Proceedings of the

Electroporation-based Technologies and Treatments

International SCIENTIFIC WORKSHOP and POSTGRADUATE COURSE

Ljubljana, Slovenia
November 10-16, 2019

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Edited by:

Peter Kramar
Damijan Miklavčič
Lluis M. Mir

Organised by:

University of Ljubljana
Faculty of Electrical Engineering

Institute of Oncology, Ljubljana

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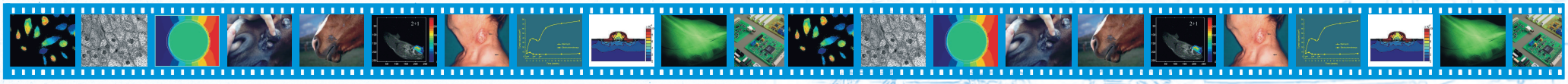

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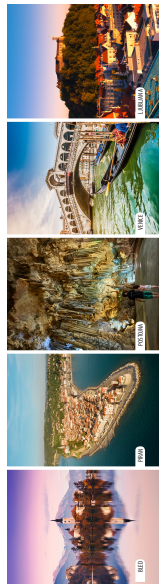

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Welcome note

Dear Colleagues,

Dear Students,

The idea of organizing the Workshop and Postgraduate Course on Electroporation Based Technologies and Treatments at the University of Ljubljana had been developing for several years. After preliminary discussions, the Workshop and Course was organised for the first time in 2003. In 2019 the Course is organised for the 13th time! In these fourteen years, the Course has been attended by 772 participants coming from 41 different countries. And this year again we can say with great pleasure: "with participation of many of the world leading experts in the field".

The aim of the lectures at this Workshop and Course is to provide the participants with sufficient theoretical background and practical knowledge to allow them to use electroporation effectively in their working environments.

It also needs to be emphasized that all written contributions collected in the proceedings have been peer-reviewed and then thoroughly edited by Peter Kramar. We thank all authors, reviewers and editors. Finally, we would like to express our sincere thanks to colleagues working in our and collaborating laboratories for their lectures and for the preparation of the practical trainings delivered during the course, to the agencies that have been sponsoring our research work for years, and to Slovenian Research Agency and Centre National de la Recherche Scientifique (CNRS), to the Bioelectrochemical Society and to the International Society for Electroporation-Based Technologies and Treatments ISEBTT for supporting the Workshop and Course. We also would like to thank Educell (Slovenia), IGEA (Italy), Iskra Medical (Slovenia), Iskra PIO (Slovenia), Jafral (Slovenia), Kemomed (Slovenia), Leroy Biotech (France), Meditech (Slovenia), Medtronic (USA), Micro+Polo (Slovenia), Omega (Slovenia), and Pulse Bioscience (USA) whose financial support allows us to organize EBTT and assist several students participating in this Workshop and Course.

Thank you for participating in our Workshop and Course. We sincerely hope that you will benefit from being with us both socially and professionally.

Sincerely Yours,

Damijan Miklavčič and Lluis M. Mir

INVITED LECTURERS

Tumour ablation with electroporation: Thinking outside the membrane.

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INTRODUCTION

Ablation is performed with heat or cold based techniques in select patients to treat small solitary tumours[1]. Pulsed electric fields have been developed for tumor ablation via irreversible electroporatio (IRE), nanoporation (nsPEF) or electrochemotherapy (ECT), classified based on the pulse parameters used for treatment and the mechanism underlying cell death[2]. PEF based tumor ablation provide many advantages over conventional heat or cold based ablative techniques, such as the ability to treat tumors adjacent to large blood vessels without being affected by the heat sink effect, as well as treating tumors involving delicate structures such as the bile duct[3] or renal pelvis with minimal risk of severe complications such as perforation or fistula formation. Despite differences in their mechanism of action, all ablation techniques place emphasis on inducing rapid or acute cell death (within 24 hours of treatment) and the on achieving complete local tumor control. This has limited the application of ablation to tumors smaller than 3cm as coverage of larger tumors requires large numbers of probe placement and/or increased energy delivery which poses safety issues. Moreover, tumors involving complex anatomy, such as bone metastases, cannot be completely ablated without damaging healthy tissue, limiting clinical use of ablation.

