ELECTROPORATION AS A TOOL FOR BIOTECHNOLOGY AND MEDICINE WITH SPECIFIC EMPHASIS ON ITS APPLICATION FOR DRUG AND GENE DELIVERY. REVIEW

Saulius Šatkauskas, Gintautas Saulis
Vytautas Magnus University, Biology Department, Vileikos 8; LT–3035 Kaunas, Lithuania;
Tel.: +370 37 45 13 69 ; E-mail: absasa@vaidila.vdu.lt

Summary. It is known that short and sufficiently strong electric field pulses may temporary increase permeability of the cell membranes providing facilitated access of exogenous molecules into the cells and tissues. The phenomenon is known as electroporation or electropermeabilization. Because of its physical nature and easiness to use, electroporation has gained wide application in cell biology, biotechnology, human and veterinary medicine. In the first part of this review the main in vitro applications of electroporation: electrostereilization, electroladong, electrofusion, electroinsertion are presented. Recently electroporation was applied to the tumor tissues to introduce nonpermeant cytotoxic drugs (like bleomycin) into tumor cells in vivo. Such electrochemotherapy allows one to obtain very high responses of the tumor treatment with highly reduced bleomycin doses. The most recent application of in vivo electroporation is delivery of genes to various tissues. The method has been shown to be effective to electrotransfer plasmid DNA to muscles, liver, skin, tumors, mouse testis, arteries and nervous tissues. It is believed that such method of DNA electrotransfer to tissues can be applied in the gene therapy to treat various acquired and congenital diseases. Thus, in the second part of this review, current status of the electroporation for drug and gene delivery into tissues is discussed.

Keywords: electroporation, electropermeabilization, electrochemotherapy, electroganetherapy, gene therapy, gene transfer, drug delivery.

ELEKTROPORACIJOS METODO TAIKYMAS BIOTECHNOLOGIJOJE IR MEDICINOJE ATSKRIRAI APTARIANT JO PANAUDOJIMAY VAISTŲ IR GENŲ SULEIDIMUI Į LĄSTELES IR AUDINIUS. APŽVALGA


Raktąžodžiai: elektroporacija, elektropermeabilizacija, electrochemoterapija, elektrogenoterapija, genų terapija, genų įvedimas, vaistų įvedimas.

Introduction. It is know that short and sufficiently strong electric field pulses may temporary increase permeability of the cell membranes providing facilitated access of exogenous molecules into the cells and tissues (Kinosita and Tsong, 1978b; Neumann et al., 1989; Chang et al., 1992; Jaroszeski et al., 2000). If the electric pulses are chosen appropriately the membrane elektropermeabilization is fully reversible and barrier functions of the membrane can be restored (Kinosita and Tsong, 1977; Saulis, 1997). Membrane permeabilization depends on various physical-chemical parameters like ionic strength, pH, osmotic pressure, temperature, composition of the membrane, but first of all on the parameters of the electric field pulses (Neumann et al., 1989). It is believed that membrane elektropermeabilization is consistent with the formation of the pores in the lipid phase of the membrane. Therefore the phenomenon is more often termed as electroporation (Kinosita and Tsong, 1977; Kinosita and Tsong, 1978b; Abidor et al., 1979; Neumann et al., 1989).

The more detailed description of fundamentals of electroporation (electropermeabilization) is described in our previous paper of this issue. Here we will shortly review main biotechnological and biomedical applications of electroporation (electrosterilization, electroladong, electrofusion, electroinsertion) giving specific emphasis on the applications our group is involved, namely to drug and gene delivery into cells in tissues. Drug delivery into tissues has yielded to antitumor electrochemotherapy, while gene transfer into tissues is directed to be applied for gene therapy.

Electrostereilization. Because of its ability to damage irreversibly cell membranes, electroporation was first suggested to use for cell killing for sterilization purposes.
It has been shown that up to 99.99% of cells can be killed by applying enough strong electric pulses (Hulsheger, 1981). Recently Bakker Schut and coworkers developed a modified flow cytometer in which one can electroporate individual cells selected by optical analysis (Baker and Shulman, 1988). In this electroporating sorter each individual cell is optically analyzed in a quartz flow channel; if it is an unwanted cell it is killed by applying an electric field pulse at the moment the cell passes the orifice.

