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Cell death due to electroporation – A review

Tina Batista Napotnik, Tamara Polajžer, Damijan Miklavčič *

University of Ljubljana, Faculty of Electrical Engineering, Tržaška cesta 25, 1000 Ljubljana, Slovenia

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ABSTRACT

Exposure of cells to high voltage electric pulses increases transiently membrane permeability through membrane electroporation. Electroporation can be reversible and is used in gene transfer and enhanced drug delivery but can also lead to cell death. Electroporation resulting in cell death (termed as irreversible electroporation) has been successfully used as a new non-thermal ablation method of soft tissue such as tumours or arrhythmogenic heart tissue. Even though the mechanisms of cell death can influence the outcome of electroporation-based treatments due to use of different electric pulse parameters and conditions, these are not elucidated yet. We review the mechanisms of cell death after electroporation reported in literature, cell injuries that may lead to cell death after electroporation and membrane repair mechanisms involved. The knowledge of membrane repair and cell death mechanisms after cell exposure to electric pulses, targets of electric field in cells need to be identified to optimize existing and develop of new electroporation-based techniques used in medicine, biotechnology, and food technology.

Contents

* Corresponding author.
E-mail address: damijan.miklavcic@fe.uni-lj.si (D. Miklavčič).

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1. Introduction

Electroporation is a phenomenon that occurs when cells are exposed to electric field of sufficient amplitude: their plasma membrane permeability becomes increased [1]. On one hand, in reversible electroporation, the increased permeability is only temporary, after a certain time cells repair their plasma membrane and re-establish homeostasis. Reversible electroporation is used in biotechnology and medicine for delivery of otherwise impermeant molecules to cells such as chemotherapeutics in electrochemotherapy (ECT) [2] or nucleic acids in gene electrotransfer (GET) [3]. On the other hand, in irreversible electroporation (usually with the use of higher number of electric pulses and of higher amplitude), the cells are damaged beyond repair and they die [4]. Irreversible electroporation is already used as a focal ablative technique for treating tumours, especially those unsuitable for surgery or thermal ablation because of their specific anatomic location [4]. Irreversible electroporation (called also as pulsed field ablation) is resurging as efficient, safe and fast ablation modality [5]. This technology is expected to represent a major advance in the field of treating heart arrhythmias [6]. The mechanisms of cell death also due to expanding interest in cardiac ablation are therefore of significant interest.

Irreversible electroporation leads to cell death of different types, namely necrosis, apoptosis, and also types of immunogenic cell death such as necroptosis and pyroptosis that have gained attention in recent years. It is important to know cell death pathways and how electric pulses of different parameters influence them in order to control and optimize therapeutic protocols such as tumour ablation and pulsed field ablation in heart. Different types of cell death have also different systemic responses in terms of abscopal effects and long-term immune response which is _especially important in oncology. Moreover, in gene electrotransfer, EP itself can also stimulate immune response [7]. Therefore, there is a need for better understanding of triggering cell death and immune response by electroporation especially considering the expanding interest in applying this technology clinically. And the last but not least, with the increasing knowledge of specific repair and cell death mechanisms after electric field exposure, direct and indirect targets of electric field in cells can be identified which can lead to a development of new electroporation-based techniques used in medicine, biotechnology, and food technology [8].

1.1. Cell injury and cell death

Cells constantly adapt to physiological demands to maintain their viability and homeostasis. The term cell injury is used to describe the situation when the stimulus/insult, external or internal, is excessive or when the cell is no longer capable to adapt without suffering some form of damage. Cell injury can be reparable/reversible (non-lethal damage which can generally be corrected) or irreversible (lethal damage) resulting in cell death. The transition between reversible and irreversible damage, commonly referred to as the "point of no return" is of major interest and importance for devising therapeutic strategies to prevent or trigger cell death after therapeutic intervention [9,10].

The main mechanisms of cell injury are: 1) membrane damage, 2) DNA and protein damage, 3) increase of ROS, 4) entry of Ca^{2+}, 5) mitochondrial damage, and 6) ATP depletion. They are schematically depicted in Fig. 1. We can observe the complexity of cell injury biochemical mechanisms. They are interconnected and overlapping, sometimes one injurious agent (insult) can trigger multiple pathways, therefore it is not always possible to determine a specific target or prevention mode of injury of a particular insult [9–12].

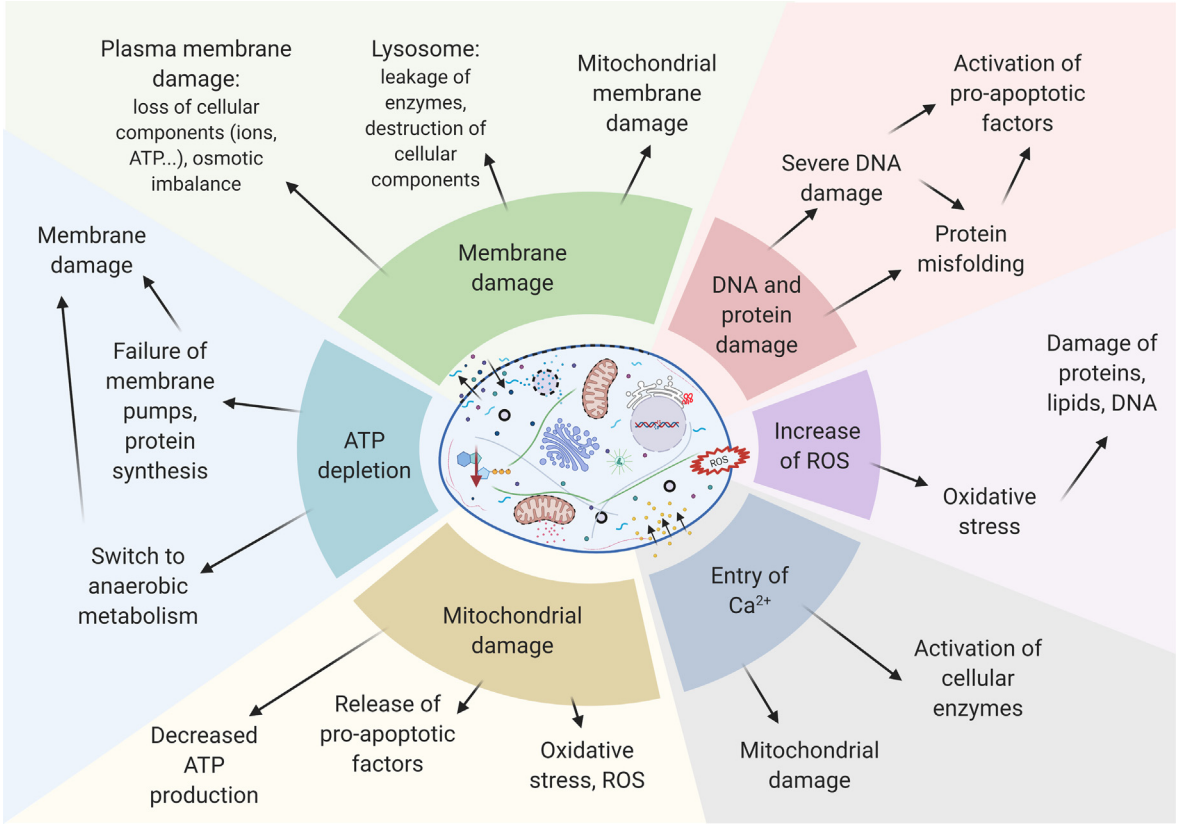

Fig. 1. Mechanisms of cell injury (derived from [9,10,12]). Created with BioRender.com.

1.2. Modes of cell death

When a cell is injured beyond repair, it dies. Historically, cell death was classified into three forms, with respect to morphological changes: type I: apoptosis (programmed cell death, cell shrinkage, caspase activation, DNA condensation, fragmentation into apoptotic bodies, absence of immune response), type II: autophagy (massive vacuolization of the cytoplasm), and type III: necrosis (accidental cell death, swelling, plasmalemmal blebs and rupture, cell lysis, immune response) [13]. This classification is still widely used, however, over the past decade new definitions of cell death emerged based on morphological, enzymological (involvement of nucleases and caspases), functional (programmed/regulated or accidental) and immunological characteristics (immunogenic or non-immunogenic) [14]. A Nomenclature Committee on Cell Death formulated guidelines for the definition and interpretation of cell death from morphological, biochemical, and functional perspectives [11]. With extensive study of cell death mechanisms, several new pathways of immunogenic cell death – a programmed/regulated cell death that can exhibit necrotic or apoptotic morphology and elicit immune response – were identified, e.g. necroptosis, ferroptosis, pyroptosis, and others [11–15].