The emphasis on immediate and complete tumor destruction has led PEF therapies to focus on membrane permeabilization, leveraging it for maximizing cell death either via direct (IRE) or indirect (through drug delivery in ECT) means. Work by us and others has shown that PEF can induce a spectrum of biological responses in the tumor microenvironment, which can be harnessed to effect anti-cancer response beyond what is feasible in the conventional approach to tumor ablation. PEF parameters required to induce these responses are often sub-lethal, and therefore can be extended to large volumes of tissue without concern for ablative effects. Our focus is on understanding the effect of PEF on tumor vasculature and extracellular space, the extracellular matrix (ECM) and inflammatory response, as alternate means for tumor control.

EFFECT OF PEF ON TUMOR VASCULATURE AND EXTRACELLULAR SPACE

Sersa G et al[4] were amongst first to observe and report on the effect of PEF on normal and tumor vasculature. Depending on the pulse parameters used, PEF elicit dynamic effects from the vasculature including vasospasm, increased permeability and in some cases, permanent occlusion of the vessel wall. We have taken advantage of these biological effects to demonstrate PEFs can be used as a vehicle to enhance nanoparticle delivery[5-6], as well as, increase treatment effect of nanoparticles by increasing acute dosing of the therapeutic drug. We have also shown that such effects can be monitored with reported nanoparticles. In addition to the effect on blood vessels, PEF mediated cell swelling and locoregional osmotic changes can alter diffusion behaviour in the extracellular space[7]. We have taken advantage of both these actions to enable delivery of therapeutic agents to tumors independent of membrane permeabilization.

EFFECT OF PEF ON THE EXTRACELLULAR MATRIX AND INFLAMMATORY RESPONSE

The lytic effect of PEF is restricted to biological entities possessing a membrane potential, and in the absence of heating the ECM at the treatment site remains largely undisturbed. The release of chemokines from cells injured by PEFs as well as the presence of intact ECM and blood vessels promote the recruitment and migration of inflammatory and immune cells into the treated tumor. The destruction of ECM during thermal ablation often restricts these cells to the outer periphery of the treatment site, while our work demonstrates widespread presence of these cells throughout the ablation zone following IRE. This work demonstrates the specific application of PEF to change the immunological status of tumor, switching it from cold to hot, thereby priming it for subsequent immune mediated clearance using immunomodulatory drugs.

POST-PEF WOUND HEALING

Ablation wounds elicit a robust wound healing response, wherein PEF treatment of tumors or tissue abutting or adjacent to luminal structures can affect function or patency[8]. Modulation of scar forming can be necessary for both achieving a desired effect (cardiac ablation) or avoiding complications. Activity of wound healing cells may also promote regrowth of residual tumors Our investigations reveal major cytokine and signalling pathways activated following PEF ablation and how that can be controlled for achieving specific outcomes.

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Multiscale modelling of electroporation

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INTRODUCTION

Electroporation is being successfully used in biology, medicine, food processing, biotechnology, and in some environmental applications. Recent applications include in addition to classical electroporation, where cells are exposed to micro- or milliseconds long pulses, also exposures to extremely short nanosecond pulses, i.e. high-frequency electroporation. Electric pulses are applied to cells in different structural configurations ranging from suspended cells to cells in tissue. Understanding electroporation of cells in tissues and other complex environments is key to its successful use and optimization in various applications and requires a multi-scale approach, where we seek information from molecular models and simple lipid systems up to *in vitro* and *in vivo* experiments. Studying electroporation in such "simple" environment is inevitable as it allows basic research on different aspects of electroporation. Our understanding of electroporation in simple systems is then transferrable to electroporation of cells in more complex systems [1].

