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Review

Electroporation in Clinical Applications—The Potential of Gene Electrotransfer and Electrochemotherapy

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Abstract: Electroporation (EP) allows for the transport of molecules into the cytoplasm with significant effectiveness by forming transient pores in the cell membrane using electric pulses. This can be used for cellular transport (RE—reversible electroporation) or ablation (IRE—irreversible electroporation). The first of described options fortifies medicine with novel possibilities: electrochemotherapy (ECT), which creates promising perspectives for cancer treatment, and gene electrotransfer (GET), a powerful method of DNA delivery as well as immunogen electrotransfer. The review constitutes a comprehensive explanation of the mechanism of EP in the case of GET, its present and prospective employment in medicine, including gene delivery, vaccinations, therapy, and transfection, are also presented.

Keywords: electroporation; gene delivery; electrovaccines; immunogen electrotransfer

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1. Introduction

Crossing the lipid cell membrane, a physical barrier separating the intracellular from the extracellular environment, is challenging for laboratory and clinical practice. However, the supply of specific molecules and medicaments to particular cells is fundamental for most treatment pathways [1,2]. For this reason, medicine is trying to design drugs to allow their active ingredients to connect with the plasma membrane or cross it, thereby influencing particular signaling pathways. The selectivity of the barrier challenges healthcare, especially regarding effective gene delivery. Plenty of nonviral techniques, although safer than the vectors, in past years have resulted in unsatisfactorily low levels of gene delivery and expression in contradistinction to their viral equivalents [3]. The development of EP shed light on a described problem, serving us with a novel, highly effective strategy for transporting molecules into living cells [4].

Electroporation induces transient pore formation in the cell membrane using high-voltage, short-duration electric pulses, which allow the molecules of drugs or nucleic acids to enter the intracellular environment [5]. The described method is characterized by a wide range of advantages, within which we are able to enumerate its universality, simplicity, and efficacy. For instance, when it comes to introducing DNA to the cell cytoplasm, the employment of EP guarantees a lower rate of its mutation as the molecule is not exposed to an endosomal and lysosomal acid environment, which would affect it in any other method [6–9].

Trying to understand the mechanism that underlays the phenomenon of electroporation and discovering its difficulty is inevitable. As the transformations of the plasma

membrane structure during EP are not macroscopically substantial, the mechanism of the described process might seem unclear. Transmembrane voltage changes from physiological (which amounts to approximately 0.1 V) to 0.5–1 V, which leads to the formation of indentation and then transient hydrophobic pores, the diameter of which ranges from 2 to several nanometers [10,11]. As the transmembrane voltage increases, maintaining hydrophobic pores is energetically more expensive than maintaining hydrophilic pores, which results in the fact that some hydrophobic pores are transmutable into hydrophilic ones. A molecular mechanism that underlays mentioned transition includes reorienting the polar headgroups of lipids towards water molecules [12,13] (Figure 1). The use of a transmembrane electric field in the range of 0.5 V/nm to 1.0 V/nm induces EP of the lipid bilayer, manifested by the formation of water channels. While at 0.5 V/nm the first water fingers develop within a nanosecond, the same process is much faster (200 ps) for a 1.0 V/nm run. The formation of water fingers penetrates the bilayer hydrophobic core from either side when the bilayer is subject to a transverse electric field. As long as the electric field is present, water fingers expand toward the opposite interface or join other water fingers to form a mature pore that extends from one interface to the other of the bilayer hydrophobic core. This causes the reorganization of lipids. Polar lipid head groups migrate from the membrane–water interface to the interior of the bilayer, forming hydrophilic pores that surround and stabilize the water columns [14,15]. Ensuring change is the foundation of sustaining the transport after the voltage reverts to its physiological value, as novel pores have extended half-life value and can be stabilized by connection to the cytoskeleton [16–21]. The lifetime of pores is very short; therefore, reclosing these pores seems arbitrary and can be prolonged while the cells are kept at 0 °C [6]. Not only does electrical stimulus induce the formation of pores, but also it influences single membrane lipids properties. Induced chemical variations, such as peroxidation, deform lipid tails (Figure 1) and therefore increase the permeability of the plasma membrane, which reinforces the previously described effect of EP [14,22–24]. Furthermore, electrical modulation also impacts the function of membrane proteins, like voltage-gated ion channels (VGICs). These transmembrane proteins respond to changes in transmembrane voltage with rearrangement of their conformation, which causes either opening or closure of the pore. As VGICs are sensitive to changes in transmembrane voltage, pulses induced by EP that range up to several hundreds of millivolts perturb their function [25].

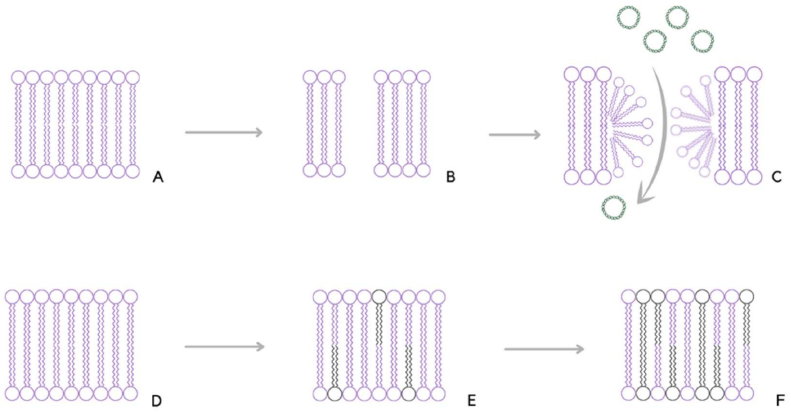


Figure 1. Schematic visualization of molecular mechanism in which plasma membrane permeability is changed due to electric stimulus, allowing genes to be transferred to the intracellular environment. A change of the physiological voltage (**A**) induces the formation of indentation and then transient hydrophobic pores, the diameter of which ranges from 2 to several nanometers (**B**). Hydrophobic pores are transmutable to hydrophilic ones by reorienting polar headgroups of lipids towards water molecules. In this way, plasmid DNA can enter the intracellular environment (**C**). The electric stimulus also influences single membrane lipids properties by inducing chemical variations, such as peroxidation, deforming lipid tails, and therefore increasing the permeability of the plasma membrane (**D**–**F**) [10,12,25].

