Electroporation-Based Technologies for Medicine: Principles, Applications, and Challenges

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pulsed electric field, electropermeabilization, electrochemotherapy, gene electrotransfer, DNA vaccination, irreversible electroporation

Abstract

When high-amplitude, short-duration pulsed electric fields are applied to cells and tissues, the permeability of the cell membranes and tissue is increased. This increase in permeability is currently explained by the temporary appearance of aqueous pores within the cell membrane, a phenomenon termed electroporation. During the past four decades, advances in fundamental and experimental electroporation research have allowed for the translation of electroporation-based technologies to the clinic. In this review, we describe the theory and current applications of electroporation in medicine and then discuss current challenges in electroporation research and barriers to a more extensive spread of these clinical applications.

Contents

Electrochemotherapy (ECT): a combined treatment using electroporation of membranes and facilitating transport of cytotoxic drugs such as bleomycin and cisplatin that have a hindered transport across the membrane and intracellular target (i.e., DNA), thus potentiating the cytotoxic effect of such drugs

Gene electrotransfer (GET): one of the gene therapy modalities using specific electric pulses for cellular uptake of naked plasmid DNA, encoding for a specific therapeutic protein with the means of molecular targeted therapy of cancer

1. INTRODUCTION

Electroporation is the increase of cell membrane permeability due to externally applied pulsed electric fields. Although observation of the effects of pulsed electric fields on biological material dates back more than 250 years, only in the past two decades have practical applications of electroporation emerged in food processing, pharmaceutics, and medicine (1).

The history of electroporation most likely begins in the middle of the eighteenth century when Nollet (2) reported the first systematic observations of the appearance of red spots on animal and human skin exposed to electric sparks. Over the next two centuries, Ritter in 1802 (see 3), Frankenhaeuser & Widén (4) in 1956, and Stampfli & Willi (5) in 1957 reported that nerve membrane electroporation may explain the electrical conductivity changes in nerves that have been damaged by electric fields. The medical application of electroporation began in 1982 with the seminal work of Neumann and colleagues (6).[1] Those authors used pulsed electric fields to temporarily permeabilize cell membranes to deliver foreign DNA into cells. In the following decade, the combination of high-voltage pulsed electric fields with the chemotherapeutic drug bleomycin and with DNA yielded novel clinical applications: electrochemotherapy (ECT) (7–9) and gene electrotransfer (GET) (10, 11), respectively. In recent years, nonthermal irreversible electroporation (NTIRE) for the ablation of solid tumors has emerged as a new medical application of electroporation technology (12, 13).[2]

[1] Neumann and colleagues also coined and first used the term electroporation in 1982.

[2] The terms pulsed electric field (PET) treatment, ECT, GET (alternatively defined as gene electrotherapy), electrogene therapy, electroplasmolysis, and NTIRE are sometimes used interchangeably, but also for better acceptance by consumers.

2. FUNDAMENTAL PRINCIPLES OF ELECTROPORATION

2.1. Events at the Molecular Level

Nonthermal irreversible electroporation (NTIRE): an electroporation-based nonthermal tissue ablation method, leading to cell death either directly through excessive damage inflicted to cells and membranes observed as necrosis or via apoptosis

Since the discovery of electroporation, several competing theoretical descriptions of the events underlying the phenomenon have been proposed, assuming either a certain type of deformation of the lipids (14–16), their phase transition (17), breakdown of interfaces between the domains with different lipid compositions (18), or denaturation of membrane proteins (19). However, all of these descriptions suffer from obvious flaws (20), and today there is broad consensus that electroporation is best described as the formation of aqueous pores in the lipid bilayer (20–23). This description also explains the prevalent choice of the term electroporation, as opposed to the broader term electropermeabilization. The latter term describes the consequence rather than the underlying mechanism and is thus applicable to any of the alternative explanations of the phenomenon as well.

According to the theory of aqueous pore formation, which is based largely on thermodynamic considerations, formation of aqueous pores is initiated by the penetration of water molecules into the lipid bilayer of the membrane, which leads to reorientation of the adjacent lipids with their polar head groups pointing toward these water molecules (**Figure 1**). Unstable pores with nanosecond lifetimes can generally form even in the absence of an external electric field, but an exposure of the membrane to such a field reduces the energy required for penetration of water into the bilayer. Within the first nanoseconds, this penetration is caused mostly by the transfer of the external field to the membrane, and then within a microsecond, the transmembrane field is amplified by polarization, resulting in the buildup of an induced transmembrane voltage (ITV) (24, 25). The entrance of water increases the probability of pore formation, resulting in a larger number of pores formed in the bilayer per unit of area and per unit of time, with the pores

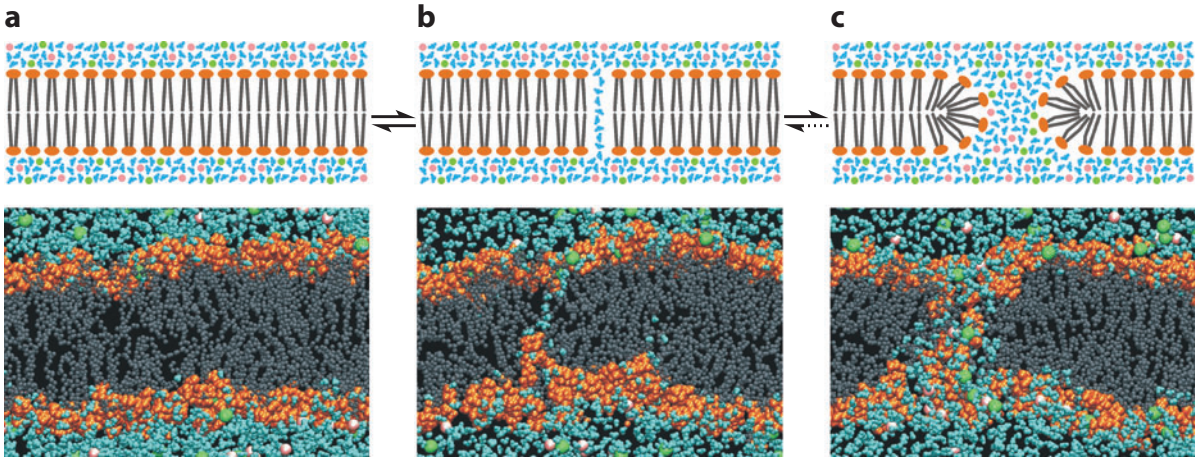

Figure 1

An idealized molecular-level scheme (*top*) and an atomic-level molecular dynamics simulation (*bottom*) of electroporation with the electric field perpendicular to the bilayer plane. In the simulation, a 1-palmitoyl 2-oleoyl phosphatidylcholine (POPC) bilayer surrounded by a saline solution is exposed to a field of 4 MV/cm, with the snapshots taken 0, 0.15, and 0.50 ns after the field is turned on. (*a*) The intact bilayer. (*b*) Water molecules start penetrating the bilayer, forming a water wire. (*c*) The lipids adjacent to the water wire start reorienting toward the wire with their polar head groups, stabilizing the pore and allowing more water, as well as other polar molecules and ions, to enter. The atoms of the lipid head groups and tails are shown in orange and gray, respectively; water molecules in cyan; sodium ions in green; and chloride ions in pink. (Top row reprinted with permission from 181; bottom row reprinted with permission from 23.)

also being more stable than those formed in the absence of an electric field. For transmembrane voltages of hundreds of millivolts, the number of pores becomes large enough, and their average lifetimes long enough (from milliseconds up to minutes), for a detectable increase of membrane permeability to molecules otherwise unable to cross the membrane.

