Parameters Affecting Cell Viability Following Electroporation In Vitro

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Abstract

Cell membrane permeabilization to nonpermeant molecules can be obtained by applying electric field pulses, which parameters have to be adapted to each cell type according to the purpose of the experiments. Electropermeabilization is associated with an uptake or release of molecules between external medium and cell interior and may cause cell death. Roughly speaking, any parameters that will increase either the size of membrane defects, leading to the permeable state of the cell membrane, or their lifetime, regulating the amount of exchanged molecules, will have severe effect on cell viability. However, other parameters, not directly correlated to the electric pulses conditions, can also have dramatic effect and jeopardize cell viability. Indeed, the ways experiments are conducted (temperature, pulsing buffer, electrode material) and the characteristics of the cells (size, shape, orientation, ATP content, normal primary cell or cancer cells, sensitivity) have direct consequences on membrane permeabilization and cell viability. Short-term direct effects, usually associated with strong electric pulses, and long-term effects, linked to the ability of the cells to repair or not the damages, can be present, which all have consequences not only on cell survival but to cell responses to electric pulses. Optimization of the protocols of electroporation, according to the objectives of the experiments, is therefore a necessity.

Keywords

Electroporation • Cell death • Cell viability • Electric pulses • Cell sensitivity

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Introduction

Electroporation-based treatments are powerful medical and biotechnological tools. They are based on the use of well-defined electric field pulses that can cause the reversible or irreversible permeabilization of the cells. Reversible electropermeabilization of the cell membrane is used for electrochemotherapy and gene therapy, allowing to deliver cytotoxic drugs or plasmid DNA into tissues. The aim is to permeabilize the cell membrane while preserving the cell viability. Irreversible electropermeabilization resulting in cell death can be useful and is under clinical evaluation for treating tumors. Electroporation is also of high importance in food industry and bioeconomy. Pulsed electric fields (PEF) and high-voltage electric discharges can efficiently assist extraction of bio-compounds from fruits, grapes, and crops (▶ Chap. 134, "Pulsed Electric Field Treatment for Fruit and Vegetable Processing"). They can also be used to inactivate microorganisms.

Electropermeabilization, also named electroporation, does not just consist on "pushing a button on a generator" but can be controlled by a number of parameters such as the electric field strength, the pulse duration and number, the interval between pulses, and the shape of the pulse and its polarity. For reversible electroporation, the electroporation parameters must be carefully selected to preserve the viability of the cells. Cells may lose their viability due to direct or indirect effects.

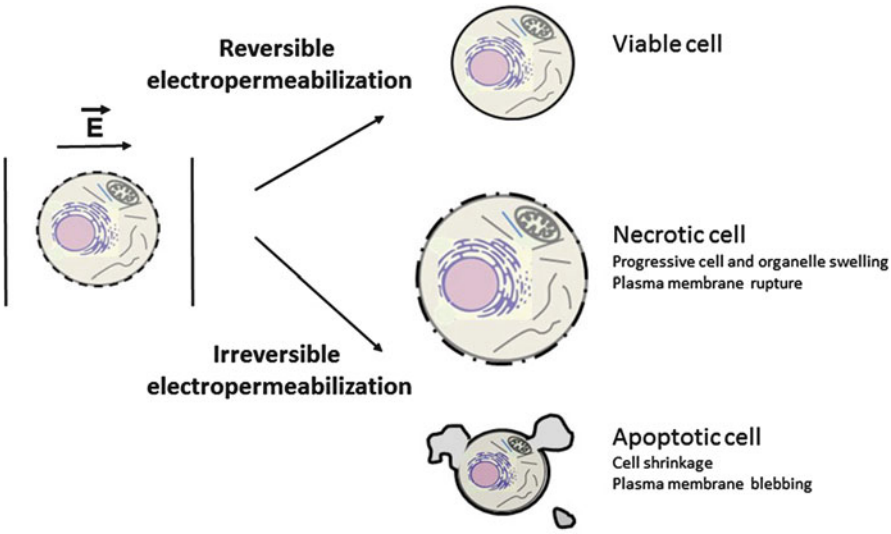

Fig. 1 Effects of electric pulses on cell permeabilization and viability. A cell is placed between two parallel electrodes. Electric pulses induce the permeabilization of the cell. Different scenarios can be observed: reversible permeabilization leading to viable cell; irreversible permeabilization leading to cell apoptosis or necrosis

Permanent damages to the plasma membrane can occur during pulse delivery. Indirect metabolic long-term effects, from which cells are not able to recover, can also occur up to hours following pulse delivery. After resealing, the recovery of cells depends on their ability to achieve biochemical balance after the induced intense influx and efflux of molecules. If that balance is not attained, cell viability may be lost. Other factors may contribute to a reduced survival rate such as the increase in temperature, the production of reactive oxygen species, and/or the release of electrolytes from electrodes. The survival of the cells may be influenced by the properties of the cells as their size and shape and their sensitivity to the electric pulses.