Damaged, dying or dead cells resulting from trauma, ischemia, cancer, and other settings of tissue damage in the absence of pathogenic infection communicate a state of danger to the organism by releasing damage-associated molecular patterns (DAMPs), i.e. endogenic danger molecules. DAMPs activate the immune system by interacting with pattern recognition receptors (PRRs) on cells of innate immune system (the same PRRs that interact also with pathogen-associated molecular patterns PAMPs) and thereby trigger non-infectious inflammation and tissue repair [16–18]. DAMP molecules are passively or actively released from cytoplasm, nucleus or other cellular compartments into extracellular space (adenosine triphosphate ATP, chromatin-binding protein high mobility group B1 HMGB1, DNA etc.) or become exposed on the cell surface (calreticulin, heat shock proteins) [13,19]. DAMPs interact with PRRs (such as receptor for advanced glycation end products RAGE, Toll-like receptors TLRs, NOD-like receptors NLRs etc.) on the surface of immune cells (dendritic cells, macrophages, T cells and neutrophils) and trigger innate and adaptive immune responses via various pathways (NF-κB etc.) [17–19]. A selective interception of immunogenic cell death pathways can be a promising new tool for diagnosis, prevention and treatment of human diseases in which cell loss must be avoided (inflammatory diseases) or amplified (cancer) [13,19–22].

2. Materials and methods

2.1. Literature search strategy and selection

For the purpose of our review of electroporation-related cell death, a semi-systematic search through Pub Med database (The National Institutes of Health) was performed by employing keywords "Electroporation cell death apoptosis", "Electroporation cell death necrosis", "Electroporation cell death necroptosis", "Electroporation cell death pyroptosis", "Electroporation cell death pyroptotic", "Electroporation nanosecond apoptosis", "Electroporation nanosecond necrosis", "Electroporation calcium cell death", and "Electroporation immune". Besides that, papers from previous reviews and publications [23–25] were included to the list of papers analysed. Among them, 113 papers related to cell injury and cell death after EP with electric pulses of different parameters (ns-ms range of square monopolar or bipolar pulses) without addition of any chemical compounds (chemotherapeutics, Ca^{2+} ions, DNA) were analysed for cell injury (Tables 1 and 2).

3. Cell death and electroporation

3.1. Electroporation and cell injury

Electroporation (EP) can be considered as a membrane damage (structural and dynamical reorganization of the plasma membrane) with cell recovery as an active cellular process, which involves cellular machinery [1,26]. Membrane pore formation by itself is an injury, moreover, EP causes also lipid peroxidation [27,28] and damages membrane embedded proteins [29–31]. Since many pathways of cell injury and cell death overlap, the main reason for cell death after electroporation is still unspecified. Nevertheless, some information can be gathered from the literature. Unfortunately, it is not possible to infer if the cell death following EP is a result of multiple cell injuries or a single specific pathway following a deleterious effect acting on a specific target, or if various pathways are initiated by different electric pulses used.

An injury in plasma membrane allows Ca^{2+} ions to enter the cell from extracellular space, disrupting the intracellular calcium homeostasis [32,33]. Since Ca^{2+} is a universal carrier of biological information [34], EP can trigger numerous pathways of cell signalling including stress or cell death pathways. Pore formation causes osmotic imbalance and cell swelling which lead to necrosis and this mechanism may be calcium-dependent [35–38]. The absence of extracellular Ca^{2+} prevents cells from dying from apoptosis via endoplasmic reticulum (ER) pathway [39] or commits cells more to apoptosis than to necrosis [37]. The increase of internal Ca^{2+} can be further amplified with store-operated (capacitive) Ca^{2+} entry [40,41] or especially, Ca-induced Ca^{2+} release from internal stores [42,43].

A massive influx of Ca^{2+} after EP causes depletion of intracellular ATP due to the activation of Ca-ATPases and inhibition of ATP production in mitochondria and is linked to cell death (mostly necrosis) in calcium electroporation (Ca EP) [32,33,44–49]. Moreover, ATP and other energetically rich molecules can leak through permeabilized plasma membrane from the cell [25,50–52]. In fact, ATP leak was even one of the first assays to detect cell membrane permeabilization [53]. Also to be noted, ATP depletion switches cell death from apoptosis to necrosis [54].

Electric pulses induce production of reactive oxygen species (ROS) and oxidative damage of unsaturated lipids that are associated to cell membrane permeability, membrane resealing time, and cell damage [1,26,27,55,56] and may therefore contribute to increased permeability post-pulse [28]. Electric pulses can initiate ROS production inside the cell [57–59], mostly in mitochondria, that can damage cell molecules, trigger oxidative stress, and lead to cell death by apoptosis, necrosis, necroptosis, or pyroptosis, which appears to depend on cell type and pulse parameters [12,58,60].

Mitochondrial damage by electroporation is intricate due to complex structure and function of this cellular organelle. The effects of electric pulses on mitochondria were mainly studied using electric pulses of nanosecond duration. In contrast to conventional electroporation using μs and ms pulses it was first believed that nanosecond pulse electroporation (nsEP) can create small pores only in membranes of cell organelles like mitochondria, with negligible impact on plasma membrane [61]. Moreover, it was discovered that electroporation with nsEP induce apoptosis where mitochondria play a major role [62,63]. It was shown that nsEP cause loss of mitochondrial membrane potential (MMP) that is crucial for mitochondrial activity [35,64–70]. However, it is still not known if this is a direct or indirect (apoptosis-related) effect of nsEP. Due to Ca^{2+}-dependency of MMP dissipation it was suggested that this was not due to electroporation of the inner mitochondrial membrane [65,71]. How-

Table 1
Published papers on cell death, reporting on cell injury *in vitro*. Most commonly used electric pulse parameters (number, duration and voltage to distance ratio) for each EP-based treatment are stated in column headers.

Cell injury	ns pulses (nsEP) (mostly 10–100 pulses of 10–300 ns, 20–150 kV/cm)	μs pulses (IRE) (mostly 20–200 pulses of 70–100 μs, 1000–2000 V/cm)	ms pulses (mostly single pulses of 1–20 ms, 1000–2000 V/cm)	Bipolar (H-FIRE) (mostly 50–200 bursts containing 25–300 pulses of 1–2 μs, 500–4000 V/cm)
Membrane damage	Yes [35–38,40,62,63,65,67,69–71,71,81,104–119]	Yes [25,53,80,82,111,122–126]	Yes [62]	Yes [123]
	No [63,120,121]			No [122]
ATP depletion	Yes [51,127,128]	Yes [25,50,52,53,129]	Yes [55]	Yes [58]
Elevation of Ca^{2+}	Yes [35,37–40,65,67,69,104,105,108,109,113,117,130]			
Mitochondrial damage	Yes [35,62,65,67–71,71,79,91,112,117]			
	No [39,131]			
Increase of ROS	No [67,69]		Yes [55]	Yes [58]
DNA damage	Yes Indirect [68,71,79,81,91,132–134]	Yes Indirect [80,82]	Yes Indirect [90]	
	Direct [76,77,106]			
	Direct/indirect not clear [109]			
Protein damage	Yes [71,103]			

Table 2
Published papers on cell death, reporting on cell injury *in vivo*. Most commonly used electric pulse parameters (number, duration and voltage to distance ratio) for each EP-based treatment are stated in column headers.

Cell injury	ns pulses (nsEP) (mostly 10–100 pulses of 10–300 ns, 20–150 kV/cm)	μs pulses (IRE) (mostly 20–200 pulses of 70–100 μs, 1000–2000 V/cm)	ms pulses (mostly single pulses of 1–20 ms, 1000–2000 V/cm)	Bipolar (H-FIRE) (mostly 50–200 bursts containing 25–300 pulses of 1–2 μs, 500–4000 V/cm)
Membrane damage		Yes [78,83,89,135–145]	Yes [146]	Yes [136]
ATP depletion				
Elevation of Ca^{2+}				
Mitochondrial damage	Yes [91]	Yes [142–145]		
Increase of ROS				
DNA damage	Yes Indirect [63,91,93,105]	Yes Indirect [73,78,83–89,141,142,144,147–149]	Yes Indirect [92]	
Protein damage				No [58]

ever, the involvement of the most likely candidate for the loss of MMP, mitochondria permeability transition pore (mPTP) complex is still under debate [71]. Nevertheless, it is clear that Ca^{2+}, ATP, and ROS all influence mitochondrial physiology. Moreover, they exist in an interdependent network, with each having the ability to affect the others. It is therefore difficult to determine which effect is the cause and which is the consequence of a pathological stimulus [72].