Aqueous pores in the bilayer have radii of at most several nanometers, which is too small to be observable by optical microscopy, and the sample preparation required for electron microscopy of soft matter (vacuumization, fixation, and/or metallic coating) is too harsh for reliable preservation of semistable structures in the bilayer, such that pores cannot be clearly distinguished from artifacts. Nevertheless, there is rather convincing evidence in favor of the theory of aqueous pore formation in the form of molecular dynamics simulations. These simulations largely confirm the hypothesized view of the sequence of molecular-scale events and also show a clear increase in the rate of pore formation with an increase in the electric field to which the membrane is exposed—first through the direct action of the external field, and then augmented by the inducement of transmembrane voltage resulting from polarization (26).

2.2. Transmembrane Voltage and Electroporation

The exposure of a cell to an external electric field can be achieved either by bringing the cell into direct contact with the electrodes generating the field (the approach used in the patch clamp technique) or by placing the cell into the field without such contact. In the latter case, the voltage formed by the field on the membrane (the ITV) represents only a part of the voltage delivered to the electrodes; moreover, the ITV varies with position on the membrane, and also with time, reaching its steady-state value after a gradual buildup.

For regularly shaped cells (close to spheres, spheroids, cylinders, etc.) sufficiently far apart (i.e., in dilute suspensions), the spatial distribution and time dependence of the ITV can be derived analytically and expressed by explicit formulae (27–31). Thus when a single spherical cell with radius R is exposed to a homogeneous electric field of strength E, the first-order approximation of the time course of its ITV (sometimes referred to as the Schwan equation) reads

$$\mathrm{ITV} = f\,ER\,\cos\theta(1 - e^{-t/\tau}),$$

where f is a dimensionless factor, θ is the angle measured from the center of the cell with respect to the direction of the field, t is the time elapsed since the onset of the field, and τ is the time constant of membrane charging. [f and τ can be expressed as functions of the electrical and geometrical properties of the cell and its surroundings (28, 30), and under physiological conditions, $f \approx 1.5$ and $\tau \approx 0.5$ μs.] The ITV is thus proportional to R and E, and its cosine spatial distribution implies that the ITV reaches its peak values at the poles of the cell facing the two electrodes and is equal to zero at the equator of the cell that lies on the cell's plane of symmetry parallel to the electrodes.

For irregularly shaped cells, as well as for cells close to each other (in tissues, clusters, and dense suspensions), the ITV cannot be derived analytically, and numerical methods have to be used instead (32–34). An alternative to both analytical derivation and numerical computation is experimental determination of the ITV using a potentiometric dye (35, 36).

As pore formation is governed by statistical thermodynamics (20), it is strictly speaking not a threshold event, in the sense that the pores would form only at E exceeding a certain fixed value. Nonetheless, electroporation-mediated transport across the membrane is strongly correlated with the ITV generated by the external electric field (37), which is, in turn, proportional to this field. The correlation between the ITV and the electroporation-mediated transport across the membrane can

be demonstrated particularly clearly by combining potentiometric measurements and monitoring transmembrane transport on the same cell, with two examples shown in **Figure 2**.

2.3. Pore Formation, Resealing, and Thermal Damage

There are four general contiguous ranges of the electric field strength (**Figure 3**) that can be best characterized with respect to the related molecular transport and the functioning of the cell:

- No detectable electroporation: This is the lowest range and the one in which there is no detectable molecular transport observed.
- Reversible electroporation: Here, temporary and limited pathways for molecular transport are formed, but after the end of the electric pulse, the transport ceases, and the cells remain viable.
- Nonthermal irreversible electroporation: Here, the transport through the membrane—particularly the leakage of intracellular content—is too extensive and/or the resealing too slow for the cells to recover, resulting in their death and eventual disintegration; still, there is generally no thermal damage to the cell nor to the released molecules.
- Irreversible electroporation accompanied by thermal effects: In sufficiently strong fields, the electric currents cause a temperature increase sufficiently high for thermal damage to the cell as well as to the released molecules.

These ranges depend on the duration of the exposure to the field (i.e., on pulse duration), and they partly overlap, as pore formation is a stochastic process and as the cells within the population generally vary in size and orientation with respect to the field direction, which affects the ITV induced by a given field (for the overlapping of nonthermal and thermal range, see below). The bounds of these four ranges also vary with the type of the cells exposed. For smaller cells, they are generally shifted upward—that is, toward higher fields (as smaller cells require higher field strengths for generation of a given ITV)—and this is also the case for cells enveloped by a wall (i.e., plants, fungi, algae, bacteria, and some archaea), as poration of this additional layer requires extra induced voltage. The ranges are also affected by the properties of the medium surrounding the cells; in particular, the range of thermal effects depends strongly on the electrical conductivity of the medium, as the density of the electric current generated by a given field is proportional to this conductivity, and so is the heat dissipated by the current per unit time. Thus the lower bound of the range of thermal effects is shifted rightward and upward for extracellular conductivities below the physiological level and in the opposite direction for higher extracellular conductivities, thus widening or narrowing, respectively, the range of nonthermal irreversible electroporation. Furthermore, the minimal temperature at which thermal damage occurs is both organism and molecule dependent. Proteins are generally the most sensitive and start to denature at relatively small temperature increases (at ~43–45°C in human cells). DNA melting occurs only above ~70°C, whereas most lipids and simpler saccharides are not affected even by boiling.

Similar to pore formation, pore resealing is a stochastic process, but it proceeds on a much longer timescale. Specifically, the formation of electropores takes nano- to microseconds, whereas their resealing—as revealed by the return of the membrane's electric conductivity to its preporation value and by termination of detectable transmembrane transport—is often completed only within seconds, or even minutes, after the end of the exposure (38). More detailed measurements reveal that the resealing proceeds in several stages with time constants ranging from micro- and/or milliseconds up to tens of seconds (39, 40). Unfortunately, neither the existing theory nor the experiments can provide a reliable picture of specific events characterizing each of these distinctive

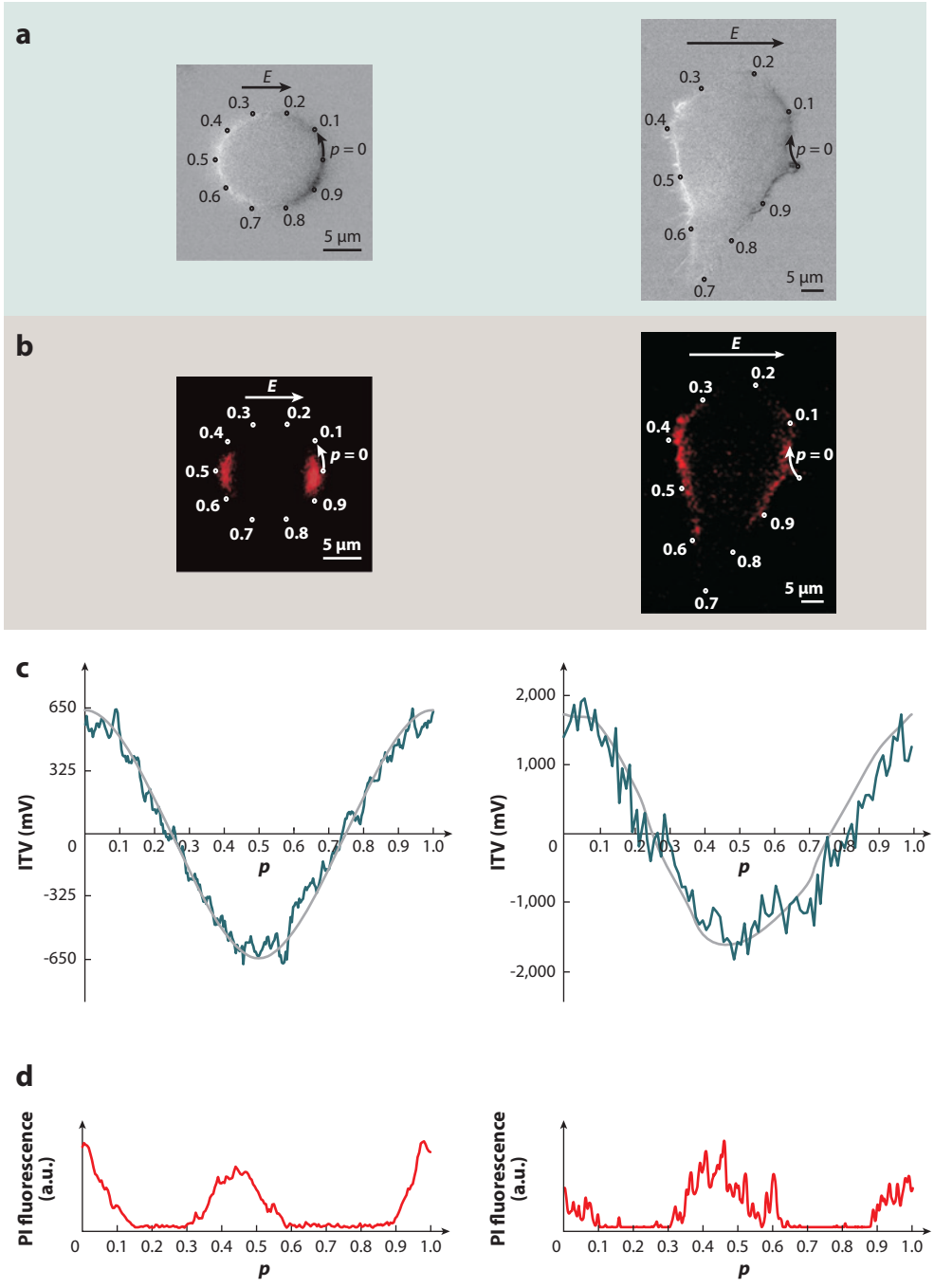
a
E
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
p = 0
5 µm
b
c
ITV (mV)
650
325
0
-325
-650
2,000
1,000
-1,000
-2,000
p
d
PI fluorescence (a.u.)