**Electroloading.** As electrically induced pores are relatively stable at temperatures below 15°C and can readily reseal at higher temperatures, e.g., 37°C, it has been suggested that electroporation could be used to load living cells with normally nonpermeant molecules (Kinosita and Tsong, 1978a; Zimmermann et al., 1980). The method of electroporpermabilization to load cells with various bioactive molecules (electroloading) provides a number of significant advantages: i) it is rapid, and large number of cells can be simultaneously exposed to an electric field, ii) it can be applied to virtually every cell type, iii) it is compatible with cell survival, iv) the permeabilization process is selective for the plasma membrane and does not affect the intercellular organelles, v) the plasma membrane can be leaky for small molecules while retaining its impermeability to the essential proteins/enzymes within the cell, vi) it changes the plasma membrane only to a limited extent, so that it can participate in the physiological response, and vii) the permeability changes can be fully reversible (Knight and Scrutton, 1986; Dagher et al., 1992).

As one of the most promising application of electroloading of erythrocytes with bioactive molecules (Zimmermann et al., 1980). Such electrically loaded cells could subsequently be used as carriers to target-specific drug administration with a controlled drug release in time and space (Zimmermann et al., 1980; Crawford and Chronos, 1996). It was shown that using erythrocytes as drug carrier systems, metotrexate could be directed exclusively to the liver (Zimmermann et al., 1980).

**Electrofusion.** The next biological use of electroporation was to induce cells to fuse via their plasma membranes. There exist a great variety of the applications of electrofusion including the formation of hybridomas (Glassy, 1988; Glassy, 1993), the production of monoclonal antibodies (Lo et al., 1984; Fong and Perkins, 1989), studying membrane fusion mechanisms (Sowers, 1987; Sowers, 1989; Sowers, 1993) and examining cytosolic events (Ozawa et al., 1985; Chakrabarti et al., 1989; Orlowski and Mir, 1993). In addition, electrofusion has proved to be a valuable tool in examining membrane interaction between two cells or within a single cell (Frederik et al., 1989; Sowers, 1990). Cell-tissue electrofusion represents another electrofusion area of interest (Heller and Grasso, 1990). In this process individual cells are incorporated into intact tissue (Grasso et al., 1989). Cell-tissue electrofusion has been performed in vivo and has been shown to be useful for the interspecies transfer of membrane surface components (Heller and Grasso, 1990).

**Electroinsertion.** Cell electroporation offers an interesting possibility to exploit membrane destabilization for the electroinsertion of proteins and foreign receptors into living cells (Teissie, 1998). This can be applied for basic studies of purified receptors as well as to take advantage of electroinsertion to graft viral receptors on erythrocyte surface to lute AIDS virus (Mouneimne et al., 1989; Nicolau et al., 1990; Zeira et al., 1991; Teissie, 1998). In brief, electroinsertion consists of the application of electric fields pulses on a suspension of cells in the presence of a selected membrane protein having a membrane spanning sequence. This procedure results in the implantation of the protein in the cell's plasma membrane (Mouneimne et al., 1989; Teissie, 1998). It has been shown that insertion is taking place only in the electroporated part of the cell membrane (Raffy and Teissie, 1995). This method has been applied to the insertion of CD4, glycoporphin and Interleukin-1 receptor into a variety of red blood cells and other cultured eucaryotic cells as well as liposomes (Mouneimne et al., 1989; Mouneimne et al., 1993; Nicolau et al., 1993; Raffy and Teissie, 1995). Electroinsertion of full-length, recombinant CD4 into the red blood cell membrane has yielded an entity capable of preventing HIV-1 infection of target cells in vitro (Nicolau et al., 1990; Zeira et al., 1991).

**Transdermal drug delivery.** In the experiments carried out on preparations of viable frog skin, Powell et al. (1989) have demonstrated that electroporation could be made to occur repeatedly in a tissue, without apparent damage. Subsequent studies have shown that the electroporation of skin could be used to enhance transdermal drug delivery (Prausnitz et al., 1993; Prausnitz et al., 1994; Zewert et al., 1995; Vanbever et al., 1996; Prausnitz, 1997; Lombry et al., 2000; Denet and Preat, 2003). Prausnitz et al. (1993) examined the possibility of electroporating the skin to enhance transdermal delivery of drugs at therapeutic levels. They have observed flux increases up to 4 orders of magnitude with human skin in vitro and hairless rat skin in vivo for three polar molecules having charges between -1 and -4 and molecular weights up to slightly more than 1 kDa (calcein, Lucifer yellow, and an erythrosin derivative) (Prausnitz et al., 1993). Using this approach, transdermal transport of a highly-charged macromolecule heparin across human skin in vitro occurred at therapeutic rates, sufficient for systematic anticoagulation. In contrast, fluxes caused by low-voltage iontophoresis having the same time-averaged current were an order of magnitude lower (Prausnitz et al., 1995).

