade of permeabilization. The peremeabilization was 99,6% in the control, 99,8% in cholesterol/arachidonic acid pretreated cells and 100% in α-tocopherol pre-treated cells. Figure 3 shows permeabilization of cells pre-treated with arachidonic acid. Almost all were permeabilization at selected electric pulse parameters. Similar results were obtained for control treatment (cells grown without additives) and cells pre-treated with cholesterol and α-tocopherol. No PI positive cells were detected before electric pulse application in control, and cells pretreated with cholesterol/α-tocopherol, while slight permeabilization (3%) was obtained for CHO cells pretreated with arachidonic acid. However, the observed percentage is within experimental error of the selected experimental method.

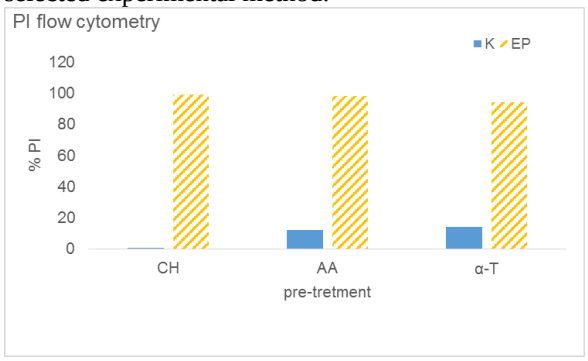

Figure 2: Permeabilization of CHO-K1 cell line with PI. Electric pulse parameters were 8×100 µs, 1.2 kV/cm, 1Hz. (A) permeabilization determined by flow cytometry. 12% of the CHO cells were permeabilized in control (no electric pulses). Abreviations: CH-cholesterol; AA-arachidonic acid; α-T-α-tocopherol.

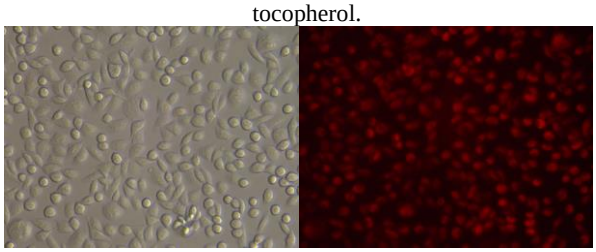

Figure 3: In situ permeabilization of CHO cells pretreated with arachidonic acid (AA) 48 before electroporation: Left panel: phase contrast. Right panel: fuorescence image of the same cells. Images were acquired at 40× objective magnification under phase contrast and fluorescence setup.

3.2 Effect of pre-treatment of CHO cells with cholesterol, arachidonic acid and α-tacopherol on cell survival and electrofusion

The survival of the cells pre-treated for 48h with cholesterol, arachidonic acid and α-tocopherol was 85% and more. Electroporation slightly reduced cell survival; nevertheless, the observed differences are within the experimental error (Figure 4A).

In control treatment, cells not exposed to electroporation nor pretreated with selected agents, 0,6 % ± 0,1 of the cells were fused. Pre-treatment of CHO-K1 cells with cholesterol resulted in 1,1 % ± 0,3; arachidonic acid in 1,4 % ± 0,4 and α-tocopherol in 1,3 % ± 0,1 of spontaneously fused cells. Electroporation increased the percentage of fused cells: 20 % ± 8 % in the control treatment and between 15 and 18 % in the pre-treated cells (Figure 4B). Pre-treatment with the selected agents did not significantly affect electrofusion (Figure 4B).