Figure 2

The induced transmembrane voltage (ITV) and electroporation of two Chinese hamster ovary (CHO) cells in a physiological medium: one suspended and almost spherical (left-hand sides of panels *a–d*) and the other attached to a surface and irregularly shaped (right-hand sides of panels *a–d*). (*a*) Changes in the fluorescence of di-8-ANEPPS, a potentiometric dye reflecting the ITV, with dark and bright regions corresponding to membrane depolarization and hyperpolarization, respectively. (*b*) Fluorescence of propidium iodide (PI), a dye fluorescing only inside the cell and thus reflecting its electroporation-mediated influx into the cell. (*c*) ITV along the path shown in panel *a* as measured (*green*) and as predicted by numerical computation (*gray*). (*d*) Fluorescence of PI along the path shown in panel *a*. The spherical cell was electroporated by a single 1.5-ms, 650-V/cm pulse, and the attached cell by a single 200-μs, 1,000-V/cm pulse. Additional abbreviations: *E*, electric field strength; *p*, normalized arc length along the membrane. (Figure reprinted with permission from 37.)

stages, and reliable molecular dynamics simulations, even in their most simplified versions (e.g., coarse grained), cannot yet cover timescales that extensive.

2.4. Transmembrane Mass Transport

Direct microscopic observations have revealed that the mass transport mechanisms during electroporation depend on the nature of molecules (41, 42). Small charged molecules, for example propidium iodide (PI), enter the cells from both poles exposed to the electrodes during and after the application of electric fields (**Figure 4**). Negatively charged small interfering RNA (siRNA) molecules, however, transfer into the cell cytoplasm exclusively on the side facing the cathode only when the electric field is applied (**Figure 4**). When added after electroporation, siRNA was unable to silence genes because it did not penetrate the cells, as reported by Paganin-Gioanni et al.

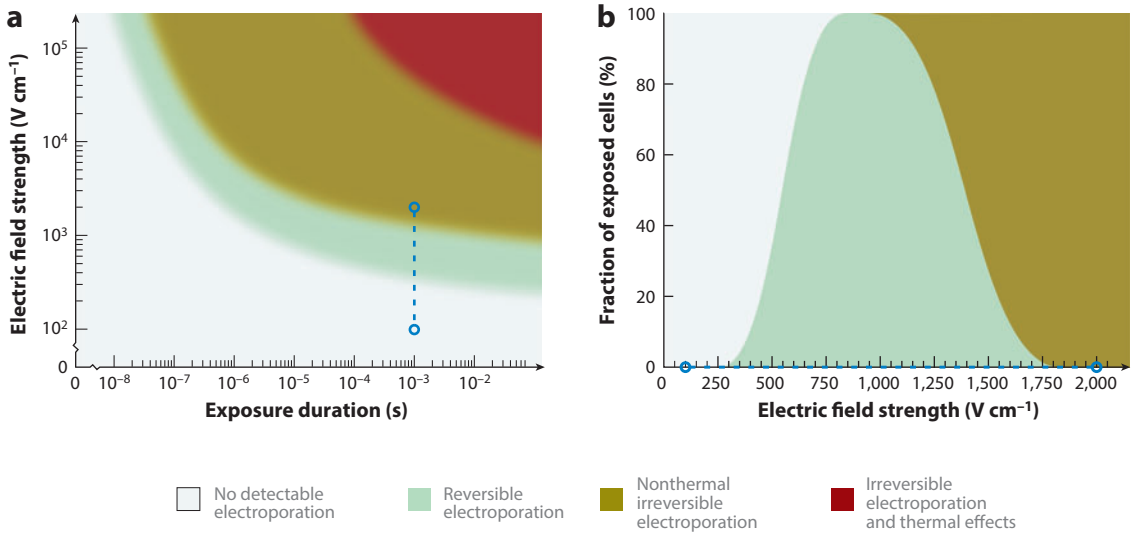

Figure 3

Electroporation and thermal effects caused by exposure of cells to electric fields. (*a*) Reversible electroporation, irreversible electroporation, and thermal damage as functions of electric field strength and duration. (*b*) The fractions of nonelectroporated, reversibly electroporated, and irreversibly electroporated cells as functions of electric field strength, for a fixed exposure duration of 1 ms (i.e., along the blue dashed vertical line in panel *a*). Note that the field scale is logarithmic in panel *a* but linear in panel *b* where it covers a much narrower range. (Panel *a* adapted with permission from 182 and 183.)

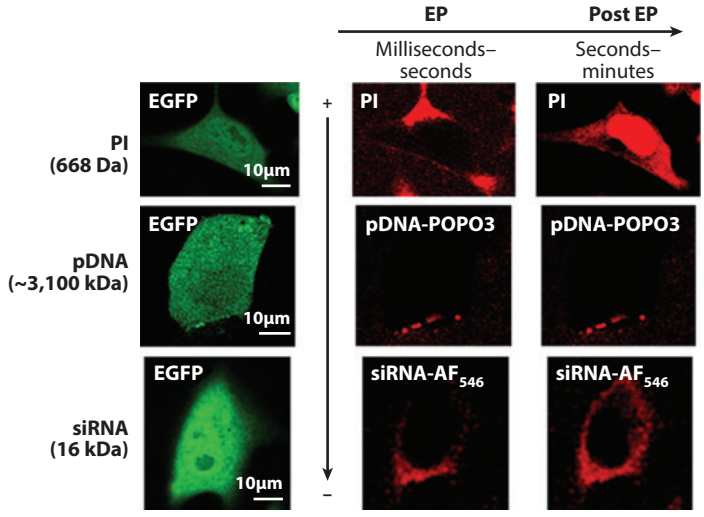

Figure 4

Direct microscopical observation of electrotransfer of PI, siRNA, and pDNA into murine melanoma cells (10 pulses, 5 ms, 300 V/cm, 1 Hz). The column on the left shows the EGFP-expressing cell. The electroporation (*center column*) in the top row is the first step of the electrotransfer of PI: During electroporation, penetration can be observed on both sides facing the electrodes. Post electroporation (*right column*), diffusion and nuclear labeling can be observed. The electroporation (*center column*) in the middle row is the first step of the electrotransfer of pDNA labeled with POPO3: A localized interaction with the plasma membrane (*fluorescent spots*) occurs on the side facing the cathode and continues post electroporation (*right column*) before translocation into the cytoplasm (not detected on this timescale). The electroporation (*center column*) in the bottom row is the first step of the AF_{546} siRNA electrotransfer: A rapid and free penetration into the cytoplasm on the side facing the cathode occurs during the electric pulses. Post electroporation, the AF_{546} siRNA is localized diffusely throughout the cytoplasm with no further entry. Abbreviations: AF_{546}, an Alexa Fluor dye; EGFP, enhanced green fluorescent protein; EP, electroporation; pDNA, plasmid DNA; PI, propidium iodide; POPO3, an intercalating dye; siRNA, small interfering RNA. (Figure adapted with permission from 41.)

