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Review

Interaction of living cells with electric pulses: Parameters and possible applications

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ABSTRACT

Remarkable improvements have been seen recently towards the applications of pulsed electric fields in permeabilizing the cell membranes. Under the normal functional activities of a living cell, the transmembrane potential is about -30 to -90 millivolts. External electric fields can induce on a living cell an extra component of transmembrane voltage. The magnitude of the added component is proportional to the strength of the external field. Both the resting and the induced component are superimposed during the incidence of the external field. The induced component will be dependent on the position, shape, and orientation of the cells with respect to the electric field. In suspensions, it also depends on the volume fraction occupied by the cells. The latent period between exposure and induction is in the order of microseconds but it can last longer if cells are in low conductivity medium. It should be also mentioned that as a consequence of this latent period, pulsed electric fields with frequencies more than 1 MHz or pulse durations shorter than 1 µs the induced transmembrane potential is inversely proportional to the frequency of the applied field and directly proportional to the pulse duration. In gigahertz range, or pulses in nanosecond range, induced voltage inside the cell become comparable or even greater than that induced on the plasma membrane. Low intensity and long duration electric pulses increase the electrophoretic lateral mobility of charged molecules in the membrane. In this review, principles and some applications of pulsed electric fields with their biological end points are discussed.

Keywords: Pulsed electric fields, testing potential, transmembrane potential, electrochemotherapy, bleomycin, electroendocytosis.

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INTRODUCTION

The biological cell is the basic structural unit of the living organism which can be considered from various aspects. Our review will be related mainly on two of them the electrical one and the geometrical one.

From the electrical point of view, the cell can be considered as an electrolyte (the cytoplasm) surrounded by an electrically insulating shell (the plasma membrane), and under physiological conditions, the surroundings of the cell are also an electrolyte. In the first approximation, one thus treats the membrane as purely dielectric, and the cytoplasm and the extracellular space as purely conductive. This approach becomes an oversimplification for rapidly time-varying electric fields, such as waves with frequencies in the gigahertz range (GHz), or electric pulses with durations in the nanosecond range (ns). To analyze the exposures of the cell to such fields, both the membrane and its surroundings have to be treated as materials with both a non-zero electric conductivity and a non-zero dielectric permittivity.

In the resting state (without external stimulation), a voltage up to –90 mV is always present on the cell membrane (Cole, 1972; Atwood and Mackay, 1989). This voltage is caused by the unbalanced ions produced from Na/K ATPase pumps and the counter transport system Na^+/H^+ (Skou, 1957; Aronson, 1985; Seifter and Aronson, 1986; Costa-Casnellie et al., 1987).

The unbalanced ions responsible for the resting transmembrane voltage represent a very small fraction of all the ions in the cytoplasm, so that the osmotic pressure difference generated by this imbalance is negligible. Also the membrane acts as a charged capacitor, with the unbalanced ions accumulating close to its surface, so that the cytoplasm can in general be viewed as electrically neutral.

From the geometrical point of view, the cell can be characterized as a geometric body (the cytoplasm) surrounded by a shell of uniform thickness (the membrane). For suspended cells, the simplest model of the cell is a sphere surrounded by a spherical shell. For augmented generality, the sphere can be replaced by a spheroid (or an ellipsoid), but in this case, the requirement of uniform thickness complicates the description of the shell substantially. Fortunately, this complication does not affect the voltage induced on the plasma membrane of such cells, which can still be determined analytically (Kotnik, 2007).

Exposure of the living cell to an external electric field

When a biological cell is exposed to an external electric field, due to the low membrane conductivity, in the vicinity of the cell the field is concentrated in the cell membrane, where it is several orders of magnitude larger than in the cytoplasm and outside the cell. These results in a so-called induced transmembrane voltage, which: only exists as long as an external electric field is present, superimposes to the resting transmembrane voltage, is position-dependent, hyperpolarizing the cell on one side and depolarizing it on the other side, and increases with both the field strength and the cell size.