Electroporation finds its application not only in cellular transport. In contradistinction to RE, in which the eventual cell's destruction comes from its incapacity to restore the previous plasma membrane structure, IRE intentionally leads to cell death and is used, for example, in tumor ablation [9,26–29]. Understanding the mechanism that underlays the phenomenon of EP allows the comprehension of the significance of its employment in gene delivery, therapy, and transfection. The described biotechnological process has changed the world of medicine inter alia by initiating electrochemotherapy (ECT) and gene electrotransfer (GET), the application of which has become more and more extensive and constitutes a promising perspective for future therapies. When it comes to the first term, ECT describes the supply of medicine to the cell's cytoplasm by using electric impulses. This method allows for reducing the concentration of administered cytostatics because an additional way of its transport is created [30–34]. On the other hand, GET is a method that utilizes electric pulses to increase permeation through plasma membranes; as a result, some molecules can be placed inside a cell's cytoplasm. The spectrum of particles that can be used is remarkably broad—for instance—plasmid DNA (pDNA), RNA, inhibitors, antibodies, or antitumor drugs [35]. Considering only plasmid DNA molecules—there are a lot of possible modifications because pDNA molecules might be liberally recombined and, for this reason, may contain various therapeutic proteins or antigens [36]. Due to the comprehensive application of EP, this method is believed to develop some areas of medical treatment, such as DNA vaccines or cancer medication [37,38]. The juxtaposition of GET and ECT allows us to find significant differences between those two terms. While GET designates the combination of gene transfer and EP, ECT is the name of the procedure when EP is combined with the injection of cytotoxic drugs, therefore enhancing their diffusion. All the practical applications of the wide potential of EP will be discussed.

Electroporation has shown some significant assets in the case of gene delivery. Indeed, it evinces some superiority in comparison to other gene-delivery methods. Primarily, an electric field used in GET is only minimally aggressive, as this method utilizes a small amount of energy—250–500 V/cm for 1 ms, depending on the properties of the system of electrodes, such as the distance between electrodes and the applied voltage—to induce the permeation of specific particles into cells. It should be emphasized that the same electric field level finds its application in ECT. In contrast, an automated external defibrillator (AED) pulses 90 times more considerable energy, and there are no side effects [39]. EP is regarded as an attractive alternative to viral-mediated gene delivery because it does not interfere with the host genome [40–44]. There was also observed that viral vectors are more likely to induce toxicity and immunogenicity [45].

2. Molecular Mechanism of Gene Electrotransfer

The pores that are shaped after using a transmembrane electric field are large enough to serve as channels for ions and small molecules. This process occurs within a few nanoseconds and is more rapid for the highest field applied.

Once the electric field is turned off, pore annihilation begins, and phospholipid head groups move out of the bilayer interior. The head groups separate again into two distinct layers, rapidly removing water. The relocalization of lipid molecules is independent of their initial location and achieved within a few nanoseconds [14].

Gene electrotransfer (GET) is a multistep process and an efficient method for introducing genes into cells in vitro and in vivo. GET involves the following steps: (1) the electropermeabilization of the plasma membrane, (2) the electrophoretic migration of the DNA towards the membrane, (3) DNA/membrane interaction, (4) DNA translocation across the membrane, (5) the intracellular migration of DNA through dense cell cytoplasm, (6) DNA passage through the nuclear envelope, and (7) gene expression (Figure 2). It occurs successfully when both electropermeabilization and viability are optimal, depending on the electrical field strength, pulse number, and duration. The plasma membrane is impervious to hydrophilic molecules, such as nucleic acids. DNA molecules cannot access the cytoplasm because negatively charged plasmid molecules cannot interact with

the cell plasma membrane that bears the same charge [46]. The field strength has been observed to play a fundamental role. GET is only detected for electric field values leading to membrane permeabilization (Ec > Ep). Plasmid molecules, negatively charged, migrate when submitted to an electric field. In the case of a "low" electric field (i.e., Ec < Ep), the plasmid simply electrophoretically flows along the cell membrane towards the anode [47]. Long pulses (1 ms to 5 ms) and a combination of high- and low-voltage pulses have been suggested in vitro. It has been demonstrated that short HV pulses are crucial for the efficient permeabilization of the cell membrane, enabling transfection, and are sufficient to successfully deliver DNA into cells at optimal plasmid concentrations [48]. Therefore, it is generally difficult to separate the role of HV pulses as being only permeabilizing and LV pulses as being electrophoretic since both effects can be present during HV pulses. Whereas for in vivo applications, where suboptimal plasmid concentration is the limiting factor for efficient transfection, cell viability is better preserved with shorter duration pulses (od 100 μs do 500 μs) [48–51].

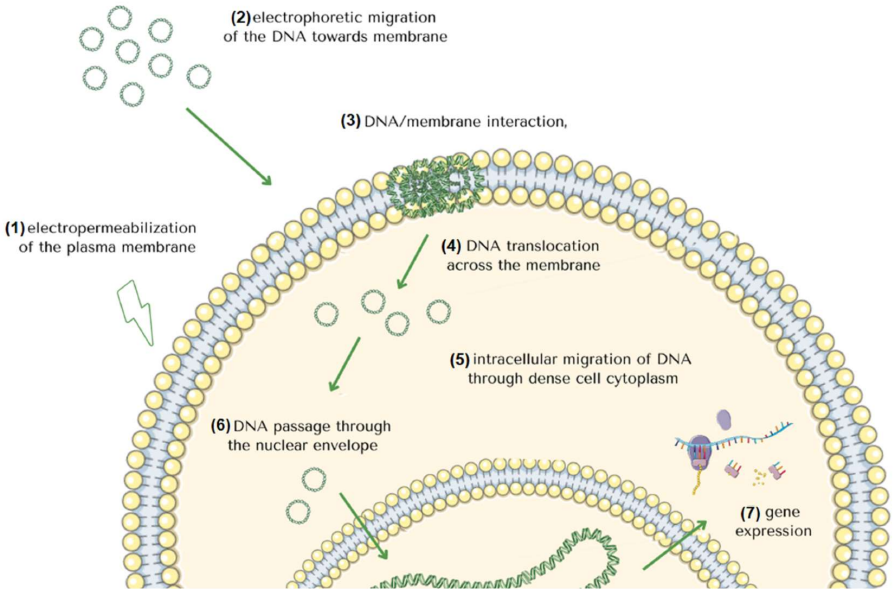


Figure 2. The chain of events that take place while inserting plasmid DNA into the cell to provoke gene expression. Once the plasma membrane is subjected to electropermeabilization, DNA electrophoretically migrates toward the membrane and interacts with it. DNA is translocated to the cytoplasm and migrates to the nucleus, leading to the expression of particular genes [12,14,19]. The Figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license.

