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# Effect of electroporation and recovery medium pH on cell membrane permeabilization, cell survival and gene transfer efficiency *in vitro*

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ABSTRACT

Electroporation is a method which uses an adequate number of electric pulses of enough amplitude, duration and number applied to cells, thus inducing transient permeabilization of the cell membrane. Due to possibility that microenvironment in applications of *in vivo* electroporation is slightly acidic, we studied the effects of slightly acidic electroporation and recovery medium. We observed no difference in the permeabilization threshold, detected by propidium iodide, of cells which were electroporated and allowed to recover in growth (pH 7.8) or acidic (pH 6.5) medium. In contrast, statistically significant difference was observed in survival of cells that were exposed to pulse amplitudes greater than permeabilization threshold. Survival of cells was greater if acidic electroporation and recovery medium were used, but acidic extracellular pH decreased gene electrotransfer efficiency. We also observed differences in morphology between cells that were electroporated and left to recover in growth medium and cells that were electroporated and left to recover in acidic medium. Our results imply that slightly acidic extracellular pH allows more efficient repair of damage that is induced on cell membrane during electroporation with high pulse amplitudes.



## 1. Introduction

Electroporation also named electropermeabilization is a method in which the application of an appropriate number of electric pulses of sufficient amplitude and duration causes transient permeabilization of the cell membrane, allowing ions and molecules to enter and/or leave the cell. Electroporation can be used for treatment of tumors, since increased membrane permeability due to electroporation enables entering of chemotherapeutic drugs into tumor cells, as a method named electrochemotherapy (ECT) [1,2]. It can also be used as delivery method for a large variety of molecules such as ions [3], dyes [4], tracers [5], antibodies [6], and also oligonucleotides [7], RNA [8] and DNA [9–11]. Electroporation is used in medicine [12], as ECT [13], irreversible electroporation (IRE) as a method of tissue ablation [14–16], gene electrotransfer (GET) as gene therapy [17,18] and DNA vaccination [19,20], transdermal drug delivery [21,22], as well as in biotechnology [23] and food and biomass processing [24,25].

ECT is an extremely effective, physical technique for elimination of cutaneous and subcutaneous solid tumors and also deep-seated tumors [26]. Commonly used drugs for ECT are bleomycin and cisplatin. Low rate of side effects and low systemic toxicity are the advantage of ECT. Response rates of 77–87% have been reported with bleomycin [1]. The efficacy of ECT depends on a variety of factors, mostly of physical quantities, which should be chosen with care. Among those factors are electric field intensity, pulse duration and the number of pulses [27].

GET is a method of DNA delivery into cell nucleus in order to achieve therapeutic effect. For effective DNA delivery and expression several steps must be overcome, among which are membrane electroporation, DNA-membrane interaction, transfer of DNA into the cell, intracellular trafficking of DNA through cytosol and nuclear import of DNA [28]. With electrotransfered DNA there is a possibility of correction of a defective gene by silencing it or with its functional replacement, electrotransfered DNA can encode a therapeutic protein or a protein that induces cell death [29]. GET efficiency depends on several factors such as electrophoretic movement of the plasmid [28,30,31], plasmid concentration [32] and pH changes [33] since medium pH can affect plasmid stability [34]. GET efficiency also highly depends on tissue type. GET in muscle can achieve high percentage of transfected cells [10] while tumors are extremely hard to transfect [11]. *In vitro* GET of tumor cells is efficient, however gene expression in tumor cells *in vivo* is weak. GET in tumors *in vivo* usually results in only a few percent of transfected cells [11,35].

One of major differences between tumor cells and surrounding normal cells is the nutritional and metabolic environment. Microenvironment in tumors tend to be acidic (pHe: 6.2–6.9) due to the overproduction of lactate, while the intracellular pH (pHi: 7.1–7.6) may remain neutral or become alkaline due to compensation mechanisms. In contrast, normal cells pHe tends to be slightly or highly alkaline (7.3–7.7) and while pHi is a little bit lower (6.9–7.2) [36].

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Regulation of pHi is one of the most important physiological functions of homeostasis, because activity of most chemical reactions *via* enzyme proteins is dependent on pH. Membrane proton pumps and transporters whose activity is controlled by intra-cytoplasmic pH sensors maintain pHi within narrow range. Intra-cytoplasmic pH sensors recognize changes in pHi and induce cellular responses to maintain the pHi, often at the expense of acidifying the pHe. On the other hand, pHe acidification impacts cells *via* specific acid-sensing ion channels and proton-sensing G-protein coupled receptors [37]. During electroporation, increase in plasma membrane permeability leads to equilibration of pH between pHe and pHi [38].

Due to acidified pHe and alkalinized pHi the reversal of pH gradients across plasma membrane is present in many tumors and is becoming one of the most significant and selective hallmarks of cancer [39]. The extracellular pH can affect numerous biological functions, such as endocytosis [40,41], lysosomal trafficking [42], gene expression [43–45], proliferation and viability [46].

Most *in vitro* electroporation and GET experiments are done in electroporation medium or growth medium with pH 7.4, while extracellular pH at least in tumors *in vivo* is acidic. In this study we investigated if acidic electroporation and recovery medium have any effect on cell processes during electroporation and GET and consequently could have an impact on effectiveness of ECT and GET. We performed electroporation and GET experiments *in vitro* in electroporation buffers of different acidity to test the effects of acidic conditions in *in vitro* experiments which however differs from extracellular composition in *in vivo* treatments. Electroporation and GET were performed in growth medium which is an approximation of the *in vivo* extracellular fluid. However, there are many more factors *in vivo* that cannot be taken in account *in vitro* such as, blood supply, immune system, electric field shielding, cell crowding. In this study we focused on effects of electroporation and recovery medium pH on membrane permeabilization, cell viability and recovery after electroporation, and GET efficiency.

## 2. Materials and methods

### 2.1. Cells

Chinese hamster ovary cell line (CHO-K1; European Collection of Cell Cultures, Great Britain) was used in our experiments. Cells were grown in 150 $mm^2$ culture flasks (TPP, Switzerland) for 2–4 days in an incubator (Kambič, Slovenia) at 37 °C, in a humid atmosphere of 5% $CO_2$ in air. CHO cells were cultured in HAM-F12 growth medium (PAA, Austria) supplemented with 10% fetal bovine serum (Sigma Aldrich, Germany), L-glutamine (StemCell, Canada) and antibiotics penicillin/streptomycin (PAA, Austria), and gentamycin (Sigma Aldrich, Germany).

Cell suspension for experiments was prepared from cells in exponential growth phase by trypsinization using trypsin – EDTA (5 g trypsin/2 g EDTA in 0.9% NaCl; SigmaAldrich) 10 x diluted in Hanks' Balanced Salt solution (Sigma-Aldrich). From the obtained cell suspension, trypsin and growth medium were removed by centrifugation at 270 RCF for 5 min at 4 °C (Sigma 3–15 K, UK). The cell pellet was resuspended in cell growth medium to obtain a final cell density of $2 \times 10^7$ cells/ml.

