

Research article

**DECREASING THE THRESHOLDS FOR ELECTROPORATION
 BY SENSITIZING CELLS WITH LOCAL CATIONIC ANESTHETICS
 AND SUBSTANCES THAT DECREASE THE SURFACE NEGATIVE
 ELECTRIC CHARGE**

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Abstract: The recently described method of cell electroporation by flow of cell suspension through localized direct current electric fields (dcEFs) was applied to identify non-toxic substances that could sensitize cells to external electric fields. We found that local cationic anesthetics such as procaine, lidocaine and tetracaine greatly facilitated the electroporation of AT2 rat prostate carcinoma cells and human skin fibroblasts (HSF). This manifested as a 50% reduction in the strength of the electric field required to induce cell death by irreversible electroporation or to introduce fluorescent dyes such as calcein, carboxyfluorescein or Lucifer yellow into the cells. A similar decrease in the electric field thresholds for irreversible and reversible cell electroporation was observed when the cells were exposed to the electric field in the presence of the non-toxic cationic dyes 9-aminoacridine (9-AAA) or toluidine blue. Identifying non-toxic, reversibly acting cell sensitizers may facilitate cancer tissue ablation and help introduce therapeutic or diagnostic substances into the cells and tissues.

Key words: Selective sensitization of cells, Local cationic anesthetics, Cationic dyes, Irreversible electroporation, Reversible electroporation, Loading with fluorescent dyes, Cell viability, Flow through an electric field, Direct current electric field, Focused electric field

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Abbreviations used: 9-AAA – 9-aminoacridine; dcEF – direct current electric field; EthBr₂ – ethidium bromide; FBS – fetal bovine serum; FDA – fluorescein diacetate; HSF – human skin fibroblasts; IRE – irreversible electroporation; PBS – phosphate buffered saline with or without calcium and magnesium ions; RE – reversible electroporation

INTRODUCTION

In the past few decades, reversible electroporation (RE) has been broadly used to introduce substances that normally do not penetrate the cell membrane, such as dyes, drugs, proteins and nucleic acids, into cells [1-3]. Since 2005, the non-thermal killing of cells via irreversible electroporation (IRE) has been used to ablate neoplastic tissue without scar formation and local bleeding [4-6].

The aim of this study was to identify substances that affect the properties of the cell membrane in such a way as to allow a reduction in the electric field strength required for effective RE and IRE. The previously reported method involved cell sensitization by hypotonic shock-facilitated electroporation of cultured cells [8], but there were a number of methodological constraints to its application *in vivo*. Another method recently described by Pakhomova *et al.* [9] involved sensitization of melanoma B16 and CHO cells by splitting eight 100 μ s pulses into two trains of four pulses, separated by a 5-min interval. This approach was shown to facilitate the uptake of bleomycin and propidium iodide by the cells.

We used the recently described the method of cell electroporation using a flow of cell suspension through a localized electric field [10] to examine the effects of substances known to modify cell membrane properties. This method allows the numbers of reversibly and irreversibly electroporated cells to be determined as a function of dcEF applied to the cells for a given period of time (time duration). This method also allows quantitative evaluation of the effects of drugs that are introduced through the electroporation solution and are known to modify cell membrane properties, on the threshold values of dcEFs and the duration of cell exposure required for IRE and RE.

Surgical procedures are usually carried out with the aid of common local anesthetics, such as procaine, lidocaine or tetracaine. These substances not only act on neurons but also affect the surrounding cells and tissues. Modulation of cell membrane properties can affect a variety of cell functions [11, 12]. External dcEFs act on cells interacting with cell membranes. The electric charge on the cell membrane is known to influence the physical and chemical properties of the cell membranes and can be expected to affect the cell responses to dcEFs acting on cells. In particular, the cationic local anesthetics procaine and lidocaine were shown to influence the electric charge on cell surfaces as determined with cell electrophoresis [13]. Therefore, in our experiments we examined the effects of local cationic anesthetics on cell sensitivity to dcEFs causing IRE and RE. These substances are harmless for cells and can be selectively and locally applied to the chosen cells or tissues, which is of potential significance in clinical practice.

Here, we identify a few substances that sensitize cells and decrease the threshold values of dcEFs required for effective IRE and RE. The experiments were carried out on a rat prostate cell line and human skin fibroblasts (HSF) using anesthetics at lower concentrations than commonly used in surgical anesthesia.