For an exposure to a DC homogeneous electric field, the transmembrane potential difference induced by the electric field, $\Delta\Psi_i$ is a complex function g(λ) of the specific conductivities of the membrane (λ_m), the pulsing buffer (λ_o) and the cytoplasm (λ_i), the membrane thickness and the cell size (r). Thus,

$$\Delta\Psi_i = f\, g(\lambda)\, r\, E \cos\theta, \quad (1)$$

where *f*, is a shape factor (a cell being a spheroid), *E* is the electric field in the region where the cell is situated, *r* is the cell radius, and θ is the polar angle measured from the center of the cell with respect to the direction of the field. This formula tells that the maximum voltage is induced at the points where the electric field is perpendicular to the membrane, that is, at θ = 0° and θ=180° and these physical predictions were checked experimentally by videomicroscopy by using potential difference sensitive fluorescent probes (Gross et al., 1986; Lojewska et al., 1989; Hibino et al., 1991). When the resulting transmembrane potential difference $\Delta\Psi$ (that is, the sum between the resting value of cell membrane $\Delta\Psi_o$ and the electro-induced value $\Delta\Psi_i$) reaches locally 200 to 500 mV, that part of the membrane becomes permeable for small charged molecules (Teissié and Rols, 1993; Teissié and Tsong, 1981).

MAIN PARAMETERS CONTROLLING PULSED ELECTRIC FIELD INFLUENCE ON THE LIVING CELLS

Electric field parameters

Field Intensity

Studies of electric field effects on cells have been carried out in three major domains of high, low, and extremely low field intensities. The application of high-pulsed electric fields to cells, leading to the induction of a transmembrane potential >~200 mV, has been associated mostly with the phenomenon of electroporation (Kinosita and Tsong, 1977; Mir et al., 2006; Impellizeri et al., 2016). Electric field intensity E is the deciding parameter inducing membrane permeabilization and controls the extent of the cell surface where the transfer can take place. The electric field is generally created by the application of a potential difference between metallic electrodes immersed in the medium containing the cells. For the simple electrode geometry of two parallel plates separated by a distance d (cm), the applied voltage to the electrodes distance is calculated from the applied voltage “U” as: E=U/d (V/cm). Field intensity larger than a critical value ($E_{p,\,r}$) must be applied to the cell suspension. From Equation 1, (assuming the absence of the resting transmembrane potential) permeabilization is first obtained for θ close to 0 or π such that:

$$\Delta\Psi_{i,perm} = f\, g(\lambda)\, r\, E_{p,r} \quad (2)$$

Permeabilization is therefore a local process on the cell surface. The extent of the permeabilized surface of a spherical cell, A_{perm}, is given by:

$$A_{perm} = A_{tot}(1-E_{p,r}/E)/2 \quad (3)$$

Where A_{tot} is the cell surface and E is the applied field intensity. Increasing the field strength will increase the part of the cell surface, which is brought to the electropermeabilized state. These theoretical predictions are experimentally directly supported on cell suspension by measuring the leakage of metabolites (ATP) (Rols and Teissié, 1990) or at the single cell level by digitized fluorescence microscopy (Gabriel and Teissié, 1997, 1999). Permeabilization, due to structural alterations of the membrane, remained restricted to a cap on the cell surface. The area affected by the electric field depends also on the shape (spheroid) and on the orientation of the cell with respect to the electric field lines. Changing the

field orientation between the different pulses increases the fraction of the cell surface which is permeabilized (Kotnik and Miklavcic, 2000).

Exposure of cells to extremely low electric and magnetic fields, which can induce a transmembrane potential difference <1 mV, have been associated with environmental exposures of humans to low-frequency electromagnetic fields on the one hand, and on the other, with possible application of medical therapeutic devices (Ahlbom et al., 2001; Lacy-Hulbert et al., 1998; Adair, 1999). The risks as well as the underlying mechanisms of these very weak electric fields are still poorly understood and highly debated (Weaver et al., 2000; Foster, 2003; Wyszkowska et al., 2016).