### 2.2. Medium pH

The pH of cell growth medium was lowered using acetic acid or HCl. HAM-F12 growth medium (G) at room temperature had the pH of 7.8. pH was lowered to 6.72, 6.38, 6.13 and 5.47 by adding 0.021%, 0.033%, 0.042% and 0.066% weight of acetic acid respectively and to pH 7.3, 7.0, 6.5 and 5.9 by adding 0.0004%, 0.0008%, 0.0016% and 0.0032% weight of HCl respectively. The interim pH values were calculated with cross calculation, since pH in this range was dropping in a linear way. pH was measured using SevenGo-SG2 (Mettler-Toledo) pH meter and measuring electrode Inlab Routine Pro (Mettler-Toledo). Medium with the pH of 6.5 was used as an acidic medium (A) as pH 6.5 is an approximation of tumor pHe.

Conductivity of acidic medium was measured with conductometer (MA 5950, Metrel). Conductivity of acidic (pH 6.5) medium was 14.52 mS/cm and conductivity of growth medium (pH 7.8) was 14.24 mS/cm.

### 2.3. Cytotoxicity

We exposed CHO cells to medium with different pH values (lowered either with acetic acid or HCl) for 24 h. Cell survival was determined with the MTS-based Cell Titer 96 AQueous One Solution Cell Proliferation Assay (Promega, USA). Absorption at 490 nm wavelength (A490) was measured with a Tecan Infinite M200 spectrophotometer (Tecan, Switzerland). An average absorption obtained in the samples containing only growth medium was subtracted from the absorption measured in cell samples. To calculate the percentage of viable cells the absorption of each sample was divided by an average absorption of the control samples.

### 2.4. Electroporation

Four combinations of electroporation and recovery medium were tested: cells were electroporated in growth medium and recovered in growth medium (GG), cells were electroporated in growth medium and recovered in acidic medium (GA), cells were electroporated in acidic medium and recovered in growth medium (AG) and cells were electroporated in acidic medium and recovered in acidic medium (AA).

30 μl of cell suspension was mixed with 30 μl of double concentrated acidic medium or 30 μl of growth medium. A drop of prepared cell suspension (50 μl) was pipetted between two parallel stainless steel electrodes with the distance between them being 2 mm. The cell sample-electrodes contact surface was not measured. It can be estimated from the equation for the volume of the cylinder with height 2 mm (the distance between electrodes) to be 25 $mm^2$; *i.e.* surface = 50 μl (*i.e.* volume of the sample)/(2 mm) = 25 $mm^2$. The total electrode surface is 200 $mm^2$ = 10 mm × 20 mm. Therefore the ratio of the contact surface in relation to the total electrode surface is 1:8. All the cells were thus exposed to approximately the same electric field, which was estimated as the voltage applied divided by the distance between the electrodes. Cells were exposed to a train of eight rectangular electric pulses with 100 μs duration and 1 Hz pulse repetition frequency, generated by Betatech electroporator (Electro cell B10; Betatech, France) for permeabilization experiments where lower voltages were applied, and a laboratory prototype pulse generator [47] for applying higher voltages for cell survival experiments. The amplitude of the pulses and consequently the applied electric field was varied from 0 to 3 kV/cm. During each experiment voltage and current applied to sample were measured with differential probe (ADP305, LeCroy, USA) and current probe (CP030, LeCroy, USA) and monitored on oscilloscope (Wavesurfer 422, 200 MHz, LeCroy, USA). At the same applied voltage, measured current did not differ between samples in acidic and growth medium. Also, the pulse generators produced the same current at the same applied voltage. The electrodes were washed with sterile 0.9% NaCl and dried with sterile gauze between electroporated samples. After electroporation cells were left for 10 min to recover either in growth or in acidic medium at room temperature.

### 2.5. Cell viability

After pulse application as previously described, 40 μl of cell suspension was transferred into a 1.5 ml microcentrifuge tube containing 40 μl of growth medium or double concentrated acidic medium and left for 10 min at room temperature to allow for cell membrane resealing. Then, 20 μl of cell suspension ($10^5$ cells) was transferred in 80 μl of growth medium prepared previously in each well of 96-well plates

(TPP, Switzerland). Three samples were taken from each electroporated droplet. Cells were placed in the incubator (37 °C, 5% $CO_2$) for 24 h. Cell viability was measured using the MTS-based Cell Titer 96 AQueous One Solution Cell Proliferation Assay (Promega, USA) as described above. To calculate the percentage of viable cells after electroporation the absorption of each sample was divided by an average absorption of the control samples, *i.e.* the samples that were exposed to electric field of 0 V/cm.

### 2.6. Fluorescence microscopy

Cell recovery after electroporation was followed by adding propidium iodide (PI; 5 μl of 1.5 mM) immediately before pulse application or 5 min after it. Again four combinations of electroporation and recovery medium were tested: GG, GA, AG and AA. Cells were electroporated according to above described protocol. Cell suspension of CHO cells between the electrodes was exposed to electric field of 2.4 kV/cm. After pulse application, 40 μl of cell suspension was transferred into a small petri dish where 320 μl of recovery medium was prepared. If PI was added before application of electric pulses, the sample was left for 10 min at room temperature prior to the capture of images. If PI was not added before application of electric pulses, the sample was left for 5 min, then PI was added and the images were captured after additional 10 min. The phase contrast and fluorescent images (Ex 562/40, Em 624/40) of the treated cells were captured using cooled CCD camera (Visicam 1280, Visitron, Puchheim, Germany) mounted on a fluorescence microscope (Zeiss AxioVert 200, objective 20×, Zeiss, Oberkochen, Germany) using MetaMorph 7.0 software (Molecular Devices Corporation, Sunnyvale, CA, USA), exposure time 100 ms. For each sample, three phase contrast and corresponding fluorescent images of a distinct area were acquired. The number of electroporated cells was determined by manually counting the cells in fluorescence and phase contrast images. The percentage of electroporated cells in a given sample was determined as the ratio between the average number of fluorescent cells counted in the fluorescence images and the average number of all cells counted in the corresponding phase contrast images.

### 2.7. Light microscopy

Using light microscopy cell morphology changes were observed after electroporation with electric field of 1.8 kV/cm, under 100 x oil immersion objective (Zeiss AxioVert 200, 100 x oil immersion objective, Zeiss, Oberkochen, Germany). Cells electroporated and recovered in AA and GG combinations were observed during recovery (6 min after electroporation) and after recovery (11 min after electroporation). Images were acquired using the VisiCam 1280 camera (Visitron, Germany) and the MetaMorph PC software (Molecular Devices, USA).

