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The electropermeome: cellular response to electroporation

Elektropermeom: celični odgovor na elektroporacijo

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Abstract

The increased permeability of a cell membrane due to exposure of cells/tissues to an electric field is called electroporation. Electroporation induces a range of changes in the cell - from structural and chemical changes in the cell membrane, structural changes in proteins or protein complexes, transport of substances in and out of the cell, activation of signalling pathways, and repair mechanisms; it also triggers cell death under certain conditions. The term electropermeome is used to describe both the permeabilised cell during or immediately after the delivery of electrical pulses and all subsequent processes that remain active for some time after the increased transmembrane transport of substances for which the cell membrane is normally impermeable has ceased, i.e. even after the membrane has resealed. Electroporation is used in many areas, including tissue ablation, gene electrotransfer for plasmid delivery into cells and electrochemotherapy. Medical applications of electroporation are effective and safe, but the action of the electrical pulses can cause certain adverse side effects, notably muscle contractions and acute pain. Further elucidation of the underlying mechanisms of electroporation and the effects of individual electric field parameters on the electropermeome is crucial to optimise the parameters of electroporation and consequently the results of electroporation-based therapies. The aim of the present paper is to provide a comprehensive overview of the mechanisms of electroporation and the electropermeome, i.e. the cellular response to electroporation.

Izvleček

Povečano prepustnost celične membrane zaradi izpostavitve celic oz. tkiv električnemu polju imenujemo elektroporacija. Povzroči vrsto sprememb v celici, od strukturnih in kemijskih sprememb v celični membrani, strukturnih sprememb proteinov oz. proteinskih kompleksov, prenosa snovi v celice in iz njih do aktiviranja signalnih poti in popravljalnih mehanizmov; ob določenih pogojih sproži tudi celično smrt. S pojmom elektropermeom označujemo tako permeabilizirano celico med ali tik po dovajanju električnih pulzov kot tudi vse poznejše procese, ki ostanejo aktivni še nekaj časa potem, ko ni več mogoče opaziti povečanega transmembranskega transporta snovi, za katere je celična ovojnica običajno neprepustna, torej tudi po času, ko že ugotavljamo, da je zaceljena. Elektroporacija se uporablja na številnih področjih, vključno z ablacijo tkiv, gensko elektrotransfekcijo za vnos plazmidov v celice ter elektrokemoterapijo. Aplikacije elektroporacije v medicini

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Ključne besede: elektropermeabilizacija; električni pulzi; transmembranski transport; peroksidacija lipidov; celična smrt

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so učinkovite in varne, vendar so zaradi delovanja električnih pulzov lahko prisotni tudi določeni neželeni stranski učinki, predvsem mišično krčenje in akutna bolečina. Za optimiziranje parametrov elektroporacije in s tem rezultatov na elektroporaciji temelječih terapij je ključnega pomena nadaljnja razjasnitev osnovnih mehanizmov elektroporacije in vplivov posameznih parametrov električnega polja na elektropermeom. Namen prispevka je predstaviti celovit pregled mehanizmov elektroporacije ter elektropermeoma, tj. celičnega odgovora na elektroporacijo.

1 Introduction

Through a system of ion channels and pumps, cells maintain a difference in electrical potential between the inside and outside of the cell membrane, which is called transmembrane potential. In eukaryotic cells, this usually ranges between -40 and -70 mV. The induced transmembrane potential present during the cell's exposure to the electric field is added to the transmembrane potential. Exposing cells to a sufficiently strong electric field can impose a significantly higher transmembrane potential than the resting transmembrane potential. Such a high transmembrane potential and, as a result, a strong electric field in the membrane, leads to a series of changes, including structural membrane changes and modifications of membrane molecules. Therefore, molecules to which the membrane is normally impermeable can pass through. Increased permeability of the cell membrane, i.e. permeabilization of the membrane, due to the exposure of cells/tissues to an electric field, is called electroporation or also electropermeabilization (1). The electric field during electroporation is established by applying electrical pulses through electrodes that are in contact with the sample or tissue.

Electroporation is used in many areas including tissue ablation, gene electrotransfer for plasmid delivery into cells, and electrochemotherapy, which is a local method of cancer treatment using a combination of standard chemotherapy and short electrical pulses (2). Medical applications of electroporation are effective and safe. In electrochemotherapy and electroporation ablation, cell death is not mainly caused by thermal injury but by increased permeability of the cell membrane. Therefore, the healthy surrounding tissue is not injured by treatment (3,4). However, due to electrical pulses, some unwanted side effects can occur, particularly muscle contractions, which can cause unpleasant sensations and even pain; in some cases, it is necessary to coordinate the delivery of pulses with the electrocardiogram to prevent arrhythmias (e.g. ventricular fibrillation). To improve results of electroporation-based applications, it is crucial to evaluate the influence of individual parameters – from electrical pulse parameters (e.g. voltage, duration and number of pulses) to electrode geometry and position – on success of treatment (2). Traditionally, electroporation procedures use monophasic pulses with a duration in the order of micro- and milliseconds, but in recent years the possibility of using nanosecond pulses and high-frequency biphasic pulses lasting only a few microseconds has been studied, as there it is possible to overcome certain limitations that appear in conventional electroporation with monophasic milli- and microsecond pulses. Nanosecond pulses reduce muscle contraction (5) and cause less tissue heating due to less energy being delivered (6). This reduces the possibility of thermal injury while the use of biphasic pulses reduces muscle contractions (7,8) and arrhythmia risk (7,9).

The comprehensive optimization of electrical pulse parameters for individual applications is limited by an incomplete understanding of the basic mechanisms of electroporation. The purpose of this paper is to provide a comprehensive overview of the cell membrane permeability increase mechanisms due to the action of electrical pulses and all subsequent changes and processes triggered by electroporation – from chemical changes in membrane lipids and modulation of protein function to changes in gene expression and protein synthesis, as well as the activation of cell death and the immune response.

2 Mechanisms of permeabilization of the cell membrane

Experimental results show that an increase in membrane permeability can occur in less than 10 ns, suggesting a direct rearrangement of membrane components (10). The currently established explanation of electroporation is based on the formation of water pores in the lipid bilayer. Molecular dynamics simulations indicate that (with a sufficiently high potential on the bilayer or a sufficiently high electric field) the pore formation begins with the orientation of water molecules in the direction of the electric field and their penetration into the lipid bilayer from both (intra- and extracellular) sides (Figure 1A). Water molecules, oriented in the