During the application of the electric field, the plasma membrane is permeabilized. DNA is electrophoretically pushed onto the cell membrane side facing the cathode, which results in DNA–membrane interactions. The involvement of the electrophoretic component leads to an asymmetrical interaction of the DNA with the membrane, which remains together for about ten minutes. After the application of the electric field and resealing of the membrane, DNA is mainly internalized by endocytosis and caveolin/raft-mediated endocytosis. The insertion of DNA into the membrane and the relatively large size of DNA aggregates may exert a sufficiently large curvature on the membrane to be recognized by the endocytic machinery, which would then puncture the membrane and, eventually, the vesicle. DNA uptake by several pathways would likely be based on aggregate size [46]. DNA is internalized by endocytosis with the following approximate contributions: 50% caveolin/raft-mediated endocytosis (caveolin/raft-ME), 25% macropinocytosis, and 25% clathrin-mediated endocytosis (clathrin-ME) [52].

The hypothesis that DNA enters the cell via electroendocytosis has already been investigated [46]. Cells were stained with membrane dye FM 1-43FX, used for observation of endocytosis, and then exposed to electric pulses. The authors aimed to analyze whether endocytosis was stimulated by applying electrical pulses below and above the threshold of GET. There was no increase in endocytosis from 20 min and even up to 2 h after the impulse, regardless of the electric field strength [53]. Concurrently, it confirms the thesis that electroendocytosis is not the dominant mechanism of GET using short high-voltage pulses, but endocytosis has recently been proven to be a valuable alternative model of DNA translocation, given the increase in experimental evidence for its implications [52,53].

If DNA is internalized by other means than endocytosis, actin participation may take the shape of bursts of polymerization. While being actively transported in the cytoplasm, DNA aggregates pass through the different endosomal compartments. Free DNA must interact with some adapter protein to be transported by motor proteins. For gene expression to occur, DNA must escape from endosomal compartments. Once in the perinuclear region, DNA must cross the nuclear envelope to be finally expressed and yield proteins released into the cytoplasm [46]. In one of the studies, M. Tarek (2005) used for the DNA/lipid system simulation a realistic DNA strand 12-basepair 59-cgcgaattcgcg-39 ecor1 DNA duplex treated by a 1.0 V/nm transverse electric field during 2 ns [12]. Consequently, the DNA duplex diffused toward the interior of the membrane through pores formed in the bilayer. As a result of DNA migration through the water pores, it comes into contact with lipid headgroups and lines the pore boundaries, giving rise to a stable DNA/membrane complex. Additionally, laterally displaced DNA was also considered, resulting from a lack of water wires just beneath the DNA. In this case, the translocation of the plasmid was not observed, which suggests that the local EP of the bilayer is a requisite to the transmembrane transfer of species.

3. EP in Immunogen Electrotransfer

Electrochemotherapy (ECT) represents one of the possible utilizations of EP and applies EP to deliver cytotoxic drugs, such as bleomycin and cisplatin into the cell [54]. In situ vaccination elicits an immune response against tumor-associated antigens (TAA), which are released from the tumor cells by ECT [55,56]. EP also induces inflammation through cytokine release, which results in immune cell recruitment [57]. Another technique based on EP is gene electrotransfer (GET), which constitutes a powerful method of DNA delivery that offers numerous medical applications. For example, it allows plasmids with encoded genes to be transferred into the tumor cells [56]. GET can also transmit immunomodulatory genes into the target tumor tissue, which boosts the antitumor effect [58]. Systemic administration of cytokines is associated with high toxicity, which might even lead to death, whereas GET allows the accumulation immunomodulatory cytokines in the vicinity of the tumor without systemic severe adverse effects [54,59,60].

The specific combination of ECT and immunogen electrotransfer leads to significant changes in the tumor microenvironment. The electrochemotherapy and the IL-12 production create a proinflammatory environment. Because of that, immune cells embark on infiltrating the tumor tissue, and the tumor cells begin to die by immunogenic death, which contributes to the release of TAA and danger-associated molecular pattern molecules (DAMP). Dendritic cells uptake TAA and relocate to the tertiary lymphoid structures, where they can induce the formation of tumor-specific effectors and memory cells. T cells then migrate to both the primary tumor site and metastases, where they can initiate regression of the tumor (Figure 3). It was noticed that Treg lymphocytes, which are present in the tumor microenvironment, have a massive impact on the immune response. They make this environment immunosuppressive, and it is crucial to inhibit their activity to increase the treatment's effectiveness [58]. The effectiveness of in situ vaccination may differ depending on the size and immunogenicity of the tumor. Electrochemotherapy is more effective in high-immunogenic tumors but immunomodulatory gene electrotransfer significantly im-

pacts low-immunogenic types [61]. GET increases a tumor's immunogenicity by activating immune cells, which may elicit a systematic response [62].