### 2.8. Flow cytometry

Cells were electroporated as described above in four combinations of growth and acidic medium (pH 6.5); AA, AG, GA and GG. Immediately before electroporation 5 μl of PI was added to cell suspension. After electroporation cells were left to recover for 10 min in 100 μl of growth or acidic medium. Incorporation of PI in cells was detected using flow cytometer (Attune® NxT, Life Technologies, Carlsbad, CA, USA). Cells were excited with a blue laser at 488 nm, and the emitted fluorescence was detected through a 574/26 nm band-pass filter. The measurement ended when 10,000 events were recorded. Obtained data was analyzed using the Attune NxT software.

### 2.9. Gene electrotransfer

$5 \times 10^4$ cells were seeded in 24-well plates as a monolayer culture at 37 °C in a humidified atmosphere in a 5% $CO_2$ incubator 24 h before GET. Prior to electric pulses delivery growth medium was removed and 150 μl of growth or acidic medium with plasmid pEGFP-N1 (10 μg/ml) (Clontech Laboratories Inc., MountainView, CA, USA) encoding green fluorescent protein (GFP) was added to cells. Cells were incubated with plasmid for 2 min, then electric pulses were applied using two parallel stainless steel wire (diameter of 1 mm) electrodes with a length of 10 mm which were 4 mm apart. Three different pulse protocols were tested; Protocol 1: 8 pulses, 100 μs, 1 Hz and 1.3 kV/cm; Protocol 2: 4 pulses, 200 μs, 1 Hz and 1.2 kV/cm, and Protocol 3: 4 pulses, 1 ms, 1 Hz and 0.8 kV/cm. Pulses were generated by Betatech electroporator (Electro cell B10, France). Immediately after electric pulses delivery 150 μl of medium with pEGFP-N1 was removed and replaced with 150 μl of growth or acidic medium with 25% of fetal bovine serum (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany). Cells were incubated for 10 min at 37 °C in a humidified atmosphere in a 5% $CO_2$ incubator. Then 1 ml of growth medium was added and cells were incubated for 24 h at 37 °C in a humidified atmosphere in a 5% $CO_2$ incubator. Three images per well on an area between electrodes were recorded using a fluorescent microscope (Zeiss 200; Axiovert) with excitation wavelength 488 nm, emitted fluorescence through a bandpass filter 525/50 nm, and counted by ImageJ program for image analysis. Transfection rate was determined as a number of GFP positive cells divided by all cells in each image, expressed in %.

### 2.10. Statistical analysis

All results are reported as a mean value of 3 to 5 experiments. The spread of the data is given by standard deviation. The significance between the experimental groups was analyzed in SigmaPlot 11.0 (Systat Software Inc., Chicago, IL, USA), and determined using Two way ANOVA test. The statistically significant difference was indicated by $p < .05$.

## 3. Results

### 3.1. Cytotoxicity of acidic medium

According to MTS assay, exposure of CHO cells to medium with pH values of 7.3, 7.0, 6.5 and 5.9, lowered with adding acetic acid or HCl, affects cell viability. After 24 h incubation in the medium with pH of 5.9, lowered with adding HCl, 86.5 ± 5.9% of cells are viable, using shorter incubation times or medium with higher pH the survival of the cells is even better. With lowering medium pH with acetic acid we obtained similar results (data not shown). We tested HCl and acetic acid to see if there is any difference in cytotoxicity of acidic medium depending on the acid used for acidification. We observed no difference in cell survival between the two acids used.

### 3.2. Cell viability after electroporation

The viability of CHO cells following electroporation with different combinations of electroporation and recovery medium is presented in Fig. 1. We observed statistically significant correlation between applied electric field and electroporation and recovery medium pH. CHO cells survival after electroporation at 1.2 kV/cm, 1.8 kV/cm and 2.4 kV/cm was better when cells were electroporated and recovered in acidic medium (AA) compared to survival of cells that were electroporated and recovered in growth medium (GG). The biggest difference was observed after electroporation at 1.8 kV/cm. Survival of cells that were treated with AA combination was 34% better than survival of cells that were treated with GG combination. Following electroporation with AA combination 73.4 ± 7.9% cells is viable in contrast to only 39.1 ± 23.4% of cells treated with GG combination ($p = .047$). Cell viability of GA treatment (48.1 ± 5.7%) is also lower compared to AA treatment ($p = .011$). When electric pulses with 2.4 kV/cm are applied, different pH of electroporation and recovery medium used caused smaller variations in cell viability. Again the highest survival was observed with AA combination (31.8 ± 5.2%)

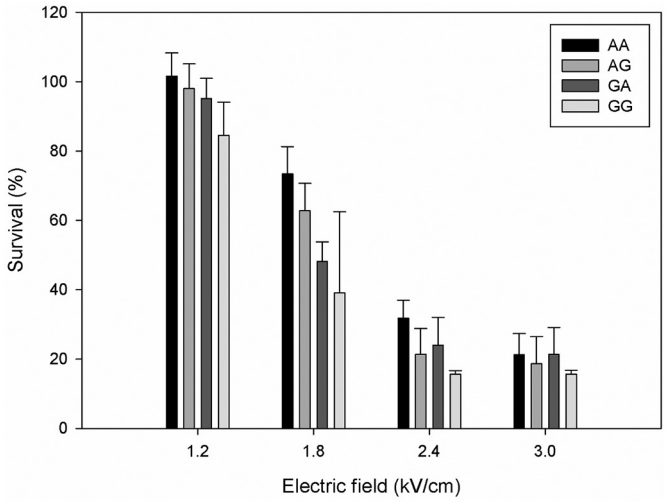


**Fig. 1.** Viability of CHO cells after electroporation with growth or acidic electroporation and recovery medium. Vertical bars represent standard deviation.

followed by GA, AG and GG combination (24.0 ± 8.0%, 21.4 ± 7.4% and 15.6 ± 1.0%, respectively). Survival of CHO cells at 3 kV/cm is low – around 20% - regardless of electroporation and recovery medium pH. With all four tested electric field strengths cell viability was always the highest with AA and the lowest with GG combination used. Results also show that values of applied electric field >1.8 kV/cm, especially 2.4 kV/cm and 3.0 kV/cm seem to approach IRE.

### 3.3. Light microscopy

When cells were exposed to growth or acidic medium without electroporation we did not observe any differences in cell morphology. There was no morphological differences between cells in growth or acidic medium after 6 or 11 min of observation. Small membrane blebs can be seen on the cell surface in either growth or acidic medium (Fig. 2). Membrane blebs are rounded membrane protrusions caused by the detachment of the lipid bilayer from the underlying cytoskeleton. Typically membrane blebs are viewed as a sign of apoptosis, but they are often observed also during the life cycle of intact cells [48], during cytokinesis [49], migration [50], and during cell detachment and spreading [51]. Short-lived membrane blebs were also observed when cells underwent spin/wash cycles and media changes [52]. Membrane blebs as can be seen in Fig. 2 are familiar features of the initial spreading process [53]. In contrast cells that were electroporated show extensive bleb formation (Fig. 3). Blebs can grow several seconds or minutes, while remaining attached to the cell [52]. Cells electroporated with GG combination had more membrane blebs that formed faster, had larger diameter and were more stable (Fig. 3; marked with arrows) than membrane blebs formed after electroporation with AA protocol. Many blebs that formed after electroporation with AA protocol already retracted in first 6 min after electroporation.