The intermediate domain of low electric fields, which may lead to the induction of transmembrane potential alteration in the range of 1 to 100 mV, has been routinely applied in electrophysiological studies of ion transport through channels. It was realized quite early that physiological electric fields in tissues are at the lower end of this range (Jaff, 1977; Nuccitelli and Jaffe, 1974; Borgens et al., 1977). These endogenous electric fields were associated with the processes of development, regeneration and wound healing (Nuccitelli, 2003). An attempt was made to understand the role of these electric fields under situations imitating physiological and pathological conditions by applying low DC electric fields and examining the phenomenon of electrophoresis of charged membrane components in the plane of the cell membrane. Lateral electrophoretic displacements of charged membrane proteins and lipids resulted in segregation of these components at the cell surface (Poo and Robinson, 1977; Poo et al., 1979; Poo, 1981). Recent studies of the effect of low DC electric fields on altering basic cellular activities such as cell cycle, directional growth, and mobility explored the underlying cellular pathways involved in inducing these changes (Wang et al., 2003).

Pulse duration

Studies of pulsed electric field influences on cells have been carried out in three major domains of millisecond, microsecond, and nanosecond pulse durations. The application of pulsed electric field in the range of microsecond to millisecond pulse duration leads to induction of short-lived permeability changes in the membrane (“electropores”) enabling the diffusion of molecules across the membrane along their electrochemical gradients in case of using high field intensity in the range of kV/cm.

Shorter pulse durations in the nanosecond range nondestructively perturb the intracellular environment, causing calcium bursts (Vernier et al., 2003; White et al., 2004), eosinophil sparklers (Schoenbach et al., 2001), vacuole permeabilization (Burgoyne and Morgan, 2003), nuclear chromatin rearrangement (Sun et al., 2006), activation of excitable cells (cardiac myocytes and adrenal chromaffin cells) (Vernier et al., 2005), and the appearance of apoptotic indicators such as release of cytochrome c into the cytoplasm (Beebe et al., 2002), loss of mitochondrial membrane potential, and caspase activation (Beebe et al., 2003; Vernier et al., 2003). Nano-electropulse induced killing of cancer cells and shrinking of tumors has been demonstrated *in vitro* and *in vivo* (Nuccitelli et al., 2006; Garon et al., 2007). In addition to these responses in the cell interior, nanoelectropulse exposure also induces phosphatidylserine (PS) externalization (translocation of PS from the cytoplasmic face of the plasma membrane to the cell exterior) a normal event in platelet activation and blood coagulation (Balasubramanian and Schroit, 2003), a diagnostic feature of apoptotic cells which serves as a physiological semaphore for their phagocytic removal (Fadok et al., 2001), and a means of intramembrane signal transduction in lymphocytes (Elliott et al., 2005). The ability to activate this signal remotely, with non-ionizing, non-thermal (high power, but low energy), non-invasive electric pulses may be useful in both research and clinical settings. All of these effects are mediated by the generation of perturbative potentials on cellular structures for periods shorter than the charging time constant of the plasma membrane (tens to hundreds of nanoseconds for mammalian cells of various sizes and shapes) (Schoenbach et al., 2001; Sher et al., 1970). Although the electrical power associated with these pulse exposures is high (megawatts), nanosecond pulse durations limit the total energy delivered to Nano-joules per cell, and there is no immediately apparent physical damage at the cellular level. Despite these low energies, the initial effects are abrupt, well-defined, and apparent (using fluorescence microscopy) within milliseconds.

It is of particular importance to chart the minima of the pulse parameters- electric field, pulse rise time and duration, pulse count and repetition rate- required to produce these biological responses. Large pulse doses are likely to produce multiple effects, which may be useful for cancer therapy or other applications where cell killing is the overriding desired outcome. In order to draw and delineate the molecular dis-equilibrations caused by nanoelectropulse exposure it will be necessary to minimize the perturbations to the cellular machinery instead of delivering an overwhelming dose. Previous studies indicate that nanosecond pulse effects may be absent when the electric field amplitude is less than about 1 MV/m (Vernier et al., 2004). Other variables may affect the level of observed threshold (for example, electropermeabilization with 4 ns pulses is observed only at high pulse repetition rates (Vernier et al., 2006).

