ELECTROPORATION AS A TOOL FOR BIOTECHNOLOGY AND MEDICINE WITH SPECIFIC EMPHASIZE 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: electrosterilization, electroloading, 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, electrogenetherapy, gene therapy, gene transfer, drug delivery.

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

Santrauka. Jau trejetą dešimtmečių žinoma, kad, biologines ląsteles paveikus trumpalaikiais, tačiau stipriais elektriniais laukais, vyksta ląstelės plazminės membranos elektropermeabilizacija (elektroporacija), dėl to laikinai pakinta jos pralaidumas įvairiems cheminiams junginiams. Reiškinys vadinamas elektroporacija, arba elektropermeabilizacija. Dėl fizikinės prigimties ir paprastumo elektroporacija pradėta plačiai taikyti ląstelės biologijoje, biotechnologijoje ir medicinoje. Darbe pateikiama pagrindinių elektroporacijos *in vitro* pritaikymų – elektrosterilizacijos (electrosterilization), ląstelių apkrovimas bioaktyviosiomis medžiagomis (electroloading), elektrosuliejimo (electrofusion), elektroįvedimo (electroinsertion) – apžvalga. Neseniai elektroporacija buvo pritaikyta priešvėžinių vaistų (pvz., bleomicino) *in vivo* leidimui į navikines ląsteles. Naujas vėžio gydymo būdas – elektrochemoterapija – leidžia pasiekti stiprų gydymo atsaką net ir naudojant daug mažesnes bleomicino dozes. Visiškai neseniai elektroporacija *in vivo* buvo pritaikyta efektyviai genus suleidus į įvairius audinius (raumenis, kepenis, odą, navikus, pelių sėklides, blužnį, kraujagysles, nervinį audinį). Manoma, kad naujas genų suleidimo į audinius būdas gali būti taikomas genų terapijoje. Straipsnyje apžvelgiami naujausi *in vivo* elektroporacijos pasiekimai į audinius leidžiant vaistus ir genus.

Raktažodžiai: elektroporacija, elektropermeabilizacija, elektrochemoterapija, 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 electropermeabilization 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 electropermeabilization 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, electroloading, 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.

Electrosterelization. Because of its ability to damage irreversibly cell membranes, electroporation was first suggested to use for cell killing for sterilization purposes

(Sale and Hamilton, 1968; Hulsheger, 1981) 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 ^{o}C and can readily reseal at higher temperatures, e.g., 37 ^{o}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 electropermeabilization 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; Foung 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 lure 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, glycophorin 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 electropermeabilized 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; Rodriguez-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 chose 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 responses 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 permabilization 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

delivered either at 1 Hz or at 5 kHz. It is believed that the pulses delivered to the tumors at the frequency of 5 kHz would induce only one sensation to the patient instead of eighth sensations in case when 8 pulses are delivered at 1 Hz.

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 subcutaneus 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 rage of tumors.

DNA electrotransfer (electrogenetherapy). The most resent and very exiting 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 electrogenetherapy 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; Kolle *et al*., 2003; Martinez and Hollenbeck, 2003).

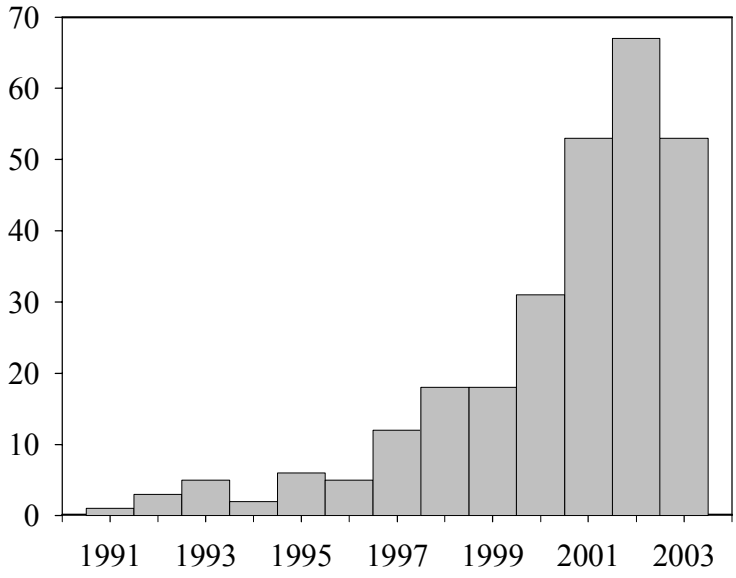

Fig. 1. **Number of publication related to gene therapy using electroporation per year. Data were collected on November 15th 2003 using PubMed Internet engine**

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 electropermeabilized 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 on *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).

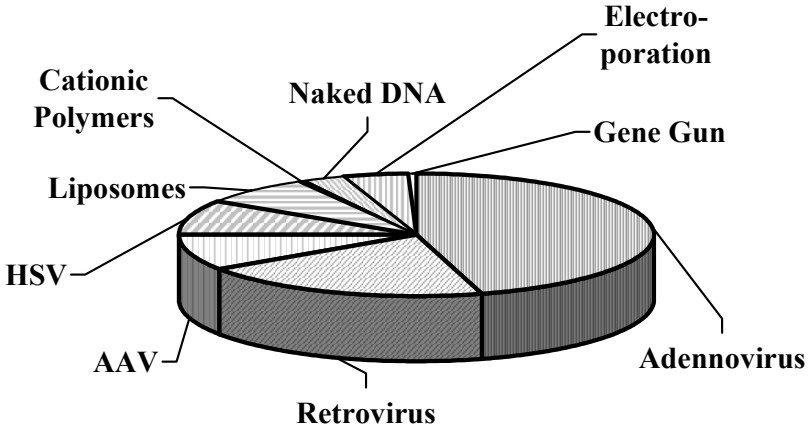

Fig. 2. **Number of publication in 2003 year related to various vectors for gene therapy. Data were collected on November 15th 2003 using PubMed Internet engine.**

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.

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