### 3.4. Permeabilization

Using flow cytometry we measured permeabilization level (PI uptake) of cells exposed to various electric field strengths. Again we used

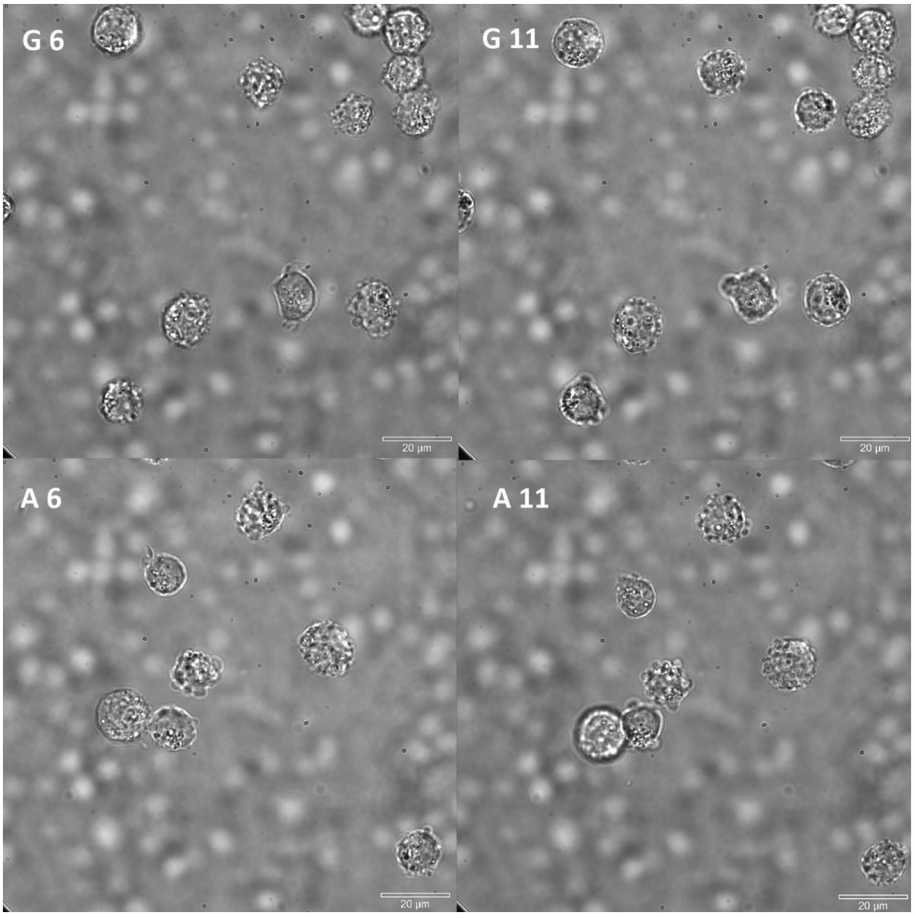


**Fig. 2.** CHO cells under light microscope, 100× oil immersion objective. G 6 – cells in growth medium after 6 min of observation, G 11 – cells in growth medium after 11 min of observation, A 6 – cells in acidic medium after 6 min of observation, A 11 – cells in acidic medium after 11 min of observation.

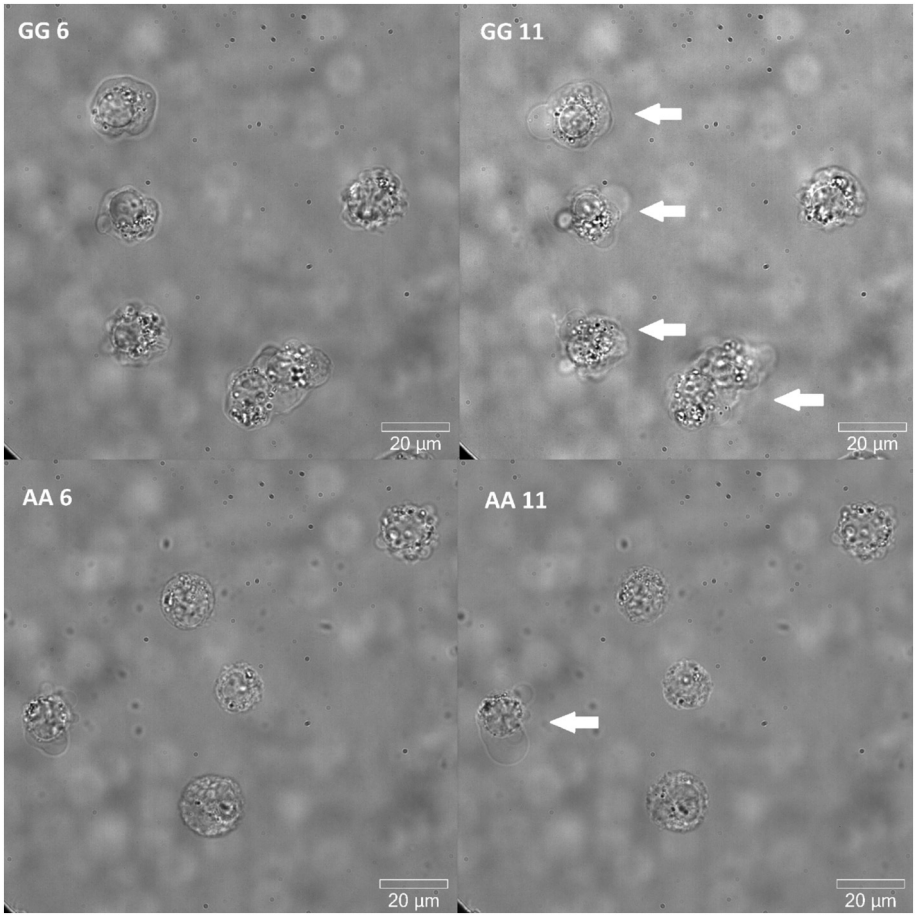


**Fig. 3.** CHO cells under light microscope, 100× oil immersion objective. Cells were electroporated with 1.8 kV/cm. GG 6 – cells electroporated with GG combination 6 min after electroporation, GG 11 – cells electroporated with GG combination 11 min after electroporation, AA 6– cells electroporated with AA combination 6 min after electroporation, AA 11 – cells electroporated with AA combination 11 min after electroporation.

AA, AG, GA and GG combinations of electroporation and recovery medium pH values. Percent of PI containing cells is presented in Fig. 4. Results show that electroporation and recovery medium pH value had no effect on percent of permeabilized CHO cells nor on their fluorescence intensity (data not shown). We observed no difference in permeabilization at any electric field strength applied (0–1.4 kV/cm) and at any combination of electroporation and recovery medium pH used.