Pulse number (or repetition frequency)

An increase of the number of pulses leads to an increase of local permeabilization (Teissié, 2007). This was

already observed in the case of low molecular weight molecules. But, as far as macromolecule transport is concerned, pulse durations in the millisecond range are required (Chu et al., 1987; Berkó et al., 2016), suggesting a contribution of electrophoretic forces (Sukharev et al., 1992). For simplicity, let us assume that we apply to the cell suspension a series of *n* square-wave electric pulses, defined by the duration *t* of each pulse and the intensity $E_{p,r}$. When either *t* is increased from about 10 µs (that is, above the time necessary for the local induction of the transmembrane potential) to a few milliseconds, or the number *n* is increased, there is no effect on the permeabilizing threshold value, but the induced membrane permeability increases (Rols and Teissié, 1990; Serpersu et al., 1985; Schwister and Deuticke, 1985). Moreover, keeping the total duration *nt* constant, the permeability induced by electric fields above the threshold value increases on increasing the number of pulses (Rols and Teissié, 1990).

In electrochemotherapy, the number of pulses, 4, 6, or 8, is not a crucial parameter provided that bleomycin is in excess in the treated tissue. If bleomycin is injected at limiting doses, better antitumor effects are obtained with 8 pulses (Mir et al., 1991). Moreover, Sersa and colleagues showed that changing the electrode orientation during the delivery of the 8 transcutaneous pulses improved the efficacy of electrochemotherapy of solid subcutaneous tumors in mice. In other words, they compared the delivery of either 4 pulses in a row, or 8 pulses in a row, or “4+4” pulses, that is two rows of 4 pulses with the orientation of the external electrodes during the second run of 4 pulses being perpendicular to the orientation during the first run. This last setting gave the best results. This can be explained by a better coverage of the tumor volume actually exposed to the permeabilizing electric fields (Sersa et al., 1996).

Irreversible electroporation is a newly developed non-thermal tissue ablation technique in which certain short duration electrical fields are used to permanently permeabilize the cell membrane, presumably through the formation of Nano-scale defects in the cell membrane. The results achieved with the different sets of electrical parameters indicate that the main parameter affecting the results is the electric field strength. The best results were obtained using plate electrodes to deliver across the tumor 80 pulses of 100 µs at 0.3 Hz with an electrical field magnitude of 2500 V/cm. These conditions induced complete regression in 12 out of 13 treated tumors, (92%), in the absence of tissue heating (Al-Sakere et al., 2007).

SOME BIOLOGICAL APPLICATIONS OF PULSED ELECTRIC FIELDS (PEF’s)

Electroendocytosis

The exposure of cells to low electric fields, of strengths smaller than that applied for electroporation, can generate, among others, electrophoretic lateral mobility of charged proteins and lipids in the plane of the cell membrane and the induction of a low amplitude (below electroporation levels) trans-membrane potential difference across the membrane (Poo, 1981; Brumfeld et al., 1989; Farkas et al., 1984). Attempts have been made to apply a low electric field, following electroporation, in order to increase the diffusion rate of DNA through electropores via electrophoretic mobility (Klenchin et al., 1991). Also, it has been shown that, after cell electroporation, the endocytosis was stimulated for 5 to 120 min (Glogauer et al., 1993) and a long-lived macropinocytosis observed for up to 60 min (Rols et al., 1995). Recently, electroporation-induced endocytosis was confirmed by Rosazza et al. 2016. They observed that endocytosis contributes in DNA electrotransfer by caveolin mediated endocytosis (~50%), clathrin dependent endocytosis (~25%), and macropinocytosis (~25%) (Rossazza et a., 2016). Moreover, lower, non electroporating either monopolar (Figure 1) or bipolar symmetric (Figure 2) electric pulses (1.5 to 100 V/cm), applied for longer durations, were reported to induce an endocytotic-like process into different cell types (Antov et al., 2004; Antov et al., 2005; Mahrour et al., 2005). Bipolar asymmetric electric pulses (Figure 3) examined by Abd-Elghany and Mir showed an increase (~35%) of an all-or-nothing type response that occurred above a threshold value of the electric field intensity (9.3 to 9.8 V/cm) (Abd-Elghany and Mir, 2015) which was nearly the same as obtained by bipolar symmetric electric pulses (Figure 2). The most powerful electric pulses were the monopolar pulses (Figure 1) which showed an increase of about 8 folds. These types of low intensity electric pulses were used to internalize proteins and drugs without killing the cells. An interesting study was performed to kill cancer cells using low intensity electric pulses LEFs as that used in Electroendocytosis. The physical idea behind this was in making low intensity electric pulses energy equivalent to irreversible electroporation energy (Shawki and Farid, 2014). This could be obtained by increasing field intensity not more than 20 V, pulse numbers to 320 pulses, duration till 2000 ms, and repetition frequency to 106 Hz.