Cells can die by two main physiological different processes, necrosis and apoptosis, that have different characteristics. While apoptosis is associated with cell shrinkage, chromatin condensation, nuclear fragmentation, and the formation of apoptotic bodies, necrosis is associated with cell swelling and plasma membrane rupture. Depending on the reversibility or irreversibility of electropermeabilization, different outcomes can be induced as shown in Fig. 1.

A high number of cell viability assays can be used to assay membrane permeability, metabolic activity, cell function (motility, deformability, ATP (adenosine triphosphate) level), or colony formation. Simple observation of cells under wide field microscope (cell shape, ability to adhere and divide) may be also indicative of inflicted damage. Morphological changes in electrically treated cells have indeed been investigated many years ago by light and scanning electron microscopy. The application of 100-μs rectangular pulses leads to the formation and growth of

spherical and hemispherical protuberances of the cell membrane. The formation of such electro-induced blebs was however not associated with the cells' death and was reversible. Increasing the tonicity of the medium prevented the bleb formation, indicating the osmotically dependent nature of the processes involved (Gass and Chernomordik 1990). Questions about cell organelle alterations remain unanswered for years. A recent publication gives evidence for a number of ultrastructural alterations in cells exposed to electric pulses. Specifically, cells subjected to trains of 5 ms, i.e., under conditions leading to gene transfer and expression, were observed to undergo morphological alterations of the mitochondria and nucleus. These modifications, detected in the minutes following pulse delivery, were transient and have direct long-term consequences on molecule delivery and therefore may explain various aspects of the mechanisms of DNA electrotransfer (Phez et al. 2016).

The aim of this chapter is to discuss how experimental conditions can affect cell viability. Among electric pulse parameters, pulse amplitude, duration, number, and repetition frequency can significantly affect electroporation. When these parameters exceed their optimal values, cell viability is affected and irreversible electroporation takes place. For introduction of small and large molecules, different electric pulse parameters need to be used. Small molecules are efficiently introduced into the cell by application of short electric pulses in range of tens to hundreds of microseconds. The transport of small molecules takes place predominately after the pulse by diffusion. On the other hand, for macromolecules, long 5–10 ms pulses with relatively low pulse amplitude are used. Besides, for successful gene electrotransfer, DNA has to be present in the medium before electric pulses are applied, while small molecules can enter the cell even if added after the pulse. For irreversible electroporation that is used for inactivation of microorganisms, the electric pulse parameters should exceed critical value, as cell death is the desired result of such application.

Basics Aspects of Electropermeabilization

Square-wave electric pulse generators are the most popular and commonly used in electropermeabilization protocols. Compared to exponential decay electric pulse generators, they permit to adjust the different pulse parameters (E, pulse strength; T, pulse duration; N, number of pulses; and f, repetition frequency) independently one to each other and whatever the ionic strength conditions. Electric pulses are delivered through a set of electrodes connected to the generator. As will be described in this chapter, in addition to the electric pulse parameters, the choice and the placement of electrodes have to be carefully selected with respect to the characteristics of the cells.

The distribution of the electric field is dependent on the electrode geometry. The most applicators used for cells in culture are plate electrodes. These electrodes can be used both for cells grown in cultures on Petri dishes and in suspension. If the cells to be treated are grown on a surface that is larger than the interelectrode distance, the entire surface will obviously have to be treated by successive placements of the electrodes to cover the entire surface by repetitive application of electric pulses.

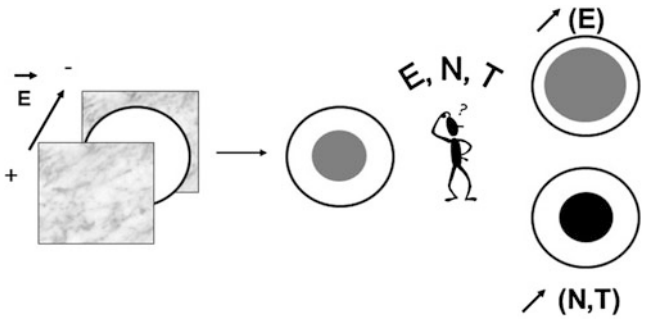

Fig. 2 Effects of electric pulse parameters on permeabilization extent and efficiency. A cell is placed between two parallel electrodes. Electric pulses induce the permeabilization of the membrane area facing the anode (*gray area*). Increasing the field intensity *E* further increases this area, while, at constant *E*, increasing the pulses number *N* or duration *T* has no effect in the size of this area but increases its permeability (*darker gray*)

Permeabilization of cells can therefore be conducted on different ways. For cells grown on Petri dish, culture medium can be replaced by a so-called pulsation medium that, as will be detailed later, has to preserve cell viability. The bottom of the Petri dish serves as an electropulsation chamber. For cells in suspension, centrifugation allows to replace culture medium by pulsation one. Special cuvettes can be purchased, but "home-made" chambers can be easily designed by the plate electrodes placed directly on the bottom of the Petri dish (▶ Chap. 23, "Gene Delivery by Electroporation In Vitro: Mechanisms").