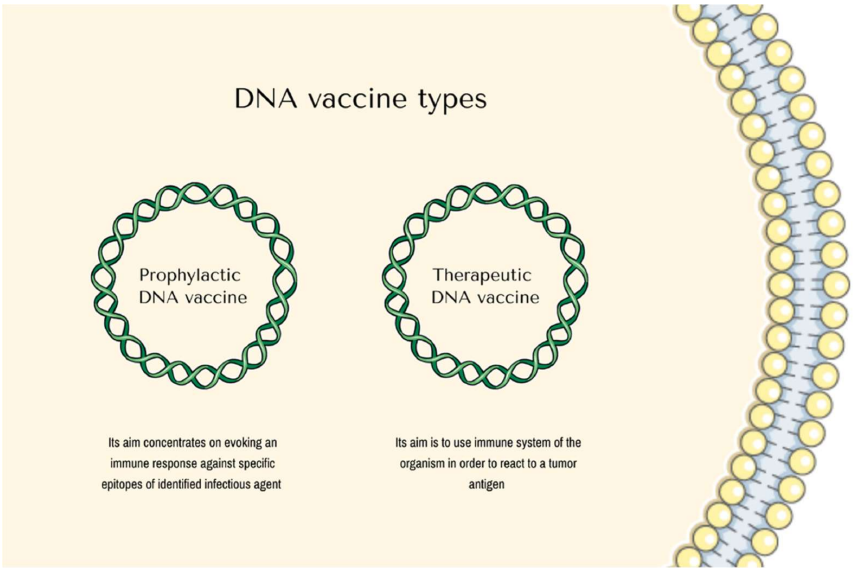


Figure 3. Schematic visualization of the chain of events that arises while deploying intratumoral ECT and peritumoral immunogen electrotransfer [63]. The Figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license.

4. Preclinical Studies

A study by Jaroszeski et al. revealed that electroporation augments the cytotoxicity of some anti-cancer drugs by increased uptake due to plasma membrane permeabilization. The experiment was conducted on carcinoma cells using chemotherapeutic agents—bleomycin, carboplatin, and cisplatin. The results are very auspicious because the cytotoxic effect of some drugs was remarkably heightened. For example, it was shown that the IC50 of bleomycin is significantly diminished (100–5000 times) when exposed to electric pulses. Cisplatin also showed immense progress—while transfected with electrical pulses, it presented improved cytotoxicity, and its IC50 was decreased in six of seven used cell lines. Carboplatin might also be considered as a choice due to its enhanced outcome in four of five examined carcinoma cell types [64]. Likewise, the study conducted by Cemazar et al. showed superior cytotoxicity against human microvascular endothelial cells (HMEC-1) while cells were being exposed to electric pulses in comparison without them. A utilization of electroporation resulted in a 5000-fold curtailed IC50 value of bleomycin and a 10-fold diminished IC50 value of cisplatin [65].

Furthermore, Fiorentzis et al. and Girelli et al. found the analog effect. The first research on uveal melanoma cells revealed elevated chemotherapeutic drugs' cytotoxicity and simultaneously highly diminished cancer cell viability. Additionally, it was proven that electroporation alone is capable of reducing melanoma cells' feasibility of survival by 25–29% [65]. The latter showed that the usage of electric fields on two different lines of pancreatic cancer cells—PANC1 and MiaPaCa2—resolved their heightened sensitivity for desired drugs along with decreased IC50 of them and lower cell's viability as well [66]. Another engaging study advocating the use of electrochemotherapy was performed by Scuderi et al. The research group tried to determine the effect of cisplatin's action on mouse skin cell melanoma (B16-F1) with and without an electric field. They proved that with the force used, cisplatin's effect on cells was stronger, even though the used therapeutic dosage was small. Furthermore, the higher the concentration of the drug was, the less neoplasm's cells survived, whereas the same increase of chemotherapeutic dose without the electric field barely changed the survival ratio. Additionally, they found that a few different types

of electric pulses (like short bipolar (HF-EP) or monopolar pulses) are able to effectively increase cell membrane permeability and decrease cell's survival as well [67].

Numerous in vitro and in vivo studies also successfully demonstrated the efficiency of immunogen electrotransfer. The in vitro research concerning GET of plasmid encoding IL-12 in combination with IL-2 in the B16.F10 murine melanoma cell line substantiated its feasibility. The EP led to lowered survival of the melanoma cells, whereas the cytotoxicity of the IL-2 and IL-12 plasmids was comparable to control plasmids, by using it can be stated that the presence of external DNA mainly induces the cytotoxic effect. Moreover, IL-12 and IL-2 mRNA and proteins were ascertained only in cells subjected to GET of these plasmids. The level of transcripts and proteins was more elevated after using EP1 protocol with longer and lower voltage pulses. These pulses are more noxious to the cells and augment the uptake of the plasmids. Due to the high cytotoxicity of EP1, it may be more efficient in tumor elimination than the EP2 protocol, based on short and high voltage pulses commonly used in ECT. However, the cytotoxicity and expression efficacy estimated in vitro may differ from the in vivo results [68].

Although the outcomes of both in vitro and in vivo research concerning immunogen electrotransfer were promising, many of them were performed with the use of plasmids containing an antibiotic resistance gene, which decreases the safety profile of GET therapies because of its risk of spreading and providing bacteria with the resistance to antibiotics used in clinical practice. Moreover, antibiotic resistance genes increase the plasmid size, which diminishes the effectiveness of plasmid expression due to their decreased capability of reaching the cell nucleus [69]. Considering the above, the most challenging aspect of the latest studies is to design a plasmid devoid of the antibiotic resistance gene. The first evaluations in vitro of antibiotic resistance gene-free plasmid encoding human IL-12 were already performed, and the results encourage further in vivo testing and human clinical trials. It was proven that GET of these plasmids outcomes with the production of biologically active IL-12 in the human squamous carcinoma cell line FaDu and the murine colon carcinoma cell line CT26, and its transcription level showed a time-dependent diminution. Furthermore, combining it with another stimulus, such as radiation, could increase the IL-12 expression [70].

Another research also resulted in the efficient expression of IL-12 after transfection. Additionally, the survival of B16-F10 and the human malignant melanoma cell line SK-MEL-28 was substantially decreased after using antibiotic resistance gene-free plasmids compared to commercial ones. Nonetheless, the plasmid production ought to be optimized before in vivo transfection to progress the quality of the plasmid by reducing the number of dimers and other destabilizing topoisomerase emerging due to the appliance of the recombinant strains of bacteria required in this process [4].