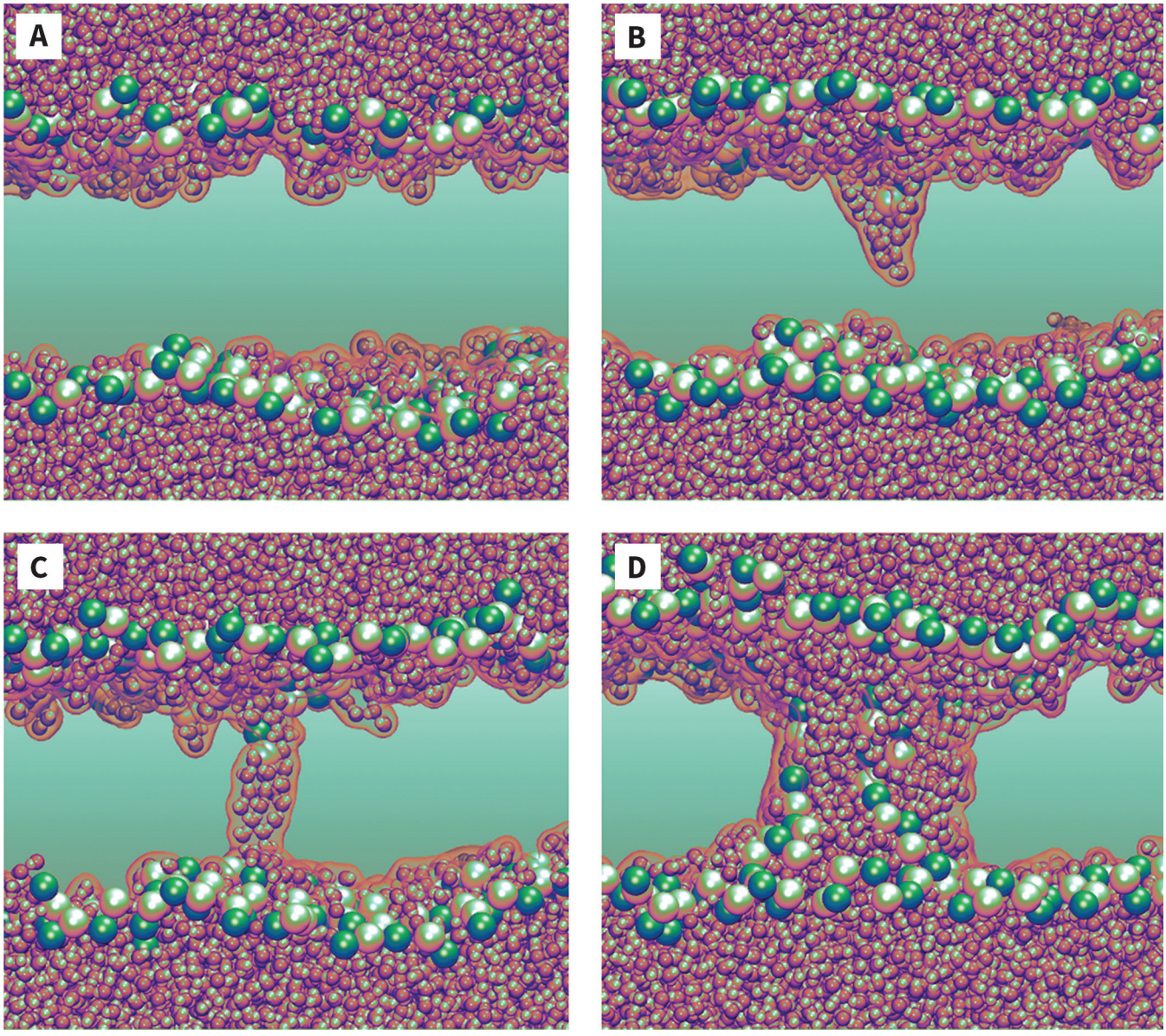

Figure 1: Pore formation in the lipid (phosphatidylcholine) bilayer.
(A) orientation of water molecules in the direction of the electric field and penetration into the lipid bilayer, (B) the appearance of water fingers, hydrogen-bonded clusters of water molecules that protrude into the core of the lipid bilayer, (C) the joining of water fingers into a water channel called a hydrophobic pore that connects the intra- and extracellular side of the lipid bilayer, (D) reorientation of phospholipids by turning their polar heads towards the water channel, which at this stage is called a hydrophilic pore. The polar phosphatidylcholine heads are shown as green and white circles, but the lipid tails are not shown for clarity.

direction of the electric field, are connected by hydrogen bonds into small clusters. These clusters, called water fingers, grow in size and increasingly protrude into the hydrophobic core of the lipid bilayer (Figure 1B) until they connect both sides (intra- and extracellular) and form a water channel (Figure 1C). Such a structure is called a hydrophobic pore. Phospholipids reorient in the presence of water channels by turning their polar head groups toward the resulting water channel to "shield" the nonpolar tails from water molecules. The reorientation of phospholipids stabilizes the pore, which at this stage is called a hydrophilic pore (Figure 1D). Stabilization of the pore allows even more water and other polar molecules to enter the water channel (1).

In the absence of an electric field, the pores begin to close. Pore closing occurs in the reverse order of the analogous stages of pore formation. While the time required for pore formation decreases exponentially with

increasing electric field strength (11), the time required for the pores to close is practically independent of the strength of the electric field that triggered their formation; the closing of pores in lipid bilayers in molecular dynamics simulations always takes from a few tens to a few hundred nanoseconds, which suggests that the pores are not stable. In simulations, the estimated time required for pore closure is several orders of magnitude shorter than the experimentally determined time required for membrane closing (i.e. the time during which increased transmembrane transport is observed) (1). The increased permeability of the cell membrane is observed for a few minutes after the electric field is no longer present, even when using pulses with a duration of only a few nanoseconds. However, the duration of the increased permeability of the cell membrane depends on the temperature (12-15), which suggests that electroporation of cell membranes is a more complex process than the mere formation of short-lived pores in the lipid bilayer. Several studies indicate the importance of chemical changes in membrane lipids and modulation of protein function in increasing membrane permeability during electroporation (1).

Electroporation causes a series of changes in the cell membrane (formation of lipid and protein pores, oxidation of membrane lipids), depolarization, formation of reactive oxygen species (ROS), release of ATP and K^+ from the cell, influx of Ca^{2+} into the cytoplasm, entry of extracellular molecules into the cell, osmotic imbalance, protein reorganization or protein structural changes, including opening of ion channels, cytoskeleton disruption, activation of various signalling pathways, changes in gene expression and protein synthesis, as well as activation of several cellular repair mechanisms (Figure 2). Changes in the permeabilized cell membrane, as well as all subsequent processes that are active even when increased transmembrane transport of substances to which the cell membrane is normally impermeable is no longer observed, are denoted by the term electropermeome (16).

2.1 Chemical changes of membrane lipids

Electroporation pulses trigger the formation of extra- and intracellular ROS (17-22). Lipid oxidation due to exposure to electrical pulses such as those used in electroporation alters the composition and properties of both lipid bilayers and cell membranes. Chemical changes in membrane lipids, particularly peroxidation, could explain the longer-lasting permeabilization of cell membranes after electroporation. Lipid peroxidation is the oxidative degradation of lipids. It involves the formation and breakdown of dioxygen adducts of unsaturated lipids called lipid hydroperoxides (Figure 3). The reaction is initiated by a strong oxidizing agent (e.g. hydroxyl radical), which removes the weakly bound allylic hydrogen from the lipid. Further degradation of hydroperoxides, the primary products of lipid peroxidation, is a complex process in which many secondary products are formed, e.g. aldehydes, ketones, alcohols, hydrocarbons, esters, furans, lactones, and peroxides. Hydroperoxides and some of their decomposition products, e.g. mutagenic malondialdehyde (MDA), react with amino acids, DNA and membranes (23).

It has been demonstrated that ROS concentration and the extent of lipid peroxidation increase with electric field intensity, pulse duration and pulse number in bacterial, plant and animal cells as well as in liposomes, and that lipid peroxidation is associated with increased cell membrane permeability, time required for membraneresealing, and cell damage (17-20,22,24).

2.1.1 Peroxidation of membrane phospholipids

Cell membranes consist of a bilayer of phospholipids and sterols, in which proteins and other molecules are placed. The presence of oxidized lipids in lipid membranes disrupts the lipid order, leads to lateral expansion and thinning of the bilayer, lowers the temperature of the phase transition, changes the hydration of the bilayer, increases lipid mobility and the frequency of flip-flops, affects the lateral membrane organization and promotes the formation of membrane defects. Therefore, bilayers with oxidized lipids are significantly more permeable and conductive than unoxidized bilayers (25-29). Experiments showed the presence of conjugated dienes in the membranes of electroporated cells or vesicles, which indicates the presence of hydroperoxides, the primary products of lipid peroxidation (17,22,24), as well as MDA, indicating the presence of secondary peroxidation products (24).

Hydroperoxides are stable enough to be present in the lipid bilayer for some time after oxidation. Using molecular dynamics simulations, Rems et al (26) quantified the permeability and conductivity of a lipid bilayer with a different proportion of hydroperoxides, i.e. of primary products of lipid peroxidation. They showed that even a small proportion (around 1%) of hydroperoxides affects the conductivity of the bilayer. However, they also found that the increase in the conductivity and ion permeability of the lipid bilayer due to the presence of hydroperoxides alone is too small to fully explain the