Cell Electroporation

As more detailed in other chapters of this handbook, electropermeabilization occurs only on the part of the plasma membrane where the potential difference has been brought to a critical value close to 200–300 mV (▶ Chap. 2, "Critical Electric Field and Transmembrane Voltage for Lipid Pore Formation in Experiments"). Membrane permeabilization is controlled by the electric strength. This means that field intensity E larger than a critical value, Ep, must be applied. Ep is dependent on the size of the target cells. Large cells are permeabilized at lower electric field values than smaller cells and appear more "sensitive" because the induced transmembrane voltage is higher for large cells than for small ones. Therefore, electric field values have to be carefully adapted to each cell line in order not to affect their viability. In addition, for a given cell line, all the cells don't have the same size due to size variation along the cell cycle.

As tentatively cartooned in Fig. 2, the field strength triggers membrane permeabilization: when $E > Ep$, it controls the area of the cell surface, which is affected. Therefore, only the localized parts of the membrane surface facing the electrodes are affected and will, eventually, be permeabilized. Within these regions, the extent

efficacy of permeabilization (i.e., the flow rate of molecules across the membrane) is controlled by the number and duration of electric pulses. So, membrane permeabilization only occurs for electric field values E higher than the threshold value Ep, whatever the number and duration of electric pulses. Increasing E, above Ep, leads to increase in the extent of membrane area where permeabilization can take place, and in that specific area, the extent efficacy of permeabilization is determined by the number and duration of electric pulses (Rols and Teissie 1990).

Electropermeabilization of the cell membrane can therefore be quantified in terms of the flow of molecules S (F_s), which cross the plasma membrane. For small molecules, Fs obeys Fick's law but occurs only on the permeabilizable cell surface:

$$\mathrm{Fs}(t) = \mathrm{Ps}\, y(N,T) A t/2 \left(1 - \frac{\mathrm{Ec}}{E}\right) \Delta S \exp k(N,T) t \tag{1}$$

Where Ps is the permeation coefficient of the solute S, y is a function that depends on the duration and number of pulses, N is the number of pulses, T is the pulse duration, A_t is the cell surface, E is the electric field intensity, ΔS is the concentration difference of S between cytoplasm and external medium, k is the resealing process constant (in the case of small molecules), and t is the time after the pulse. $A_{t/2}$ $(1 - \mathrm{Ec}/E)$ defines the size of "macro-domains" where electrotransfer can take place; y (N, T) defines the size of "micro-domains" where electrotransfer takes place.

The electro-induced permeabilized state of the cell membrane is transient and disappears progressively after the application of the electric field pulses. Its lifetime is under the control of the electric field parameters. The rate constant of the annealing phase is shown to be dependent on both pulse duration and number but is independent of electric field intensity which creates the permeabilization. The phenomenon can be described in terms of membrane organization transition between the natural impermeable state and the electro-induced permeable state of the membrane.

Membrane electropermeabilization to small molecules depends on the different parameters of the electric field. The electric field intensity E is the deciding parameter inducing membrane permeabilization which controls the extent of the cell surface where the transfer can take place. An increase in the number of pulses enhances the rate of permeabilization. The pulse duration parameter is crucial for the penetration of macromolecules into cells under conditions where cell viability is preserved (Rols and Teissie 1998). The main difference between the electrotransfer of small size molecules and macromolecules is the duration of the pulses. Microsecond pulses are sufficient to obtain cytotoxic drug transfer. Millisecond pulses are required to obtain efficient gene expression with a good cell viability. Long pulse durations limit electric field intensities required when short pulses are used. DNA is a large molecule; therefore the size of membrane areas allowing its transfer must be sufficient enough. This is obtained by applying pulses of low intensity but long pulse duration. Under that optimized conditions, cell viability is generally preserved.

So, the optimization of electroporation protocols highly depends on the objective of the experiments: the size of the molecules to be electrotransferred and the effect on

cell viability. If the objective is to affect a large area of cell, then high electric field values have to be used. If cell viability has also to be preserved, this can be obtained by using short pulse duration.

Cell Permeability Measurements

It is obvious that, under "classical" conditions of electroporation, cell membrane permeabilization precedes cell viability loss. Therefore, detection and quantification of permeabilization have to be first evaluated to define the electrical conditions that will be further used to determine cell viability. The use of fluorescent dyes allows detecting membrane permeabilization (▶ Chap. 68, "Fluorescent Indicators of Membrane Permeabilization Due to Electroporation"). Visualization can be performed at the single-cell level under fluorescent microscopes, while flow cytometry permits to quantify the uptake of molecules on a large number of cells. Quantification can be subject to artifacts as the sensitivity of the detection method highly depends on the fluorescent dyes (size, fluorescent quantum yield). Consequently, the statement that a cell has been successfully permeabilized or not requires additional information including the type of molecule used in the assay.