(41). By contrast, plasmid DNA (pDNA) penetration begins with the induction of local physical interactions between the nucleic acid and the cell membrane during the application of electric fields; it is followed by a slow intracellular release into the cytoplasm after exposure to the electric field is terminated (42), and then by trafficking to the nucleus. These differences are important for ECT and GET treatment planning.

3. ELECTROPORATION APPLICATIONS IN MEDICINE

3.1. Electrochemotherapy

Electrochemotherapy (ECT) has reached an established position among local treatments in oncology, both human and veterinary (43, 44). More than 3,000 patients were treated with this application in the European Union in 2012 (45). The basic principle of ECT is the use of electroporation for delivery of non- or poorly permeant chemotherapeutic drugs into the cells, facilitating their transmembrane transport with the aim of increasing their cytotoxicity. In preclinical studies, bleomycin, used by Mir et al. (9, 46), and cisplatin, used by Serša et al. (47), were identified as the most suitable drugs for clinical testing (7, 9, 46–48). The effectiveness of ECT has been demonstrated on different solid tumor models, with the treatment inducing complete or partial

responses (49). It should be noted that ECT has an excellent therapeutic index; that is, for antitumor effectiveness, very low drug concentrations of the chemotherapeutics are needed, thus avoiding toxicity.

During preclinical studies, several mechanisms that contribute to the overall effectiveness of ECT have been demonstrated. Besides the basic mechanism of increased membrane permeability leading to increased drug cytotoxicity (50), reduced tumor blood flow (vascular lock) is observed (51) and results in a vascular disruption effect of ECT (52–55); an enhanced immune response is also observed (56–58). Thus, combining ECT with an immunostimulatory approach using recombinant cytokines or GET-delivered plasmids encoding these cytokines (e.g., IL-2, IL-12, IL-15, GM-CSF, and TNF-α) can give rise to a significant potentiation of ECT treatment (59–62).

The first clinical study on ECT was published in 1991 by Mir et al. (7) and demonstrated the feasibility, safety, and effectiveness of ECT. This first study stimulated groups in the United States (Tampa), Slovenia (Ljubljana), and France (Toulouse and Reims) to perform further clinical studies (63, 64), which were then compiled in a joint paper published in 1998 (65). The coalescence of the field was marked by the report of a European project called the European Standard Operating Procedures on Electrochemotherapy (ESOPE) (**Figure 5**). Based on the results of a clinical study published in 2006 (66) together with the standard operating procedures (SOP) for ECT using the electric pulse generator Cliniporator (67), ECT was widely accepted for clinical use throughout Europe. Clinical indications were published in 2008 (68), and a systematic review and meta-analysis (69) recently analyzed the results of all the published studies through 2012. Data analysis confirmed that delivery via ECT has a significantly ($p < 0.001$) higher effectiveness (by more than 50%) than bleomycin or cisplatin injection alone. The overall effectiveness of ECT was 84.1% objective responses (OR, which include both complete and partial responses), and from these, 59.4% were complete responses (CR, which indicate complete regressions of tumors after therapy) after a single ECT treatment. The procedure can, however, be repeated with similar effectiveness (70). Another recent review and clinical study (71) suggested that the SOP may need refinement, as the current SOP for ECT may not be suitable for tumors bigger than 3 cm in diameter, which require multiple, consecutive ECT treatments. Several ongoing ECT studies are targeting superficial tumors, predominantly melanoma but also chest wall breast cancer recurrences (72–74) and head and neck cancers (75). Furthermore, the technology is also being adapted for the treatment of deep-seated tumors, such as colorectal tumors; soft tissue sarcomas; and brain, bone, and liver metastases (45, 52).

Currently, ECT is being used for treatment of metastases and primary tumors in more than 130 cancer centers in Europe and has been accepted in line with other local tumor treatments (76). In addition, ECT is also used in veterinary oncology, for treatment of metastases as well as primary tumors (44, 77–82). The success rate of primary tumor treatment with ECT also provides good evidence for translation of ECT into treatment of less-advanced tumors in humans.

Vascular lock: transient reduction of perfusion, at a greater extent in tumors than in normal tissues, that occurs after application of electric pulses to tissues

Vascular disruption effect of ECT: a cascade of tumor cell death, surrounding obstructed tumor vessels, resulting from the drug used in the electroporation of endothelial cells and contributing to the overall effectiveness of ECT

3.2. Nonthermal Tissue Ablation

Nonthermal irreversible electroporation (NTIRE) is an emerging minimally invasive surgical procedure to ablate tissue. In NTIRE, externally applied pulsed electric fields cause irreversible damage to cells by affecting the cell membrane, while sparing the tissue scaffold, large blood vessels, and other tissue structures (83–85). The preservation of tissue architecture is an important and unique property of NTIRE ablation, and it probably contributes to the reduced scarring observed (as reported in 83, 84, 86).

The first work on NTIRE was published by Davalos et al. (12) in 2005. Using mathematical models, the authors predicted NTIRE would result in the nonthermal ablation of clinically relevant

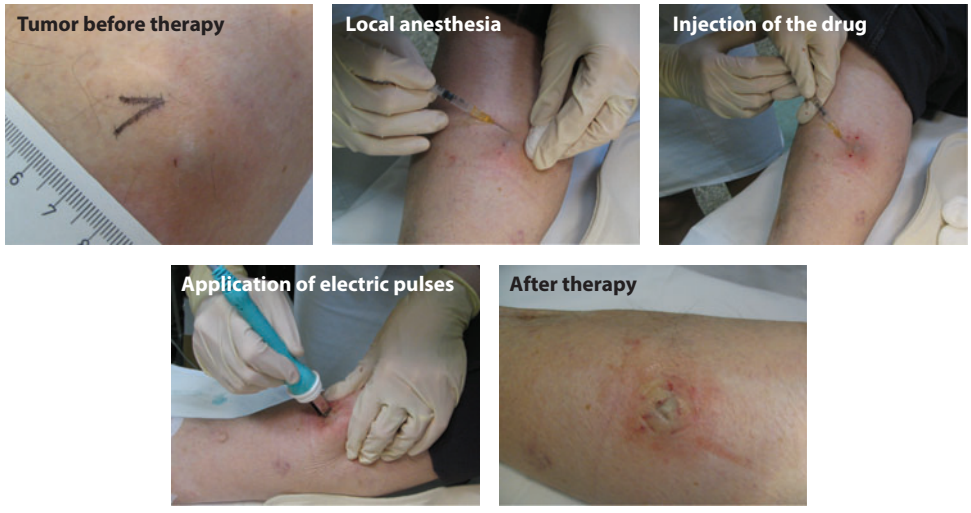

Figure 5

Tumor treatment with electrochemotherapy (ECT). Cutaneous or subcutaneous tumor nodules are treated by ECT either with intratumoral bleomycin or cisplatin administration or with intravenous bleomycin. Immediately after intratumoral administration or 8 min after intravenous bleomycin administration, electric pulses are applied using plate or needle electrodes. If the tumor is larger than the gap between the electrodes, electric pulses are delivered by multiple applications, so that the whole tumor volume is exposed to a sufficiently high electric field, thereby assuring cell membrane electroporation. (Figure adapted with permission from 63.)