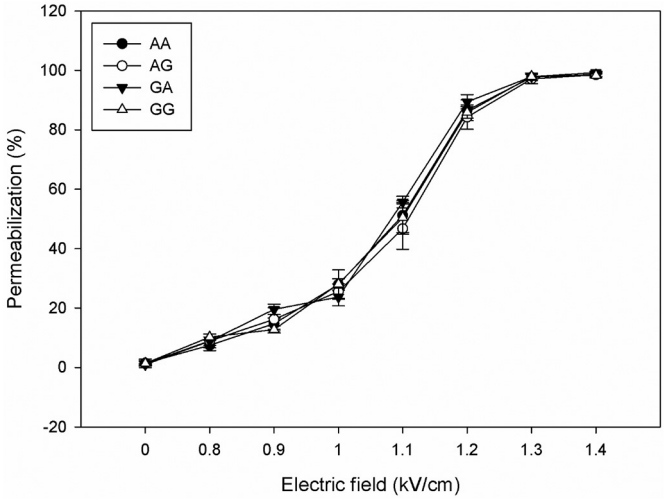


**Fig. 4.** PI uptake in CHO cell line after electroporation with growth or acidic electroporation and recovery medium. Vertical bars represent standard deviation.

### 3.5. Recovery of cells after electroporation

In these experiments, PI was added immediately before (0) exposure to electric pulses or 5 min after it. Since we wanted to test cell membrane repair capacity of cells we choose electric field strength at which all the cells were permeabilized *i.e.* > 1.4 kV/cm (Fig. 4) and also decreased cell survival was observed (Fig. 1). In cell viability experiments we observed a slight difference, although not significant, in survival of cells after electroporation at 2.4 kV/cm. Based on this results we choose electric field of 2.4 kV/cm for our cell recovery experiments. The percentages of CHO cells, which were exposed to electric field of 2.4 kV/cm in growth or acidic cell medium, with incorporated PI are shown in Fig. 5. If PI was added before exposure to electric pulses, the acidity of electroporation medium and acidity of recovery medium did not affect the percentage of fluorescent cells significantly which is consistent with permeabilization assay (Fig. 4). If exposure of the cells to electric pulses and recovery were done in growth medium, the cells did not recover within 5 min time interval after electroporation. Namely the percentage of fluorescent cells remained 66.6 ± 19.1% also if PI was added 5 min after exposure to electric pulses. The graph in Fig. 5 shows that recovery after exposure to electric pulses is much better in acidic medium than in growth medium, independent of whether electroporation is done in growth or acidic medium. This can be seen in cells in which PI was added 5 min after exposure to electric pulses and later recovered in acidic medium. Here, only 4.8 ± 2.3% of cells electroporated in growth and 4.7 ± 3.1% of cells electroporated in acidic medium, incorporated PI. If cells were exposed to electric pulses in acidic medium and recovery was proceeded in growth medium, 39.0 ± 6.1% of cells were still

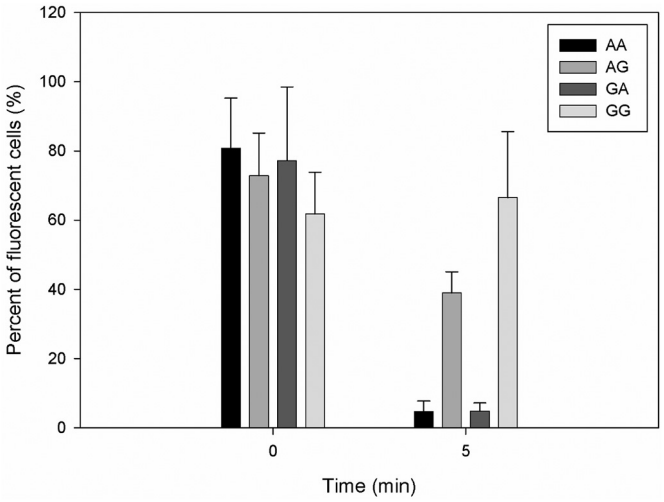


**Fig. 5.** Percentage of flourescent CHO cells after electroporation at 2.4 kV/cm and recovery in growth or acidic medium. Vertical bars represent standard deviation.

unrecovered 5 min after exposure to electric pulses. When comparing percentage of fluorescent cells with PI added 5 min after electroporation statistically significant differences are observed between all pairs except GA-AA pair (AA-GG: $p < .001$; GG-GA: $p < .001$; GG-AG: $p = .023$; AG-AA: $p = .002$; AG-GA: $p = .002$).

### 3.6. Gene electrotransfer (GET)

Same as electroporation experiments, also GET was performed in AA, AG, GA and GG combinations of electroporation and recovery medium. We tested three different pulse protocols; protocol 1 (8 pulses, 100 μs, 1 Hz and 1.3 kV/cm) for comparison with electroporation experiments and two pulse protocols with longer pulses, which are reported in the literature to be used for GET; protocol 2 (4 pulses, 200 μs, 1 Hz and 1.2 kV/cm) and protocol 3 (4 pulses, 1 ms, 1 Hz and 0.8 kV/cm) (Fig. 6).

With all three pulse protocols the lowest percentage of transfected cells was obtained when electroporation and recovery were done in acidic medium (AA) (Protocol 1: 9.5 ± 1.3%; Protocol 2: 7.3 ± 2.7%; Protocol 3: 11.1 ± 5.9%) (Figs. 7 and 8). The highest percentage of transfected cells was obtained when electroporation and recovery were done in growth medium (GG) (Protocol 1: 33.7 ± 5.2%; Protocol 2: 23.7 ± 5.6%) except for pulse protocol 3 where the highest percentage of transfected cells was obtained with GA combination (26.8 ± 10.5%), however not statistically significant. When comparing different electroporation and recovery medium combinations statistically significantly higher transfection was achieved with GG combination compared to AA for pulse protocols 1 ($p < .001$) and 2 ($p = .001$). In these two pulse protocols transfection was also statistically significantly higher with AG (Protocol 1: $p = .007$; Protocol 2: $p = .049$) and GA (Protocol 1: $p = .003$; Protocol 2: $p = .021$) combinations compared with AA combination. Pulse protocol 3 gave statistically significant different result only between GG and AA combinations ($p = .014$), again GG being higher than AA. We observed no statistically significant difference in GET efficiency among the same electroporation and recovery medium combinations between three tested pulse protocols. In agreement with results presented in Fig. 1 no statistical differences in cell survival after GET with all three pulse protocols (electric field: 0.8, 1.2 and 1.3 kV/cm) among different electroporation and recovery medium combinations were observed (data not shown).