Cell Viability Measurements

Cell viability can be assayed by different methods (▶ Chaps. 63, "Mathematical Models Describing Cell Death Due to Electroporation," and ▶ 73, "Different Cell Viability Assays Following Electroporation In Vitro"). However, results can be inconsistent if assays are performed without any idea of the process of electropermeabilization, i.e., the fact that membrane can stay permeable after a while following pulses application and that both short-term and long-term effects can be present. In their study, Satkauskas and collaborators compared different and commonly used cell viability assays after mammalian cell electroporation in the presence of bleomycin. Cell viability was measured in vitro using clonogenic assay, propidium iodide, and MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), employing flow cytometry modality. Results showed that although clonogenic cell viability drastically decreased, PI assay performed a few minutes after the treatments indicated nearly 100% cell viability. Assays performed at 6–72 h time points revealed that cell viability is highly dependent on evaluation time point and decreased with later evaluation time points. In comparison to clonogenic cell viability, MTT cell viability was significantly higher, except if used at later times, 2–3 days after the treatment, allowing reliable evaluation of cell viability. In overall, their results showed that in order to estimate cell viability after electroporation, the most reliable method is clonogenic assay. Improper use of PI and MTT assays can lead to misinterpretation of the experimental results (Jakstys et al. 2015).

Effects of Physical Parameters on Cell Viability

Different effects of pulsed electric field are present during cell electropermeabilization. In addition to the transmembrane potential increase, that is mandatory to induce membrane permeabilization, other effects can be induced that may have direct or indirect consequences on cell viability.

Orientation

The transmembrane potential of the cell is critical for successful permeabilization. In that context, cell shape and orientation are two parameters that therefore may impact both permeabilization efficiency and cell viability. The transmembrane potential of prolate and oblate spheroidal cells has been calculated for various orientations with respect to the electric field direction, both numerically and analytically. The induced transmembrane potential is maximum when the longest axis of the cell is parallel to the electric field and is minimum when the longest axis of the cell is perpendicular to the electric field (▶ Chap. 2, "Critical Electric Field and Transmembrane Voltage for Lipid Pore Formation in Experiments"). The dependency on orientation is highly pronounced for elongated cells. It is negligible for spherical cells. The biological relevance of these theoretical results was confirmed with experimental results of the electropermeabilization of plated Chinese hamster ovary cells, which are elongated. Both theoretical and experimental results showed that permeabilization is not only a function of electric field intensity and cell size but also of cell shape and orientation (Valic et al. 2003). Since in practical use of electropermeabilization, especially for gene transfection, we need to use the electric field high enough to achieve permeabilization but at the same time low enough to keep the viability of the cells, the orientation of the cells in the applied electric field is an important parameter that has to be taken into account.

Temperature

Another key parameter that can be easily adjusted is the temperature. Experiments are usually performed at room temperature, but incubating the cells on ice before exposing them to electric pulses or during the minutes following it can improve the uptake of molecules. This is due to the fact that membrane can stay permeable for a certain delay following pulse delivery and that this delay is increased by decreasing the temperature. However, for preserving cell viability and depending on the goal of the experiment, one of the tips to preserve cell viability can be to place the cells immediately after the pulses at 37 °C.

This is specially recommended for gene electrotransfer experiments. Former protocols in the literature prescribed to perform electropulsation at low temperature. This was before knowing the process of DNA delivery into cells, where only DNA present during pulses will eventually enter the cells. There is no rational need to let

the cells at low temperature following pulse delivery that instead of enhancing DNA delivery, affect cell survival and as a consequence jeopardize gene expression. The effect of the preincubation temperature has indeed been shown not to significantly affect the survival rate of cells while strongly affecting the transfection efficiency. Lowering the temperature from 37 °C to 4 °C can induce up to a tenfold increase in the percentage of cells expressing the electrotransferred activity. When cells are preincubated at 4 °C before pulsation, transfection is shown to highly increase with post-pulse incubation temperature, while viability is affected at a lower extent. Optimum conditions thus are a preincubation on ice, followed by a post incubation at 37 °C, which is in conflict with what is currently performed, but in line with reports where post-pulse incubation at room temperature has been reported to increase transfection yield. These observations can be explained as follows. Preincubation of cells with plasmid DNA before pulsation increases the interfacial concentration of plasmid at the cell membrane. Performing this step at low temperature limits DNA degradation by extracellular nucleases. As it has been shown that transfection only occurs when DNA is present during pulsation, stability of the transient permeable structures with time by lowering the temperature is not required to increase transfection of cells. It appears that, associated with the increase in cell viability, a 37 °C post-pulse temperature highly increases transfection efficiency. The data indicate that the post-pulse incubation at low temperature which is routinely performed is detrimental to transfection (Rols et al. 1994).

In a more recent publication, Heller and collaborators evaluated high and low electric field conditions on cells preheated up to 45 °C. Gene expression and viability were measured. They clearly demonstrated that heat can in fact assist in increasing gene expression levels. Their results indicated a two- to fourfold increase in gene expression that was temperature and field dependent. By doing that, electric field can be reduced 20–30% while maintaining or slightly increasing gene expression and increasing viability (Donate et al. 2015).