**Antitumor electrochemotherapy.** Recently the method of electroporation has been applied in vivo to introduce anticancer drugs to the tumor tissue in order to obtain therapeutic effects (Okino and Mohri, 1987; Mir et al., 1991b). This has resulted in a development of a novel antitumor treatment known as antitumor electrochemotherapy (Mir et al., 1991b). Electrochemotherapy (ECT) could be defined as combined antitumor treatment that consists of systemic or local administration of cytotoxic drug (e.g. bleomycin) followed by local delivery of electric pulses to the tumor (Mir et al., 1991b). Consequently the main factors that play crucial role in obtaining high responses of the treatment are the drug used in the treatment and the electric pulses delivered to the tumor.
Drugs for electrochemotherapy. The ‘ideal’ drug for antitumor ECT should possess at least two main properties. First, the drug should possess very high intrinsic cytotoxicity, i.e. cytotoxicity only when the drug is inside of the cells. Secondly, the drug should not cross easily the plasma membrane of the cell at physiological conditions, i.e. the highly cytotoxic drug should be nonpermeant. Additionally to that, the ‘ideal’ drug in ECT should not have any side effects under physiological conditions.

First experiments in screening several anticancer drugs in vitro revealed that bleomycin exhibit both previously described properties: it is almost nonpermeant and it is potent cytotoxic when inside of the cells (Poddevin et al., 1991). Because of these properties cytotoxic effect of bleomycin on electropereamalized cell can be increased from hundred to thousand times (Poddevin et al., 1991; Mir et al., 1996). These findings lead to widespread application of bleomycin in ECT trials in vivo as well as in clinics (Belehradek et al., 1991; Mir et al., 1991b; Salford et al., 1993; Belehradek et al., 1993; Serša et al., 1994; Heller et al., 1995; Domenge et al., 1996; Glass et al., 1996; Šatkauskas et al., 1998b; Gehl and Geertsen, 2000; Rols et al., 2000; Rodríguez-Cuevas et al., 2001). Additional advantage of the use of the bleomycin in ECT is the ability of the bleomycin to induce larger cytotoxic effect on dividing cells in respect to nondividing (Mekid et al., 2003). Therefore, it brings a possibility of safe treatment of large margins around the treated nodules (Mir, 2001; Mekid et al., 2003).

Another successful candidate as a drug for ECT is cisplatin. It seems that translocation of this drug across the cell membrane can be facilitated by electric pulses; consequently increased cytotoxic activity of the drug is obtained (Melvik et al., 1986). Therefore, cisplatin was demonstrated to be also effective in ECT experiments treating various tumors in vivo (Serša et al., 1995; Čemažar et al., 1998; Čemažar et al., 1999) as well as in clinical trials (Serša et al., 1998; Serša et al., 2000b).

Electric pulses for electrochemotherapy. The second crucial requirement for effective ECT is a proper choice of the parameters of the electric field pulses needed for tissue permeabilization as well as a proper time at which the pulses are delivered to the tumor. The proper time of the pulse delivery is consistent with the maximal accumulation of the cytotoxic drug in the tumor tissue and depends on the type of administration of the bleomycin. Usually systemic (intravenous) administration is used, however other intratumoral injections are feasible and result in high antitumor response when combined with delivery of the electric pulses (Heller et al., 1997). It was shown that highest responses are obtained when pulses are delivered 3–4 min. later the intravenous administration (Mir et al., 1991b). In case of intratumoral administration of bleomycin electric pulses are delivered 10 min. later (Heller et al., 1997).

A crucial step for effective ECT is a proper choice of the parameters of electric pulses. The main point here is to use the electric pulses at the parameters needed for permeabilization of the vast majority of the tumor cells, since even several unpermeabilized cells may give recurrences. On the other hand, the electric pulses must not be to strong or operate too long as irreversible damage of tumor and surrounding tissues may take place what is out of the scope of ECT. So far in most laboratories 8 electric pulses of 1300 V/cm strength, 100 µs duration and repeated at frequency of 1 Hz are used in antitumor ECT. Indeed, these pulses applied to the tumor several minutes after intravenous injection of bleomycin resulted in very high antitumor response, as it is demonstrated in various laboratories (Okino and Mohri, 1987; Mir et al., 1991b; Serša et al., 1994; Heller et al., 1995; Hofmann et al., 1999; Kuriyama et al., 2001; Kitamura, 2003) as well as by our group (Šatkauskas et al., 1998a; Šatkauskas et al., 1998b). It was shown that because of tumor electroporation effective bleomycin doses can be reduced more than hundred times (Mir et al., 1991b; Heller et al., 1995; Šatkauskas et al., 1998a).