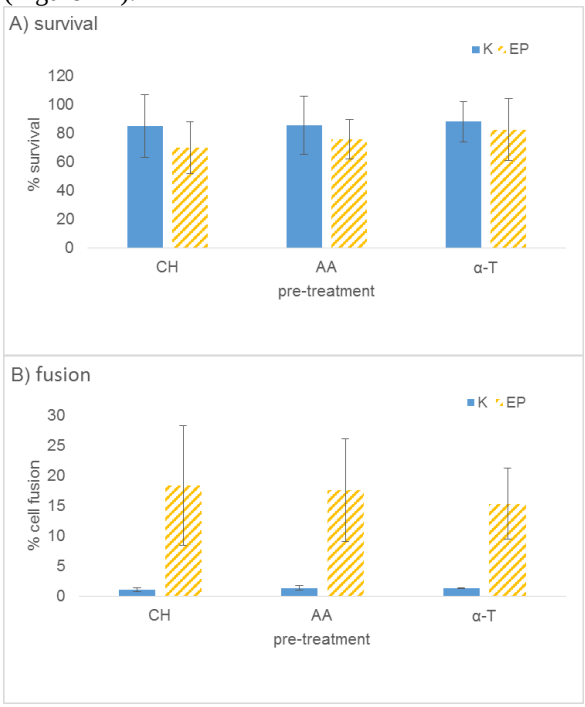

Figure 4: Cell survival (A) and cell fusion (B) of CHO cells grown in the medium containing cholesterol, arachidonic acid or α-tocopherol for 48h. Abreviations: K cells not exposed to electric pulse treatmen, EP electroporation with 8×100 µs, 1.2 kV/cm, 1Hz; CH-cholesterol; AA-arachidonic acid; α-T-α-tocopherol.Values are means of at least 3 independent experiments ±STD.

4 Discussion and conclusions

The effect of cholesterol, arachidonic acid and lipo-soluble vitamin E on cell membrane electropermeabilization, cell-cell fusion and cell survival of CHO-K1 cell line was addressed in this study. In the living organisms, those agents are present in the circulations and are available to cells and tissue. To mimic this situation "*in vitro*" cell culture media was supplemented with physiologic concentrations of cholesterol (50 µM), arachidonic acid (5 µM) and α-tocopherol (50 µM). To determine the effects of lipids

and lipo-soluble vitamin E in normal cells a non-malignant Chinese hamster ovary cell line CHO-K1 was used for experiments.

The obtained results demonstrated that cell membrane permeabilization was not affected by pre-treatment of CHO-K1 cell with cholesterol, arachidonic acid and α-tocopherol (Figure 2). The pre-treatment with arachidonic acid, cholesterol and α-tocopherol affect cell membrane fluidity of cultured cells [8], [11], [13]. Arachidonic acid enhance damage [7] while α-tocopherol protects [12] membrane lipids from peroxidation after electroporation. If lipid peroxidation was involved in cell membrane permeability [7] the differences in permeabilization between arachidonic acid and α-tocopherol pre-treated cells should be observed (Figure 2). In accordance with our previous results [6] differences in cell membrane fluidity caused by cholesterol and arachidonic acid pre-treatment did not affect cell membrane permeabilization (Figure 2).

The liposoluble α-tocopherol protected cell viability in mouse melanoma cell line [14]. In CHO-K1 cell line the protective effect of α-tocopherol was not observed (Figure 4A). The differences could be explained by different grades of malignant transformation (melanoma vs ovary cells) and different origins of the cell lines used in the studies.

The supplementation of cell culture media with cholesterol, arachidonic acid and α-tocopherol slightly increased the spontaneous cell fusion form 0,6% in untreated to 1% in pre-treated cells. Pre-treatments did not affect electrofusion percentage (Figure 4 B). Lipid composition of the cell membrane determines the propensity of lipid bilayers to form fusion pores [16] in biologic cell fusion. Lipid composition does not have a significant effect on electrofusion of CHO-K1 cell line. The selected concentrations of the tested agents were in the range of physiologic values found in the circulation of healthy, adult organism where biologic cell fusion is a rate event.

To conclude: physiologic concentrations of cholesterol, arachidonic acid and α-tocopherol do not affect cell membrane premeabilization, cell survival or cell fusion of non-malignant cell line CHO-K1.

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