volumes of tissue. This theoretical work was later confirmed by experimental studies in vitro using cell cultures (87), small-animal models (88), and large-animal models (89) (**Figure 6**) and recently by the first human studies (90, 91). The exact mechanisms of cell death after NTIRE are as yet unknown, and the reports on pathways that lead to cell death are somewhat contradictory. For example, Guo et al. (92) reported extensive caspase-3 activation 24 h after NTIRE of rat hepatocellular carcinoma, which suggests apoptosis. By contrast, José et al. (93) did not detect any caspase-3-positive cells in the treated area of pancreatic carcinoma. The latter group of authors argued that the differences observed may be related to the tumor model used; however, whether NTIRE affects the cell membrane as a final event or induces long-term programmed cell death is still unresolved. It is important to point out that even at the single-cell level in culture, NTIRE-induced cell death is a statistical phenomenon (94, 95). In vivo, only limited attention has been devoted to the inflammatory response to NTIRE. Al-Sakere et al. (96) concluded that NTIRE does not induce substantial infiltration of immune cells into the treated tissue, which would suggest that the immune response is not essential for successful NTIRE. By contrast, Onik et al. (97) observed an immunologic reaction in the lymph nodes draining the prostate area ablated by NTIRE. Most recently, Neal et al. (98) showed an improved system response to NTIRE ablation of renal carcinoma tumors in immunocompetent mice as compared with immunodeficient mice.

Most recent clinical studies have investigated the safety and efficacy of NTIRE treatment of pancreatic adenocarcinoma (90, 99), renal cell carcinoma (100, 101), lung malignant tumors (101, 102), and hepatic malignant tumors (101, 103, 104). One drawback of NTIRE is that the strong electric fields induce muscle contractions, therefore requiring special anesthesia (105). Cardiac arrhythmias developed during NTIRE in several patients have been managed by electrocardiographically synchronized delivery of pulses. Recently, a case report (106) claimed that a patient

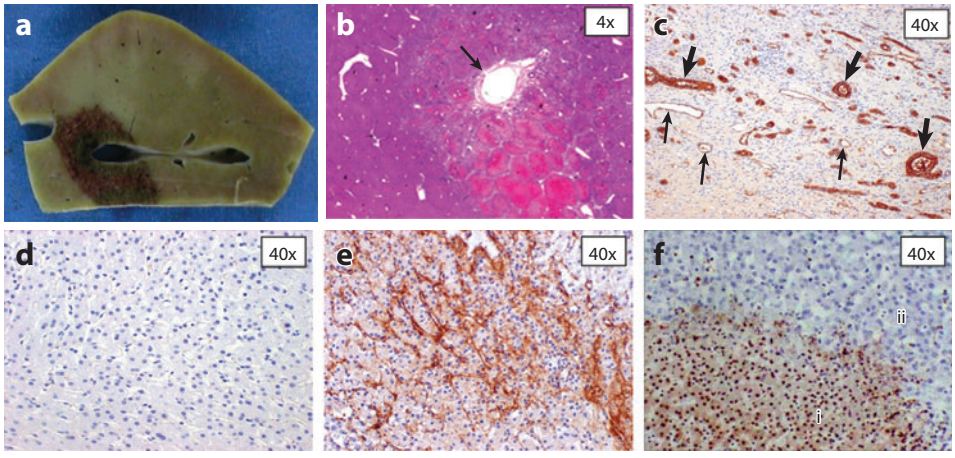

Figure 6

Swine liver histopathology after NTIRE. (*a*) Gross pathologic sectioned specimen of NTIRE-ablated liver shows areas of discoloration caused by ablation. (*b*) Preserved large vessel (*arrow*) in area of NTIRE ablation stained with H&E. (*c*) Numerous capillaries (*thin arrows*) and bile ducts (*thick arrows*) are stained with vWF, revealing structural preservation. (*d*) The normal liver tissue shows mild vWF staining of vessels and bile ducts. (*e*) The ablated zone shows markedly increased vWF-positive staining along the sinusoids, as well as staining of vessels and bile ducts. (*f*) The ablated zone (i) shows increased apoptotic markers in a TUNEL assay, compared with normal liver (ii). (Figure adapted with permission from 84.) Abbreviations: H&E, hematoxylin and eosin; NTIRE, nonthermal irreversible electroporation; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; vWF, von Willebrand Factor.

with a pancreatic tumor treated by NTIRE developed melena and hematemesis. The origin of this complication is still not known, but it may be attributable to proteolytic pancreatic enzyme leakage resulting from vascular injury during surgery or to still unknown mechanisms related to ablation. The efficacy of the procedure varies among studies and treated organs. In pancreatic and prostate cancer, studies have reported 100% success in tumor ablation (90, 91, 99); however, no treated lung tumors have been successfully ablated to date (101, 102). In liver, the response varied from 50 to 98.1% successful ablation of tumor lesions in different studies (101, 103, 104). In addition to human studies, NTIRE has also been successfully used for tumor ablation in veterinary medicine (107–109).

3.3. Gene Therapy and DNA Vaccination

DNA vaccination: a technique for protecting an organism against disease by injecting it with genetically engineered DNA to produce an immunological response more safely than with viral vectors

Another biomedical application of electroporation involves gene electrotransfer (GET) to cells and tissues for gene therapy and DNA vaccination. This technique has been used mainly for DNA vaccination against infectious diseases, cancer, arthritis, multiple sclerosis, and inflammation following organ transplantation and to genetically modify cells for regenerative medicine applications (110–113).

In animals, GET was demonstrated first for skin by Titomirov et al. (11), then for liver, muscle, and tumor tissue (114–116). Since then, GET use has been expanded to other targets, including brain, kidney, testis, cartilage, arteries, prostate, and cornea (117–119), though transfection efficiency varies considerably across different tissues. Skeletal muscle fibers, which are polynucleated, are easily accessible and readily transfected, whereas rapidly dividing tumor cells in solid tumors are the most difficult to transfect (120).

In cancer treatment, most of the GET preclinical studies have concentrated on delivering immunomodulatory genes, such as IL-12, IL-2, IL-15, and GM-CSF (110, 111). In addition, several studies have documented the use of genes that target the tumor vasculature, such as vasostatin, endostatin, and vascular endothelial growth factor (VEGF) (121–124). Recently, GET of an antiangiogenic metargidin peptide (AMEP) targeting integrins and GET of siRNA targeting endoglin have been shown to exert antiproliferative and antiangiogenic effects on melanoma tumors (for AMEP) and mammary carcinoma tumors (for siRNA) (125, 126). Two clinical studies that have used GET successfully have demonstrated the safety and effectiveness of GET to tumors. The first study, by Daud et al. (10), demonstrated that IL-12 GET to cutaneous melanoma nodules in humans was very efficient, as tumor necrosis was observed in most of the treated lesions; furthermore, in two of the patients, the response was also observed in distant untreated metastases. The other clinical study (127), which examined intratumoral GET of plasmid AMEP in patients with cutaneous melanoma metastases, demonstrated not only the safety of transferring the molecule into tumors but also some effectiveness.

GET is also being successfully used in veterinary oncology for treatment of tumors in companion animals and in horses. To date, reports on the successful use of IL-12 GET for treatment of sarcoids in horses and different primary tumors in dogs are available (128, 129). In addition, one study describes the successful combination of ECT and GET for treatment of tumors in dogs (130).

GET in DNA vaccination, introduced by Nomura et al. (131) in 1996, aims to address two major factors that are thought to limit clinical success of human DNA vaccines: (*a*) low transfection efficiency and (*b*) insufficient recruitment of antigen-presenting cells to the injection site (132). GET has been shown to enhance DNA vaccine efficacy up to three orders of magnitude (132) and to achieve responses comparable to those achieved with protein vaccines (133). Higher efficiencies have been obtained with GET than with the gene gun or by direct intramuscular injections (134, 135).