MATERIAL AND METHODS

Chemicals

9-Aminoacridine (9-AAA), ethidium bromide, diacetate fluorescein, Alexa Fluor 488 Phalloidin, gentamicin, calcein, lidocaine HCl, Lucifer yellow, procaine HCl, tetracaine HCl, toluidine blue and trypsin-EDTA were all obtained from Sigma. Fetal bovine serum (FBS) was from Gibco, Invitrogen. Carboxyfluorescein was from Fluka-biochemist, culture medium RPMI 1640 with L-glutamine from Lonza, NaCl and sucrose from Merck, and PBS without calcium and magnesium ions and PBS with calcium and magnesium ions (composed of: CaCl_2 – 9 μM , MgCl_2 – 1 mM, KH_2PO_4 – 1.5 mM, $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ – 7.5 mM, KCl – 2.7 mM, NaCl – 138 mM) were from Biomed.

Cells

Experiments were carried out on the well-characterized AT-2 rat prostate cancer cell line grown in 25-cm² flasks (Sarstead) as described previously [14]. For some experiments, normal human skin fibroblasts (HSF) were used [15]. Before electroporation, cells were washed in Ca^{2+} - and Mg^{2+} -free PBS by centrifugation and suspended in an electroporation solution. The electroporation solution was 9.5% sucrose and PBS with Ca and Mg in the ratio 19:1, unless stated otherwise. In the cell sensitization experiments, the cells were incubated in electroporation solution containing various concentrations of lidocaine HCl, procaine HCl, tetracaine HCl, 9-AAA or toluidine blue for 10 min. Following the incubation, the cells were centrifuged for the second time and re-suspended in the electroporation solution.

Cell electroporation and viability examination

Cell electroporation was carried out with the setup described in [10], in a solution containing calcein, carboxyfluorescein or Lucifer yellow. The effectiveness of the method was established by scoring the number of fluorescent cells found 15-30 min following the procedure.

A fluorescent viability test using fluorescein diacetate (FDA) and ethidium bromide (EthBr_2) was carried out to determine the number of live cells [10, 16-20]. During the electroporation procedure, the electroporated cells remained in the electroporation medium with or without added substances for no more than one hour. Then the cells were transferred either to PBS or to the cell culture medium for examination under a fluorescence microscope. In each experiment, 1250 to 2250 cells were examined under a Jenavert epi-fluorescent microscope (Carl Zeiss Jena) to determine the effect of established exposure time on cells of the dcEFs. At least 250 cells were observed to determine the position of one point in the plot. The FDA/ EthBr_2 iodide test was applied. Green fluorescent cells were counted as alive and red fluorescent cells as dead. The cells that showed the uptake of calcein, carboxyfluorescein or Lucifer yellow were counted as reversibly electroporated, while the cells that were not fluorescent were counted as not reversibly electroporated. All of the curves presented in Figs 1-4 were

drawn as matched with experimentally determined points. The values of LD₅₀ and ED₅₀ were calculated with Stephen Wolfram's Mathematica program.

In experiments testing the effects of local anesthetics on the RE and IRE thresholds, the drugs were applied at much lower concentrations than used in clinical practice. For example, in order to obtain a local anesthesia lasting for 1 to 3 h, procaine HCl and lidocaine HCl are used at concentrations of 1 to 2%, while tetracaine is used at 0.05 to 0.5%. In our experiments, we used procaine HCl and lidocaine HCl at 10 mM concentration and tetracaine at 0.6 mM (corresponding to concentrations of 0.2% and 0.02%, respectively). One experiment carried out to electroporate the cells at given time of cell exposure to dcEFs lasted less than 1 h. The efficiency of RE or IRE as a function of dcEFs was determined after the transfer of cells to anesthetic-free and fluorescent dye-free media.

RESULTS

We applied the recently described method of cell electroporation by flow of cell suspension through a localized electric field [10]. The experiments were carried out on a rat prostate cell line and human skin fibroblasts (HSF) using anesthetics at lower concentrations than commonly used for surgical anesthesia.