In spite of very high antitumor responses of ECT using these (1300 V/cm, 100 µs) pulses some tumors show recurrences from the sites that most probably were not affected by electric pulses (Mir et al., 1991b; Serša et al., 1994; Heller et al., 1995; Šatkauskas et al., 1998b). The electric component of ECT treatment (8 electric pulses of 1300 V/cm strength, 100 µs duration) was chosen from in vitro studies, where cells are in suspension and therefore are dispersed homogeneously. In tissue, however, the situation is much more complicated. Therefore, the pulses that are permeabilizing 100 % of tumor cells in vitro may not be as effective for permeabilization of tumor cells in vivo. These considerations clearly show necessity of electric pulse optimization for in vivo conditions. However, so far there are no systemic in vivo studies on optimization of electric pulses for more effective ECT. Thus, optimization of the parameters of electric pulses is still needful. This statement is supported by our resent results, which show that longer (up to 1 ms) and stronger (up to 1500 V/cm) pulses are more effective treating Lewis lung carcinoma in mice (unpublished results).

In spite of the lack of optimization of electric pulses for effective ECT, some progress in optimization of electric conditions has been done. For example, several novel construction of needle electrodes used for permeabilization of the tumor were designed (Salford et al., 1993; Gilbert et al., 1997; Šatkauskas et al., 1998b). These electrodes in some cases were needed to reach internal tumors (Salford et al., 1993), however in some cases replaced plate electrodes in order to improve coverage of the tumor volume by the electric fields. (Gilbert et al., 1997; Šatkauskas et al., 1998b). With the same intention Serša et al., (1996) performed tumor electroporation at different orientation of the plate electrodes and obtained significant improvement of the treatment (Serša et al., 1996). Additional improvement of the design of the electrodes as well as optimization of the parameters of electric pulses for effective ECT may come from the appearance of the numerical models that calculate distribution of the electric fields inside the tissues (Gilbert et al., 1997; Miklavčič et al., 1998; Gehl et al., 1999; Miklavčič et al., 2000).

Recently a new protocol of electric pulses for the ECT was proposed by Pucihar et al. (2002) (Pucihar et al., 2002). Authors showed that the uptake of small hydrophilic molecule Lucifer Yellow by the cells in vitro is similar when permeabilizing electric pulses are
Clinical trials of electrochemotherapy. Encouraging results of ECT obtained already in the first animal studies has stimulated transition of the method from laboratory to clinics (Mir et al., 1991a; Belehradek et al., 1993). Since then the method has been investigated in clinics in France, Slovenia, United States, Japan, Denmark and Mexico (Mir et al., 1991a; Belehradek et al., 1993; Serša et al., 1997; Kubota et al., 1998; Gehl and Geertsen, 2000; Rols et al., 2000; Rodriguez-Cuevas et al., 2001). Electrochemotherapy has been applied to treat cutaneous and subcutaneous tumors of basal cell carcinoma, malignant melanoma, adenocarcinoma, head and neck carcinoma. Clinical complete responses were achieved in 56% of treated tumors (Mir et al., 1998b). Later progress of ECT resulted in an efficacy close to that of the surgery (Jaroszeski et al., 2000) and showed complete preservation of the tissues (ears, lips, neck, etc.) the tumors were localized in. (Glass et al., 1997). This clearly demonstrates efficiency of ECT and stimulates its further development in clinics. Additional improvements of the method may come from the possibilities to exploit the fact that electric pulses induce temporal vascular lock (Gehl et al., 2002), to combine ECT with immunostimulation (Mir et al., 1995; Serša et al., 1997), as well as with other classical therapeutic approaches like radiotherapy (Serša et al., 2000a; Kranjc et al., 2003).

In conclusion to this part several advantages of electrochemotherapy over other standard tumor treatment methods should be stressed. They include the very high effectiveness of electrochemotherapy and the absence of side effects (cytotoxic drugs like bleomycin and cisplatinum (drugs currently used in oncology clinics) in ECT appear to be very effective even at very reduced doses), preservation of the treated organs where the tumor is located. Moreover, since any type of tumor cell can be affected by the electroporation, electrochemotherapy is applicable to treat a wide range of tumors.

DNA electrotransfer (electrogenetherapy). The most recent and very exciting among other biomedical applications of electroporation is DNA electrotransfer to various tissues (Jaroszeski et al., 2000; Somiari et al., 2000; Mir, 2001; Scherman et al., 2002).