Electrochemical Reactions

Electrochemical reactions take place at the electrodes. Electropermeabilization by symmetrical bipolar rectangular pulses may therefore cause some electrolytic contamination due to the electrodes. This can have direct consequences on cell viability. A comparative study of the contamination of a cell suspension by ions released from aluminum cuvettes (Al^{3+}) and stainless steel electrodes (Fe^{2+}/Fe^{3+}) during cell membrane electropermeabilization by unipolar and by symmetrical bipolar rectangular electric pulses revealed that the released concentrations of Al^{3+} and Fe^{2+}/Fe^{3+} were always more than one order of magnitude lower with bipolar pulses than with unipolar pulses of the same amplitude and duration. Electrolytic contamination and its detrimental effects can therefore be largely reduced with no loss in efficiency of electropermeabilization, if bipolar rectangular pulses of the same amplitude and duration are used instead of the commonly applied unipolar pulses (Kotnik et al. 2001).

Electroporation Medium

In addition to the modulation of the transmembrane potential, electric pulse delivery to cells may induce significant increase of the temperature due to Joule effect. As an electrical current is flowing, Joule heating is taking place and the temperature of the sample is going to increase. When the temperature reaches values above 42 °C, cell viability may be affected. Temperatures between 46 °C and 60 °C are associated with irreversible cellular damage, proportional to the exposure time. Between 60 °C and 100 °C, protein coagulation occurs instantly with irreversible damage of key cytosolic and mitochondrial enzymes and nucleic acid-histone complexes. Temperatures above 105 °C result in tissue boiling, vaporization, and carbonization. Therefore, one of the key aims of any experimental protocols for electropermeabilization should be to control temperature. This is particularly the case when long duration pulses are applied. This deleterious side effect can be controlled by using a low ionic content pulsing buffer and by delivering a limited amount of energy. A commonly used so-called pulsation medium is a low ionic, isoosmotic buffer that allows to limit the Joule effect. The composition of this medium is: 10 mM phosphate buffer, 250 mM sucrose, and 1 mM $MgCl_2$, pH 7.4.

As one of the steps involved in gene electrotransfer is the interaction of DNA with the cell membrane, divalent cations may influence the anchoring of DNA to the cell membrane and therefore control gene electrotransfer efficiency. The effect of different concentrations of Mg^{2+} on electropermeabilization, gene electrotransfer, and viability of cells has been studied. For that, different pulsation media with increasing Mg concentrations have been used and revealed that high Mg concentrations lead to both high electropermeabilization and viability while causing low gene electrotransfer. This was due to the fact that Mg^{2+} ions can bind DNA at cell surface at such strength that cannot pass into the cell during application of electric pulses, which can lead to lower gene transfection (Haberl et al. 2010).

Presence of DNA

The permeability of cell membranes submitted to electric pulses was shown to increase noticeably if the cells are pulsed in the presence of DNA (Klenchin et al. 1991). Therefore, plasmids can be toxic in combination with electric pulses. This can lead to a decrease in survival and transfection efficiency directly linked to the physical size and concentration of each individual molecule of plasmid: a single copy of a large plasmid is more toxic and harder to introduce into the cell than a single copy of a small plasmid or than the number of copies of a small plasmid equivalent in mass to the single copy of the large plasmid (Lesueur et al. 2016). This toxicity of large plasmids electrotransfer is not caused by plasmid expression. This is of remarkable practical importance to reach high levels of transfection and survival for both small and large plasmids. In another study, electrotransfer of pDNA (plasmid devoid of therapeutic genes) was shown to lead B16F10 melanoma tumor cells to secrete IFNβ (interferon) and to die by necrosis (Znidar et al. 2016).

B16F10 cells in culture indeed express cytosolic DNA sensors, and the expression of IFNβ mRNA (messenger ribonucleic acid) and protein is increased after pDNA electrotransfer. These changes in mRNA and protein levels are accompanied by DNA concentration-dependent cell death. These effects may be due to the activation of signaling pathways mediated by the upregulated cytosolic DNA sensors. Electrotransfer of pDNA can cause necrotic and/or apoptosis cell death. Not only pulse parameters but also plasmid DNA can be responsible for triggering cell death.

Effects of Cell Parameters on Cell Viability

Cells have all the equipment and expertise necessary to carry out the functions of life. But, despite these similarities, cells are not equal. Cells by essence have different morphological and metabolic characteristics. They can in particular differ in size and in shape and can be obtained from cancer cell lines or from primary cell cultures; all these parameters may have direct or indirect consequences on their response and sensitivity to electric pulses.

Size and Shape Effect

Computer simulations predicted that large cells would be specifically permeabilized in a mixture with smaller cells. This was examined on a mixture of Chinese hamster ovary (CHO) cells and erythrocytes. CHO cells were permeabilized to Trypan blue without any occurrence of hemolysis. A similar "size" specificity was observed on blood samples. This agreement between prediction and experimental observation indicates that induction of electropermeabilization is mainly under the control of the size of the target cell. Its physiology plays only a minor role, if any. Treating blood induced the permeabilization of 70% of the leucocytes (polymorphs and monocytes) but did not affect erythrocytes (Sixou and Teissie 1990). Therefore, in agreement with theory, the larger the cells, the more sensitive to electric pulses both in terms of permeability and, as a direct consequence, loss in viability.