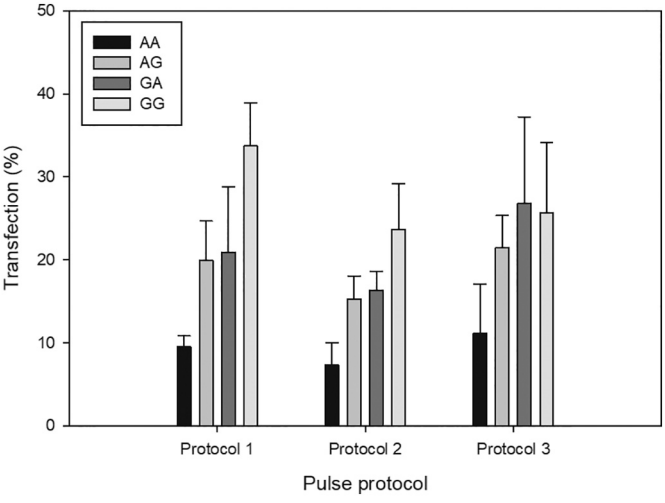


**Fig. 6.** Percentage of transfected CHO cells after GET with different pulse protocols (Protocol 1: 8 pulses, 100 μs, 1 Hz and 1.3 kV/cm; Protocol 2: 4 pulses, 200 μs, 1 Hz and 1.2 kV/cm, and Protocol 3: 4 pulses, 1 ms, 1 Hz and 0.8 kV/cm) and recovery in growth or acidic medium. Vertical bars represent standard deviation.

## 4. Discussion

*In vitro* electroporation and GET experiments are most often performed in medium with neutral or slightly alkaline pH. Since extracellular environment of most tumors is acidic, applications of electroporation *in vivo* are thus done in acidic conditions. With the goal of most effective transfer of findings *in vitro* to *in vivo* treatments, we investigated if acidic electroporation and recovery medium have any effect on cell membrane permeabilization, cell survival after electroporation and on GET efficiency.

Our results show that electroporation and recovery medium pH had no effect on CHO cell membrane permeabilization threshold. The similarity of PI uptake curves implies that induced transmembrane voltage and accompanying process of pore formation are not affected by acidity of electroporation or recovery media. However, we observed differences in cell viability depending on the pH of electroporation and recovery medium. Cells electroporated and recovered in acidic medium had significantly higher percentage of survival compared to cells electroporated and recovered in growth medium. When electroporation was done in medium with different pH than the pH of the medium in which cells recovered (GA and AG combinations), the differences in survival were less pronounced, although cells exposed to AG combination had higher survival, which might suggest that electroporation in acidic medium allows better survival. Medium pH had small influence on cell viability if low or very high electric fields were applied (Fig. 1). With fluorescence microscopy we observed that in growth medium less cells completely recovered within 5 min than in acidic medium. (Fig. 5), which implies that in cells recovering in growth medium cell membrane resealing is slower. Electroporation and recovery medium pH also has effect in GET efficiency. Acidic electroporation and recovery medium (AA combination) significantly decreased GET efficiency compared to growth electroporation and recovery medium (GG combination). When GET was performed in medium with different pH than the pH of the medium in which cells recovered (GA and AG combinations) GET efficiency was lower than with GG combination and higher than with AA combination. No differences in GET efficiency between GA and AG combinations were observed.

Electric pulses trigger different processes in cells and on their membranes. Apparently these processes depend on characteristics of electroporation and recovery medium and can influence the response of cells to applied electric field. Pulse parameters for efficient electroporation treatments are determined on the basis of results obtained in *in vitro* experiments which are mostly performed in selected electroporation buffers. Electroporation medium composition can have a profound effect on electroporation effectiveness [54]. There are contradictory reports of medium composition and conductivity effects on cell membrane permeabilization

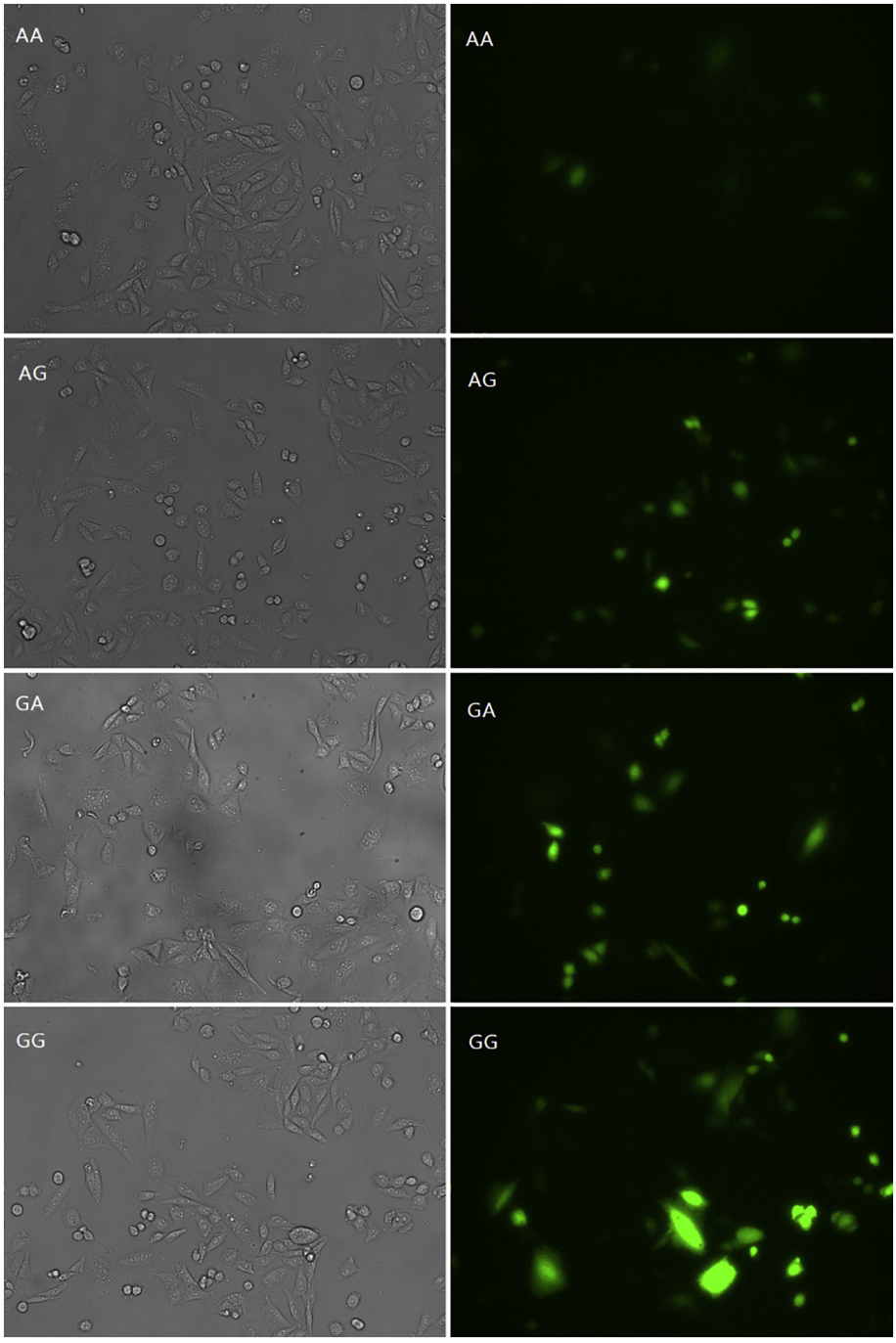


**Fig. 7.** Bright field images (left) and fluorescence images (right) of CHO cells 24 h after GET with pulse protocol 1 (8 pulses, 100 μs, 1 Hz and 1.3 kV/cm) with electroporation and recovery in growth or acidic medium.