In cell-based regenerative medicine, electroporation is an alternative nonviral method for introducing DNA and RNA into cells. Although viral-mediated transfection is the most efficient method for genetic manipulation of mammalian cells, virus-related immunogenicity, cytotoxicity, and possible mutations that may disrupt tumor suppressor genes, activate oncogenes, or interrupt essential genes are major drawbacks of viral methods for clinical applications (136). Multiple GET protocols have been published for various cell types with commercially available solutions for optimizing the electroporation procedure (137, 138). Defining the optimum conditions for specific-cell-line electroporation can be a labor- and cost-intensive procedure. To avoid this problem, a high-throughput method has been introduced to identify electroporation parameters for cell electroporation in a single step using a concentric electrode configuration (139).

Microfluidics provides a convenient and cost-effective way to design miniaturized devices to electroporate large volumes of cells, which has been a challenge for translating cell therapy to clinical application (140). In contrast to the classic bulk electroporation system, microfluidic platforms can provide a controlled microenvironment for single-cell transfection (141). Recently, Geng et al. (142) reported a ~75% transfection efficiency using a microfluidic system with a flow rate of ~20 mL/min, which may be sufficient for clinical cell-therapy applications.

3.4. Transdermal Drug Delivery

The barrier property of skin provides obvious survival benefits in the context of variable and harsh environments (i.e., in the presence of environmental threats of a chemical, physical,

or biological nature). The skin also undergoes continuous regeneration and repair and is composed of several layers: the stratum corneum, viable epidermis, dermis, and subcutaneous tissue (hypodermis). Owing to its size and accessibility, the skin represents a convenient entry element for drugs. In particular, transdermal delivery has potential for introducing drugs that are not suitable for intravenous or oral administration, and can overcome many of the disadvantages of other delivery routes. By using the transdermal route of administration, we can avoid the liver's first-pass effect (via hepatic metabolism), obtain better pharmacokinetic profiles, reduce side effects, and achieve reasonable patient compliance. However, simple transdermal delivery often offers limited flux owing primarily to the impermeable outermost layer of the skin—the stratum corneum. Overcoming this barrier can be achieved by different enhancement methods, both passive and active (143), one of them being electroporation (144, 145).

Electroporation has been shown to create aqueous pathways across the stratum corneum and thus to enhance transdermal drug delivery. The stratum corneum's electrical resistance is orders of magnitude higher than that of deeper tissues, and the high electric field resulting from the application of electric pulses remains mostly in the stratum corneum. As a result of electroporation, the resistance of the stratum corneum rapidly decreases, and the electric field distributes into deeper tissue layers (**Figure 7**) (146, 147).

Exponentially decaying pulses and square wave–shaped pulses are the most commonly used pulse types in electroporation. In general, increasing the pulse amplitude, duration, and number results in increased permeability of the stratum corneum, allowing for better drug transport (148). Consequently, molecular flux is enhanced until it reaches a maximum value beyond which increasing pulse parameters has negligible effect on flux (149). Electroporation has been used successfully to enhance skin permeability for molecules with differing lipophilicity and size (small molecules, proteins, peptides, and oligonucleotides), including drugs such as fentanyl, insulin, and methotrexate (144, 150, 151).

Molecular transport through electroporated skin occurs through different mechanisms, mainly (*a*) enhanced diffusion during and after application of electric pulses and (*b*) electrophoretic movement with very slight electroosmosis during application of a pulse (152). It should be stressed that reproducibility of drug delivery (i.e., dose control) using electroporation is rather poor and represents a major obstacle in using the technique as a drug delivery enhancer. Electroporation can, however, be readily used for applications such as gene delivery. As discussed above, electroporation can be used for applications in which, after intradermal injection, therapeutic molecules need to be inserted in viable skin cells, a process that is now increasingly being tested for DNA vaccination (153, 154).

Electroporation can also be used for transdermal delivery of low-molecular-weight peptide vaccines and, thus, for cancer and infection disease in a type of needleless vaccination (155, 156). Electric pulses and electrodes must be carefully selected in order to avoid skin damage and/or irritation as well as pain.

4. TREATMENT PLANNING AND IMAGING

Treatment planning is essential for successful electroporation. Efficient cell membrane electroporation depends on establishing a sufficiently high electric field locally (i.e., in the target tissue). The ultimate goal of treatment planning is, therefore, to model electrode position and number; electric field amplitude; and pulse duration, number, and frequency to nonthermally ablate only the targeted tissue. The current treatment planning models focus on (*a*) electrode position, to cover the target tissue by electric fields and spare nontarget tissue (89); (*b*) electric field protocol

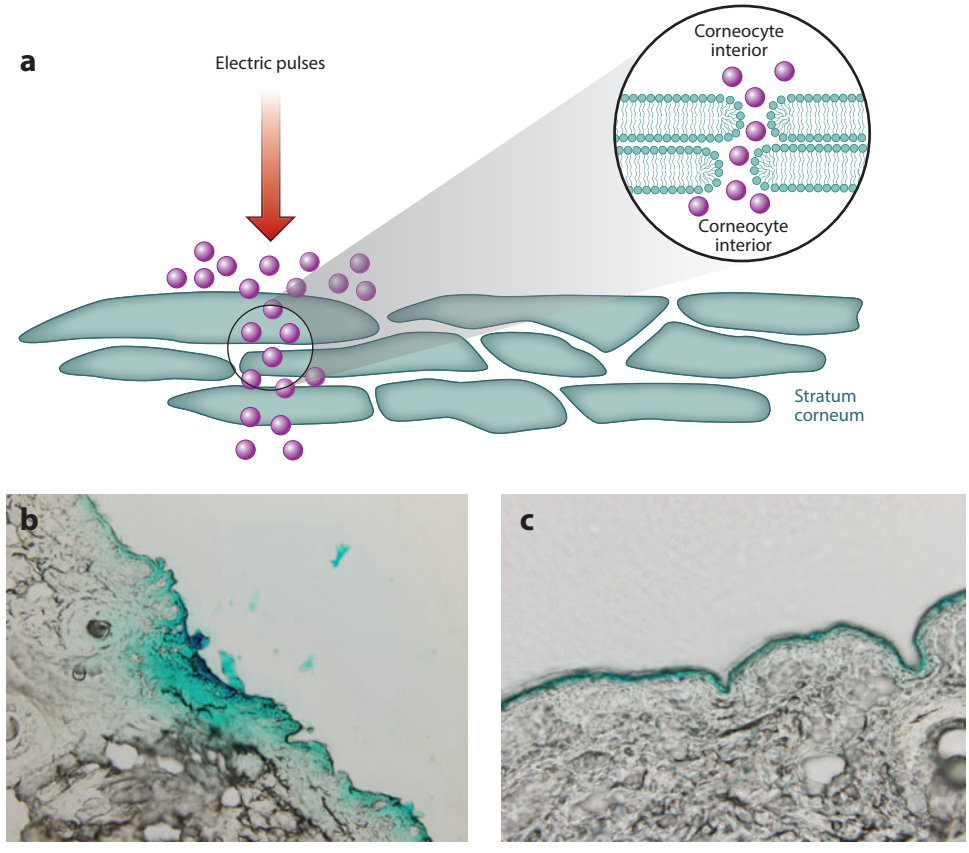

Figure 7

Electroporation of the stratum corneum. (*a*) Electric pulses create pathways through lipid bilayers of corneocytes in the stratum corneum, making a transcellular route amenable for drug transport. (*b*,*c*) In an experiment using fresh full-thickness pig ear skin, a patch with 100 μL of 2.5% patent blue solution was placed on the skin for 5 min before pulse delivery. During skin electroporation treatment, electric pulses of 200 V and 100-μs duration were delivered continuously for 30 s. Peak current during the pulses, measured using digital oscilloscope, was ~20 mA. After treatment, the patent blue patch was applied for another 15 min. Tenfold magnification of the histological section of the skin sample is shown (*b*) after electroporation treatment and (*c*) after passive diffusion. (Panel *a* adapted with permission from 140; panels *b* and *c* reproduced with permission from 184.)

optimization, to delineate the thermal damage from NTIRE and avoid irreversible electroporation in the case of GET or ECT (157); and (*c*) integration of mathematically derived treatment planning with diagnostic imaging (158, 159). An example of a treatment plan used in ECT in a case of liver colorectal metastasis (52) is shown in **Figure 8**.