Electrochemotherapy (ECT)

It is the combination of cell permeabilizing pulsed electric field and non permeant drugs like bleomycin (Mir et al., 1991). The electric pulses used (8 monopolar pulses of 100 µs and 800 to 1300 V/cm with a frequency of 1 Hz) provoke the electropermeabilization (“electroporation”) of the cell membrane allowing for the uptake of molecules such as bleomycin that are unable to diffuse through the normal cell membrane (Cemazar et al., 1998; Mir and Orlowski, 1999; Gehl, 2003; Satkauskas et al., 2005). Preclinical results show an enhancement in cytotoxicity of

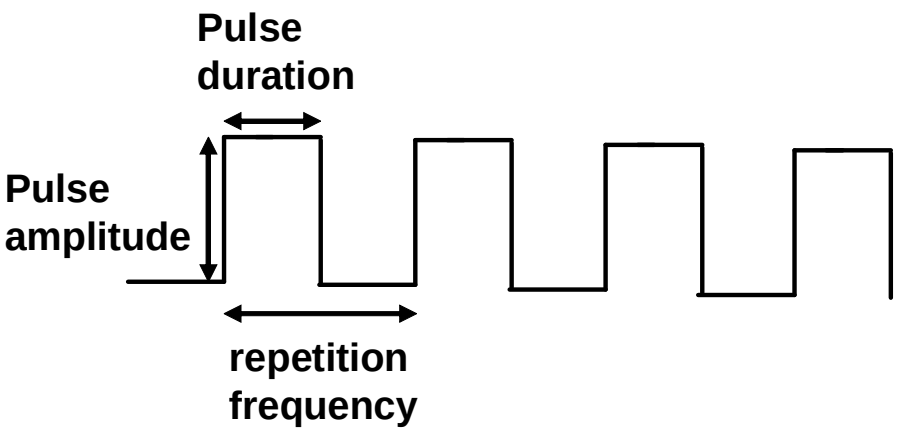

Figure 1. Monopolar electric pulses used in the study of Antov et al. (2005).

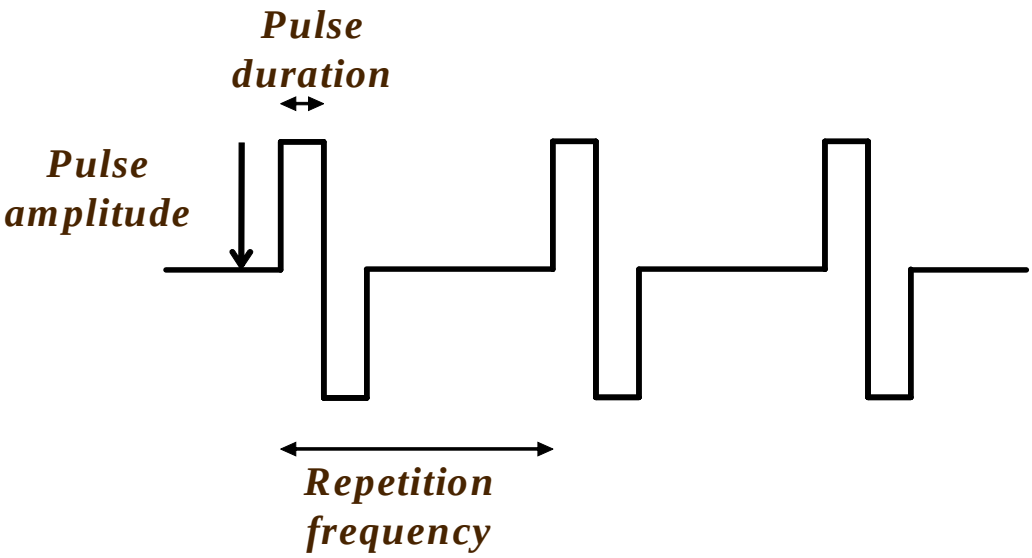

Figure 2. Bipolar symmetric square pulse used in the study of Mahrour et al. (2005).