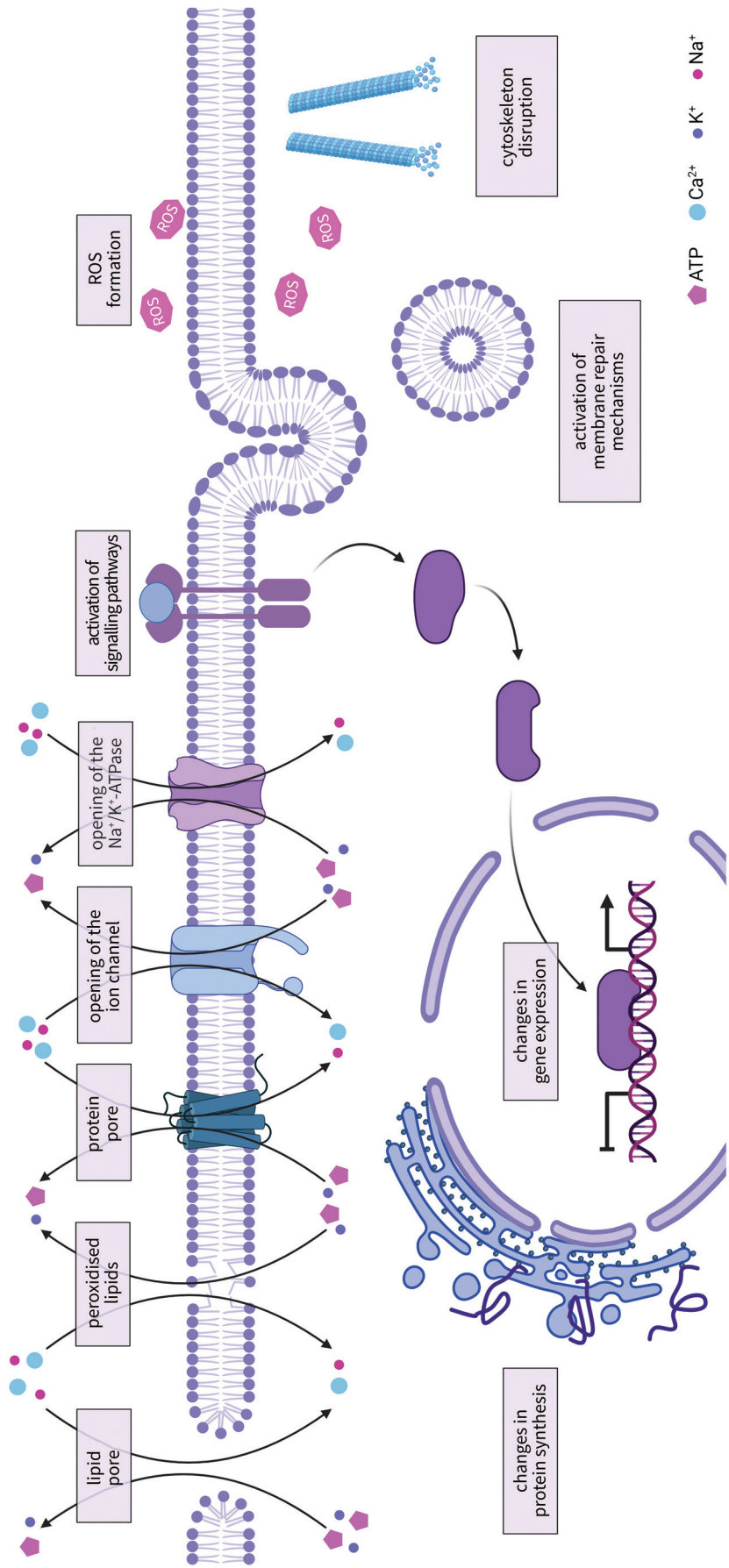

Figure 2: Electropermeome scheme - changes and processes that occur in the cell during and after electroporation.
Among other things, electroporation causes the formation of lipid and protein pores, peroxidation of membrane lipids, opening of ion channels and Na^+/K^+-ATPase, influx of Ca^{2+} into the cytoplasm, ATP and K^+ release from the cell and resulting osmotic imbalance, formation of reactive oxygen species (ROS), cytoskeleton disruption, activation of signalling pathways and repair mechanisms, and changes in gene expression and protein synthesis.

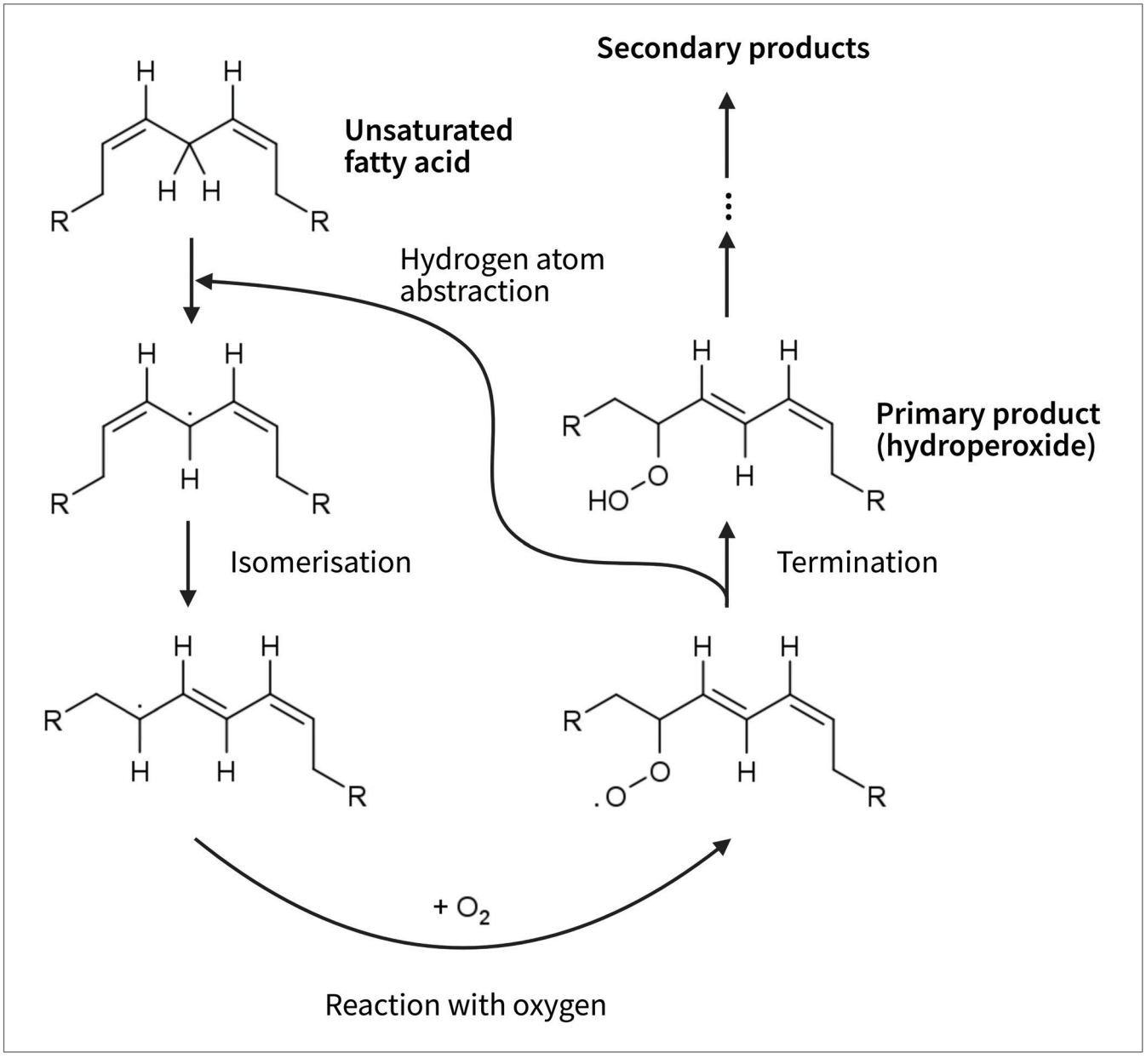

Figure 3: Scheme of lipid peroxidation reactions.
The reaction is initiated by a strong oxidant, which removes a weakly bonded allylic hydrogen from the unsaturated fatty acid - this is how the alkyl fatty acid radical is formed. The addition of molecular oxygen to an alkyl radical can lead to the formation of a conjugated peroxyl radical. The latter abstracts the allylic hydrogen from another unsaturated fatty acid, leading to the formation of hydroperoxide, the primary product of lipid peroxidation, and a new alkyl radical. With a sufficient amount of molecular oxygen and non-oxidized unsaturated lipids, this step can be repeated many times, but it also competes with several termination reactions at the same time. Termination involves a reaction between two alkyl radicals, between two peroxyl radicals, between an alkyl and a peroxyl radical, or the reaction of an alkyl/peroxyl radical with a non-lipid substrate (e.g. phenolic antioxidants, ascorbate, glutathione, or amino acid residues). Further degradation of hydroperoxides, the primary products of lipid peroxidation, is a very complex process in which many secondary products are formed, some of which have important biological effects.

experimentally determined values. Spontaneous pore formation was not observed in lipid bilayers with hydroperoxides (26,30,31).

Phospholipids with aldehyde groups on acyl tails (secondary products of phospholipid peroxidation) disrupt the lipid bilayer more than hydroperoxides. In experiments (27,32) and molecular dynamics simulations (28,30-33), a significant increase in membrane permeability with a certain proportion of phospholipids with aldehyde groups was observed, as well as the spontaneous

organization of aldehydes into pores (27,30-34). The pores formed as a result of the presence of lipid peroxidation products are not the same as the pores formed in a non-oxidized lipid bilayer under the influence of an electric field. In molecular dynamics simulations, pores from lipid peroxidation products with aldehyde groups were opened for a few microseconds, and in the presence of cholesterol for the entire duration of the simulation (5 μs) (33).