Phase and fluorescent microscopic studies have led to a partial understanding of the mechanisms behind cell membrane electroporation. These studies have shown that different types of molecules—small molecules, RNA, and DNA—have different transmembrane transport mechanisms during electroporation (41). However, in clinics an important advantage of electroporation-based technologies is the ability of physicians to use existing imaging methods—ultrasound (US), magnetic resonance imaging (MRI), computed tomography (CT), and positron

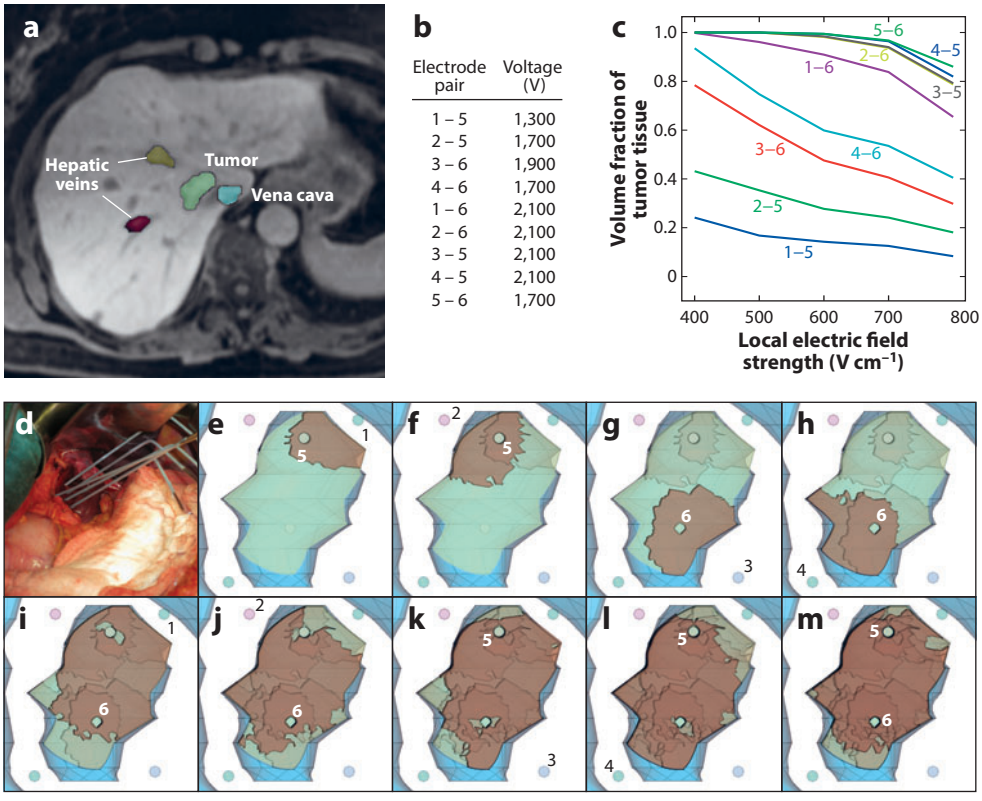

Figure 8

Treatment plan used in a case of colorectal liver metastasis (52). The tumor was located between major hepatic veins and vena cava and was not amenable to surgical resection or RFA, so electroporation was performed using six needle electrodes 20 cm in length with an insulating sleeve, an active tip of 4 cm, and a diameter of 1.2 mm. The electrodes were introduced using ultrasound during open surgery, and high-voltage pulses, as given in panel *b*, were delivered between pairs of electrodes, assuring coverage of the whole tumor volume with sufficiently high electric field. The required electric field for the tumor was 400 V/cm for eight pulses of 100-μs duration. Panel *c* shows the fraction of tumor volume covered by 400+ V/cm produced with each pair of electrodes. Actual placement of the electrodes during open surgery is shown in panel *d*. Panels *e* through *m* provide graphical representations of the tumor fraction covered with 400 V/cm in a selected cross section for each pair of electrodes, with the corresponding electrode pairs marked with Arabic numerals. The area exposed to an electric field higher than 400 V/cm appears dark/brown, whereas the tumor is light/green.

emission tomography (PET)—for procedure guidance. Below, we briefly describe use of these imaging methods to monitor clinical electroporation effects and mechanisms.

4.1. Ultrasound

US has been used in animal and clinical studies for real-time electrode positioning and observing both the immediate and posttreatment effects of electroporation. The US findings after electroporation are dynamic (**Figure 9*a***) and tightly correlated with histological observations. In liver, the irreversibly electroporated region is immediately visualized as a hypoechoic area (**Figure 9*a***, *left*) (85); however, after 90–120 min, an external hyperechoic rim appears, probably owing to hemorrhagic infiltration (160). The hypoechoic area transitioned fully to hyperechoic 24 h after treatment (85). The tightest correlation between the size of histological findings of ablated tissue and the hyperechoic rim was observed 90–120 min after NTIRE treatment (160).

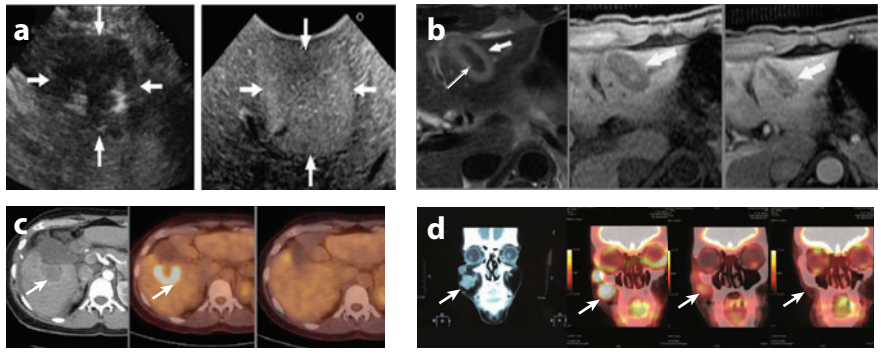

Figure 9

(*a*) US imaging of NTIRE of a normal pig liver. The ablated zone (*arrows*) is shown (*left*) immediately after the procedure and (*right*) 24 h later. (Panel adapted with permission from 85.) (*b*) MRI of NTIRE of a human liver. (*left*) T2-weighted axial MRI of the liver 2 days post NTIRE shows a hyperintensive reactive rim (*bold arrow*) surrounding the ablated region with a hypointense necrotic center (*thin arrow*). (*center*) T1-weighted axial MRI 2 days post NTIRE demonstrates mixed signal intensity (*arrow*) composed of a hypointense ablated zone containing a hyperintense central signal. (*right*) Post-contrast-enhanced hepatic-arterial-phase axial T1-weighted MRI shows a nonenhancing linear ablation zone and hyperenhancing peripheral rim (*arrow*), which corresponds with the hyperemic rim in the left-hand side of panel *b*. (Panel adapted with permission from 165.) (*c*) CT and PET imaging of NTIRE of a human liver. (*left*) A contrast-enhanced CT image shows the ablation zone (*arrow*) immediately after NTIRE. (*center*) The peripheral zone of the NTIRE-ablated region (*arrow*) shows increased FDG uptake 3 days post NTIRE. (*right*) At 1 month post NTIRE, a PET image does not show increased FDG uptake within the ablated zone or at the hyperemic reactive rim. (Panel adapted with permission from 165.) (*d*) CT and PET imaging of ECT of face melanoma. (*far left*) A pretreatment CT scan with evidence of a sandglass-shaped mass (*arrow*) on the right cheek. (*center left*) An FDG-PET scan shows an SUVmax of 19.5. (*center right*) Post-ECT FDG-PET scan shows that the SUVmax decreased to 5. (*far right*) Post-ECT FDG-PET scan showing the SUVmax having decreased to 1.3. (Panel adapted with permission from 166.) Abbreviations: CT, computed tomography; ECT, electrochemotherapy; FDG, fluorodeoxyglucose; MRI, magnetic resonance imaging; NTIRE, nonthermal irreversible electroporation; PET, positron emission tomography; SUVmax, maximum standardized uptake value; US, ultrasound.