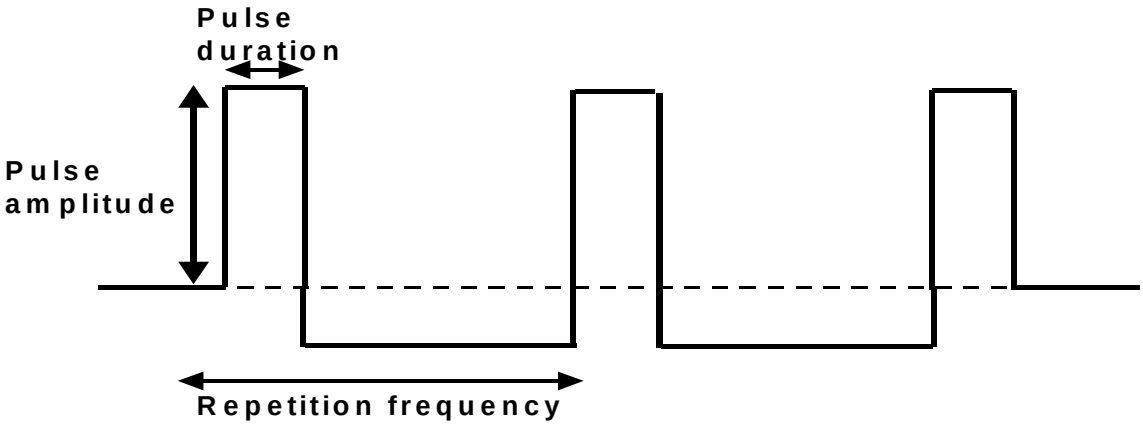

Figure 3. Bipolar equilibrated asymmetric electric pulses used in the present study.

300 to 700 folds at the level of 50% cell kill by electroporation (Mir et al., 1991; Gehl et al., 1998).

Data collected from preclinical studies were translated into a clinical electrochemotherapy investigation by Mir's group (Belehradek et al., 1993) at the institute Gustave-Roussy, Villejuif, France. This first electrochemotherapy trial enrolled patients with recurrent or progressive permeation nodules (cutaneous metastasis) of head and neck squamous cell carcinoma located in the anterior cervical region or in the upper part of the thorax. All patients enrolled in the study had been previously treated with radiation therapy, surgery, and/or chemotherapy. None of the patients had been exposed to bleomycin before the trial. This trial utilized a protocol that was similar to the preclinical methods for administrating electrochemotherapy. Bleomycin was used as the chemotherapeutic agent. A 10-mg/m^2 bolus dose was administered to patients. Beginning 3.5 min after injection, pulses were administered to the first tumor. Subsequent tumors were treated in series with a 1 min interval between electrical treatments. Mir utilized two parallel stainless steel strips as electrodes. Either four or eight pulses were administered with electric field strength of 1300 V/cm. The rectangular direct current pulses delivered to the tumors were 100 µs in duration with a duty cycle of 1s. The majority of the tumors treated were 2 × 2 cm in size or less. Nodules that were too large to fit between the 6 mm fixed gap of the electrodes were electrically treated in sections. Eight patients were enrolled in the study; a total of 40 nodules were treated with electrochemotherapy. Objective responses were seen in 29 (72%) of the nodules, and 23 (57%) of the 40 tumors responded completely. Nodules that did not receive electric pulses did not respond even though they were subjected to the same bleomycin dose as the electrically treated tumors. These response rates were extremely encouraging based on the facts that they were from the first electrochemotherapy trial ever performed in humans, the treatment was administered only once, and that the enrolled patients had recurred or failed other therapies prior to electrochemotherapy treatment.