Electric pulses can also cause DNA damage, however, it is not clear [63,73] if the effect is direct [74–77] or indirect as a consequence of apoptotic cell death [78–93].

A direct protein damage after EP was not yet extensively studied. MD and other simulations revealed that electric field exposure may result in direct detrimental effects on structure (unfolding, modifying of H-bonding, conformational changes, and/or disruption of secondary structures such as α-helix or β-sheet) of proteins,

e.g. myoglobin [94,95], tubulin [96], kinesin [97], soybean hydrophobic protein [98] or small peptide V3-loop [99], and even ion channels that exhibit pores in voltage-sensor domains after electric field exposure [31]. In experiments with purified proteins exposed to electric fields, Raman spectroscopy, dynamic light scattering and atomic force microscopy imaging, or X-ray crystallography were used to demonstrate that the intense electric fields can affect protein conformation and structure [100–102]. It thus seems that electric pulses may cause direct protein damage in biological systems [29,30,71,103].

Published papers on cell death, reporting cell injury *in vitro* and *in vivo* are listed in Tables 1 and 2. An empty cell of the table means that there was no published data on the subject found in literature search.

As evident from Tables 1 and 2, electric pulses have detrimental effects on many cellular structures and functions, directly or indirectly through different pathways of cell functions and encompass all of the mechanisms of cell injury depicted in Fig. 1. When the injuries are severe and beyond repair, cells undergo one of several types of cell death. Therefore, different therapeutic strategies have evolved over the past few decades to ablate tissues such as irreversible electroporation (IRE) that use microsecond (mostly 20–200 pulses of 70–100 μs, 1000–2000 V/cm voltage to distance ratio) or ms (mostly single pulses of 1–20 ms, 1000–2000 V/cm) electric pulses [4], high-frequency irreversible electroporation (H-FIRE) that uses bursts of bipolar microsecond electric pulses (mostly 50–200 bursts containing 25–300 pulses of 1–2 μs, 500–4000 V/cm) [58], pulsed field ablation to treat cardiac arrhythmias with a large range of pulse parameters combinations [150], nanosecond pulsed electric field (nsEP) ablation (mostly 10–100 pulses of 10–300 ns, 20–150 kV/cm) [109], as well as electrochemotherapy (ECT) that combines electric pulses of lower electric field strength with cytotoxic chemotherapeutic drugs [2] and calcium electroporation (Ca EP) that combine electric pulses (mostly 8 pulses of 100 μs, 1000 V/cm) with high doses of calcium [32] to treat cancer.

3.2. Membrane repair after electroporation

Electroporation results in an injury of plasma membrane (and also internal membranes such as in the case of nsEP) and most of detrimental effects on cells that can trigger cell death (such as Ca^{2+} influx, ATP depletion, ROS increase and mitochondrial damage) can be considered a consequence of membrane damage [151]. Therefore it is of utmost importance that cell restores its plasma membrane integrity quickly after injury to maintain cell homeostasis that depends on plasma membrane selective permeability [152].

In general, only tiny membrane injuries (lipid pores of nm range) may reseal spontaneously, for injuries larger than a few nm different active mechanisms for membrane repair have evolved in eucaryotic cells [151,153,154]. Special signalling mechanisms help cells to identify the nature, magnitude (size and number of wounds) and location of the plasma membrane injury and coordinate the appropriate repair responses [151,154]. Membrane repair response occurs in seconds to minutes (mostly within 30 s after membrane injury) therefore, all the components of membrane repair mechanisms must be ready to be activated without *de novo* protein synthesis [151,152]. Ca^{2+} influx acts as a key trigger for plasma membrane repair: membrane injury results in localized and transient increases of cytosolic free Ca^{2+} concentration which trigger repair mechanisms at the site of the injury [154].

Membrane repair mechanisms are an area of active research and are reviewed in several papers [151–157]. All of membrane repair mechanisms are Ca^{2+} dependent [155]. Cells employ multiple mechanisms simultaneously for efficient membrane repair [151]. Large wounds (several μm in diameter) are repaired by patching: cytoplasmic vesicles fuse together and form a patch to fill the wound [153,154,157]. Small or medium size holes (μm to few μm scale) are repaired by clogging with annexins and other proteins (e.g. dysferlin), followed by membrane shedding (pinching out) [154]. Small wounds (smaller than 100 nm) are repaired by two mechanisms: exocytosis followed by endocytosis and membrane shedding via ESCRT (Endosomal Sorting Complex Required for Transport) proteins [154]. In exocytosis, lysosomes migrate immediately after Ca^{2+} influx towards the injured site and fuse with plasma membrane with a help of proteins involved in membrane repair (e.g. SNARE proteins, synaptotagmins, calpains, dysferlin) [151,153,155,156,158]. Exocytosis and patching also reduce membrane tension that appear in membrane injury and allow faster spontaneous resealing of small lipid pores [153,157]. Exocytosis is however not sufficient to eliminate persistent wounds such as pore-forming proteins. Therefore, the release of lysosome content into extracellular space triggers massive endocytosis [155]. Lysosomal enzyme acidic sphingomyelinase initiates production of ceramide domains in plasma membrane near wounded site and ceramide-dependent endocytosis (through caveolar vesicles) that internalizes and later degrades the lesions/pore-forming toxins [152,154,155].

Following exposure of cells to electric pulses, the aqueous pores formed in plasma membrane (or rather increased membrane permeability) persist for several minutes or, if incubated in low temperatures, e.g. 4 °C, even hours before they reseal [1,159–161]. Closing of pores and resealing of the membrane – re-establishing its full barrier function consists of several stages: a rapid stage that lasts only microseconds after the pulse with a rapid decrease in pore size, and several slower stages that can last minutes after the pulse with slow decrease in pore size and number of pores which lead to gradual reseal [161–166]. Therefore, the resealing times obtained experimentally can be of various duration, depending on the detection method, e.g. membrane conductance relaxation (50 ms – 2 s) [162,167–169] and restoration of barrier function to ions or molecules (120 ms – 20 h) [159,161,170–173]. Moreover, the pore closure time in molecular dynamics (MD) simulations is about nine orders of magnitude shorter than typical experimentally determined membrane resealing times [1] which suggests that the pores in cell membranes are more complex (e.g. involving both membrane lipids and proteins) than those in simple bilayers in MD [31,174] or that electropermeabilization of cell membranes may involve other mechanisms than electroporation such as lipid peroxidation [1]. Considerable efforts have been made in attempt to describe resealing theoretically [166,175,176].

Depending on exposure conditions, electric pulses produce heterogeneous populations of membrane pores, with sizes ranging from 1 to 100 nm [177]. Small, nanometer scale pores that occur in lipid bilayer after EP may reseal spontaneously [153,178]. However, the resealing of EP pores is mostly an active process that requires extracellular calcium: plasma membrane integrity after EP in calcium-depleted medium requires much longer time to reestablish than in medium containing calcium ions [179–183]. The resealing is affected by pulse parameters (higher the pulse amplitude and number, longer the resealing time) [53,55,172,184], temperature [159,161], calcium concentration in extracellular medium [179], generation of reactive oxygen species [55] and medium composition (e.g. sucrose concentration, Mg^{2+}) [55,185]. Although Ca^{2+} ions are required for active repair mechanisms they also interact with lipids and may therefore have a direct effect on the dynamics of pore formation, size and resealing [182].

In a simple (spontaneous) lipid pore closure model, the fate of a pore lies in the ratio between two opposing dynamic forces: contractile line tension and expansive membrane surface tension. Electropore line tension energy is proportional to the pore radius,

therefore, the resulting total energy for a given pore (and fate: closure or expansion) is strongly dependent on the pore size [157,186–188]. The cytoskeleton, especially the actin cortex under the plasma membrane has an important effect on the stability and resealing of pores [187]. Anchoring of the actin cortex to the plasma membrane alters electropore dynamics in a manner that allows for electropore stability – opposing spontaneous closure and allowing molecule transport after pulse cessation [153,189] but also opposing pore expansion and the pores may therefore be more manageable by the cell's active wound healing mechanisms [187,189,190].