The additional determining aspect of IL-12 plasmid production is to opt for the appropriate promoter controlling IL-12 expression. Studies have shown that p21 is an efficient promoter used in these plasmids. The p21 is the promoter of the cyclin-dependent kinase inhibitor 1 (CDKN1a) gene. It determines the low expression in normal cells and high expression in tumor cells. Furthermore, it is stress-induced, which implies that the expression can be enlarged by either radiotherapy or chemotherapy [70,71]. It allows combining the IL-12 GET with other therapies, which may elicit the ameliorated response of the tumor cells. IL-12 GET combined with radiation was proven to induce a significant antitumor effect with the tumor growth delay, although it was similar to the therapy with a constitutive promoter. Regardless, the p21 is a prospective promoter in cancer treatment due to its endogenous provenance because, as a result, it is not susceptible to transcriptional inactivation [72].

5. Clinical Studies

Gene electrotransfer can achieve long-lasting gene expression and may be used in different tissue types in various species. Reconstructive surgery widely uses skin flaps to repair large and deep skin injuries. The significant difficulty in this type of treatment is the

formation of ischemia and subsequent necrosis of the transplanted tissue. To prevent this, exogenous vascular endothelial growth factor (VEGF) can be delivered to insufficiently ischemic areas. An increase in VEGF level causes the upregulation of endothelial nitric oxide synthase, increasing NO concentration, which signals smooth muscles to relax, leading to vasodilation, increasing blood flow, and better perfusion. Regrettably, the delivery of VEGF presents some difficulty. However, the research has shown that EP is suitable for delivering plasmid DNA encoding VEGF to ischemic skin and rat skin flap. Compared to injection alone, electroporation resulted in a 6.6-fold increase in VEGF protein on day 2 after treatment in the distal area of skin flaps [73]. Using such gene therapy presents better results while applying EP, either in skin flap healing and survival or ischemic wound treatment. A major challenge for medicine is the treatment of diabetic skin wounds. It is patients with diabetes who particularly experience nonhealing skin lesions and injuries. LL-37 peptide promotes wound healing and inhibits bacterial growth. Unfortunately, there are difficulties in delivering it into the tissues. However, the delivery of plasmid-encoding hCAP-18/LL-37 by EP has turned out to be effective and resulted in the increased expression of LL-37 in the epithelium. The electroporation of phCAP-18/LL-37 restored delayed wound healing in diabetic mice. Wound closure occurred in 61 ± 8% of animals treated with LL-37 delivered through EP, whereas in the control group, the wound was closed in 43 ± 9% of individuals [74]. These results clearly indicate that the application of treatment with EP was more successful (Figure 4).

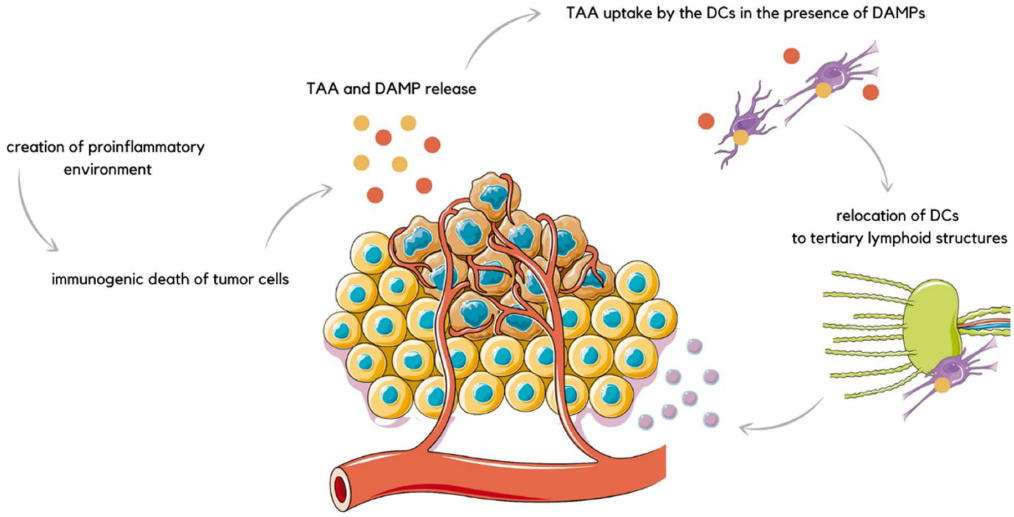


Figure 4. Schematic visualization of the role that EP plays in wound healing, allowing plasmid DNA to enter the cell via GET and therefore inducing an increase in either LL37 or VEGF level [75]. The Figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license.

Gene therapy is a promising strategy for treating heart disease. Unfortunately, most current strategies in gene delivery to the heart are ineffective. Nevertheless, EP is a method that significantly increases the potential for the gene transfer and induction of gene expression at a clinically relevant level in this organ [75]. In addition, while there have been doubts about whether using electrical pulses near the heart is dangerous, studies have shown that EP can be a safe and, at the same time, effective method for delivering genes to a working heart [76]. Other research shows that gene delivery to the cardiac muscle tissue by plasmid injections only results in lower gene expression than the electroporated tissue [77].

A fair number of gene delivery methods into the lungs have been developed; however, most have severe limitations. EP exceeds the major problem since it shows a low inflammatory response in lung tissue. In the study, purified plasmid DNA was introduced into the lungs and subjected to EP in mice bodies. The expression of the injected gene was

detected as early as day one after the procedure, mainly in alveolar epithelial cells type I and II. In contrast, no clinically significant expression was detected with DNA injection without EP [78]. Another electroporated gene, hepatocyte growth factor (HGF), is able to affect alveolar type II epithelial cells and may improve the recovery process of the lung tissue. The study of bleomycin-induced lung fibrosis consisted of plasmid encoding HGF injection and EP in rats. The expression of HGF after the procedure reached the appropriate level, and pulmonary fibrosis was reduced, confirming that EP could also be used to repair alveolar wounds and stop the process of pulmonary fibrosis [79].