The cationic anesthetics procaine HCl and lidocaine HCl were present in the electroporation medium at a concentration of 10 mM and tetracaine was present at 0.6 mM (corresponding to concentrations of 0.2% and 0.02%, respectively). Such exposure had no effect on the viability or uptake of the used fluorescent dyes in cells incubated for 1 h in their presence and not electroporated. More than 90% of the cells remained alive and unloaded with fluorescent dyes. Toluidine blue present at 15 μM and 9-AAA at 30 μM also had no effect on cell viability. In other experiments on the morphology, proliferation and movement of various cells grown for days in the presence of substances tested in these experiments, procaine HCl was found to have the least influence (data not shown). The experiments demonstrated that all three tested local cationic anesthetics significantly decreased the dcEF strength required for effective IRE in AT2 cells. Fig. 1A-C show the percentage of AT2 cells killed by the exposure to dcEFs in the range 300 to 1200 V/cm for 0.62 s (IRE) in the electroporation medium alone (control) and in the electroporation medium supplemented with added local cationic anesthetics, 9-AAA or toluidine blue.

All three tested anesthetics significantly decreased the dcEF strength required for the effective IRE in AT2 cells (Fig. 1A, B). As shown in Fig. 1A, 10 mM procaine HCl caused a decrease in the LD₅₀ dcEF from 784 to 426 V/cm. The two cell viability tests (the calcein [10, 20, 21] and FDA/ethidium bromide tests [18]) gave compatible results.

We then studied the effects of the cationic local anesthetics tetracaine, procaine and lidocaine on cell viability using the calcein uptake test. Tetracaine was used at a concentration of 0.6 mM since higher concentrations decreased cell viability

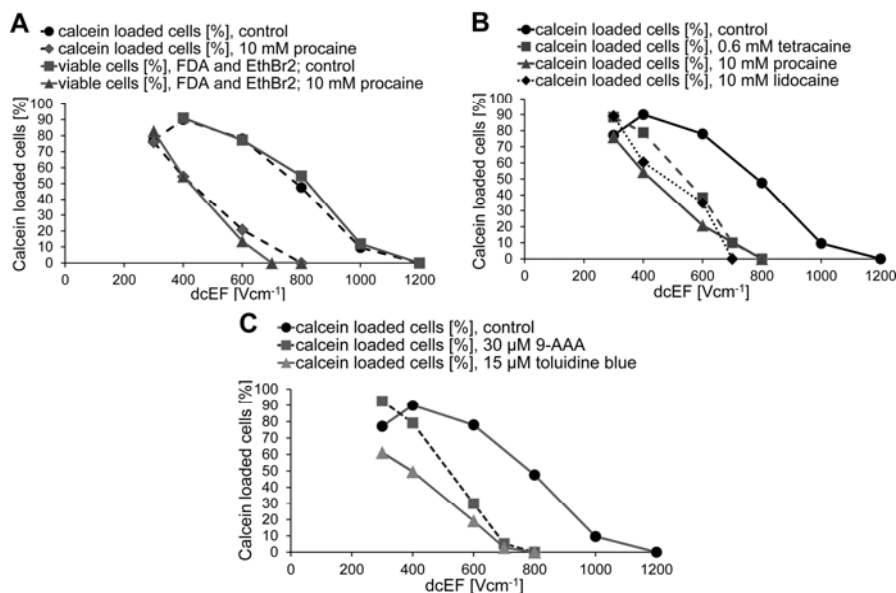


Fig. 1. Irreversible electroporation with cationic local anesthetics or cationic dyes was used to decrease the threshold values of dcEFs that cause non-thermal cell killing of AT2 cells. A – The effect of procaine on the viability of AT2 cells exposed to dcEF for 0.62 s. Viability was examined using the FDA/ethidium bromide test and the calcein uptake test. X-axis – dcEF in V/cm, Y-axis – percentage of viable cells as a function of dcEF strength (calcein-loaded cells – control $LD_{50} = 784$ V/cm; LD_{50} in the presence of 10 mM procaine = 426 V/cm). B – Cationic local anesthetics (procaine, lidocaine, and tetracaine) decrease the threshold values of dcEFs that cause non-thermal cell killing by irreversible electroporation (calcein-loaded cells – control $LD_{50} = 784$ V/cm; LD_{50} in the presence of 0.6 mM tetracaine = 543 V/cm; LD_{50} in the presence of 10 mM procaine = 426 V/cm; LD_{50} in the presence of 10 mM lidocaine = 482 V/cm). Cell viability was determined using the calcein test. C – The effects of cationic dyes (9-aminoacridine and toluidine blue) on the decrease in the threshold values of dcEFs that cause cell killing by irreversible electroporation (calcein-loaded cells – control $LD_{50} = 784$ V/cm; LD_{50} in the presence of 30 μ M 9-AAA = 519 V/cm; LD_{50} in the presence of 15 μ M toluidine blue = 397 V/cm). Cell viability was determined with the calcein test.

after 1 h incubation. The results are shown in Fig. 1B. Both lidocaine and tetracaine also significantly decreased the threshold values of dcEFs required for IRE, when compared with the values of dcEFs required for cell killing by IRE in the control experiments in the absence of the tested cationic anesthetics.