It was shown that the membrane repair kinetics after EP follows an exponential dynamics that is interrupted by abrupt recovery steps consistent with a membrane patch model [191]. The authors also suggested two other active cellular mechanisms of repair: i) a removal of leaky patches of membranes by endocytosis and ii) a calcium-induced vesicle exocytosis that reduces the plasma membrane tension and thereby enables pore repair by constriction and bilayer resealing [191]. Indeed, Huynh et al. detected a lysosomal-associated membrane protein 1 (Lamp-1) on the cell surface indicating a lysosomal exocytosis in response to EP wounding [192,193] that was correlated to a degree of damage induced by different pulse parameters [192]. Moreover, a CHMP4B subunit of ESCRT-III complex, involved in repair of small membrane wounds (less than 100 nm) mainly by plasma membrane shedding [194] was localized not only at plasma membrane after EP but also at nuclear envelope after exposure to electric pulses that affect both plasma membrane and nuclear envelope [195]. The calcium binding protein ALG-2 may also contribute to membrane repair and cell survival after EP [196]. Membrane recovery and cell survival in slightly acidic medium were better than in physiological one which can be attributed to more efficient membrane repair mechanisms (possibly exocytosis) in acidic environment [197]. A less efficient membrane repair system in cancer cells may account for higher susceptibility to EP compared to normal cells [198].

Experiments with double wounding by EP [180] revealed similar results as those with double wounding by mechanical puncture [199]: the resealing after the second wounding was faster than after the first one. Authors suggested that the membrane wound was repaired by exocytosis and the second wound resealed faster due to calcium-influx-mediated activation of Golgi apparatus (GA) and its formation of new vesicles. The increasing delay (1, 2, and 3 min delay) between two pulse trains led to a decreased molecular transport [180]. In a study with similar results (the delivery of target molecules decreased with increasing delay time between pulses; high repetition rate is more efficient for permeabilization, i.e. leading to higher permeabilization than low repetition rate) the authors suggest that the second pulse re-porates the weakened cell membrane (already containing nano-sized pores or defects) [163]. Higher EP efficiency of higher pulse repetition rate is usually attributed to the temporal summation of brief sub-threshold effects/lesions which can recover without consequences if the interval between pulses is sufficiently long [200]. However, some studies revealed that low pulse repetition rates are more efficient for permeabilization than high repetition rates, e.g. the study on both microsecond and nanosecond electric pulse duration [201]: applying a pulse on a permeabilized cell membrane is likely to be less effective since the existing conducting structures prevent the formation of equally high transmembrane potential [200–202]. The authors speculate that in this case, the type of damage induced by both μs and ns pulses is similar and that the resealing of such damage happens through identical pathways [201]. However, for the similar damage, much higher number and amplitude of ns pulses compared to μs pulses was applied. Nevertheless, the effect of pulse repetition rate is still puzzling (and leading to different results, for review see the Introduction of Pakhomova et al. [200]) and suggests complex permeabilization and resealing processes [163,201].

Since the application of single nanosecond electric pulses (nsEP) cause smaller pores (with a diameter $\leq$ 2 nm) than longer, micro- or millisecond electric pulses or multiple nsEP [177,203] it was always a question whether repair mechanisms that heal nsEP disruptions differ from those that restore the membrane after longer pulses (the size of pores/membrane damage affects the mode of membrane repair [153]). Lysosomes are known to contribute to membrane repair by exocytosis [158]. However, it was shown that nsEP in the presence of extracellular calcium cause inhibition of intracellular migration of lysosomes which can be a result of calcium-induced disruption of the microtubules [203,204]. Therefore, the repair mechanisms for restoring the membrane after nsEP exposure remain unknown.

3.3. Types of cell death after electroporation

Electroporation can trigger different types of cell death in treated tissues, namely apoptosis, necrosis, necroptosis, and pyroptosis. EP-based treatments lead to typical types of cell death (such as apoptosis after IRE or nsEP, necrosis after Ca EP) however, each treatment can result in many different types of cell death. The type of cell death triggered by EP depends on pulse parameters, cell and tissue type, treating conditions and other factors. *In vivo* EP-treated cells undergo a spectrum of different cell death types depending on the location in different treatment zones where they encounter specific electric field parameters. Cells close to electrodes are exposed to electric fields with the highest amplitudes and therefore die of necrosis or even coagulative necrosis with denatured proteins as a consequence of thermal damage, whereas in other treated areas cells die of other modes of cell death such as apoptosis [83,89,138]. Cells at the margins of treated area undergo reversible EP and eventually survive. However, by adding adjuvant molecules and/or chemotherapeutics that are taken up by reversibly electroporated cells at treatment margins the efficacy of tumour treatment by irreversible electroporation can be further increased (a combination of IRE and electrochemotherapy) [205–207]. Different tissues may also respond to electric fields of the same parameters with different types of cell death [140]. In the following sections different types of cell death that occur after different electroporation treatments will be presented and discussed.

3.3.1. Apoptosis

Apoptosis is a programmed, regulated, non-inflammatory cell death which is generally characterized by distinct morphological characteristics and energy-dependent biochemical mechanisms [208]. According to existing literature it is a type of cell death most commonly occurring in electroporation-based ablations such as irreversible electroporation (IRE) [83,85–89,125,135,138,141,142,144,147,148,209,210], high-frequency irreversible electroporation (H-FIRE) [58,123,136,211,212], electrochemotherapy (ECT) [205,207,213–215], electroporation combined with electrolysis [216] and nsEP ablation [63,91,93,120]. Apoptosis was confirmed also in numerous *in vitro* studies using ms [217], μs (IRE, H-FIRE) [80,82,123,125,126,218] and ns pulses [39,62,65,66,69,79,111,114,120,130,131].

Cells that undergo apoptosis after IRE with μs pulses exhibit typical morphology: nuclear condensation and fragmentation, cell shrinkage and fragmentation to apoptotic bodies [83,141,148,207]. Apoptosis in IRE treated cells and tissues was also detected by activation of executioner caspases (proteases that play essential roles in apoptosis, coordinating the destruction of cellular structures), namely caspases −3 and −7 [88,125,126,135,138,139,141], and by DNA fragmentation, typical for apoptosis, detected by Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL)

assay [73,83,85–89,141,142,144,147,148]. A clinical study of IRE in patients with colorectal liver metastases revealed that IRE induces apoptotic cell death in colorectal liver metastases and the ablation zone shows a sharp demarcation between avital and vital tissue [143]. However, apoptosis does not occur in all regions of tissue treated with IRE: at regions close to the electrodes where electric fields reach high amplitudes and even thermal damage cells die from necrosis [83,89,138]. The occurrence of apoptosis and necrosis *in vivo* is time-dependent, but the results are sometimes difficult to discern what comes first [125,135,144,148]. Sometimes, apoptosis is absent from IRE treated tissues; cells undergo other cell death pathways such as necrosis, pyroptosis, and necroptosis [73,137,219–223].

H-FIRE affects cells and tissues in a similar way as IRE (apoptosis, necrosis) [58,123,136,210–212], although with some differences in caspase-dependency in some cells [123]. H-FIRE seem to have more effect on intracellular structures, e.g. nuclei, and less on plasma membrane than IRE [122]. H-FIRE can also induce pyroptosis [58].