From historical point of view, the first in vitro electroporative gene transfer into living cells with the subsequent actual expression of the foreign gene was obtained by Neumann et al. (1982). Since then, electroporation has become popular as an effective technique for introduction of foreign DNA into cells of any origin (Potter, 1988; Neumann et al., 1989).

The easiness of the electroporation technique and its applicability to various types of cell has stimulated to initiate investigations of DNA electrotransfer into various tissues. The first report on DNA electrotransfer to newborn mice skin appeared in 1991 (Titomirov et al., 1991). From then a number of publications devoted for the in vivo gene therapy using cell and tissue electroporation has been progressively increasing (Fig.1). The break point for the development of electrogene-therapy occurred in 1998 and 1999 when several reports on DNA electrotransfer were published (Suzuki et al., 1998; Aihara and Miyazaki, 1998; Rols et al., 1998; Mir et al., 1998a; Mir et al., 1999; Mathiesen, 1999; Rizzuto et al., 1999). These and others reports demonstrated feasibility and effectiveness of electroporation to facilitate plasmid DNA transfer to various tissues in vivo including: muscle (Mir et al., 1999; Mathiesen, 1999), tumors (Nishi et al., 1996; Rols et al., 1998; Heller et al., 2000; Wells et al., 2000; Lucas et al., 2002), liver (Heller et al., 1996; Suzuki et al., 1998), skin (Titomirov et al., 1991; Zhang et al., 2002), spleen (Tupin et al., 2003), lung (Dean et al., 2003), mouse testis (Muramatsu et al., 1997), arteries (Young et al., 2003), and nervous tissues (Murphy and Messer, 2001; Haas et al., 2002; Kolley et al., 2003; Martinez and Hollenbeck, 2003).
Particular interest has been devoted to DNA electrotransfer into skeletal muscles (Mir et al., 1999; Lu et al., 2003). Muscle tissue possesses specific cellular, anatomical and physiological properties that made it particularly interesting target for gene therapy, especially for production of proteins as systemic therapeutic agents (Bettan et al., 2000; Martinenghi et al., 2002; Lu et al., 2003; Sun et al., 2003; Wang et al., 2003). Other possible targets of muscle gene therapy are vaccination and the treatment of congenital diseases such as Duchenne's muscular dystrophy (Vilquin et al., 2001; Gollins et al., 2003; Wells et al., 2003).

Muscle gene electrotransfer consists of direct injection of the plasmid DNA into the muscle followed by delivery of permeabilizing electric pulses to site of injection. This results in increase of expression level of the gene from ten to thousand times in respect to the injection of the plasmid alone (Mir et al., 1999; Mathiesen, 1999; Hartikka et al., 2001). Muscle electroporation results not only in tremendous increase of gene expression but also increase predictability of the expression and therefore can be more precisely controlled.

The mechanism of translocation of large DNA molecule through electropemabilized membrane is different from simple diffusion of small hydrophilic molecules and is not completely understood. Milestones leading to better understanding of the mechanism of DNA electrotransfer come from the facts that effectiveness DNA electrotransfer increases when longer electric pulses are used (Mir et al., 1999). As DNA is a polyanionic molecule it is believed that longer pulses may act not only on membrane inducing permeabilization, but also on DNA causing its electrophoresis (Sukharev et al., 1992). This double effect of electric pulses has been demonstrated in vivo experiments using two different types of electric pulses: HV (high voltage, short pulses), and LV (low voltage, long pulses) (Bureau et al., 2000; Šatkauskas et al., 2002). Using long pulses Zaharoff et al (2002) directly showed that DNA mobility in extracellular matrix is indeed greatly facilitated due to electrophoretic forces of the electric pulses (Zaharoff et al., 2002). Taking into account all these considerations, it was proposed that the mechanism of DNA electrotransfer in muscles in vivo is multistep process that includes: i) injection and distribution of the DNA in the tissue, ii) cells permeabilization, iii) probably, an improved DNA distribution in the permeabilized tissue, and iv) DNA transfer facilitated by DNA electrophoresis in the tissue (Šatkauskas et al., 2002).

In conclusion, the utility of electroporation for DNA electrotransfer into various tissues is already proven and therefore DNA vectorization using electroporation starts to find its place among other viral and nonviral vectors for gene therapy (Fig. 2).

Fig. 2. Number of publication in 2003 year related to various vectors for gene therapy. Data were collected on November 15th 2003 using PubMed Internet engine.

The main advantages of electroporation that includes: i) easiness and safety of the technique, ii) no necessity to manipulate with viruses or to complex DNA with other chemical agents, iii) possibility to target DNA to specific sites of the organism provide further guides for the efficient development of electroporation for gene therapy.

References