Next, we tested whether other non-toxic cationic substances, such as toluidine blue (15 μ M) [13] or 9-AAA (30 μ M) [22, 23], would influence the threshold values of dcEFs required for cell killing by IRE. Toluidine blue was more effective than procaine but caused some calcein leakage from calcein-loaded cells if they were later incubated in its presence for 1 to 3 h. The values of LD_{50}

in the control experiments without the addition of tested sensitizers and in their presence are included in the legends to the figures.

In a previous study [10], we found that briefly exposing cells to dcEF allowed for more effective calcein loading and that cell viability was improved when exposure to dcEFs did not exceed 0.1 s. Therefore, in order to introduce fluorescent dyes that normally do not permeate cell membranes, we applied single exposures to dcEFs lasting for 0.08 s. The results are shown in Fig. 2A. A dcEF of 50 V/cm was sufficient to load approximately 90% of AT-2 cells with calcein in the presence of procaine, whereas in the control experiments without procaine, a dcEF of 400 V/cm was required (Fig. 3). The cells flowing through the system but not exposed to dcEFs were not loaded with the fluorescent dyes (0 dcEF strength). Lidocaine and tetracaine also decreased the dcEFs required for calcein loading but were less effective than procaine. In the presence of cationic dyes that decrease the negative electric charge on the cell surface (toluidine blue and 9-AAA), the uptake of calcein by AT2 cells required much lower dcEFs than in the control experiments with the cells electroporated in the electroporation solution without the addition of these cationic substances (Fig. 2B).

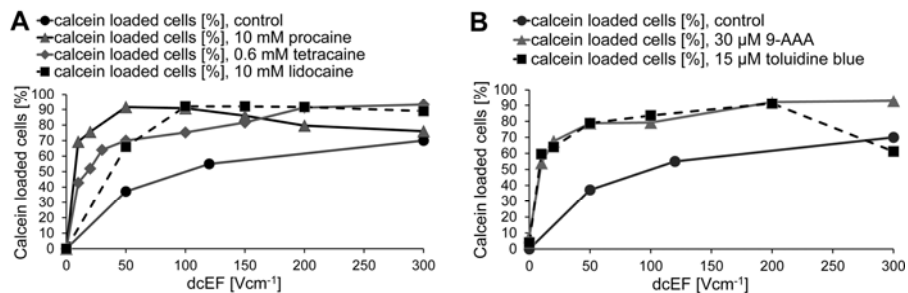


Fig. 2. Decreased dcEF strength is required for the uptake of calcein by AT2 cells when cationic anesthetics or cationic dyes are used. A – The effect of the supplementation of the electroporation solution with local cationic anesthetics (procaine, lidocaine and tetracaine) on the dcEF strength required for the uptake of calcein by AT2 cells after a single 0.08 s exposure to dcEFs (ED_{50} for calcein-loaded cells under control conditions = 100 V/cm; ED_{50} in the presence of 10 mM procaine is 7 V/cm; ED_{50} in the presence of 0.6 mM tetracaine = 18 V/cm; ED_{50} in the presence of 10 mM lidocaine = 38 V/cm). B – The effect of cationic dyes (aminoacridine and toluidine blue) on the sensitization of cells to dcEFs and decrease in dcEF strength required for loading cells with calcein (ED_{50} for calcein-loaded cells under control conditions = 100 V/cm; ED_{50} in the presence of 30 μ M 9-AAA = 9 V/cm; ED_{50} in the presence of 15 μ M toluidine blue = 8 V/cm). The dcEFs in the range 0 to 300 V/cm were examined. Other notations as in Fig. 1A.

The effects of anesthetics on calcein and carboxyfluorescein loading of cells were identical (data not shown). Interestingly, the threshold values of dcEFs required for loading of cells with Lucifer yellow were lower than those required for calcein loading and were further decreased in the presence of procaine (Fig. 3A and B).