4.2. Magnetic Resonance Imaging

MRI techniques are often used for detecting changes in tissue structure and assessing functionality after ECT and NTIRE in animals and in human subjects. MRI imaging of NTIRE is shown in **Figure 9*b***. Systematic MRI studies of NTIRE-ablated tissue demonstrated dynamic behavior of various MRI sequences detected in the ablated tissue. Indeed, studies have shown that the ablated volumes detected by T1-weighted (T1W), transverse relaxation time (T2), T2-weighted (T2W), fluid attenuated inversion recovery (FLAIR), and diffusion-weighted magnetic resonance imaging (DW-MRI), for the apparent water diffusion coefficient (ADC), are similar to the volumes detected by histological observations. Additionally, T1-Thrive5-3D-GE urogram scans, together with intravenous urography, were used to validate the functionality of NTIRE-ablated swine kidney (161). The usefulness of a particular MRI sequence depends on the type of ablated tissue. In a murine model, although the ADC allowed the visualization of early and rapid changes in ECT-treated tumors (162), no significant changes in ADC were observed during blood-brain-barrier NTIRE ablation up to 30 min post treatment (163). Contrast agents such as Gd-DOTA have already been successfully used for the purpose of observing reversibly electroporated areas either in muscle tissues or in the brain (163, 164).

4.3. Computed Tomography and Positron Emission Tomography

Non-contrast- and contrast-enhanced CT have been used in animal experiments and in patient care for diagnosis, electrode positioning, and posttreatment evaluation of tissue ablation and tumor regression. NTIRE ablation of liver results in a hypoattenuating area with a hyperattenuating rim in the ablated area 2 days after the procedure (85) (**Figure 9*b***, *left*). Contrast-enhanced CT was used to evaluate the treatment response of liver metastases treated by intraoperative ECT. The response was deemed complete when the treatment zone appeared as a well-defined area of low attenuation without enhancement (52).

PET scans of NTIRE ablations show a time-dependent response (**Figure 9*c***). At 3 days post NTIRE, a fludeoxyglucose (FDG) enhancement in the peripheral zone surrounding the ablated region is present (**Figure 9*c***, *center*), but the peripheral increase in FDG uptake disappears within 1 month of treatment (**Figure 9*c***, *right*). (165). Other work with PET showed the effects of ECT on cheek melanoma (**Figure 9*d***) (166). PET CT was also used to evaluate the responses of large tumors (e.g., recurrent breast cancer) being treated with ECT (74).

4.4. Electrical Impedance

In tissues, electroporation leads to an immediately detectable tissue impedance decrease, which can be used for outcome assessment (167–170). Control of in situ muscle electroporation to reduce cell damage to cells while assuring successful gene transfection was demonstrated using current and voltage measurement during pulse delivery (167). Changes in tissue conductivity can be measured in real time using a galvanic electroporation cell (168, 171). In this approach, electroporation is delivered via electrodes with different electrochemical potential (e.g., Zn and Cu). The galvanic current generated by the electrodes and the treated tissue provides direct measurement of electric impedance changes during and after electroporation without using additional devices (168, 171). The two-dimensional reconstruction of tissue impedance, electrical impedance tomography (EIT), in principle allows near real-time monitoring of tissue electroporation (169, 172). In addition to EIT, magnetic resonance electrical impedance tomography (MREIT) and current density imaging (CDI) have been proposed for measurement of current and electric field distributions in tissue during the treatment (173, 174), which would allow immediate corrective action in performing electroporation-based treatments, thus further improving their efficacy.

5. POTENTIAL AND CHALLENGES

Electroporation increases cell membrane permeability through a nonthermal, chemical-free path. Therefore, it can be used to (*a*) reprogram cell and tissue function by introducing external molecules that affect different cellular pathways, (*b*) load cells with new materials, and (*c*) cause cell damage and death. Accessible imaging of the treatment and its effects, together with fast patient recovery, has generated great interest in the basic and translational aspects of this technique, spurring the emergence of data across species, from various tissues, and from various clinical applications. As described above, encouraging results have been reported in animal models and in several clinical trials using ECT, GET, and NTIRE. However, the inability to ablate lung tumors and to induce long-term immune protection with DNA vaccines, as well as the somewhat contradictory results with cell therapy, must be clarified and overcome. These problems could potentially be understood through better integrating imaging for treatment planning and by using optimization algorithms for electrode positioning. Development and integration of standard electroporation treatment planning methods are essential for therapy success (175). This integration will provide more precise treatment with fewer side effects. In addition, new treatment planning methods will

allow for precise dose control, which will lead to the destruction of large, deep-seated tumors by ECT and NTIRE. Future systematic studies are required to characterize electroporation-induced dynamic changes in various tissue types.

To use electroporation efficiently, we must understand the basic mechanisms of damage and regeneration at the molecular, cellular, and tissue levels. Presumably, molecular understanding will emerge from advanced molecular dynamics simulations and the like. Investigation of the impact of electroporation on homogeneous and, more importantly, heterogeneous tissues will address the critical question of selectivity: Can electroporation selectively target specific cell types? In addition, further studies on the role of the immune system response to electroporation-induced permeabilization are needed and will presumably provide answers concerning in vivo mechanisms of therapeutic efficacy. The combined use of electroporation and immune response stimulation may yield an even more attractive treatment approach.

An additional challenge involves the elimination of electroporation-induced pain and muscle contraction without using total-body-paralysis agents. Solving this problem will significantly simplify the use of electroporation in medicine, including its use in developing countries. Recently, a high-frequency pulse delivery regime with a special electrode configuration was proposed for NTIRE tissue ablation with reduced muscle contraction (176, 177).

Tissue decellularization using irreversible electroporation and perfusion is a promising technology for both in vitro and in vivo regenerative medicine. Although the available data show that electroporation speeds the decellularization process in vitro (178), a complete analysis of the biological scaffold that is produced is still missing. The host response to the electroporation-prepared matrix is also still not known.

The clinical data published thus far on electroporation-based applications have been quite encouraging. Reports describing the clinical advantages of ECT, NTIRE, and electroporation-based DNA vaccination continue to stimulate current basic and applied research in the field. GET and other nonviral gene delivery systems may gain more attention, as viral vectors have not proved to be optimal, owing to safety issues such as insertional mutagenesis and immunological interference (119). The transfection efficiency of GET in different tissues is still low; therefore, some attempts have been made to improve it either by scavenging reactive oxygen species (ROS) produced by electroporation (179) or by degrading the extracellular matrix (180).

Although studies have reported electroporation to be safe, additional large-scale-outcomes research is needed for various clinical conditions, as are comparative studies with other therapeutic approaches. Long-term observations on tumor recurrence, tissue repair, and long-term immunization will contribute to greater acceptance of electroporation in medical practice.

6. CONCLUSION

Electroporation is a multidisciplinary platform technology with multiple medical applications. Successful application of electroporation technologies requires close collaboration between physicians, life and computer scientists, and engineers. Current knowledge of electroporation suggests that it has vast potential for a number of important global medical challenges, including cancer treatment, infection disease treatment, and vaccination. Although a full understanding of the fundamental mechanisms at the cellular and tissue levels has yet to be developed, we will undoubtedly see an increase in medical applications of electroporation over the coming years.

DISCLOSURE STATEMENT

D.M. holds patents on electrochemotherapy that have been licensed to IGEA S.p.A. D.M. also consults for IGEA.

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