and resealing. In some studies, increasing the ionic strength of the medium caused cell membrane electroporation at lower electric field intensities [55] in others, at the unchanged medium conductivity, ionic composition and strength of the medium had almost no effect on electroporation but, when medium conductivity was decreased, increased electroporation efficiency was observed [56]. On the contrary, the resealing of the membrane was independent of medium ionic composition or conductivity [57]. Electroporation medium osmolarity also has an effect on electroporation. In a hypertonic medium, cells are permeabilized at a lower voltage than cells in isotonic medium. In contrast, increasing the osmotic pressure of the recovery medium (hypertonic) facilitates the resealing of electroporated cells [58].

Higher survival of cells electroporated and recovered in acidic medium can be a consequence of more efficient pore resealing in acidic environment. Membrane repair process is started with $Ca2+$ influx through plasma membrane within <30 s after injury. Resealing involves exocytosis of lysosomes followed by massive endocytosis. Endocytosis and injury removal are triggered by extracellular activity of the lysosomal enzyme acidic sphingomyelinase [59,60]. Glunde et al. (2003) showed that in human mammary epithelial cells (HMECs) and breast cancer cells of different degrees of malignancy acidic pHe values of pH 6.8 and pH 6.4 cause a significant displacement of lysosomes from the perinuclear region to the cell periphery. They also observed higher number of lysosomes in cells exposed to extracellular acidity [42]. It was demonstrated that exposure of the

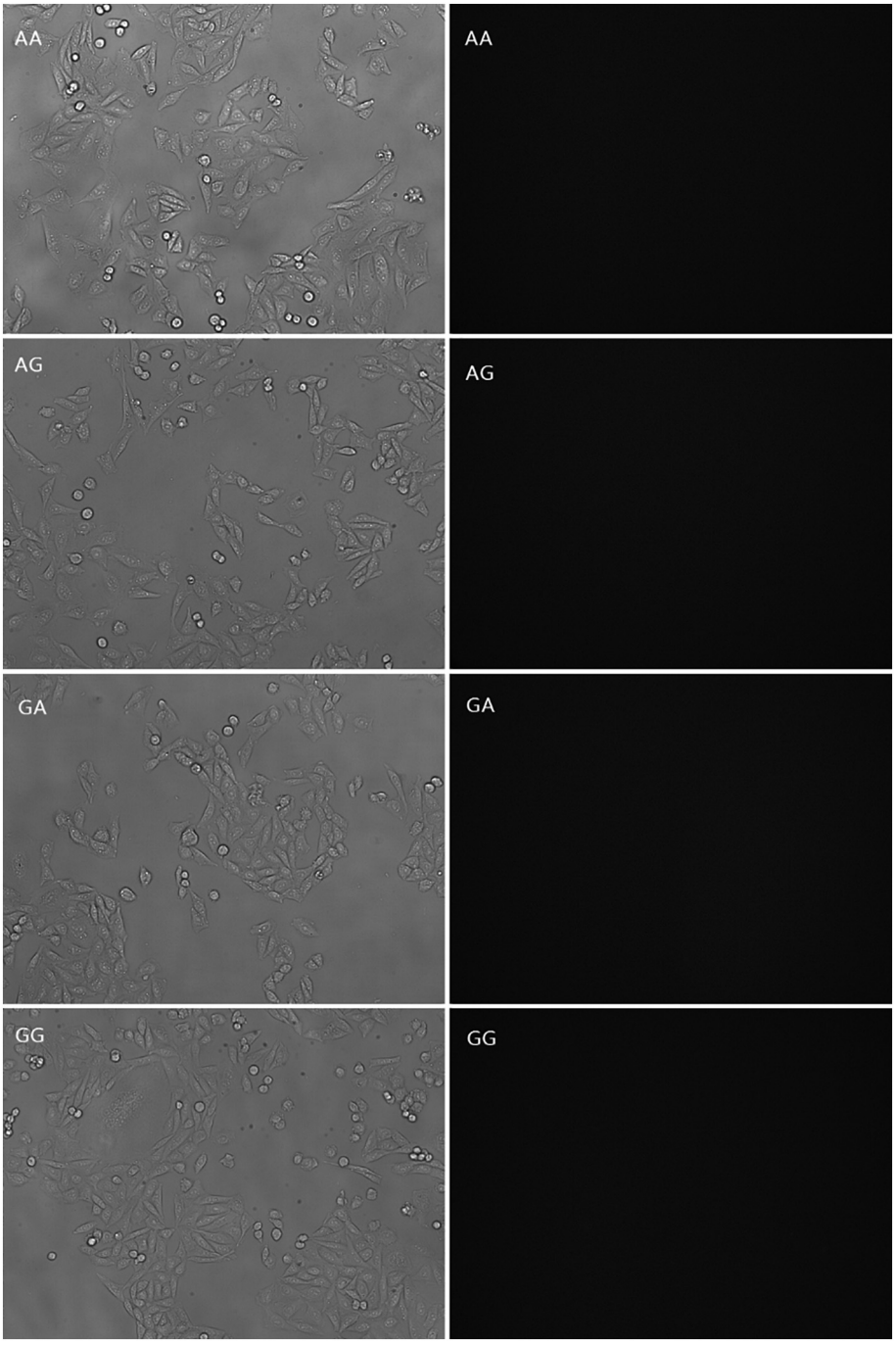


**Fig. 8.** Bright field images (left) and fluorescence images (right) of control CHO cells 24 h after exposure to growth or acidic electroporation and recovery medium without pulse application.

cell surface to a high concentration of protons stimulates the formation of inward membrane invaginations and vesicles, accompanied by an enhanced uptake of macromolecules [40,41]. Membrane invaginations and displacement of lysosomes from the perinuclear region to the cell periphery, driven by extracellular acidosis, could increase exocytosis of lysosomes and thus facilitate faster and more efficient plasma membrane damage repair which enables better cell survival in acidic environment. [61] showed that cells which are adapted to grow in acidic conditions express higher number of lysosomal proteins. The most upregulated protein was LAMP2 which protects lysosomal membranes from acid proteolysis. Interestingly more LAMP2 protein was found in cell plasma membrane compared to lysosomal membranes, which is in contrast with cells that grow in neutral pH. Higher expression of LAMP2 protein in cell plasma membrane triggered by acidosis was confirmed *in vitro*, *in vivo* and in patient samples. Increased number of LAMP 2 proteins in plasma membrane could thus present an advantage in cell membrane repair after electroporation in acidic environment [61].