Regarding immunogen electrotransfer, the most extensively used cytokine gene in GET is IL-12 [80]. Interleukin 12 is a proinflammatory agent, which mainly enhances cytotoxicity of various immune cells, such as NK cells and T lymphocytes. It evokes an IFNγ production and increases the expression of MHC molecules [62]. IL-12 boosts the proliferation of CD8+ cells and reduces PD1 expression [81,82]. Furthermore, the IL-12 enhances its secretion by dendritic cells, which creates a positive feedback loop [83]. Preclinical studies on IL-12 GET using rodents have been very successful, as 47% of mice with the complete regression of murine malignant melanoma B16.F10 was achieved after IL-12-plasmid intertumoral injection followed by EP. Additionally, five of seven mice remained healthy for 100 days after being challenged with tumor cells [60]. Another research showed that 80% of mice were cured for over 100 days. Furthermore, only 37.5% of mice developed lung nodules, whereas in a control group, these nodules appeared in 87.5%, which shows that the following treatment may prevent metastases [84]. The high effectiveness of IL-12 GET treatment on murine SA-1 fibrosarcoma was also proven, while 90% complete response rate was obtained after using an intertumoral injection and 60% of cured animals remained resistant to tumor cells [85].

IL-12 GET may be combined with other methods, such as an electrotransfer of IL-12 plasmid with IL-2 plasmid. Interleukin 2 is responsible for the proliferation and differentiation of T lymphocytes, such as CD8+ cells. It also constitutes a strong mitogen and may prevent the activation of Treg cells. IL-2 is used in the treatment of renal carcinoma and metastatic melanoma, but in GET therapy, the dosage of this cytokine is much lower, which allows us to avoid the toxic systemic effect. The usage of the described combination in research resulted in an increased concentration of dendric cells and macrophages in the tumor tissue and the development of anti-tumor immune memory. A total of 71% of treated mice obtained the complete regression of the B16.F10 melanoma and 80% rejected tumor cells after secondary challenge [68]. IL-12 can also function as an adjuvant when it is combined with electrochemotherapy because it enhances the antitumor effect of cytotoxic drugs [54,61,86]. Electrochemotherapy can induce only a local effect, whereas immunotherapy can contribute to the cure of metastases. IL-12 GET used as an adjuvant is very efficient. It intensifies the response rate and may induce a systemic effect [86]. Interleukin 12 was also used in conjunction with tumor necrosis factor-α (TNFα), a cytokine with direct cytotoxic activity. IL-12 and TNFα GET resulted in 79% of the complete responses of the tumor, which was confirmed by the vast accumulation of immune cells in the tumor [87].

The IL-12 GET was also tested in treating the canine transmissible venereal tumor (CTVT) in beagle dogs. This treatment resulted in complete tumor regression and elicited a systemic response, which led to a cure of distant tumors and prevented the appearance of a new tumor [88]. In conjunction with electrochemotherapy, it resulted in high efficacy in the treatment of mast cell tumors in dogs. The complete response rate was 72% and no side effects were noticed [89]. The phase I trial of IL-12 plasmid in vivo EP was performed on patients with metastatic melanoma. This study resulted in tumor necrosis in 76% of lesions. Treated tumors showed the infiltration of lymphocytes. Notably, 10% of patients obtained the complete regression of all lesions and another 42% demonstrated a partial response. The major side effect was temporary pain after applying electric pulses [90].

Interleukin 12 is not the only mediator used in GET. Another critical cytokine is IL-15, which regulates the activity of immune cells. The plasmid with encoded IL-15 gene has antitumor potential proven [91]. Interestingly, the combination of delivery of IL-15 gene

and its α receptor gene demonstrated a better potential than the monotherapy. The IL-15/IL-15Rα complex stimulates cytotoxic cells. In trials with IL-15 superagonist, the level of T cytotoxic cells was higher [92]. Other agents that were examined were chemokines RANTES/CCL5 and TARC/CCL17. These chemokines are proinflammatory and they are connected with the infiltration of immune cells into the tumor tissue. Although the GET of these chemokines did not show an antitumor effect, a minor prolongation of mice survival was obtained [93]. Another newly described cytokine is IL-28. It belongs to the type III interferons group, consisting of IL-28 and IL-29. The IL-28 GET inhibited murine melanoma growth for 15 days. These results encourage further research in this field [94].

Summarizing this research, it appears that the IL-12 cytokine has the significant antitumor effect, which can be boosted when IL-12 GET is combined with other techniques (Figure 5). As for other cytokines, such as IL-2, IL-15, or IL-28, further research should be conducted to assess their treatment potential in GET therapy. Considering all this research, EP could be regarded as the most advanced and effective physical, non-viral gene delivery method. Weak immune response, low-grade inflammation, and a high level of gene transfer and gene expression compared to other gene transfer methods, even those with viral vectors, are the most significant advantages of electroporation. Over the past few decades, the possibilities of EP in clinical practice have increased significantly. Today, EP can be applied to a wide range of tissues and different organs. Scientists still face various difficulties associated with EP in gene delivery; however, the results of human clinical trials and laboratory studies are auspicious.

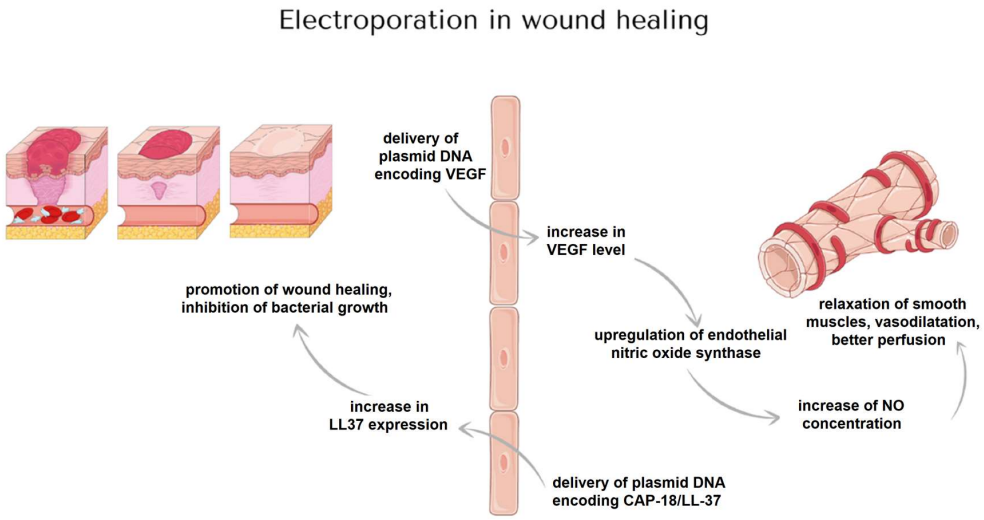


Figure 5. Schematic visualization of combinations used in immunogen electrotransfer, considering Il-12 and its combinations with other techniques, as well as Il-15 and Il-28 [78,84,94].