2.1.2 Oxidation of membrane sterols

Sterols influence the conformational order of acyl lipid chains, the thickness of the hydrophobic part of the membrane, and the lateral membrane organization and permeability. Cholesterol greatly reduces the membrane's permeability for water, oxygen, ions, and other small molecules. Most biological membranes are dominated by a single sterol. In mammalian cells, this is cholesterol (35).

Oxidation of membrane cholesterol changes the structure of the membrane. Cholesterol can be oxidized enzymatically or non-enzymatically due to direct ROS action. Oxidized cholesterol derivatives with one or more additional oxygen functional groups are called oxysterols. Oxysterols can be divided into two groups: those in which the short nonpolar tail is oxidized, and those in which the tetracycline ring is oxidized. Oxysterols with an oxidized tail have a similar effect on membranes as cholesterol, but they cause the phospholipid tails to be less ordered and do not condense the lipid bilayer as much as cholesterol does. Rapid turnovers of oxysterols with oxidized tails increase membrane permeability (e.g. 25-hydroxycholesterol is known to increase membrane permeability to calcium ions and glucose). Oxysterols with an oxidized tetracycline ring, which can adopt a different conformation, disrupt the membrane structure more than oxysterols with oxidized tails, as they increase the mobility of phospholipid tails (35).

There are few studies investigating sterol oxidation by electroporation. Kazmierska et al (36) observed a relatively low increase in the concentration of oxysterols (both those with an oxidized tail and those with an oxidized tetracycline ring) in yolks exposed to electrical pulses. By increasing the number of electrical pulses, they measured a higher concentration of oxysterols.

2.2 Modulation of protein function

Evidence of electroporation's effect on proteins and their role in increasing membrane permeability can also be found in the literature. Both experiments on lipid bilayers and molecular dynamics simulations indicated that the presence of a protein channel in the lipid bilayer stabilizes the membrane, requiring a higher electric field strength for electroporation to occur. In simulations, the formation of larger pores near the channel was not observed (37,38). However, Azan et al (39,40) using confocal Raman microspectroscopy demonstrated protein modifications in living cells exposed to electroporation pulses. Unfortunately, the method used does not allow for differentiation between the modification of membrane and cytoplasmic proteins.

2.2.1 Membrane proteins

Submicrosecond pulses cause voltage-gated calcium channels to open via a mechanism that does not involve lipid bilayer pore formation, heating, or membrane depolarization via voltage-gated sodium channels (41-43). Microsecond electroporation pulses, however, cause the Na^+/K^+-ATPase to open (44). Due to electroporation, cadherin in cell junctions is also lost (45,46). Cadherins are transmembrane proteins that play an important role in the formation of adherens junctions, a type of intercellular junction in epithelial and endothelial tissues.

Using electric fields that induce electroporation, Rems et al. (47,48) observed pore formation in the voltage-sensing domains of various voltage-gated channels in molecular dynamics simulations. In the simulations, the formation of the pore was followed by the unwinding of the voltage-sensing domain and the stabilization of the pore by membrane lipid heads. Such pores remained stable even until the end of the simulation (one microsecond after the electric field was no longer present), which is significantly longer than the pores in the lipid bilayer. Rems et al (47) concluded that in the case of major disruption of the protein channel structure, it cannot spontaneously fold back into its native conformation, but the cell repairs the damage through the mechanism of endocytic recycling.

2.2.2 Cytoskeleton

Cytoskeleton proteins (actin filaments, intermediate filaments and microtubules) and related proteins affect membrane permeability – the formation and expansion of membrane pores and membrane closing after electroporation. Disruption of the actin filament network lowers the energy barrier for membrane pore formation (15). Electroporation of cells or giant unilamellar vesicles with encapsulated actin did not cause large micrometre-diameter pores that were observed when

electroporation of empty vesicles (49). In cells and vesicles with encapsulated actin, however, a longer lasting permeabilization of the lipid bilayer was observed than in cells incubated with a toxin that destabilizes actin filaments or in empty vesicles (15,50).

Electroporation results in reversible disruption of the three-dimensional filamentous structures of actin, tubulin, and intermediate filaments, but not degradation of monomeric cytoskeletal proteins. The cytoskeleton reassembles within hours after electroporation (45,46,51,52). However, it is not yet entirely clear whether the destruction of the cytoskeleton is a direct consequence of the action of electrical pulses or fields on cytoskeleton proteins or the result of ATP release from cells, increased concentration of calcium ions in the cytoplasm, hydrolysis of phosphatidylinositol-4-5-bisphosphate (PIP2) and/or cell swelling due to electroporation (49).

Pakhomov et al (53) showed that the disruption of actin filaments after electroporation with nanosecond pulses is the result of cell swelling. Contrarily, research in which pulses with different parameters were used indicated that actin filaments can collapse even without cell swelling or vesicle formation, which indicates the direct effects of electric pulses or fields on actin (45,50). Using atomic force microscopy, Louise et al (54) showed that cell swelling is mainly due to the destabilization of the interaction between cortical actin and the membrane due to electroporation pulses, but not to the depolymerization of actin filaments. This confirmed the findings on the membrane's separation from the cytoskeleton under the influence of electrical pulses or fields (55).

Using molecular dynamics simulations, Marracino et al (56) showed that high-voltage nanosecond electrical pulses cause changes in the conformation of the C-terminal end of β-tubulin and changes in the local electrostatic properties of the GTPase domain and the binding site for most molecules that bind to β-tubulin. Their findings suggest that nanosecond electrical pulses can physically affect microtubule dynamics. Chafai et al (57) also showed experimentally that nanosecond electrical pulses change the conformation of the C-terminal part of tubulin, which polymerizes into different structures; whether the modulation of tubulin self-organization is reversible or irreversible depends on the parameters of the electrical pulses. Modulation of tubulin-associated proteins (e.g. kinesin) could also alter the microtubule network dynamics. Using molecular dynamics simulations, Průša et al (58) showed that a 30 ns electrical pulse changes the contact surface between kinesin and tubulin as well as tubulin binding sites and nucleotide hydrolysis sites on kinesin.

3 Changes in gene expression and protein synthesis after electroporation

Electroporation initiates a series of physiological cell responses, which is also reflected in gene expression and protein synthesis changes. Since electroporation pulses induce ROS formation, it is expected that cells will respond to oxidative stress. In the yeast Saccharomyces cerevisiae, exposure to electrical pulses increased the expression of genes for proteins involved in the response to oxidative stress (GLR1, SOD1, SOD2 and GSH1) (59). Michel et al (60), however, observed increased immunocytochemical staining with antibodies against superoxide dismutase SOD-2 after electroporation, incubation with cisplatin, and a combination of electroporation and cisplatin (electrochemotherapy) in metastatic pancreatic cancer cells. An increase in SOD-2 gene expression after electroporation was also measured by Dovgan et al (61) in mesenchymal adipose-derived stromal cells and umbilical-cord-derived stromal cells.

By monitoring the expression of various genes after electroporation, Morotomi-Yano et al (62) showed that nanosecond electrical pulses present a different type of stress to cells than endoplasmic reticulum injury, ultraviolet light, or heat shock. Cells respond to physiological stress by activating various mechanisms. Since protein synthesis consumes a significant proportion of cellular building blocks and energy, it is highly regulated during stress responses. Electroporation causes phosphorylation of the eukaryotic translation initiation factor eIF2a and dephosphorylation of the 4EBP1 protein, which indicates suppression of protein translation or protein synthesis in general (62), and to the reduced expression of genes involved in protein synthesis (63). Hojman et al (64) detected a reduced expression of genes involved in metabolism (e.g. genes for phosphoenolpyruvate carboxykinase and dipeptidase) in murine muscles after electroporation, which suggests a reduction in catabolism. Electroporation also causes other changes: reduced expression of histones H2A and H4, which are crucial for chromatin organization (63,65), reduced expression of cytoskeletal protein genes (64), and changes in genes and proteins associated with cell death and immune response (66).

3.1 Cell death and immune response

Cell death can be approximately divided into pathological (necrosis) and programmed. Until recently, apoptosis was considered synonymous with programmed cell death, but in recent years other types of programmed

cell death have been discovered, e.g. pyroptosis and necroptosis. Apoptosis can be triggered by several pathways, which are mainly divided into extrinsic and intrinsic pathways. The extrinsic pathway is mediated through receptors on the surface of the cell membrane (death receptors). In the extrinsic pathway, caspase-8 is activated at the cell membrane and then directly activates effector caspases (caspase-3, -6 and -7). The intrinsic pathway, on the other hand, is mediated via mitochondria and is particularly fast, as all the factors are already present and only need activation. In the intrinsic pathway, disruption of the balance between proapoptotic (e.g. Bid, Bax and Bak) and antiapoptotic (e.g. Bcl-2) proteins releases apoptosis-inducing proteins (e.g. cytochrome c and apoptosis-inducing factor, AIF) from the mitochondria, which activates caspases. Unlike apoptosis, activation of caspase-1, -4, 5- or -11 is key in pyroptosis, which then initiate the process of programmed cell death by cleaving the pore-forming protein gasdermin D. Key markers of pyroptosis are caspase-1 activation and caspase-3 non-activation (the latter is associated with apoptosis). The activation of necroptosis is influenced by the activation of the kinases RIPK3 and MLKL (67).