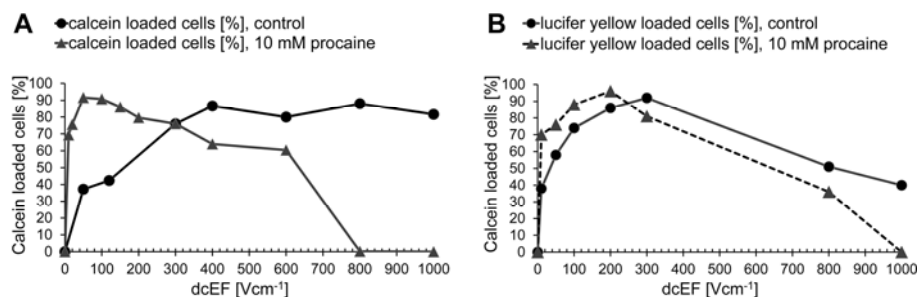


Fig. 3. The effect of procaine on AT2 prostate cancer cell loading with calcein or Lucifer yellow following a single 0.08 s exposure to dcEF. A – Cells loaded with calcein (ED_{50} under control conditions = 161 V/cm; ED_{50} in the presence of 10 mM procaine = 7 V/cm). B – Cells loaded with Lucifer yellow (ED_{50} under control conditions = 34 V/cm; ED_{50} in the presence of 10 mM procaine = 7 V/cm). The dcEFs in the range 0 to 1 000 V/cm were examined. Other notations as in Fig. 1A.

In our previous study [10], we found that the human skin fibroblasts are much more sensitive to IRE and RE than AT2 cancer prostate cells or human red blood cells. Fig. 4 shows that the presence of 10 mM procaine in the electroporation solution decreased the thresholds of dcEFs required for RE and IRE in human skin fibroblasts (HSF) after an 0.08 s exposure. The fibroblasts were so strongly sensitized to electric fields in the presence of procaine that the 0.08 s exposure of these cells to a dcEF of 10-20 V/cm appeared sufficient to load the cells with calcein, and a field strength of 300 V/cm was sufficient to kill about 50% of cells with IRE.

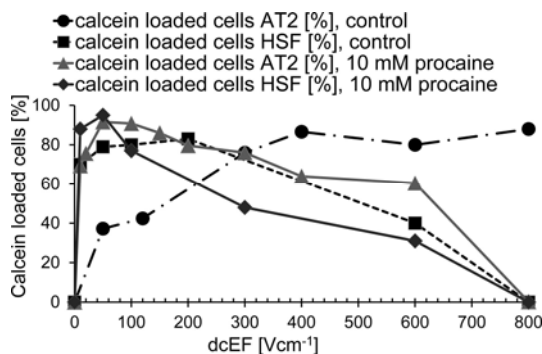


Fig. 4. Calcein loading of AT2 prostate cancer cells and human skin fibroblasts (HSF) after a single exposure to dcEF for 0.08 s in the presence and in the absence (control) of procaine in the electroporation medium. ED_{50} for calcein-loaded AT2 cells under control conditions = 161 V/cm. ED_{50} for calcein-loaded AT2 cells in the presence of 10 mM procaine = 7 V/cm. ED_{50} for calcein-loaded HSF cells under control conditions = 7 V/cm. ED_{50} for calcein-loaded HSF cells in the presence of 10 mM procaine = 6 V/cm. The dcEFs in the range 0 to 1 000 V/cm were examined. Other notations as in Fig. 1A.

DISCUSSION

Our recently described method of cell electroporation involves equipment commonly used for electrophoresis and shows versatility. It permits the determination of cell sensitivity to external electric fields under controlled experimental conditions [10]. In 2004 and 2011, we examined the applicability of the method using a short exposure of a flowing suspension of cells through a focused dcEF to study the survival of *Dictyostelium discoideum* and tissue culture cells as a function of electric field strength and time of exposure (unpublished results). To facilitate the delivery of substances into the cells and improve cell viability following the procedure, the cells had to remain in contact with the tested substances during and after the electroporation. This was achieved by the flow of cell suspension within the narrow tube and elimination of the action of electrode products on the cells, as previously described [10]. Independently, Geng *et al.* [24, 25] demonstrated an efficient transfection of Chinese hamster ovary cells with plasmids encoding green fluorescent protein by flow through a chip device ensuring a repeated exposure of cells to focused constant voltage electric fields, corresponding to the series of electric pulses.

We examined the effects of a single exposure of cells to dcEFs in the presence of various substances modifying cell membrane properties. This approach yields results that are more unequivocal and can be more easily interpreted than those from a series of electric pulses or exposures to an external electric field [8]. In this case, the time interval between the exposures to the strong electric field has great impact on cell responses [9]. The method described in this study greatly improves cell viability following electroporation and sensitizes cells to non-thermal cell killing by exposure to dcEFs.