Apoptosis was extensively studied in treatments *in vitro* and *in vivo* with nsEP since first confirmed [63]. The fact that cells undergo cell death without the use of chemical compounds (chemotherapeutics) led to nsEP-based treatment of tumours [224]. Apart from morphology, apoptosis due to nsEP exposure was detected by activation of executioner caspases −3 and −7 [62,63,66,69,79,91,107,112,114,119,120,225,226], and DNA fragmentation [62,63,79,91,120,227]. Apoptosis detection with phosphatidylserine externalization assay using Annexin-V must be implemented and results interpreted with caution since it can occur due to pore formation in EP, not related to apoptosis [228,229]. Therefore, the assay has to be employed with a sufficient delay after nsEP application when the pores are resealed [230]. Apoptosis by nsEP is executed via different pathways (Fig. 2). Loss of mitochondrial membrane potential [65,68–70], cytochrome *c* release [62,65,69,79,91,112], caspase-9 activation [65,69,91,112,226], upregulation of pro-apoptotic factors (BAX, BAK, BAD) and downregulation of anti-apoptotic factors (Bcl-2, Bcl-xL, Mcl-1) [79,91,93,109] confirmed the involvement of mitochondria, mostly through intrinsic apoptosis pathway, however, in some studies, BID cleavage also points to the activation of type II extrinsic-like apoptosis [69]. In some cells (HCT, B16F10, E4 SCC, Jurkat), apoptosis progresses also through type I extrinsic-like pathway without or with little involvement of mitochondria [67,69,79] and with caspase-8 activation and modulation of extrinsic apoptotic regulators which influence sensitivity to nsEP [131]. This means that nsEP have a prominent effect also on plasma membrane structures, too, not only on cell interior. Different apoptotic pathways are triggered in different cells [65], sometimes even in the same cells [69]. Moreover, different conditions and severity of injury in nsEP exposure lead to different forms of cell death: necrosis following extensive swelling as a predominant cell death mode in U-937 human monocyte cell line can be switched to apoptosis if swelling is prevented by adding sucrose to electroporation medium [37,38]. Extracellular Ca^{2+} also influence the mode of cell death in nsEP in a similar way as in Ca EP [130]. The balance between apoptosis and necrosis in cells exposed to nsEP may be influenced by the ability to repair the damage (e.g. ion balance, ATP supply) or the level of intracellular damage (higher in nsEP than μs pulses) [81]. Nevertheless, similarly to longer (μs and ms) pulses, more cells undergo necrosis when they are exposed to pulses of higher amplitude or pulse number, resulting in more severe damage [93,114].

The most intriguing question in understanding nsEP cell death is what is the primary target of nsEP that triggers the cascade of the programmed cell death. Intrinsic apoptosis is initiated by a variety of microenvironmental perturbations including growth factor withdrawal, DNA damage, endoplasmic reticulum (ER) stress, reactive oxygen species (ROS) overload, replication stress, microtubular alterations or mitotic defects [11]. As known, nsEP were also reported to cause direct DNA damage and mitotic defects [76,77,231]. Apoptosis is linked to DNA damage via several pathways [232] that were not yet fully studied in the case of nsEP. One possibility is via PLK-1 protein and centrosome-mediated apoptosis [77], PUMA and NOXA were not activated [91,112]. Exposure of cells to nsEP cause ER stress that could be related to ROS formation, permeabilization of the ER, or Ca^{2+} influx, and can further trigger mitochondria-mediated intrinsic apoptosis via PERK and IRE1 [39,128]. ROS formation that occur after nsEP [57] could trigger both intrinsic and extrinsic apoptosis via several cellular targets including those in mitochondria, DNA, ER and plasma membrane [60].

In electrochemotherapy (ECT), i.e. electroporation combined with chemotherapeutic drug, pulses (typically of 100 μs duration) with lower electric field strength and lower number of pulses are used to allow tumour cells to reseal and survive, therefore, cells predominantly die due to the cytotoxic action of chemotherapeutic drugs delivered into cells with EP [233]. With the use of electric pulses that cause increased permeability, intracellular drug accumulation is increased and it results in increased drug cytotoxicity [234]. The two drugs that have been used most often in ECT are bleomycin, and cisplatin which both target DNA [233]. Bleomycin is a cytotoxic antibiotic that generates DNA double-strand breaks (DSB) and DNA single-strand breaks (SSB) [214]. Apoptosis, along with mitotic cell death, is a predominant mode of cell death after ECT [205,207,214,215,235,236], however, other forms of cell death (necrosis, necroptosis, immunogenic cell death) were also identified [50,235,237]. It seems that the internal concentration (which is related to external concentration and electric pulse parameters) and, consequently, the amount of DSB and SSB and the ratio between them determine the mode of cell death [214,215,236,238]. SSB are responsible for the induction of apoptosis which occurs at high amounts of SSB and low to moderate amounts of DSB. At moderate level of DSB and low SSB, cells undergo mitotic cell death, a caspase-dependent programmed cell death which results from the abnormal passage through mitosis of cells containing unrepaired DNA breaks, similar to cell death caused by radiation [214,238,239]. However, at high amounts of DSB (at high doses of bleomycin), irrespective of SSB amount, cells die of a rapid apoptosis-like cell death (here termed as "pseudoapoptosis") [214,215]. "Pseudoapoptosis" in this case is a very fast (a few minutes) cell death process that displays the morphological and biochemical characteristics of apoptosis, however it seems that it does not require induction of cell endonucleases involved in typical apoptosis: it is rather caused by direct effect of bleomycin than via cell endonucleases and can therefore proceed faster than regular apoptosis. The hypothesis is that bleomycin at high concentrations acts directly as an endonuclease and can be considered as an apoptosis-mimetic drug [215].

Apart from apoptosis, there are other forms of regulated cell death such as necroptosis and pyroptosis that also occur after cell exposure to electric field. However, they both elicit immune response *in vivo*, therefore, they will be discussed in Section 3.3.3: Immunogenic cell death and immune response.

3.3.2. Necrosis / accidental cell death

Accidental cell death is a rapid, uncontrollable cell death caused by extreme conditions (heat, radiation, trauma, anoxia, infection) that lead to loss in cell homeostasis and is characterized by the rupture of the cell membrane. It was previously referred to necrosis which exhibits a typical necrotic morphology: cellular changes include cell swelling (oncosis) and blebbing, swelling of mitochondria, ER and nuclear envelope dilatations, random DNA

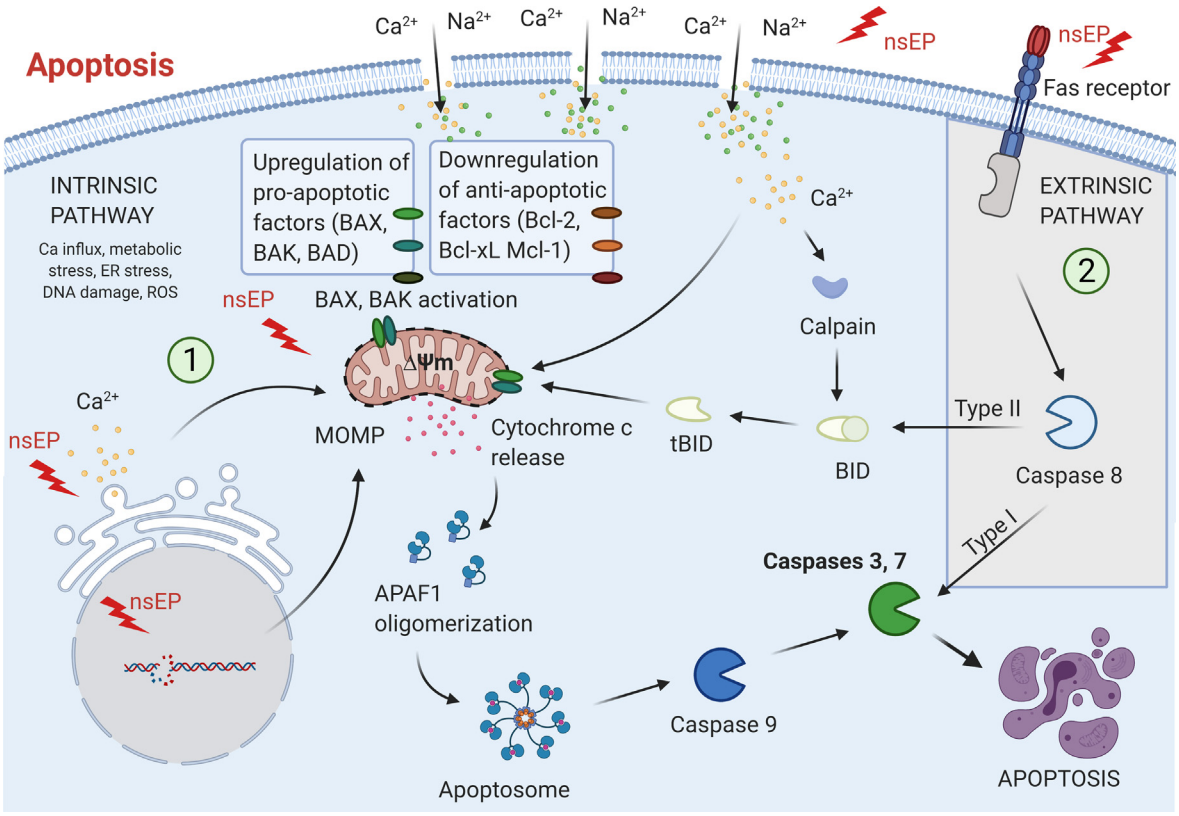

Fig. 2. Pathways of apoptosis after nanosecond pulse electroporation (nsEP). Mostly, apoptosis is executed via intrinsic pathway (1) where mitochondria play a major role. Intrinsic pathway is triggered by internal Ca^{2+} elevation, metabolic stress, ER stress, possible permeabilization of ER and mitochondrial membranes, DNA damage and ROS production that initiate upregulation of pro-apoptotic factors and downregulation of anti-apoptotic factors, dissipation of mitochondrial membrane potential, mitochondrial outer membrane permeabilization (MOMP), cytochrome *c* release, apoptosome formation, caspase-9 activation and subsequent activation of executioner caspases −3 and −7 which leads to apoptotic cell death. In some cells, apoptosis can progress via extrinsic pathway (2) where nsEP trigger aggregation of the Fas receptor, activation of caspase-8 and subsequent activation of executioner caspases −3 and −7 without (Type I) or with (Type II) amplification through mitochondrial pathway. Derived from [65,69,131]. Created with BioRender.com.