Electrochemotherapy (ECT) should be considered as an innovative way of treatment, which is seen as one strategy for overcoming multi-drug resistance (MDR), which is a considerable difficulty in cancer treatment. Metanalysis from 2022 reports very encouraging results, 53.5% complete and 77% overall response rates on a per lesion basis in the treatment of malignant skin melanoma (MM) [95]. The results of another study of ECT therapy against skin MM demonstrate clinical effectiveness. A partial response in 49% of patients and a complete response in 23% were achieved [96]. In addition, there are more and more promising research results regarding combining ECT with immunotherapy. It is thought that there may be an immunostimulant effect and even a synergistic relationship between ECT and immunotherapy [97]. This should prompt further research on the subject.

6. EP in Electrovaccines

Vaccinations constitute the tool by the use of which humanity fights infectious diseases. Even though the general concept of vaccination has not changed since 1796, the molecular

mechanism of this kind of immunization is still being improved to become safer and more effective over time. Today, medicine offers us discoveries known as DNA vaccines which, applied cutaneously or intramuscularly, are very effective, provided that an electric field is applied to the tissue subsequent to the place of DNA injection [3,34]. Otherwise, when the electric stimulus is not present, the utility of the described process is restricted by poor transduction efficiency. The uptake of DNA vaccines in both skin and muscle augments significantly through electroporation and so does its immunogenicity. Among the advantages of DNA vaccines combined with EP, safety must be pointed out, as no viral components are needed to create an immunological response. Those gene-based expression plasmids which encode the specific epitopes of the antigens mimic the immunological effects of infection or break tolerance against specific self-antigen when it comes to cancer [98,99]. What is more, the process of manufacturing them is relatively uncomplicated and inexpensive and therefore has the potential to be diffused around the world. Furthermore, the reversibility of that kind of GET has been proven, as the expression of a transfected gene can be switched off by re-electroporating the tissue and simultaneously administering an injection of calcium in the vicinity of re-electroporation localization [100].

GET is characterized by many possible deployments and can be delivered to varied destinations in the human body. Its derivative, the DNA vaccine idea, puts a new complexion on EP, which becomes a valuable utensil for transfecting nucleic acids that encode specific antigens. In this way, the response of an immune system can be intensified [101]. DNA vaccines in present clinical trials are mainly applied intramuscularly. Muscles are natural factories of proteins so, they can produce a significant amount of protein after transfection with a minute quantity of DNA. The present point of those trials does not, however, let us state whether muscles really constitute the optimal organ for performing DNA vaccinations [98,102–105]. Apart from the most commonly targeted localizations, which are the muscles, skin, tumors, liver, brain, mucosa, pancreas, and kidneys, must be considered while enumerating the destinations of DNA vaccinations [106–112]. Hopefully, the following years will prove which anatomical approach is the most effective for provoking an immune response. When it comes to the model of functioning, DNA vaccines are generally divided into two categories (Figure 6):

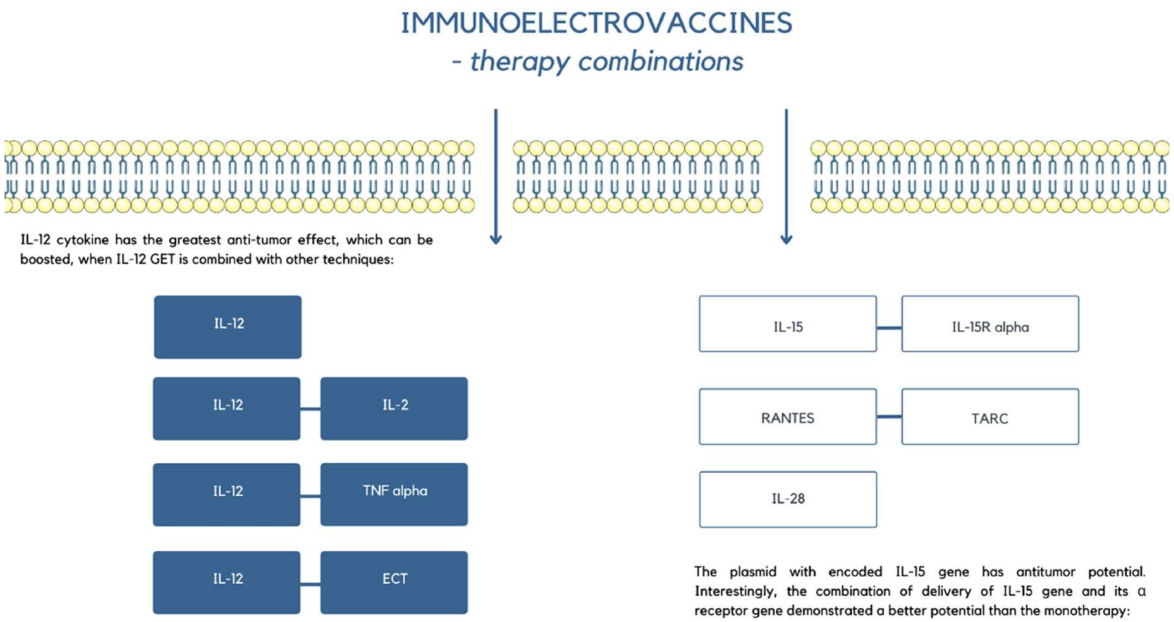


Figure 6. Types of DNA vaccines and their functions [3,110,113]. The Figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license.

The prevention and treatment of infectious diseases that are credited to the plasticity of DNA vaccine immunization open new horizons for healthcare regarding vaccinations directed against presently unmet disease targets. Preclinical data abound in examples of DNA vaccines combined with EP for malaria, HBV, Ebola, influenza, *Clostridium difficile*, and AIDS [114–121].