The differences in gene electrotransfer and membrane electropermeabilization between cells grown plated on Petri dishes or in suspension have been investigated in two different cell lines (CHO and B16F1) in addition to viability. Results showed that there is a marked difference in the efficiency of gene electrotransfer between suspended and plated cells (Marjanovic et al. 2010). CHO cells had a slightly better survival rate at higher electric fields than B16F1 cells, both in suspension and plated, that was due to differences in cell size and shape.

Normal Versus Tumor Cells

Using a 3D spheroid cell culture mode, the effect of calcium electroporation has been tested on three different human cancer cell lines as well as on primary normal human

dermal fibroblasts. The results showed a clear reduction in spheroid size in all three cancer cell spheroids. Strikingly, the size of normal fibroblast spheroids was not affected (Frandsen et al. 2015). One very important issue is the relation between tumor cell kill efficacy and normal cell sensitivity, calcium electroporation being a promising anticancer treatment method (▶ Chap. 93, "Electrochemotherapy and Its Clinical Applications").

Another difference between normal primary and cancer cell lines comes from their repair capacity that could explain their various sensitivities. Membrane repair was investigated by monitoring dye entry over time after electroporation. The normal primary cell line exhibited the slowest rate. A viability assay performed 1 day after plasma membrane electropermeabilization revealed that viability in the primary normal cell line was higher than in the three tested cancer cell lines. These data suggest more effective membrane repair in normal, primary cells and supplement previous explanations why electroporation-based therapies and other therapies permeabilizing the plasma membrane are more effective on malignant cells compared to normal cells in cancer treatment (Frandsen et al. 2016).

Reactive Oxygen Species

Exposure of cells to the electric pulses induces generation of reactive oxygen species at the electropermeabilized cell membrane level (▶ Chap. 28, "Involvement of Reactive Oxygen Species in Membrane Electropermeabilization"). This generation has been shown to be directly associated with the part of the membrane surface which is electrically restructured, as shown by its dependence on electric parameters. The electro-induced cell process is activated by Ca^{2+} and Mg^{2+} ions and by exogenous adenosine 5′-triphosphate. A metal-ion-catalyzed Haber-Weiss reaction occurs. The use of antioxidant products such as dimethylsulfoxide and sodium L-ascorbate showed that cell survival after electric treatment was directly correlated to the oxidative jump intensity. This observation had to be associated with the cell-damaging action of oxygen-reactive species that have a dramatic effect on cell viability (Gabriel and Teissie 1994).

Bystander Effect

Bystander effect, a known phenomenon in anticancer therapies, where cells release signals which cause damage to nearby cells, has been very recently explored in electroporated melanoma cells in vitro. Viability of non-electroporated cells exposed to medium from electroporated cells and the release of microvesicles have been determined as potential indicators of the bystander effect. Results showed that cells exposed to electric pulses mediated their damage to the non-electroporated cells, thus decreasing cell viability. The murine melanoma B16F1 cell line was found to be more electrosensitive and thus more prone to bystander effect than the canine melanoma CMeC-1 cell line. Shedding microvesicles may be one of the ways

used by the cells to mediate the death signals to the neighboring cells. The effect depends on pulse amplitude, repetition frequency, and cell type (Prevc et al. 2016).

Some Tips to Preserve Cell Viability

In addition to the electric pulse conditions that have to be carefully defined, according to the cell characteristics, some tips can be used to enhance cell viability for cells which are easily permeabilized but too much affected by electroporation.

Addition of Serum

Serum present during electric pulse delivery can have a positive effect on viability. Increase in cell viability is due to a protection of cells by serum that limits the flow of exchange of molecules across the permeabilized membrane. In particular, the ATP leakage is less pronounced when serum is present. ATP leakage can be reduced from 40% to 10%, thanks to the presence of 20% serum. Serum addition can therefore have a positive effect on cell transfection when added after the pulses. This depends on the physiology of the cells; the more their viability will be preserved, the more efficient transfection will be obtained. By acting as a protector of cell viability, serum indeed can help to increase gene transfer and expression (Delteil et al. 2000).

Addition of Poloxamer

Triblock copolymers as pluronic L64 are used to improve gene transfer mediated by several agents into muscle tissue. Their mechanism of action is still under investigation. The combination of electrotransfer and triblock copolymers, in allowing softening electric field conditions leading to efficient DNA transfection, could potentially represent a milder and more secure transfection method. A pretreatment of cells with L64 has been showed to significantly improve transfection efficiency. This pretreatment was indeed shown to increase cell viability, and this was partly responsible for the improvement of transfection efficiency (Wasungu et al. 2011).

Cell Centrifugation

Viability of electrotransfected adherent and suspended cells can be improved if pelleting by centrifugation is performed immediately after pulsing. The protection effect on cell viability is cell line and pellet thickness dependent. For forming CHO cell pellets, centrifugation force (300–13,000 g) and duration are not crucial; about five to ten cell layers in the pellet provided the optimal protection effect. NK-L, K-562, L1210, and MC2 cell pellets are optimally formed by centrifugation at 13,000 g. Pelleting improves the cell viability. When this pelleting method is applied

to load CHO cells with FITC-dextran (41,000 MW), not only is the success rate close to 100%, but the growth rate is similar to the control, which is far better than the conventional electroporation method. Furthermore, the transfection efficiency of the cell lines in pellet could be significantly higher than that in suspension (Li et al. 1999).