degradation, and finally, membrane rupture followed by spilling cell contents into its surrounding [12,240,241]. Contrary to apoptosis, necrosis was considered to be accidental, unprogrammed or unregulated, however, there are some molecular events and patterns (e.g. RIP1 and PARP activation) that typically occur during necrosis, therefore it is at least in part regulated [240–242]. Moreover, according to Nomenclature Committee on Cell Death new types of regulated cell death that also exhibit necrotic morphotype have been identified such as necroptosis and ferroptosis. Therefore, cell death types that exhibit necrotic morphotype can be accidental or regulated [11]. Most papers reporting necrosis as the form of cell death after EP were however published before the last recommendations of Nomenclature Committee on Cell Death that categorize types of cell death to accidental and regulated [11]. In most of the EP studies, researchers have identified necrosis morphologically. What the authors refer to as "necrosis" is probably, in most cases, what the Committee defines as accidental cell death however, it is impossible to distinguish the precise type of cell death that occurred after EP exposure. Hence, we refer here to necrosis as to all cell death types with necrotic morphology.

Cellular and molecular mechanisms underlying necrotic morphotype cell death are: ATP depletion, mitochondrial dysfunction, oxidative stress, protein kinase signalling, PARP activation, and plasma membrane injury [12,241]. ATP depletion is crucial for necrosis induction: in case of mitochondrial dysfunction indicative for both apoptosis and necrosis, a rapid loss of ATP can switch cell's fate from energy-dependent apoptosis to energy-independent necrosis [241,243]. The spillage of the contents of necrotic cells into the surrounding tissue activates inflammatory signalling pathways, which recruit diverse types of immune cells (neutrophils, macrophages, dendritic cells) involved in the immune response [242]. This renders necrosis also an immunogenic cell death. The cell death triggering of immune response by releasing danger signals (DAMP molecules, such as HMGB1, Heat shock proteins, calreticulin, and mRNA) will be discussed in Section 3.3.3: Immunogenic cell death and immune response.

Exposure to electric pulses caused necrosis in *in vitro* and *in vivo* studies using IRE [126,137,140,142,144,147,209,210,218–223] and H-FIRE pulses [58,136,210,212,244], Ca EP [33,44–49], ECT [235,245] and nsEP [37,38,70,81,93,114,130,134,246,247]. Necrosis was also determined as a mode of cell death after cardiac ablation with IRE for treating heart arrhythmias (for atrial fibrillations, *in vivo* experimental procedure in pigs) [248].

In Ca EP, electric pulses (mostly 100 μs long, but also with nsEP [249] and H-FIRE pulses [250]) are delivered with high concentrations of Ca^{2+} *in vitro* or *in vivo* (IC_{50} ranging from 0.4 to 5.0 mM Ca^{2+} concentration *in vitro*, and 100–500 mM Ca^{2+} with 20–80% tumour volume *in vivo*) [32]. A high Ca^{2+} uptake leads to cell death, mostly necrosis [33,44–49], however, a few studies also reported apoptosis [49,251,252] and necroptosis [250]. *In vitro*, most of the cells swell, rupture and lyse after CaEP, although some cells may exhibit apoptotic morphology and shrinkage (Fig. 3) [48,49]. Several studies reported an immediate, severe and long-lasting drop in cellular ATP level [33,46–49]. ATP depletion as a result of increased intracellular Ca^{2+} may be caused by highly increased activity of Ca-ATPases in plasma membrane (PMCA) and ER (SERCA) that try

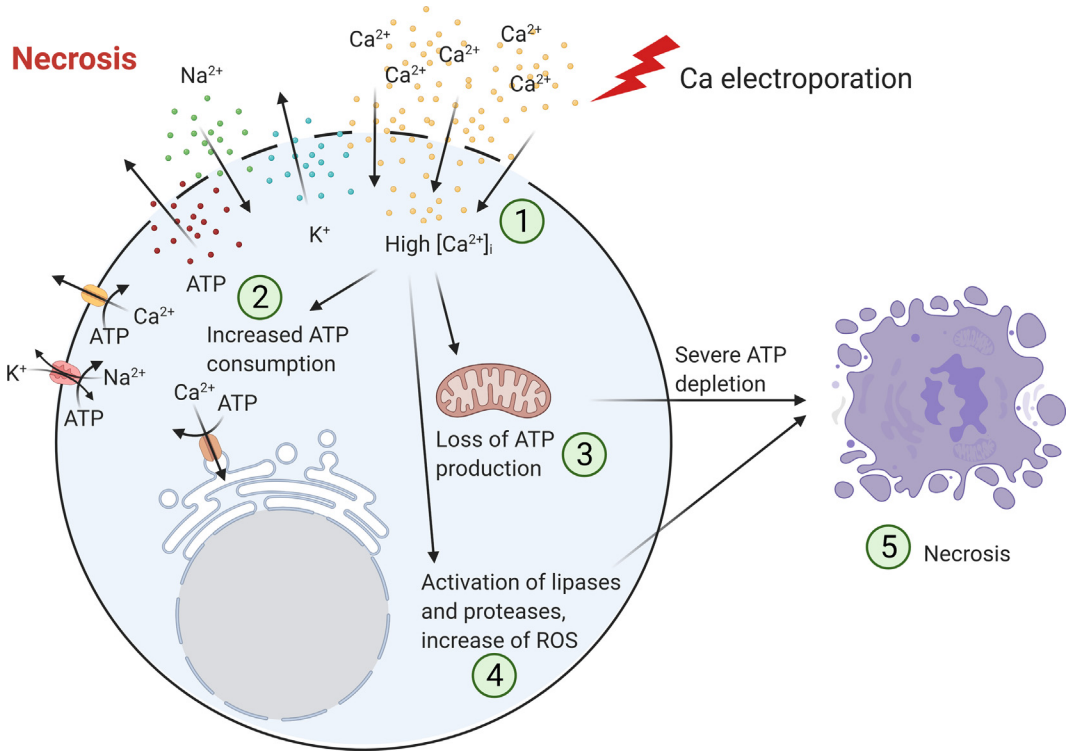

Fig. 3. Necrosis after calcium electroporation (Ca EP). Increased plasma membrane (PM) permeability causes massive Ca^{2+} influx and high intracellular Ca^{2+} concentration (1). This leads to: (2) an increased ATP consumption due to activation of Ca^{2+} pumps (PM, ER) and other pumps (such as Na^+/K^+-ATPase), and ATP loss through permeabilized PM, (3) loss of ATP production due to calcium overload in mitochondria and disruption of electrochemical gradient in mitochondria necessary for ATP production, and (4) other effects such as activation of lipases and proteases, and generation of ROS. A severe ATP depletion in cells eventually triggers necrosis (5). At the necrotic stage (5) plasma membrane is ruptured, and cell lysis occurs (this stage is symbolically depicted here on a smaller scale). Derived from [46]. Created with BioRender.com.

to restore low levels of intracellular Ca^{2+}, opening of permeability transition pores in the mitochondrial membrane, resulting in loss of ATP production, and a direct loss of ATP, i.e. leakage through permeabilized plasma membrane [32,46]. Besides the abrupt ATP depletion which is pivotal for necrosis induction, calcium overload also causes activation of lipases and proteases, and generation of ROS which may also contribute to cell death [32,46]. It has been shown that normal cells seem to be less sensitive to Ca EP than cancer cells [251–254]. Moreover, contrary to ECT, Ca EP induces cytotoxicity without any genotoxicity [33]. It was also reported to elicit immune response and long-term anti-tumour prevention mediated by DAMP molecules (HMGB1) [45].