When it comes to GET, concerning nucleic acids in vaccination, not only is DNA utilized, but RNA can also be taken into consideration. Based on either mRNA or RNA replicons, RNA vaccines outperform DNA vaccines in delivery because they do not need to pass into the nucleus, unlike their DNA counterparts, as they perform their duties in the cytoplasm. Although the less demanding passage is a tremendous advantage of RNA vaccines, a perspective of its stability leaves much to be desired, and RNA is generally known to be much more prone to degradation than DNA [98]. Nevertheless, compared with traditional vaccines, designing mRNA vaccines requires virus gene sequences instead of virus strains; the production requires neither cell cultures nor animal matric and therefore is definitely easier. It also turns out to be more flexible; if a virus mutates, it is simpler to adjust mRNA sequence than the protein structure. Another advantage of mRNA vaccines is that mRNAs are the normal components of cells and can be degraded without causing the slightest toxicity [122]. mRNA vaccinations gained popularity during the COVID-19 pandemic. Preclinical investigation evidenced that antigen-encoding mRNA vaccines are effective, as the administration of the mRNA-encoded virus-like particles in mice did provoke an antiviral-like immune response [123,124]. Afterward, mRNA encoding SARS-CoV-2′s receptor-binding domain was capsulated in a lipid nanoparticle and injected into mice as well as nonhuman primates intramuscularly. That induced specific antibody production and Th1-biased cellular response [125]. Clinical trials proved that the vaccine causes an increase in SARS-CoV-2 neutralizing titers and is effective [126,127].

7. Future Perspectives

Given the proven feasibility and safety of electroporation-based therapies, they should be considered methods with comprehensive perspectives for use in clinical practice. ECT is now mainly applied in treating cutaneous and subcutaneous tumors, and its effectiveness with limited side effects is well demonstrated in various studies. Their prevalent use is focused on palliative treatment in patients with inoperable tumors when other methods of treatment have failed [30,33,128,129]. Prospectively, ECT may be implicit in deep-seated tumor treatment; however, further investigation is required. The treatment of internal tumors needs more effort and the development of clinical devices; however, it is beginning to develop optimistically in both ECT and IRE, which will be described below. Regarding technical requirements, the design of new electrodes may be valuable in clinical applications due to the challenging procedure of placement of ECT needles. One of the important considerations is the use of ECG during the procedure on internal tumors in the vicinity of the heart. The delivery of pulses should be synchronized with ECG [33,130,131]. The studies show no major changes in the heart rhythm and clinically irrelevant changes in the heart rate during ECT of colorectal liver metastases [132]. The entire tumor should be provided with the exact extent of EP, which can be accomplished with the support of real-time imaging. The application of suitable imaging may increase the effectiveness of ECT, because of the control of electrode adjustment and tumor coverage by the electric field [130]. Although promising results were achieved using in situ imaging methods, such as electrical impedance tomography (EIT) and magnetic resonance electrical impedance tomography (MREIT), these methods are currently too complex for EP. Interestingly, current density imaging (CDI) appeared to be a convenient method of monitoring the distribution of electric fields in electroporation-based therapies [63]. Further suggestions include the implementation of ECT in combination with other techniques, such as surgery. It could be utilized as a neoadjuvant in order to facilitate the removal of tumors challenging to reach. Another approach is based on combining ECT with other EP-based therapies, such as immunomodulatory GET, especially IL-12 GET [33,131]. Although GET studies

mentioned previously have achieved auspicious results, further investigation is necessary for clinical deployment.

Another prosperous EP-based therapy is irreversible electroporation (IRE). The use of IRE in clinical practice is developing, notably in treating liver and pancreatic cancer. Additionally, the efficacy of this method was recently clinically confirmed in the treatment of unresectable hilar cholangiocarcinoma and prostate cancer. Furthermore, IRE might be applied as a therapy for lung or brain cancer in the future [63]. Irreversible electroporation has been shown to be efficient as an ablative therapy in the treatment of locally advanced pancreatic cancer. It also may prove effective in combination with intraprocedural chemotherapy or immunotherapy, which could reinforce the treatment results [133]. Despite the encouraging outcomes, more prospective randomized controlled trials are necessary in order to establish the indications of treatment with IRE [134,135].

The novel approach based on calcium electroporation, where high calcium concentrations are inserted into the cell with the use of EP and could prospectively be applied as a new efficient anticancer treatment method. The clinical studies demonstrated the systemic immune response after calcium electroporation and have asserted the safety and effectiveness of this method, which should encourage further investigations [136,137].

The further utilization of EP is to enhance the transfer of DNA vaccination into the cell. It has been stated that EP increases the immunogenicity of DNA vaccines. The immune response after delivering DNA vaccine by EP in comparison to intramuscular injection was enhanced 100 times [99]. Moreover, DNA vaccines delivered by EP may allow for a vaccination with multiple epitopes, which makes this method potentially one of the most economical [102]. Over and above the benefits of electrovaccines mentioned before, they also abate the volume of the vaccine and the number of injections. In order to make electrovaccines widespread in the future, the further improvement of the EP is significant. The evolution should include the development of EP devices regard to make them easy to operate and portable [103]. The effectiveness could also be ameliorated through the adjustment of the DNA injection method, pulse configuration, and electrode geometry [102]. Electrovaccines may be the most effective in cancer treatment due to their capability to enhance the immune response. Simultaneously, EP-delivered DNA coding immunomodulatory genes have also demonstrated efficiency in cancer eradication. However, more prospective studies are required to confirm the effectiveness of both electrovaccines and immunogen electrotransfer in clinical conditions [138].

The turning point in the clinical appliance of electroporation-based therapies was the establishment of the standard operating procedures (SOP) for electrochemotherapy using a Cliniporator designed within the ESOPE project. It was a breakthrough that implemented the electrochemotherapy of cutaneous tumors as a standard clinical practice in Europe. As a result of the spreading and development of this treatment method, the preparation of new standard operating procedures concerning deep-seated tumors and other electroporation-based therapies is needed [33].

8. Summary

Electroporation (EP) enables the efficient delivery of DNA into cells and tissues and the enhancement of the expression of particular proteins of therapeutic or immunogenic function. Gene electrotransfer (GET) constitutes a delivery system that allows us to introduce plasmid DNA (pDNA), RNA, inhibitors, antibodies, and antitumor drugs into cells. This can be applied to vaccination and the distribution of genes encoding specific therapeutic molecules. The method seems very promising, and future research should focus on optimizing GET protocols to include them in the standard therapeutic protocols.

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