Cryopreservation

New cryopreservation approaches for medically applicable cells are of great importance in clinical medicine. Current protocols employ the use of dimethyl sulfoxide (DMSO), which is toxic to cells. Trehalose is a nontoxic disaccharide that can be used as a cryoprotectant. The efficiency of combining reversible electroporation and trehalose for cryopreservation of human adipose-derived stem cells was assayed. After thawing, 80% cell recovery was obtained at 250 mM trehalose and cells' functionality was preserved. Successful cell growth and efficient adipogenic and osteogenic differentiation were achieved. Electroporation seems to be an efficient method for loading nonpermeable trehalose into stem cells, allowing long-term cryopreservation in DMSO-free and xeno-free conditions (Dovgan et al. 2016). This approach is also developed for plant. The impregnation of trehalose by the combined actions of vacuum impregnation and pulsed electric field is a technology with strong potential for improving the freezing tolerance of leaves and, therefore, keeping their fresh-like characteristics after freezing and thawing (▶ Chap. 114, "Pulsed Electric Fields in Combination with Vacuum Impregnation for Improving Freezing Tolerance of Vegetables").

Mathematical Models to Predict Cell Death

Mathematical models of survival in which the probability of cell death is predicted are of interest (▶ Chap. 63, "Mathematical Models Describing Cell Death Due to Electroporation"). Various mathematical models (first-order kinetics, Hülsheger, Peleg-Fermi, Weibull, logistic, adapted Gompertz, Geeraerd) were shown to fit to experimental data using a nonlinear least-square method. The most appropriate models of cell survival as a function of treatment time were the adapted Gompertz and the Geeraerd models and, as a function of the electric field, the logistic, adapted Gompertz and Peleg-Fermi models (Dermol and Miklavcic 2015). This approach is of interest for in vivo situations where viability is of high importance to preserve healthy tissues integrity.

Conclusions

Electroporation can be detrimental to cell viability, not only by increasing the permeability of the cell membrane but also by inducing side effects as the increase in temperature, the release of ATP, and the formation of electrolytes near the

Table 1 Examples of parameters that can preserve cell viability following electroporation

Parameters	Effect on cell viability	References
Temperature of experiment	Placing the cells at 37 °C after PEF decreases the release of ATP and preserves cell viability	Rols et al. (1994)
	Preheated cells before PEF can reduce electric field values needed for membrane permeabilization and preserve cell viability	Donate et al. (2015)
Electrodes	Electrochemical reactions can induce the release of cytotoxic compounds	Kotnik et al. (2001)
	Reversing the polarity may decrease this deleterious phenomenon	
Pulsation medium	Joule heating can damage the cells; it can be reduced by using a low ionic, isoosmotic medium	Haberl et al. (2010)
External molecules	DNA can be toxic in combination with electroporation	Lesueur et al. (2016)
	Triblock polymers can increase cell viability	Wasungu et al. (2011)
	Serum has a positive effect on cell viability	Delteil et al. (2000)
Cell size and shape	Large cells are generally more easily permeabilized and therefore affected by PEF than small cells	Sixou and Teissie 1990
		Marjanovic et al. (2010)
Normal and cancer cells	Viability in primary normal cell lines is higher than in cancer cell lines	Frandsen et al. (2015)
	Membrane repair is more effective in normal, primary cells than in cancer cells	Frandsen et al. (2016)

electrodes or reactive oxygen species near cell membranes. Some of these side effects can be partly reduced in vitro by optimizing the electric pulse parameters and the experimental conditions according to the cell characteristics and sensitivity to electric pulses (Table 1).

Therefore, long-term viability that is required for gene transfer and expression can be obtained. In in vivo environments, one must also consider cells and tissues viability, which in turn can also strongly affect gene electrotransfer efficiency due to DNA damage/denaturation or decreased cell viability. Gene electrotransfer represents a promising delivery system for introducing foreign genes into cells for a range of medical applications. Though numerous strides forward have been made in defining the mechanisms of DNA electrotransfer, several challenges remain for researchers in this field of study (Rosazza et al. 2016). It is therefore necessary to define the relationship between findings in vitro and in vivo, as cell responses to electric pulses can differ between in vitro and in vivo cell cultures, where the microenvironment itself may influence the cell response.

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Cross-References

- ▶ Critical Electric Field and Transmembrane Voltage for Lipid Pore Formation in Experiments
- ▶ Different Cell Viability Assays Following Electroporation In Vitro
- ▶ Electrochemotherapy and Its Clinical Applications
- ▶ Fluorescent Indicators of Membrane Permeabilization Due to Electroporation
- ▶ Gene Delivery by Electroporation In Vitro: Mechanisms
- ▶ Involvement of Reactive Oxygen Species in Membrane Electropermeabilization
- ▶ Mathematical Models Describing Cell Death Due to Electroporation
- ▶ Pulsed Electric Field Treatment for Fruit and Vegetable Processing
- ▶ Pulsed Electric Fields in Combination with Vacuum Impregnation for Improving Freezing Tolerance of Vegetables