3.3.3. Immunogenic cell death and immune response

In many studies using electric pulses of different parameters researchers report immunogenic cell death (ICD) eliciting immune response. The term immunogenic cell death is used here in a broader context of different types of cell death that can trigger immune response (necrosis, necroptosis, pyroptosis, and even apoptosis), not referring to a specific type of ICD characterized by apoptotic morphology and connected to ER stress [255].

In *in vivo* treatments of tumours with IRE and H-FIRE [58,73,73,85–88,92,129,135,137,142,144,212,221,222,244,256–260], ECT [50,235,261,262], Ca EP [44,45,253,261] and nsEP [51,127,226,263–266], immune response was observed. Besides innate immune response that recruits macrophages and natural killer cells to remove damaged and dead treated tumour cells and debris, the most important is adapted immune response: the activation of specific anti-tumour memory cells can lead to long-lasting protection against tumour that was treated and can, with an abscopal effect (*ab scopus* - away from target) prevent metastases to spread the disease, as was also reported in some cases after electroporation [45,51,58,127,226,256–258,261,264–266]. A strong positive correlation between up-regulation of cellular immunity-associated genes and decreased tumour diameter was shown [58]. Moreover, "vaccination" with ECT or nsEP-treated cancer cells protects animals against subsequent challenge with cancer cells [50,264]. Therefore, the immune response in treating cancer is advantageous. However, not all EP-based treatments were successful in eliciting a long-term anti-cancer protection [267], and in some cases incomplete eradication of tumours was reported to lead to even faster growth of recurring tumours [268].

EP is also a potent immunological adjuvant for genetic vaccination with gene electrotransfer (GET) due to a low-intensity tissue damage, which rapidly resolves, and pro-inflammatory cytokine release [7,269]. GET of cytokine genes can be used in combination with EP [270–273] or ECT [274,275] to boost the immune response after EP or ECT and enable to prevent recurrences and distant metastases. A combination of ECT with immunostimulating agents (e.g. interleukin-2) can also be an elegant and efficient way to cure both the ECT-treated nodules and distant nodules [276]. However, the immune response in gene therapy can also be unwanted since it may eliminate transfected cells or interfere with transgenic protein expression and function [277,278].

Immunogenic cell death is characterised by release of damage-associated molecular patterns (DAMPs) from dying cells [13,16,17,19,25]. Released DAMPs bind to pattern-recognition receptors (PRRs) of immune cells and elicit immune response [16–18]. The signalling of DAMP molecules is reviewed by Galluzzi [17]. Indeed, it was shown that IRE [25,129,256], H-FIRE [58], ECT [50], GET [92] and nsEP [51,127,128] cause release of DAMPs both *in vitro* and *in vivo*. In most of these studies, ATP, calreticulin and HMGB1 were detected as they represent the gold standard for predicting the ICD-inducing capacity of chemotherapeutic agents [22].

However, other DAMPs such as nucleic acids and uric acid were also investigated [25]. The release of DAMPs increases with increasing pulse amplitude, number and duration [25,51,53,128,129,256] which is consistent with the hypothesis that the release of DAMPs correlates with the degree of (membrane) injury inflicted to cells [25,279]. However, in a recent study *in vitro*, concentrations of DAMPs correlate strongly with cell death but only weakly with cell membrane permeabilization in the range of reversible EP which suggests greater complexity in DAMP signalling [25].

In necrosis, DAMPs are passively released from the cells (ATP, HMGB1, Hsp70, Hsp90, gp96) or, in the case of an ER protein calreticulin, translocated to the surface of the cells [241,242]. Historically, necrotic cells were considered as the most prominent, if not the only, source of DAMPs since apoptotic cells conserved an intact plasma membrane [280]. However, there are other forms of regulated cell death with necrotic and apoptotic-like morphology that also elicit a strong immune response such as necroptosis and pyroptosis. Since necroptosis and pyroptosis were only investigated in recent years it is possible that necrosis and apoptosis identified morphologically in some older studies may in fact be attributed to necroptosis or pyroptosis [145].

Necroptosis is a form of regulated and immunogenic cell death showing morphological features similar to necrosis [11,13,15,281]. Typical events for determining necroptosis (Fig. 4) is activation of receptor interacting serine/threonine kinase 3 (RIPK3) which subsequently activates mixed lineage kinase domain-like pseudokinase (MLKL), the effector of necroptosis: MLKL migrates to plasma membrane and causes membrane permeabilization, cell swelling and rupture which results in release of DAMPs [11,13,15,281,282]. A few studies have identified necroptosis *in vitro* or *in vivo* after IRE [73,145], electroporation combined with electrolysis [216], ECT [237], Ca EP [250] and nsEP [112]. Only two studies confirmed the activation of RIPK3 and MLKL [73,216], others determined necroptosis on the basis of morphology and time frame of cell death [145,237], or other biochemical characteristics such as sensitivity to necroptotic inhibitors [237], analysis of other cell death mechanisms [112] or activation of genes that may contribute to necroptosis signalling [250]. The lack of RIP3 expression in B16F10 melanoma cells may contribute to a weak antitumour immune response after treatment with nsEP [246].

Pyroptosis is a regulated, caspase-dependent cell death which differs from apoptosis (also regulated, caspase-dependent cell death) in morphology, biochemical pathways and immune response. Pyroptosis (Fig. 5) is driven by the activation of inflammatory caspases, most importantly, caspase −1 (but also 4, 5, or 11) that is activated within an inflammasome, a macromolecular protein complex composed of inflammasome-initiating sensors and inflammatory caspases [283]. Inflammatory caspases activate gasdermin D, a pore-forming protein that permeabilizes plasma membrane and promote the release of DAMPs through pores and subsequent membrane ruptures. Caspase-1 also activates inflammatory cytokines, IL-1β and IL-18 which are then released through gasdermin D and membrane ruptures into the cell surrounding [11,13,281]. The release of DAMPs and, especially, the release of inflammatory cytokines promote a strong immune response [11,13,281]. Pyroptosis exhibits a distinct morphology that includes multiple bubble-like protrusions that can produce pyroptotic bodies, and a peculiar form of chromatin condensation that differs from its apoptotic counterpart. Cell lysis after membrane rupture is also typical for pyroptosis but not for apoptosis [11,284].

Activated caspase-1 and gasdermin D were found in liver tissue treated with IRE [73] or electroporation combined with electrolysis [216] and upregulation of genes associated with pyroptosis in H-FIRE treated tumours were linked to a systemic anti-tumour immune response [58]. It is likely that DAMP molecules activate