The Villejuif trial was continued to include the treatment of larger and deeper head and neck squamous cell carcinomas in five patients along with two adenocarcinoma patients (Domenge et al., 1996). Tumors with dimensions up to 20 cm were treated; these tumors were considerably larger than any previously treated nodules. These larger nodules also had greater depth. Bleomycin was administered intravenously in the majority of the patients. Intra-arterial injection was used for two patients as a mean of allowing bleomycin to reach the treatment region more easily. For either case a dose of 10 or 15 mg/m^2 was administered for each treatment. Large antitumor effects were seen in the more superficial regions of the nodules while the deeper portions continued to grow. This indicated that the depth of effective electrical treatment was limited by the parallel plate electrodes which were positioned on the tumor/skin during pulse delivery. Deeper tumor regions were not subjected to fields of sufficient intensity to cause electropermeabilization. Therefore, these deep portions of large tumors did not respond. Complete and partial responses were obtained in spite of the fact that the electrodes were not well suited for treating large nodules. However, treatment effectiveness was lower than the previously treated small nodules. It was noted that maximum antitumor effects resulted when pulses were administered between 8 and 28 min after injection of a bolus bleomycin dose. This indicated the time when bleomycin concentrations were highest in the interstitial fluid surrounding the tumor cells. In addition, intra-arterial bleomycin injection was observed to result in increased effectiveness in two patients. Mir and collaborators reported that no significant modification of cardiological or hemodynamic parameters was noted during or after electrochemotherapy treatment. Those patients with smaller tumors were treated with oral sedatives 1h before electrochemotherapy treatment (Mir et al., 1991; Belehradek et al., 1993). Patients with larger nodules were treated under general anesthesia (Domenge et al., 1996). In general, the patients were reported as tolerating the treatment well. Slight muscle contractions were observed as a result of each pulse. These contractions were described as painless by the patients, but were also associated with an unpleasant sensation. This unpleasant feeling subsided immediately after each electric pulse. No residual sensations were noted. Reactions at the site of treatment included slight edema and erythema beginning 1 to 2 h after treatment. These conditions disappeared in less than 24 h.

Nearly identical patient tolerance was noted by Heller and co-workers (Heller, 1995; Heller et al., 1996) from a study conducted in Tampa, Florida, USA. The phase I/II study investigated the use of electrochemotherapy for treating cutaneous and subcutaneous cancers. These included three melanoma, two basal cell carcinoma (Glass et al., 1996), and one adenocarcinoma patients between ages of 39 and 65. Bleomycin was used as the chemotherapeutic agent, and a dose of 10 units/m^2 was administered intravenously at rate of 0.6 to 0.8 mg/min. Between 5 and 15 min after bleomycin infusion, eight 99 µs rectangular direct current pulses were delivered to tumors following administration of 1% lidocaine around the treatment sites. Parallel plate electrodes, 2 × 2 cm, with an adjustable gap were used to deliver the 1300 V/cm pulses at a duty cycle of 1 pulse/s. A total of 18 tumors were treated with electrochemotherapy in six patients. Objective responses were obtained in 13 (72.2%) of the tumors. Of these responding lesions, six (33.3%) were complete responses and seven (38.9%) were partial responses. Tumors treated with bleomycin alone showed no effect. Several possible reasons for not obtaining complete responses in the majority of the treated tumors were addressed. One reason was that a

10-units/m^2 bleomycin dose may have been too low. Another was that incorrect timing between drug infusion and pulse delivery may have resulted in an insufficient amount of drug in the tumors during electroporation. And, finally, poor tumor vascularity or circulatory disease may have inhibited bleomycin distribution to the treatment site. Based on these possibilities, the treatment protocol was modified to utilize intralesional bleomycin following a preclinical investigation (Heller et al., 1997). Intralesional bleomycin doses were scaled based on tumor volume. Lesions that were less than 100 mm^3 received 0.5 units, tumors that were between 100 and 150 mm^3 were given 0.75 units, and lesions that were greater than 150 mm^3 received a 1.0 unit dose. Eight patients were treated with intratumor bleomycin; all other aspects of the treatment protocol remained the same. Five patients had melanoma, two had basal cell carcinoma, and one had Kaposi's sarcoma (Reintgen et al., 1996). All patients had multiple tumors treated. A total of 25 tumors were treated with electrochemotherapy; 24 (96%) of these completely responded. The remaining tumors responded partially. Thus, the objective response rate was 100%. These response rates were considerably higher than those obtained by systemic use of bleomycin. The patients treated in the trial conducted by Heller frequently formed scars in the areas where the electrodes were positioned. These were due to a slight amount of skin damage that resulted from holding the tumor firmly between the electrodes and due to minor arcing that occurred during pulse delivery. The study also reported that flat tumors were difficult to hold between the parallel plate electrodes. Electrode designs were modified in order to curtail these complications. A modified electrode design (Gilbert et al., 1997) was tested in a murine melanoma tumor model and incorporated into the trial (Reintgen et al., 1996). The electrode design utilized six needles arranged at 60° intervals around a circle. This array was inserted around the perimeter of tumors to a specified depth, and the needles were used as electrodes to rotate the applied pulses around the tumor. A total of 49 tumors in six melanoma, four basal cell carcinoma, and one squamous cell carcinoma patients were treated using the needle electrode with intratumor bleomycin. The new needle array electrode resulted in 48 (97.9%) objective responses; 45 (91.8%) of the treated tumors responded completely. Partial responses occurred in three (6.1%) lesions, and a single tumor did not respond. The new electrode produced less scarring than the partial parallel plate design. In addition, the needle electrode was easier to apply for flat tumors.