The results presented here show that local cationic anesthetics commonly used in medicine, such as procaine, lidocaine or tetracaine, allow a 50% reduction in the strength of dcEFs required for effective IRE or RE. These effects are observed when the anesthetics are applied at concentrations much lower than used in the clinical practice. Though lidocaine appears to be the most often used local anesthetic, we observed that procaine was more effective in decreasing dcEF threshold values required for cell electroporation and preserving cell viability during and after the procedure. A similar decrease in electric field strength threshold values for IRE and RE was also observed for cationic dyes toluidine blue and 9-AAA used at non-toxic concentrations.

The local anesthetics procaine, lidocaine, and tetracaine induce changes in the cell membrane properties and functions in non-neuronal cells [11, 12], including changes in the cell surface charge and changes in the cell electrophoretic mobility [13]. Decreasing the charge density can greatly affect the physical properties of the cell membrane. Changes in protein conformation and phospholipid liquid crystal structure caused by cationic substances can sensitize cells to dcEFs, increasing the effectiveness of RE and IRE [27]. In support of this hypothesis, we observed that the substances that reduce the negative charge

of the cell surface [22, 23], such as 9-AAA and toluidine blue, also decreased the dcEFs required for IRE and RE. The precise mechanism of the effects induced by local anesthetics will require further study.

Our experiments described in this report were inspired by two groups of observations. First, the thresholds of dcEFs for RE and IRE depend on the cell size [10, 27-29]. Even when the cell size effect is eliminated and the threshold values of electric field strength are expressed in V/cell diameter, the differences in the sensitivity of various cell types are preserved [10]. This suggests that the cell membrane properties significantly influence cell sensitivity to dcEFs.

Second, in the 1960s, on the grounds of the theory of flexible electrolytes, it was discussed how changes in the membrane surface electric charge density influence dynamic membrane properties, leading to pore formation [30, 31]. It was suggested that changes in the electric charge on the cell surface may modify the physical and chemical properties of the cell membrane and affect its interactions with external electric fields and other surfaces [30, 32]. Gingell [33] demonstrated that a non-specific decrease in the negative electric charge on the surface of *Xenopus laevis* eggs introduced by adsorption of polycations, induced changes in the membrane organization, manifested by increased membrane permeability. This in turn caused contractile responses in the cortical cytoplasm of *Xenopus laevis* eggs. It was observed that the non-thermal break in the membrane of cells placed in strong dcEFs always took place at the cell pole directed to the anode [34] and that the same part of the cell showed contraction during cell galvanotaxis in weak dcEFs [35]. More recently, Teissié [36] founds that the cell reversible electroporation (RE) also starts at the cell end directed to anode. In addition, it is known that coating DNA with cationic substances improves the effectiveness of transfection [37, 38].

The findings described in this study may find application in clinical practice. Identification of non-toxic, reversibly acting cell sensitizers may facilitate cancer tissue ablation and help introduce therapeutic or diagnostic substances into the cells and tissues. The discovery of cell sensitizers for light significantly increased the effectiveness of phototherapy [39, 40] and temperature sensitizers are likely to improve thermotherapy in oncology [41, 42]. One can expect that the results presented here will stimulate further search for cell sensitizers and help open new avenues for the application of RE and IRE in biotechnology and medicine.