Triggering of apoptosis after electroporation is mainly mentioned in association with nanosecond pulses (68-70), but it has also been observed with electroporation with longer pulses. Ford et al (69) detected an electric field-dependent increase in the amount of activated caspases-3, -6, -7, -8 and -9 in murine melanoma cells after electroporation with 3 ns pulses. However, no release of cytochrome c, AIF, or Smac/DIABLO from mitochondria was observed. Their findings suggest that nanosecond pulses trigger apoptosis through a pathway similar to the extrinsic activation pathway. In contrast, Beebe et al (68) observed in electroporation with 60 ns pulses that the initiation of apoptosis depends on the activation of caspases as well as on mitochondria, as they detected the release of cytochrome c into the cytoplasm in a T lymphocyte cell line. Zhang et al (71) monitored the expression of 17 genes related to apoptosis. After only two hours after electroporation of breast cancer cells with microsecond pulses, changes in the expression of caspases and genes associated with death receptors were observed. The expression of caspase-3 was increased, while the expression of caspase-6, -7 and -9 and Bc1-2, Bid and FASLG were decreased. They concluded that the activation of apoptosis after electroporation was mainly through the intrinsic pathway.

Since caspase-3 is involved in both the intrinsic and extrinsic pathways of apoptosis initiation, the increased expression of caspase-3 may indicate that the process of apoptosis has been initiated in the cells, but the pathway itself could not be determined. Zhang et al (72) detected more caspase-3 in pancreatic cancer cells exposed to electroporation pulses, O'Brien et al (73) observed immunohistochemical staining of cleaved caspase-3 only at the edge of the pancreatic ablation zone after electroporation, Siddiqui et al (74) detected cleaved caspase-3 in the entire zone of liver ablation, and Mercadal et al (75) recorded increased expression of caspase-3 or -7 in pancreatic adenocarcinoma cells. All three studies indicated that apoptosis was initiated. Michel et al (60) observed increased immunocytochemical staining with anti-caspase-3 antibodies after incubation with cisplatin, microsecond pulse electroporation, and a combination of electroporation and cisplatin (electrochemotherapy) in metastatic pancreatic cancer cells.

An electric current flowing through a conductor (e.g. cell suspension, tissue, and so on) causes it to be heated (i.e. Joule heating). With properly selected electrical pulse parameters, it is possible to achieve a small enough increase in temperature to prevent thermal injury to cells/tissue. Faroja et al (76) wanted to determine whether high-energy pulses (i.e. a large number of pulses and/or a high electric field strength) can cause thermal injury to liver tissue. When the electroporation temperature did not exceed 39 °C, apoptotic cells with cleaved caspase-3 were observed and virtually no HSP70 heat shock proteins were detected (characterized by their expression being greatly increased by heat stress or toxic chemicals). In contrast, in cells subjected to electroporation where the temperature exceeded 60°C, distinct expression of HSP70 and only minimal expression of caspase-3 was observed. Ben-David et al (77), however, observed differences in the immunohistochemical staining of cleaved caspase-3 and HSP70 in different tissues after electroporation: strong staining for cleaved caspase-3 and limited expression of HSP70 was detected in the liver, no staining was detected in muscle cells, while minimal staining for cleaved caspase-3 and a significant increase in HSP70 in the tissue surrounding the area where the electrical pulses were delivered were observed in the kidneys. Kanthou et al (45) did not detect an increased accumulation of HSP70 after electroporation of umbilical cord endothelial cells, while Mlakar et al (63) and Dovgan et al (61) after electroporation of melanoma cells and mesenchymal adipose-derived stromal cells and umbilical-cord-derived stromal cells observed increased expression of proteins from the heat shock protein family HSP70.

Contrary to most of the literature, in the research carried out by Mercadal et al (75), pancreatic

adenocarcinoma cells died after electroporation via a pathway independent of caspase-3 or -7; however, Zhang et al (67) observed increased expression of cleaved caspase-1, gasdermin D, RIPK3, and MLKL and decreased expression of cleaved caspase-3 six hours after liver electroporation. They concluded that the results indicate the activation of pyroptosis and necroptosis, but not apoptosis. Ringel-Scaia et al (78) observed changes in gene expression consistent with apoptosis immediately after electroporation of breast cancer cells. Over time, however, they observed a change in gene expression towards inflammatory types of cell death and necrosis – after 24 hours, increased expression of genes associated with necrosis and pyroptosis was recorded. Pyroptosis is also associated with the regulation of pattern recognition receptors (PRR), so it is not surprising that they also observed increased expression of three networks associated with damage associated molecular patterns (DAMPs): ROS, ATP and HMGB1 signalling. However, they detected a decreased expression of genes related to immune system suppression, and an increased expression of genes related to the inflammatory response. The authors also observed decreased expression of genes associated with cell damage and increased expression of genes associated with regeneration.

Peng et al (79) detected increased expression of genes related to apoptosis/necrosis (caspase-8, bcl-w, Mt2 and seven genes from the cytochrome P450 family) and increased expression of several chemokine genes, including MIP-1α, MIP-1β, MIP-1γ, IP-10 and MCP-2, four hours after electroporation in murine skeletal muscle. Heller et al (80) measured elevated mRNA levels for several inflammatory chemokines and cytokines (MIP-1α, MIP-1β, IP-10, IL-6, and inducible nitric oxide synthase) after electroporation of murine melanomas. They also detected increased levels of IL-1β and IL-6 proteins after electroporation. Goswami et al (81) studied the effect of microsecond electroporation pulses on triple negative 4T1 breast cancer cells. They measured increased mRNA concentrations for IL-6 and tumour necrosis factor (TNF) and decreased mRNA concentrations for TSLP after electroporation. Lower expression of TSLP, which plays an important role in cancer progression, was also confirmed at the protein level. Zhang et al (71) observed a decreased expression of Ki-67 and TGF-β proteins after electroporation of breast cancer cells. Ki-67 is used as a marker for dividing cells and is associated with tumour growth and invasion, and TGF-β expression correlates with tumour invasiveness. Mlakar et al (63), however, showed that electroporation of melanoma cells did not change the expression of the main tumour suppressor genes and oncogenes. All of these studies indicate that electroporation is a safe and non-carcinogenic method.

4 Conclusion

Electroporation is a phenomenon that causes increased cell membrane permeability due to exposure of cells/tissues to an electric field. It causes a series of changes in the cell, including structural changes in the cell membrane, peroxidation of membrane lipids, influx of Ca^{2+} into the cytoplasm, ATP and K^+ release from the cell, osmotic imbalance, cytoskeleton disruption, changes in gene expression and protein synthesis, formation of reactive oxygen species, activation of signalling pathways and repair mechanisms; it also triggers cell death under certain conditions. The term electropermeome refers to both a permeabilized cell and what happens during or immediately after the delivery of electrical pulses, as well as all subsequent processes that remain active for some time after increased transmembrane transport of substances to which the cell membrane is normally impermeable is no longer observed, i.e. even after the time when we can already see that the membrane has resealed.

Electroporation is used in many fields, including electrochemotherapy, tissue ablation, and gene electrotransfer. Electrochemotherapy has been used in clinical practice for more than 15 years and is included in guidelines and standard clinical practice in many European countries for the treatment of various superficial tumours, including melanoma, squamous carcinoma and metastases of all histological types. Clinical studies have shown that electrochemotherapy is feasible, safe, and effective even for deep-seated tumours (2,3). While thermal techniques (radiofrequency and microwave ablation, cryoablation) are routinely used for soft tissue ablation, interest in irreversible electroporation ablation is growing. The latter is particularly interesting for use on anatomical sites where surgery and thermal ablation methods are not possible, e.g. due to the proximity of vital structures such as large blood vessels, intestine, and biliary or urinary tracts. Due to the predominantly non-thermal mechanism of action, ablation with irreversible electroporation does not damage the surrounding tissue. The efficacy and safety of irreversible electroporation ablation have been demonstrated in numerous clinical studies for ablation of deep-seated liver, kidney, pancreas and prostate tumours, as well as for pulmonary vein isolation in the treatment of atrial fibrillation (4,82). Clinical studies are also investigating the possibility of using electroporation as a method of introducing nucleic acids for gene therapy: for the treatment of cancer,

introduction of DNA or RNA-based vaccines against infectious diseases and cancer, introduction of components of the CRISPR-Cas9 system for genome editing, etc. (83).