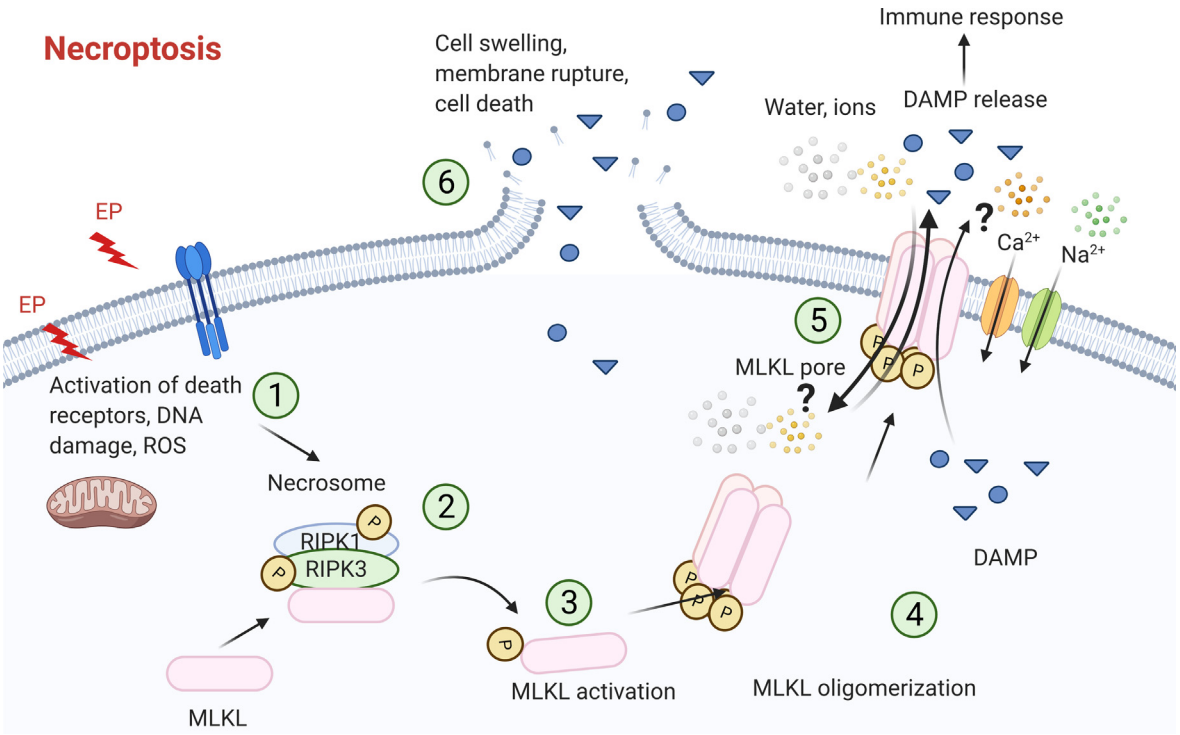

Fig. 4. Necroptosis after electroporation. Necroptosis is triggered by activation of death receptors, DNA damage or ROS production (1). The formation of necrosome enables the activation of RIPK3 kinase (2) which then activates mixed lineage kinase domain-like pseudokinase MLKL (3). Activated MLKL molecules engage in oligomerization (4) and move to plasma membrane where they possibly form pore and/or activate ion channels which allow increased transport of ions and water through plasma membrane (5). This leads to cell swelling, membrane rupture and eventually, to cell death. DAMPs released through pores and/or ruptures stimulate immune response (5, 6). Derived from [281,282]. Created with BioRender.com.

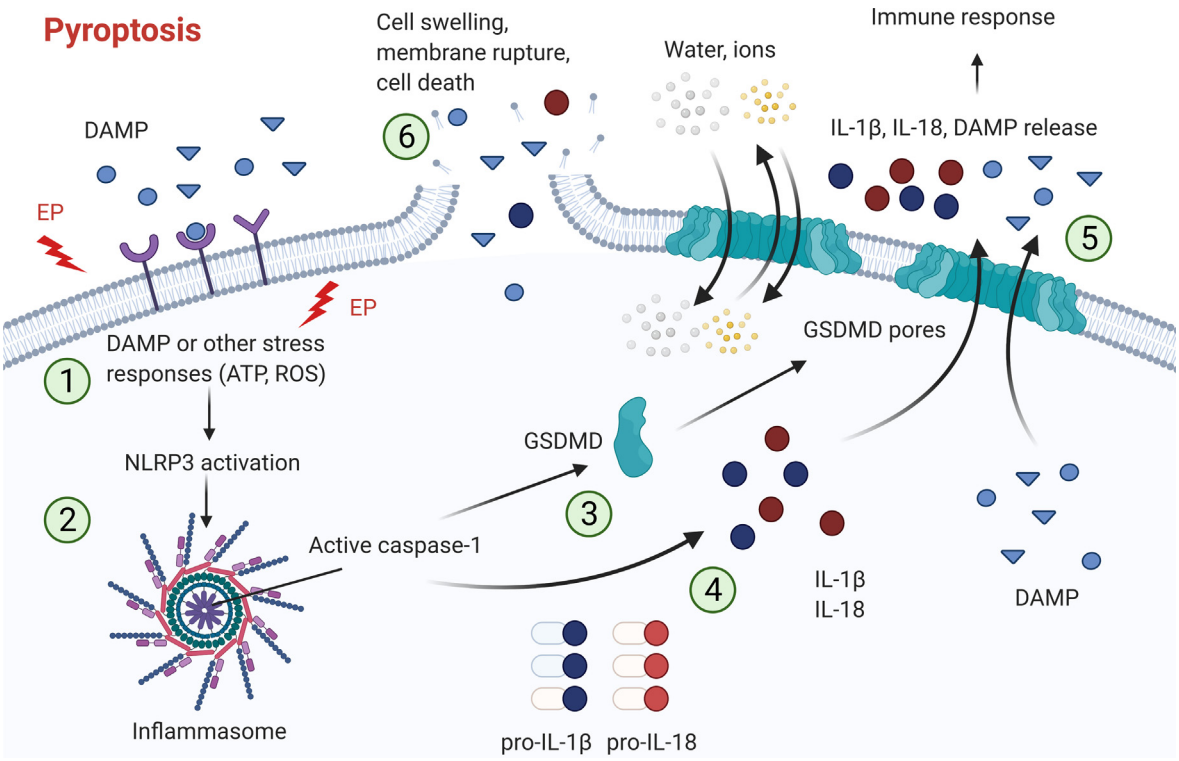

Fig. 5. Pyroptosis after electroporation. Pyroptosis is triggered by DAMPs or other stress responses (ATP, ROS) (1). Inflammatory caspases (mostly caspase-1) are activated within an inflammasome (2). Caspase-1 activates gasdermin D (GSDMD) (3), a pore-forming protein that permeabilizes plasma membrane. Caspase-1 also activates inflammatory cytokines, IL-1β and IL-18 (4). Cytokines and DAMP molecules are released through GSDMD pores out of the cell and stimulate immune response (5). Ion exchange and water influx through GSDMD pores cause cell swelling, membrane rupture, leakage of cell's constituents (including DAMPs and cytokines) and eventually, cell death (6). Derived from [281,282]. Created with BioRender.com.

NLRP3 inflammasome pathway in caspase-1-dependent pyroptosis [58].

Electroporation with a chemotherapeutic drug SN38 in the presence of free Fe^{2+} ions may lead to ferroptosis [285], a form of regulated, immunogenic cell death initiated by oxidative perturbations of the intracellular microenvironment, particularly severe lipid peroxidation, which relies on ROS generation and iron availability [11]. Since EP causes such oxidative perturbations in plasma membrane it may lead to ferroptosis [56].

Although apoptosis is considered non-immunogenic [14], nsEP and IRE treatments still elicit immune response. This can be explained by the fact that some cells in treated tumours undergo necrosis as well, especially near the electrodes, or other immunogenic forms of cell death, and it is sufficient to trigger immune response [24,112]. Moreover, some studies show that also caspase-dependent apoptotic processes can lead to immune response and exposure of DAMP molecules in pre-apoptotic stage [51,226,255,286–288], possibly through ER stress [51,128,264].

4. Conclusions

(1) There are many different forms and pathways of cell death that occur in cells and tissues. Apoptosis, necrosis, necroptosis and pyroptosis were all reported to be induced by electric pulses causing electroporation under certain conditions. The ability to trigger an immune response after electroporation-based cancer treatments is crucial for eradication of tumours on a long-term scale to prevent the recurrence. We strongly believe that with an increasing knowledge on how pulse parameters and different treatment conditions affect cell death pathways is a key to optimisation of therapies.

(2) The extent of cell death pathways needs to be evaluated in cells of different physiology to determine whether pulses are simply stimulating molecular responses or whether its effects are more specific and truly related to the electric pulse parameters. In addition, much care must be taken when comparing studies across different pulse durations and pulse shapes. Considering different types of cell death that occur after EP treatments that use a large range of pulse parameters, maybe cell death mechanisms between long and short pulses are more connected than was previously believed.

(3) Electroporated cells exhibit membrane damage, increase in intracellular Ca^{2+} concentration, mitochondrial disruption, ATP depletion, ROS production, and DNA damage, which all contribute to different forms of cell death. Nevertheless, the exact targets that may lead to different mechanisms of cell death caused by electroporation in different cells under specific conditions still need to be determined. However, this may not be an easy task, considering the cell specificity, complexity, interconnectivity and overlapping of cell injury and death pathways.

CRediT authorship contribution statement

Tina Batista Napotnik: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing - original draft, Writing - review & editing. **Tamara Polajžer:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing - original draft, Writing - review & editing. **Damijan Miklavčič:** Conceptualization, Method-

ology, Funding acquisition, Resources,Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Competing interests

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