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REFERENCES

1. Chang, D.C., Chassy, B.M., Saunders, J.A. and Sowers, A.E. **Guide to Electroporation and Electrofusion**, Academic Press Inc., San Diego, 1992.
2. Li, S. (Ed.). **Electroporation Protocols. Preclinical, and Clinical Gene Medicine**, Humana Press, Totowa, New Jersey, 2008.
3. Neumann, E., Schaeffer-Ridder, M., Wang, Y. and Hofschneider, P.H. Gene transfer into mouse lymphoma cells by electroporation in high electric fields. **EMBO J.** 1 (1982) 841-845.
4. Gissel, H., Lee, R.C. and Gehl, J. Electroporation and cellular physiology. In: **Clinical Aspects of Electroporation** (Kee, S.T., Gehl, J. and Lee, E.W., Eds.), Springer, New York, 2011, 9-17.
5. Miller, L., Leor, J. and Rubinsky, B. Cancer cells ablation with irreversible electroporation. **Technol. Cancer Res. Treat.** 4 (2005) 699-705.
6. Rubinsky, B. (Ed.). **Irreversible Electroporation**. Springer-Verlag, Berlin, Heidelberg, 2010.
7. Rubinsky, J., Onik, G., Mikus, P. and Rubinsky, B. Optimal parameters for the destruction of prostate cancer using irreversible electroporation. **J. Urol.** 180 (2008) 2668-2674. DOI: 10.1016/j.juro.2008.08.003.
8. Barrau, C., Teissié, J. and Gabriel, B. Osmotically induced membrane tension facilitates the triggering of living cell electropermeabilization. **Bioelectrochemistry** 63 (2004) 327-332.
9. Pakhomova, O.N., Gregory, B.W. and Pakhomov, A.G. Facilitation of electroporative drug uptake and cell killing by electrosensitization. **J. Cell. Mol. Med.** 17 (2013) 154-159. DOI: 10.1111/j.1582-4934.2012.01658.x.
10. Korohoda, W., Grys, M. and Madeja, Z. Reversible and irreversible electroporation of cell suspensions flowing through a localized DC electric field. **Cell. Mol. Biol. Lett.** 18 (2013) 102-119. DOI: 10.2478/s11658-012-0042-3.
11. Michalik, M., Pierzchalska, M., Pabiańczyk-Kulka, A. and Korohoda, W. Procaine-induced enhancement of fluid-phase endocytosis and inhibition of exocytosis in human skin fibroblasts. **Europ. J. Pharmacol.** 475 (2003) 1-10.
12. Pierzchalska, M., Michalik, M., Stepień, E. and Korohoda, W. Changes in morphology of human skin fibroblasts induced by local anesthetics; role of actomyosin contraction. **Europ. J. Pharmacol.** 358 (1998) 235-244.
13. Dickstein, R.A., Kiremidjian-Schumacher, L., and Stotzky, G. Effect of lidocaine on production of migration inhibitory factor and on macrophage motility. In vitro exposure of Guinea pig lymphocytes and macrophages. **J. Leukocyte Biol.** 36 (1984) 621-632.
14. Djamgoz, M.B.A., Mycielska, M., Madeja, Z., Fraser, S.P. and Korohoda, W. Directional movement of rat prostate cancer cells in direct-current electric field: involvement of voltage-gated Na⁺ channel activity. **J. Cell Sci.** 114 (2001) 2697-2705.

15. Krzysiek-Mączka, G. and Korohoda, W. Surface anisotropy orients cell divisions in contact guided cells. **Folia Biol.** 56 (2008) 13-19.
16. Karmiol, S. Cell isolation and selection. In: **Methods of Tissue Engineering**. (Atala, A. and Lanza, R., Ed), Academic Press, San Diego, 2002, 19-35.
17. Kemp, R.B., Meredith, R.W.J., Gamble, S. and Frost, M. A rapid cell culture technique for assessing the toxicity of detergent-based products in vitro as a possible screen for eye irritancy in vivo. **Cytobios** 36 (1983) 153-159.
18. Szydłowska, H., Zaporowska, E., Kuszlik-Jochym, K., Korohoda, W. and Branny, J. Membranolytic activity of detergents as studied with cell viability tests. **Folia Histochem. Cytochem.** 16 (1978) 69-78.
19. Zaporowska-Siwiak, E., Michalik, M., Kajstura, J. and Korohoda, W. Density-dependent survival of Ehrlich ascites tumor cells in the presence of various substratum for energy metabolism. **J. Cell Sci.** 77 (1985) 75-85.
20. Schlieve, C.R., Lieven, C.J. and Levin, L.A. Biochemical activity of reactive oxygen species scavengers do not predict retinal ganglion cell survival. **Invest. Ophthalm. Vis. Sci.** 47 (2006) 3878-3886.
21. Ammar, D.A., Noecker, R.J. and Kahook, M.Y. Effects of benzalkonium chloride-preserved, polyquad-preserved, and sofzia-preserved topical glaucoma medications on human ocular epithelial cells. **Adv. Ther.** 27 (2010) 837-845. DOI: 10.1007/s12325-010-0070-1.
22. Ambrose, E.J. Possible mechanisms of the transfer between small groups of cells. In: **Ciba Foundation Symposium Cell Differentiation** (A.V.S. de Reuck and J. Knight, Eds.), John Wiley&Sons, Ltd., Chichester, UK, 1967, 100-101.
23. Chow, W.S. and Barber, J. Salt dependent changes of 9-aminoacridine fluorescence as a measure of charge densities of membrane surfaces. **J. Biochem. Biophys. Methods** 3 (1980) 173-185.
24. Geng, T., Zhan, Y., Wang, H.Y., Witting, S.R., Cornetta, K.G., Lu, C. Flow-through electroporation based on constant voltage for large-volume transfection of cells. **J. Control. Rel.** 144 (2010) 91-100. DOI: 10.1016/j.jconrel.2010.01.030.
25. Geng, T., Zhan, Y. and Lu, C. Gene delivery by microfluidic flow-through electroporation based on constant DC and AC field. **34th Annual International Conference of the IEEE EMBS**, San Diego, California USA, 28 August-1 September, 2012, 2579-2582. DOI: 10.1109/EMBC.2012.6346491.
26. Giotta, G.J., Chau, D.S. and Wang, H.H. Binding of local anesthetics to phosphatidylcholine and phosphatidylserine liposomes. **Arch. Biochem. Biophys.** 163 (1974) 453-458.
27. Craiu, A. and Scadden, D. Flow electroporation with pulsed electric fields for purging tumor cells. **Methods in Molecular Biology** 22, The Humana Press, Totowa, NJ, 2008, 301-310. DOI: 10.1007/978-1-59745-194-9_22.