Medical applications of electroporation are effective and safe, but due to the action of electrical pulses, certain unwanted side effects are also possible, particularly muscle contraction and acute pain. Further clarification of the basic mechanisms of electroporation and the influence of individual electric field parameters on the electropermeome is crucial for the optimization of electroporation parameters and the results of electroporation-based applications.

Conflict of interest

D. Miklavčič is a consultant to Medtronic. A. Vižintin has no conflict of interest.

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References

1. Kotnik T, Rems L, Tarek M, Miklavčič D. Membrane Electroporation and Electropermeabilization: mechanisms and Models. Annu Rev Biophys. 2019;48(1):63-91. DOI: 10.1146/annurev-biophys-052118-115451 PMID: 30786231
2. Geboers B, Scheffer HJ, Graybill PM, Ruarus AH, Nieuwenhuizen S, Puijk RS, et al. High-Voltage Electrical Pulses in Oncology: Irreversible Electroporation, Electrochemotherapy,Gene Electrotransfer, Electrofusion, and Electroimmunotherapy. Radiology. 2020;295(2):254-72. DOI: 10.1148/radiol.2020192190 PMID: 32208094
3. Stepišnik T, Jarm T, Grošelj A, Edhemović I, Đokić M, Ivanecz A, et al. Elektrokemoterapija – učinkovita metoda zdravljenja tumorjev s kombinacijo kemoterapevtikain električnega polja. Zdrav Vestn. 2016;86(1):41-55. DOI: 10.6016/ZdravVestn.1453
4. Cindrič H, Kos B, Miklavčič D. Ireverzibilna elektroporacija kot metoda ablacije mehkih tkiv: pregled in izzivi priuporabi v kliničnem okolju. Zdrav Vestn. 2021(1-2):38-53. DOI: 10.6016/ZdravVestn.2954
5. Kim V, Gudvangen E, Kondratiev O, Redondo L, Xiao S, Pakhomov AG. Peculiarities of Neurostimulation by Intense Nanosecond Pulsed Electric Fields: Howto Avoid Firing in Peripheral Nerve Fibers. Int J Mol Sci. 2021;22(13):7051. DOI: 10.3390/ijms22137051 PMID: 34208945
6. Schoenbach KH, Beebe SJ, Buescher ES. Intracellular effect of ultrashort electrical pulses. Bioelectromagnetics. 2001;22(6):440-8. DOI: 10.1002/bem.71 PMID: 11536285
7. Siddiqui IA, Latouche EL, DeWitt MR, Swet JH, Kirks RC, Baker EH, et al. Induction of rapid, reproducible hepatic ablations using next-generation, high frequencyirreversible electroporation (H-FIRE) in vivo. HPB (Oxford). 2016;18(9):726-34. DOI: 10.1016/j.hpb.2016.06.015 PMID: 27593589
8. Dong S, Yao C, Zhao Y, Lv Y, Liu H. Parameters Optimization of Bipolar High Frequency Pulses on Tissue Ablation and InhibitingMuscle Contraction. IEEE Trans Dielectr Electr Insul. 2018;25(1):207-16. DOI: 10.1109/TDEI.2018.006303
9. O'Brien TJ, Passeri M, Lorenzo MF, Sulzer JK, Lyman WB, Swet JH, et al. Experimental high-frequency irreversible electroporation using a single-needle deliveryapproach for nonthermal pancreatic ablation in vivo. J Vasc Interv Radiol. 2019;30(6):854-862.e7. DOI: 10.1016/j.jvir.2019.01.032 PMID: 31126597
10. Vernier PT, Sun Y, Gundersen MA. Nanoelectropulse-driven membrane perturbation and small molecule permeabilization. BMC Cell Biol. 2006;7(1):37. DOI: 10.1186/1471-2121-7-37 PMID: 17052354
11. Levine ZA, Vernier PT. Life cycle of an electropore: field-dependent and field-independent steps in porecreation and annihilation. J Membr Biol. 2010;236(1):27-36. DOI: 10.1007/s00232-010-9277-y PMID: 20623350
12. Pakhomov AG, Shevin R, White JA, Kolb JF, Pakhomova ON, Joshi RP, et al. Membrane permeabilization and cell damage by ultrashort electric field shocks. Arch Biochem Biophys. 2007;465(1):109-18. DOI: 10.1016/j.abb.2007.05.003 PMID: 17555703
13. Pucihar G, Kotnik T, Miklavčič D, Teissié J. Kinetics of transmembrane transport of small molecules into electropermeabilized cells. Biophys J. 2008;95(6):2837-48. DOI: 10.1529/biophysj.108.135541 PMID: 18539632
14. Pakhomov AG, Kolb JF, White JA, Joshi RP, Xiao S, Schoenbach KH. Long-lasting plasma membrane permeabilization in mammalian cells by nanosecond pulsedelectric field (nsPEF). Bioelectromagnetics. 2007;28(8):655-63. DOI: 10.1002/bem.20354 PMID: 17654532
15. Muralidharan A, Rems L, Kreutzer MT, Boukany PE. Actin networks regulate the cell membrane permeability during electroporation. Biochim Biophys Acta Biomembr. 2021;1863(1):183468. DOI: 10.1016/j.bbamem.2020.183468 PMID: 32882211
16. Sözer EB, Levine ZA, Vernier PT. Quantitative limits on small molecule transport via the electropermeome measuringand modeling single nanosecond perturbations. Sci Rep. 2017;7(1):57. DOI: 10.1038/s41598-017-00092-0 PMID: 28246401
17. Maccarrone M, Rosato N, Agrò AF. Electroporation enhances cell membrane peroxidation and luminescence. Biochem Biophys Res Commun. 1995;206(1):238-45. DOI: 10.1006/bbrc.1995.1033 PMID: 7818526
18. Maccarrone M, Bladergroen MR, Rosato N, Finazzi Agrò AF. Role of lipid peroxidation in electroporation-induced cell permeability. Biochem Biophys Res Commun. 1995;209(2):417-25. DOI: 10.1006/bbrc.1995.1519 PMID: 7733908
19. Gabriel B, Teissié J. Generation of reactive-oxygen species induced by electropermeabilization of Chinesehamster ovary cells and their consequence on cell viability. Eur J Biochem. 1994;223(1):25-33. DOI: 10.1111/j.1432-1033.1994.tb18962.x PMID: 8033899
20. Gabriel B, Teissié J. Spatial compartmentation and time resolution of photooxidation of a cell membraneprobe in electropermeabilized Chinese hamster ovary cells. Eur J Biochem. 1995;228(3):710-8. DOI: 10.1111/j.1432-1033.1995.tb20314.xPMID: 7737168
21. Nuccitelli R, Lui K, Kreis M, Athos B, Nuccitelli P. Nanosecond pulsed electric field stimulation of reactive oxygen species in human pancreaticcancer cells is Ca(2+)-dependent. Biochem Biophys Res Commun. 2013;435(4):580-5. DOI: 10.1016/j.bbrc.2013.05.014 PMID: 23680664