References

Delteil C, Teissie J, Rols MP (2000) Effect of serum on in vitro electrically mediated gene delivery and expression in mammalian cells. Biochim Biophys Acta 1467:362–368

Dermol J, Miklavcic D (2015) Mathematical models describing Chinese hamster ovary cell death due to electroporation in vitro. J Membr Biol 48:865–881

Donate A, Burcus N, Schoenbach K, Heller R (2015) Application of increased temperature from an exogenous source to enhance gene electrotransfer. Bioelectrochemistry 103:120–123

Dovgan B, Barlic A, Knezevic M, Miklavcic D (2016) Cryopreservation of human adipose-derived stem cells in combination with trehalose and reversible electroporation. J Membr Biol. doi:10.1007/s00232-016-9916-z

Frandsen SK, Gibot L, Madi M, Gehl J, Rols MP (2015) Calcium electroporation: evidence for differential effects in normal and malignant cell lines, evaluated in a 3D spheroid model. PLoS One 10, e0144028

Frandsen SK, McNeil AK, Novak I, McNeil PL, Gehl J (2016) Difference in membrane repair capacity between cancer cell lines and a normal cell line. J Membr Biol 249:569–576

Gabriel B, Teissie J (1994) Generation of reactive-oxygen species induced by electropermeabilization of Chinese hamster ovary cells and their consequence on cell viability. Eur J Biochem 223:25–33

Gass GV, Chernomordik LV (1990) Reversible large-scale deformations in the membranes of electrically-treated cells: electroinduced bleb formation. Biochim Biophys Acta 1023:1–11

Haberl S, Miklavcic D, Pavlin M (2010) Effect of Mg ions on efficiency of gene electrotransfer and on cell electropermeabilization. Bioelectrochemistry 79:265–271

Jakstys B, Ruzgys P, Tamosiunas M, Satkauskas S (2015) Different cell viability assays reveal inconsistent results after bleomycin electrotransfer in vitro. J Membr Biol 248:857–863

Klenchin VA, Sukharev SI, Serov SM, Chernomordik LV, Chizmadzhev Yu A (1991) Electrically induced DNA uptake by cells is a fast process involving DNA electrophoresis. Biophys J 60:804–811

Kotnik T, Miklavcic D, Mir LM (2001) Cell membrane electropermeabilization by symmetrical bipolar rectangular pulses. Part II Reduced electrolytic contamination. Bioelectrochemistry 54:91–95

Lesueur LL, Mir LM, Andre FM (2016) Overcoming the specific toxicity of large plasmids electrotransfer in primary cells in vitro. Mol Ther Nucleic Acids 5:e291

Li LH, Ross P, Hui SW (1999) Improving electrotransfection efficiency by post-pulse centrifugation. Gene Ther 6:364–372

Marjanovic I, Haberl S, Miklavcic D, Kanduser M, Pavlin M (2010) Analysis and comparison of electrical pulse parameters for gene electrotransfer of two different cell lines. J Membr Biol 236:97–105

Phez E, Gibot L, Rols MP (2016) How transient alterations of organelles in mammalian cells submitted to electric field may explain some aspects of gene electrotransfer process. Bioelectrochemistry 112:166–172

Prevc A, Bedina Zavec A, Cemazar M, Kloboves-Prevodnik V, Stimac M, Todorovic V, Strojan P, Sersa G (2016) Bystander effect induced by electroporation is possibly mediated by microvesicles and dependent on pulse amplitude, repetition frequency and cell type. J Membr Biol 249:703–711

Rols MP, Teissie J (1990) Electropermeabilization of mammalian cells. Quantitative analysis of the phenomenon. Biophys J 58:1089–1098

Rols MP, Teissie J (1998) Electropermeabilization of mammalian cells to macromolecules: control by pulse duration. Biophys J 75:1415–1423

Rols MP, Delteil C, Serin G, Teissie J (1994) Temperature effects on electrotransfection of mammalian cells. Nucleic Acids Res 22:540

Rosazza C, Meglic SH, Zumbusch A, Rols MP, Miklavcic D (2016) Gene electrotransfer: a mechanistic perspective. Curr Gene Ther 16:98–129

Sixou S, Teissie J (1990) Specific electropermeabilization of leucocytes in a blood sample and application to large volumes of cells. Biochim Biophys Acta 1028:154–160

Valic B, Golzio M, Pavlin M, Schatz A, Faurie C, Gabriel B, Teissie J, Rols MP, Miklavcic D (2003) Effect of electric field induced transmembrane potential on spheroidal cells: theory and experiment. Eur Biophys J 32:519–528

Wasungu L, Marty AL, Bureau MF, Kichler A, Bessodes M, Teissie J, Scherman D, Rols MP, Mignet N (2011) Pre-treatment of cells with pluronic L64 increases DNA transfection mediated by electrotransfer. J Control Release 149:117–125

Znidar K, Bosnjak M, Cemazar M, Heller LC (2016) Cytosolic DNA sensor upregulation accompanies DNA electrotransfer in B16.F10 melanoma cells. Mol Ther Nucleic Acids 5:e322