We can observe characteristic changes in cell appearance after electroporation with 1.8 kV/cm as seen in images of cells under light microscope. Cells exposed to electric pulses show signs of granulation, loss of cell membrane integrity and long lasting membrane blebs, which could be regarded as signs of cell death. Described changes appear more often and are more pronounced in cells that were electroporated and recovered in growth medium (GG combination) compared to cells that were electroporated and left to recover in acidic medium (AA

combination) (Fig. 3). Primarily, cells electroporated in AA combination exhibit less long lasting huge membrane blebs.

It is proposed that blebbing is a by-product of electric field induced cell damage. Blebs can grow several seconds or minutes and they can reach sizes comparable to the size of the cell [52,62]. Blebs are initiated by a local disruption in the proteins that link the membrane to the cytoskeleton or local rupture of the cortex, a thin layer of the cytoskeleton directly beneath the membrane. Locally decreased pressure results in flow of the cytosol toward the area of detachment and local expansion of the cell membrane [63]. The hydrostatic pressure drops as the blebs expand. Therefore blebbing is possibly a primary self-protection process because it can rapidly releases the stress inside cells and prevents the immediate cell lysis. If the cortex gradually reassembles at the bleb membrane, the bleb retraction occurs [52]. Experimental observations were related to mathematical models of membrane blebs dynamics, but detailed mechanisms of bleb expansion and retraction are still unclear [49,64].

Sonoporation experiments performed on a site-specific basis *via* the synergized use of targeted microbubbles and single-shot ultrasound exposure showed that, membrane blebbing occurs at the sonoporation site after membrane initial resealing [65]. Although membrane integrity may be restored within tens of seconds, cytoskeleton disassembly may persist, and promotes bleb formation. Blebs were also observed at other places along the membrane periphery, because sonoporation-induced cytoskeleton disruption may not necessarily be a localized phenomenon and may propagate to the entire cell over time [66]. Moreover, it was proved that blebs physically serve as a buffer compartment to accommodate the high cytoplasmic $Ca^{2+}$ level caused by an influx of extracellular $Ca^{2+}$ due to cell membrane permeabilization [65]. If the cell membrane was permeabilized in $Ca^{2+}$ depleted media, no blebs were formed on the cell membrane, substantiating the essential role of $Ca^{2+}$ influx during cell membrane permeabilization in the membrane blebbing response.

According to our results less blebs were formed on the cells that were electroporated and left to recover in acidic medium (AA combination). It has been shown that higher number of lysosomes is present in cell periphery in acidic extracellular media and that lysosomes are recruited in the sites of injuries in a $Ca^{2+}$-dependent fashion. Based on this, lysosomes could be involved in the early stage of the blebbing processes by fusion with the cell membrane, inducing compensatory endocytosis and internalization of the injured membrane. These observations are in agreement with our results obtained with MTS assay, showing higher survival of cells that were electroporated and recovered in acidic medium (Fig. 1). It is also known that resealing of small membrane defects is faster, less energy demanding and has less negative effects on cell survival [54].

Our results show that for standard ECT pulse protocol (ESOPE pulse protocol; 8 pulses, 100 μs, 1 Hz) the effect of acidic pHe present in tumors is most likely irrelevant at lower electric field strengths. At 1.2 kV/cm we observed only small differences in cell survival between acidic and growth electroporation and recovery medium. However better survival of cells that were electroporated and recovered in acidic medium observed at 1.8 kV/cm and 2.4 kV/cm (Fig. 1) imply that acidic pHe should be considered when applying IRE. Our results show that tissueswhich have acidic pHe such as tumors, might need higher voltages applied during IRE to successfully achieve ablation.

According to our results of PI uptake measured with flow cytometry, electroporation and recovery medium pH had no effect on membrane permeabilization, since PI uptake was almost identical in all four combinations of electroporation and recovery medium pH used (Fig. 4).

In studies on effect of medium pH on process of electrofusion it was shown that the optimal pH for cell fusion is around pH 7.5. Fusion yield was higher at medium pH 7.5 and the average number of cells within a fusion chain was also larger compared to medium with lower pH. The fusion yield dropped by 40% when the pH was lowered from 7.5 to 6.0 [67]. There could be connection between lower fusion rates observed in acidic medium and faster membrane resealing and less defects formed on cell membrane after electroporation in acidic medium observed in our experiments.

In literature only the effects of pH fronts that form between electrodes during GET were studied. It was shown that pH fronts are the main reason for tissue damage observed after GET near electrodes [33]. Therefore we excluded cells that were near electrodes and analyze only cells in the middle between the electrodes. In our experiments we primarily studied GET in acidic extracellular medium which is present in numerus tumors. We observed decreased GET efficiency in acidic electroporation and recovery medium (AA) with all three pulse protocols tested (Fig. 6). The level of permeabilization as shown in Fig. 4 is the same in all medium pH combinations for all three pulse protocols. It is possible that acidic pH reduces net DNA negative charge and decreases electrophoretic movement of DNA [68] which in tumors *in vivo* is already low due to dense extracellular matrix. This hypothesis is supported also by lower GET efficiency of AG combination compared to GG combination and higher GET efficiency of GA compared to AA combination. Acidic pH also reduces endocytosis [69] which is one of proposed mechanism of DNA entry into cells during GET [70]. We did not observe any significant difference in GET efficiency between GG and GA combinations, however GET efficiency was higher with AG compared to AA combination using protocols 1 and 2. Since no difference in GET efficiency between GA and AG combinations was observed, we assume that acidic pH effects are not limited to one, but may affect several of the steps involved in GET. Although pH changes depend on pulse parameters and may induce damage near the electrodes, our results show that away from the electrodes these effects are negligible, as no statistically significant difference in GET efficiency in the same pH combinations between three pulse protocols was observed. However, we tested only three pulse protocols out of a wide range of pulse protocols used in GET and only one plasmid size so more experiments with various pulse parameters and plasmids are needed to generalize our results.

The results, obtained in experiments done on CHO cell line, imply that there is a difference in cell membrane repair that depends on extracellular pH or that extracellular pH affects the characteristics of defects that form on plasma membrane during electroporation. It is possible that acidic extracellular pH allows more efficient repair of damage that is induced on cell membrane during electroporation with high pulse amplitudes. However further studies including various cell lines are needed to confirm if our observations are general.

## 5. Conclusions

To conclude, our results indicate that there is a difference in cell membrane repair that depends on extracellular pH or that extracellular pH affects the characteristics of defects that form on plasma membrane during electroporation. It is possible that acidic extracellular pH allows more efficient repair of damage that is induced on cell membrane during electroporation with high pulse amplitudes. Our results also show that acidic extracellular pH decreases GET efficiency.

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