22. Breton M, Mir LM. Investigation of the chemical mechanisms involved in the electropulsation of membranesat the molecular level. Bioelectrochemistry. 2018;119:76-83. DOI: 10.1016/j.bioelechem.2017.09.005 PMID: 28917184
23. Frankel EN. Lipid oxidation: Mechanisms, products and biological significance. J Am Oil Chem Soc. 1984;61(12):1908-17. DOI: 10.1007/BF02540830
24. Benov LC, Antonov PA, Ribarov SR. Oxidative damage of the membrane lipids after electroporation. Gen Physiol Biophys. 1994;13(2):85-97. PMID: 7806071
25. Vernier PT, Levine ZA, Wu YH, Joubert V, Ziegler MJ, Mir LM, et al. Electroporating fields target oxidatively damaged areas in the cell membrane. PLoS One. 2009;4(11):e7966. DOI: 10.1371/journal.pone.0007966 PMID: 19956595
26. Rems L, Viano M, Kasimova MA, Miklavčič D, Tarek M. The contribution of lipid peroxidation to membrane permeability in electropermeabilization:A molecular dynamics study. Bioelectrochemistry. 2019;125:46-57. DOI: 10.1016/j.bioelechem.2018.07.018 PMID: 30265863
27. Runas KA, Malmstadt N. Low levels of lipid oxidation radically increase the passive permeability of lipidbilayers. Soft Matter. 2015;11(3):499-505. DOI: 10.1039/C4SM01478B PMID: 25415555
28. Wong-Ekkabut J, Xu Z, Triampo W, Tang IM, Tieleman DP, Monticelli L. Effect of lipid peroxidation on the properties of lipid bilayers: a molecular dynamicsstudy. Biophys J. 2007;93(12):4225-36. DOI: 10.1529/biophysj.107.112565 PMID: 17766354
29. Sabatini K, Mattila JP, Megli FM, Kinnunen PK. Characterization of two oxidatively modified phospholipids in mixed monolayers withDPPC. Biophys J. 2006;90(12):4488-99. DOI: 10.1529/biophysj.105.080176 PMID: 16581831
30. Boonnoy P, Jarerattanachat V, Karttunen M, Wong-Ekkabut J. Bilayer Deformation, Pores, and Micellation Induced by Oxidized Lipids. J Phys Chem Lett. 2015;6(24):4884-8. DOI: 10.1021/acs.jpclett.5b02405 PMID: 26673194
31. Van der Paal J, Neyts EC, Verlackt CC, Bogaerts A. Effect of lipid peroxidation on membrane permeability of cancer and normal cells subjectedto oxidative stress. Chem Sci (Camb). 2016;7(1):489-98. DOI: 10.1039/C5SC02311D PMID: 28791102
32. Lis M, Wizert A, Przybylo M, Langner M, Swiatek J, Jungwirth P, et al. The effect of lipid oxidation on the water permeability of phospholipids bilayers. Phys Chem Chem Phys. 2011;13(39):17555-63. DOI: 10.1039/c1cp21009b PMID: 21897935
33. Wiczew D, Szulc N, Tarek M. Molecular dynamics simulations of the effects of lipid oxidation on the permeabilityof cell membranes. Bioelectrochemistry. 2021;141:107869. DOI: 10.1016/j.bioelechem.2021.107869 PMID: 34119820
34. Cwiklik L, Jungwirth P. Massive oxidation of phospholipid membranes leads to pore creation and bilayer disintegration. Chem Phys Lett. 2010;486(4–6):99-103. DOI: 10.1016/j.cplett.2010.01.010
35. Kulig W, Olżyńska A, Jurkiewicz P, Kantola AM, Komulainen S, Manna M, et al. Cholesterol under oxidative stress-How lipid membranes sense oxidation as cholesterolis being replaced by oxysterols. Free Radic Biol Med. 2015;84:30-41. DOI: 10.1016/j.freeradbiomed.2015.03.006 PMID: 25795515
36. Kazmierska M, Rudzinska M, Jarosz B, Dobrzanski Z, Trziszka T. Changes of the fatty acid composition, cholesterol and cholesterol oxide contentsin whole egg after pulsed electric field treatment. Arch Geflugelkd. 2012;76(4):246-53.
37. Troiano GC, Stebe KJ, Raphael RM, Tung L. The effects of gramicidin on electroporation of lipid bilayers. Biophys J. 1999;76(6):3150-7. DOI: 10.1016/S0006-3495(99)77466-7 PMID: 10354439
38. Tarek M. Membrane electroporation: a molecular dynamics simulation. Biophys J. 2005;88(6):P4045-53. DOI: 10.1529/biophysj.104.050617 PMID: 15764667
39. Azan A, Untereiner V, Descamps L, Merla C, Gobinet C, Breton M, et al. Comprehensive Characterization of the Interaction between Pulsed Electric Fields andLive Cells by Confocal Raman Microspectroscopy. Anal Chem. 2017;89(20):10790-7. DOI: 10.1021/acs.analchem.7b02079 PMID: 28876051
40. Azan A, Untereiner V, Gobinet C, Sockalingum GD, Breton M, Piot O, et al. Demonstration of the Protein Involvement in Cell Electropermeabilization using ConfocalRaman Microspectroscopy. Sci Rep. 2016;2017(7):1-10. PMID: 28102326
41. Craviso GL, Choe S, Chatterjee P, Chatterjee I, Vernier PT. Nanosecond electric pulses: a novel stimulus for triggering Ca2+ influx into chromaffincells via voltage-gated Ca2+ channels. Cell Mol Neurobiol. 2010;30(8):1259-65. DOI: 10.1007/s10571-010-9573-1 PMID: 21080060
42. Semenov I, Xiao S, Kang D, Schoenbach KH, Pakhomov AG. Cell stimulation and calcium mobilization by picosecond electric pulses. Bioelectrochemistry. 2015;105:65-71. DOI: 10.1016/j.bioelechem.2015.05.013 PMID: 26011130
43. Burke RC, Bardet SM, Carr L, Romanenko S, Arnaud-Cormos D, Leveque P, et al. Nanosecond pulsed electric fields depolarize transmembrane potential via voltage-gatedK+, Ca2+ and TRPM8 channels in U87 glioblastoma cells. Biochim Biophys Acta Biomembr. 2017;1859(10):2040-50. DOI: 10.1016/j.bbamem.2017.07.004 PMID: 28693898
44. Teissié J, Tsong TY. Evidence of voltage-induced channel opening in Na/K ATPase of human erythrocyte membrane. J Membr Biol. 1980;55(2):133-40. DOI: 10.1007/BF01871155 PMID: 6251222
45. Kanthou C, Kranjc S, Serša G, Tozer G, Županič A, Čemažar M. The endothelial cytoskeleton as a target of electroporation-based therapies. Mol Cancer Ther. 2006;5(12):3145-52. DOI: 10.1158/1535-7163.MCT-06-0410 PMID: 17172418
46. Meulenberg CJ, Todorović V, Čemažar M. Differential cellular effects of electroporation and electrochemotherapy in monolayersof human microvascular endothelial cells. PLoS One. 2012;7(12):e52713. DOI: 10.1371/journal.pone.0052713 PMID: 23300747
47. Rems L, Kasimova MA, Testa I, Delemotte L. Pulsed Electric Fields Can Create Pores in the Voltage Sensors of Voltage-Gated IonChannels. Biophys J. 2020;119(1):190-205. DOI: 10.1016/j.bpj.2020.05.030 PMID: 32559411
48. Ruiz-Fernández AR, Campos L, Villanelo F, Gutiérrez-Maldonado SE, Perez-Acle T. Exploring the conformational changes induced by nanosecond pulsed electric fieldson the voltage sensing domain of a Ca2+ channel. Membranes (Basel). 2021;11(7):473. DOI: 10.3390/membranes11070473 PMID: 34206827
49. Graybill PM, Davalos RV. Cytoskeletal Disruption after Electroporation and Its Significance to Pulsed ElectricField Therapies. Cancers (Basel). 2020;12(5):29-32. DOI: 10.3390/cancers12051132 PMID: 32366043
50. Perrier DL, Vahid A, Kathavi V, Stam L, Rems L, Mulla Y, et al. Response of an actin network in vesicles under electric pulses. Sci Rep. 2019;9(1):8151. DOI: 10.1038/s41598-019-44613-5 PMID: 31148577
51. Harkin DG, Hay ED. Effects of electroporation on the tubulin cytoskeleton and directed migration of cornealfibroblasts cultured within collagen matrices. Cell Motil Cytoskeleton. 1996;35(4):345-57. DOI: 10.1002/(SICI)1097-0169(1996)35:4<345::AID-CM6>3.0.CO;2-5 PMID: 8956005
52. Thompson GL, Roth CC, Kuipers MA, Tolstykh GP, Beier HT, Ibey BL. Permeabilization of the nuclear envelope following nanosecond pulsed electric fieldexposure. Biochem Biophys Res Commun. 2016;470(1):35-40. DOI: 10.1016/j.bbrc.2015.12.092 PMID: 26721436
53. Pakhomov AG, Xiao S, Pakhomova ON, Semenov I, Kuipers MA, Ibey BL. Disassembly of actin structures by nanosecond pulsed electric field is a downstreameffect of cell swelling. Bioelectrochemistry. 2014;100:88-95. DOI: 10.1016/j.bioelechem.2014.01.004 PMID: 24507565
54. Louise C, Etienne D, Marie-Pierre R. AFM sensing cortical actin cytoskeleton destabilization during plasma membrane electropermeabilization. Cytoskeleton (Hoboken). 2014;71(10):587-94. DOI: 10.1002/cm.21194 PMID: 25308626

55. Titushkin I, Cho M. Regulation of cell cytoskeleton and membrane mechanics by electric field: role oflinker proteins. Biophys J. 2009;96(2):717-28. DOI: 10.1016/j.bpj.2008.09.035 PMID: 19167316

56. Marracino P, Havelka D, Průša J, Liberti M, Tuszynski J, Ayoub AT, et al. Tubulin response to intense nanosecond-scale electric field in molecular dynamicssimulation. Sci Rep. 2019;9(1):10477. DOI: 10.1038/s41598-019-46636-4 PMID: 31324834