A third clinical electrochemotherapy trial has been started in Ljubljana, Slovenia, for the treatment of melanoma. Rudolf et al. (1995) reported on the treatment of 24 tumors in the first two patients enrolled in the trial. Both patients were treated with electrochemotherapy using an intravenous bolus of 10 mg/m^2 bleomycin and parallel plate electrodes. The treatment protocol administered pulses starting 8 min after bleomycin administration. Tumors were sprayed with xylocaine several minutes before pulsation. The method for delivering the pulses followed from the preclinical work of the group (Sersa et al., 1996; Cemazar et al., 1995) which showed that delivering four pulses in two perpendicular directions, eight pulses total, was superior to delivering eight pulses in a single direction. Other electrical parameters were the same as those used by Heller and Mir. Their results showed that 22 tumors (91.6%) responded objectively; all of these were complete responses. Two tumors showed no effect. No major general or local side effects of electrochemotherapy treatment were reported for the patients treated in the Ljubljana trial. Muscle contractions that were coincident with each pulse were noted. These contractions disappeared immediately after each pulse. Patients reported the sensations during pulse delivery as an unpleasant shock or pain within the treatment region; however, the treatment was tolerable. Slight edema and erythema were noted in the treatment sites. These symptoms subsided in 24 h. These minor side effects were consistent with those reported in other trials.

A recent clinical electrochemotherapy trial has been performed in Ljubljana for deep seated tumors. Two patients with squamous cell carcinoma neck metastases were treated with ECT, but outcomes of both patients differ drastically. After ECT extensive crust formation was observed in patient 1. In the following three months tissue necrosis with suppuration and purulent discharge developed. A tracheotomy and gastrostomy were performed. Nevertheless, in 4.5 months after ECT the patient died of cancer progression. In Patient 2, complete response of the metastasis behind the left mandibular angle (metastasis No. 2) was recorded and partial response of the metastasis in the left parotid gland (metastasis No. 1). Six weeks after ECT, fine-needle aspiration biopsy of the treated area revealed necrosis and inflammatory cells, without any viable tumor cells in the specimens from both of the treated lesions. In addition, good cosmetic effect was obtained three months after ECT (Groselj et al., 2016).

CONCLUSIONS

The influence of electrical fields on living cells represents an important meeting area for the physical and biological sciences, where there remains on-going fundamental research. During the last decades, different approaches have been developed to incorporate macromolecules into cells one of them is based on an electrically driven process (electroporation) where cells are exposed to high intensity electric pulses for short durations of micro-to milliseconds. This exposure leads to the formation of electropores, which enables the diffusion of small molecules across the membrane or the uptake of

macromolecules such as DNA. The most important application for electroporation is the electrochemotherapy of cancer cells. Now, electrochemotherapy became an important outpatient modality for superficial tumors with ongoing efforts to treat deep seated tumors. The emergence of mechanistic biological studies together with improvements in electroporation setup has improved the targeting of cancer tissue through proper choice of electrodes and electrical parameters.

Low intensity electric pulses can be used as an adjuvant pulse to increase the diffusion rate of DNA through electropores via electrophoretic mobility in either bacterial transformation using electroporation or electrogenotherapy. Low intensity non electroporating either monopolar, bipolar symmetric, or bipolar asymmetric electric pulses applied for longer durations, were reported to provoke an endocytotic activity for different cell types. The most powerful electroendocytosis was induced by monopolar electric pulses.

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