28. Eppich, H.M., Foxall, R., Gaynor, K., Dombkowski, D., Miura, N. and Cheng, T. Pulsed electric fields for selection of hematopoietic cells and depletion of tumor cell contaminants. **Nat. Biotechnol.** 18 (2000) 882-887.
29. Sixou, S. and Teissié, J. Specific electropermeabilization of leukocytes in a blood sample and application to large volumes of cells. **Biochim. Biophys. Acta** 1028 (1990) 154-160.
30. Korohoda, W., Ambrose, E.J. and Forrester J.A. Some aspects of the dynamic characteristic of biological membranes. **Folia Biol.** 15 (1967) 371-393.
31. Korohoda, W., Forrester, J.A. Moreman, K.G. and Ambrose, E.J. Size changes in isolated nuclei of *Ameba proteus* on treatment with polyionic substances. **Nature** 217 (1968) 615-617.
32. Hanpft, R. and Mohr, K. Influence of cationic amphiphilic drugs on the phase-transition temperature of phospholipids with different polar groups. **Biochim. Biophys. Acta-Biomembranes** 814 (1985) 156-162.
33. Gingell, D. Contractile responses at the surface of an amphibian egg. **J. Embryol. Exp. Morphol.** 23 (1970) 583-609.
34. Korohoda, W. and Kurowska, A. Quantitative estimations of the thresholds of electrotactic responses in *Amoeba proteus*. **Acta Protozool.** 7 (1970) 375-382.
35. Korohoda, W., Mycielska, M., Janda, E. and Madeja, Z. Immediate and long-term galvanotactic responses of *Ameba proteus* to dc electric fields. **Cell Motil. Cytoskeleton** 45 (2000) 10-26.
36. Teissié, J. and Rols, M. Manipulation of cell cytoskeleton affects the lifetime of cell membrane electropermeabilization. **Ann N Y Acad. Sci.** 720 (1994) 98-110.
37. Korohoda, J. and Strzałka, K. High efficiency genetic transformation in maize induced by exogeneous DNA. **Z. Pflanzenphysiol.** 94 (1979) 95-99.
38. Madeja, Z., Rak, M., Wybieralska, E., Rożański, I., Masnyk, M., Chmielewski, M., Łysek, R., Chojnacki, T., Jankowski, W., Ciepichal, E., Świeżawska, E., Tekle, M. and Wallner, G. New cationic polyprenyl derivative proposed as a lipofecting agent. **Acta Biochim. Pol.** 54 (2007) 875-876.
39. Dougherty, T.J. An update on photodynamic therapy applications. **J. Clin. Laser Med. Surg.** 20 (2002) 3-7.
40. Nowak-Śliwińska, P., Karocki, A., Elas, M., Pawlak, A., Stochel, G. and Urbańska, K. Verteporfin, photofrin II, and merocyanine 540 as pDT photosensitizers against melanoma cells. **Biochim. Biophys. Res. Commun.** 349 (2006) 549-555.
41. Józwiak, Z. and Leyko, W. Role of membrane components in thermal injury and development of thermotolerance. **Int. J. Radiat. Biol.** 62 (1992) 743-56.
42. Kim, S.H., Kim, J.H., Alfieri, A.A., He, S.Q. and Young, C.W. Gossypol, a hyperthermic sensitizer of HeLa cells. **Cancer Res.** 45 (1985) 6338-6340.