57. Chafai DE, Sulimenko V, Havelka D, Kubínová L, Dráber P, Cifra M. Reversible and Irreversible Modulation of Tubulin Self-Assembly by Intense NanosecondPulsed Electric Fields. Adv Mater. 2019;31(39):e1903636. DOI: 10.1002/adma.201903636 PMID: 31408579

58. Průša J, Cifra M. Molecular dynamics simulation of the nanosecond pulsed electric field effect on kinesinnanomotor. Sci Rep. 2019;9(1):19721. DOI: 10.1038/s41598-019-56052-3 PMID: 31873109

59. Tanino T, Sato S, Oshige M, Ohshima T. Analysis of the stress response of yeast Saccharomyces cerevisiae toward pulsed electricfield. J Electrost. 2012;70(2):212-6. DOI: 10.1016/j.elstat.2012.01.003

60. Michel O, Kulbacka J, Saczko J, Mączyńska J, Błasiak P, Rossowska J, et al. Electroporation with Cisplatin against Metastatic Pancreatic Cancer: In Vitro Studyon Human Primary Cell Culture. BioMed Res Int. 2018;2018:7364539. DOI: 10.1155/2018/7364539 PMID: 29750170

61. Dovgan B, Miklavčič D, Knežević M, Zupan J, Barlič A. Intracellular delivery of trehalose renders mesenchymal stromal cells viable and immunomodulatorycompetent after cryopreservation. Cytotechnology. 2021;73(3):1-21. DOI: 10.1007/s10616-021-00465-4 PMID: 33875905

62. Morotomi-Yano K, Oyadomari S, Akiyama H, Yano K. Nanosecond pulsed electric fields act as a novel cellular stress that induces translationalsuppression accompanied by eIF2α phosphorylation and 4E-BP1 dephosphorylation. Exp Cell Res. 2012;318(14):1733-44. DOI: 10.1016/j.yexcr.2012.04.016 PMID: 22652449

63. Mlakar V, Todorović V, Čemažar M, Glavač D, Serša G. Electric pulses used in electrochemotherapy and electrogene therapy do not significantlychange the expression profile of genes involved in the development of cancer in malignantmelanoma cells. BMC Cancer. 2009;9(1):299. DOI: 10.1186/1471-2407-9-299 PMID: 19709437

64. Hojman P, Zibert JR, Gissel H, Eriksen J, Gehl J. Gene expression profiles in skeletal muscle after gene electrotransfer. BMC Mol Biol. 2007;8(1):56. DOI: 10.1186/1471-2199-8-56 PMID: 17598924

65. Mittal L, Camarillo IG, Varadarajan GS, Srinivasan H, Aryal UK, Sundararajan R. High-throughput, Label-Free Quantitative Proteomic Studies of the Anticancer Effectsof Electrical Pulses with Turmeric Silver Nanoparticles: an in vitro Model Study. Sci Rep. 2020;10(1):7258. DOI: 10.1038/s41598-020-64128-8 PMID: 32350346

66. Batista Napotnik T, Polajžer T, Miklavčič D. Cell death due to electroporation - A review. Bioelectrochemistry. 2021;141:107871. DOI: 10.1016/j.bioelechem.2021.107871 PMID: 34147013

67. Zhang Y, Lyu C, Liu Y, Lv Y, Chang TT, Rubinsky B. Molecular and histological study on the effects of non-thermal irreversible electroporationon the liver. Biochem Biophys Res Commun. 2018;500(3):665-70. DOI: 10.1016/j.bbrc.2018.04.132 PMID: 29678581

68. Beebe SJ, Fox PM, Rec LJ, Willis EL, Schoenbach KH. Nanosecond, high-intensity pulsed electric fields induce apoptosis in human cells. FASEB J. 2003;17(11):1493-5. DOI: 10.1096/fj.02-0859fje PMID: 12824299

69. Ford WE, Ren W, Blackmore PF, Schoenbach KH, Beebe SJ. Nanosecond pulsed electric fields stimulate apoptosis without release of pro-apoptoticfactors from mitochondria in B16f10 melanoma. Arch Biochem Biophys. 2010;497(1-2):82-9. DOI: 10.1016/j.abb.2010.03.008 PMID: 20346344

70. Beebe SJ, Fox PM, Rec LJ, Somers K, Stark RH, Schoenbach KH. Nanosecond pulsed electric field (nsPEF) effects on cells and tissues: apoptosis inductionand tumor growth inhibition. IEEE Trans Plasma Sci. 2002;30(1):286-92. DOI: 10.1109/TPS.2002.1003872

71. Zhang H, Liu K, Xue Z, Yin H, Dong H, Jin W, et al. High-voltage pulsed electric field plus photodynamic therapy kills breast cancer cellsby triggering apoptosis. Am J Transl Res. 2018;10(2):334-51. PMID: 29511429

72. Zhang Z, Li W, Procissi D, Tyler P, Omary RA, Larson AC. Rapid dramatic alterations to the tumor microstructure in pancreatic cancer followingirreversible electroporation ablation. Nanomedicine (Lond). 2014;9(8):1181-92. DOI: 10.2217/nnm.13.72 PMID: 24024571

73. O'Brien TJ, Passeri M, Lorenzo MF, Sulzer JK, Lyman WB, Swet JH, et al. Experimental high-frequency irreversible electroporation using a single-needle deliveryapproach for nonthermal pancreatic ablation in vivo. J Vasc Interv Radiol. 2019;30(6):854-862.e7. DOI: 10.1016/j.jvir.2019.01.032 PMID: 31126597

74. Siddiqui IA, Latouche EL, Dewitt MR, Swet JH, Kirks RC, Baker EH, et al. Induction of rapid, reproducible hepatic ablations using next-generation, high frequencyirreversible electroporation (H-FIRE) in vivo. HPB (xford). 2016;18(9):726-34. DOI: 10.1016/j.hpb.2016.06.015 PMID: 27593589

75. Mercadal B, Beitel-White N, Aycock KN, Castellví Q, Davalos RV, Ivorra A. Dynamics of Cell Death After Conventional IRE and H-FIRE Treatments. Ann Biomed Eng. 2020;48(5):1451-62. DOI: 10.1007/s10439-020-02462-8 PMID: 32026232

76. Faroja M, Ahmed M, Appelbaum L, Ben-David E, Moussa M, Sosna J, et al. Irreversible electroporation ablation: is all the damage nonthermal? Radiology. 2013;266(2):462-70. DOI: 10.1148/radiol.12120609 PMID: 23169795

77. Ben-David E, Ahmed M, Faroja M, Moussa M, Wandel A, Sosna J, et al. Irreversible electroporation: treatment effect is susceptible to local environmentand tissue properties. Radiology. 2013;269(3):738-47. DOI: 10.1148/radiol.13122590 PMID: 23847254

78. Ringel-Scaia VM, Beitel-White N, Lorenzo MF, Brock RM, Huie KE, Coutermarsh-Ott S, et al. High-frequency irreversible electroporation is an effective tumor ablation strategythat induces immunologic cell death and promotes systemic anti-tumor immunity. EBioMedicine. 2019;44:112-25. DOI: 10.1016/j.ebiom.2019.05.036 PMID: 31130474

79. Peng B, Zhao Y, Xu L, Xu Y. Electric pulses applied prior to intramuscular DNA vaccination greatly improve thevaccine immunogenicity. Vaccine. 2007;25(11):2064-73. DOI: 10.1016/j.vaccine.2006.11.042 PMID: 17239494

80. Heller LC, Cruz YL, Ferraro B, Yang H, Heller R. Plasmid injection and application of electric pulses alter endogenous mRNA and proteinexpression in B16.F10 mouse melanomas. Cancer Gene Ther. 2010;17(12):864-71. DOI: 10.1038/cgt.2010.43 PMID: 20706286

81. Goswami I, Coutermarsh-Ott S, Morrison RG, Allen IC, Davalos RV, Verbridge SS, et al. Irreversible electroporation inhibits pro-cancer inflammatory signaling in triplenegative breast cancer cells. Bioelectrochemistry. 2017;113:42-50. DOI: 10.1016/j.bioelechem.2016.09.003 PMID: 27693939

82. Štublar J, Žižek D, Jan M, Jarm T, Miklavčič D. Zdravljenje atrijske fibrilacije s katetrsko ablacijo. Zdrav Vestn. 2021;90:410-9.

83. Sachdev S, Potočnik T, Rems L, Miklavčič D. Revisiting the role of pulsed electric fields in overcoming the barriers to in vivogene electrotransfer. Bioelectrochemistry. 2022;144:107994. DOI: 10.1016/j.bioelechem.2021.107994 PMID: 34930678