The lecture will provide an overview of electroporation models on different spatio-temporal scales; including insights from molecular dynamics simulations, continuum electroporation models and simple models of tissues representing their underlying cellular structure. The lecture will also indicate how insights from molecular dynamics simulations could be applied to enhance the development of continuum description of membrane electroporation, and how these continuum-level models can further guide experimental work on cells and tissues. Finally, the questions which remain to be answered in order to better understand electroporation-related processes will be pointed out. Answering these questions should allow us to improve the designs of current as well as future technologies and treatments.

MOLECULAR DYNAMICS SIMULATIONS

Molecular dynamics (MD) simulation generates a trajectory of atomic positions and velocities in the considered molecular system based on integrating the classical equations of motion. MD simulations have revealed many aspects of the electroporation phenomenon. Firstly, they allowed us to study the formation of lipid pores under the influence of strong electric field and how this formation depends on the lipid architecture. They also allowed us to explore the transport of molecules across lipid pores. In addition, they allowed us to estimate the possible contribution of lipid peroxidation to cell membrane permeability [2]. Recent simulations are also indicating perturbation of membrane proteins by electric fields such as voltage-gated ion channels (Fig. 1).

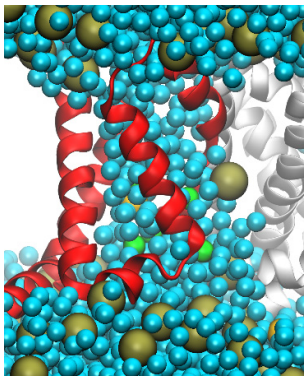

Figure 1: A pore formed in the middle of the voltage sensor of a voltage-gated ion channel, which becomes stabilized by lipid headgroups. The helices of the voltage sensor are represented with red ribbons. Gold, blue, yellow and green spheres represent, respectively, lipid P atoms, water O atoms, sodium ions and chloride ions.

CONTINUUM MODELS

MD simulations provided the first "visual" molecular-level evidence that the electric field promotes formation of pores in the lipid bilayer and confirmed some of the main hypotheses underlying the two-decades-older development of theoretical concepts of electroporation. The main motivation in developing electroporation models is to understand the experimental results, predict experimental outcomes, and to use the models for optimization of experimental protocols. For such purpose, the models need to enable prediction of the physical quantities that are accessible by experimental measurements. Results from MD often cannot be compared directly to experiments, since the system in the simulations is representative of only a tiny area of the lipid membrane. For modelling electroporation of experimental systems, e.g. planar lipid bilayers, lipid vesicles, cells, or cell assemblies, it is necessary to resort to more large-scale models, which analyse electroporation in terms of continuum approaches, which include solving ordinary and partial differential equations using different numerical methods [3].

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Effects of electroporation and electrochemotherapy on normal and tumor blood vessels

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INTRODUCTION

Electroporation/electropermeabilization (EP), i.e. application of electric pulses to cells or tissues, induces reversible permeabilization of cell membranes under suitable conditions, thus facilitating entry of exogenous molecules into cells. EP of tissues in humans is feasible, efficient, and tolerable and its most advanced routine clinical use is electrochemotherapy (ECT), where cytotoxic drugs are delivered to cells to treat tumors. Although the primary mode of action of ECT is the destruction of tumor cells due to the increased cytotoxicity of chemotherapeutic drugs, it also has different effects on the tissue level. It was shown that EP and ECT induce cellular stress [1] and have blood flow modifying effects on normal as well as on tumor vasculature.

DIFFERENTIAL EFFECTS OF EP AND ECT ON NORMAL AND TUMOR BLOOD VESSELS

Application of EP pulses to normal blood vessels increases the permeability of affected blood vessels, causes a transient vascular lock, *i.e.* decrease in perfusion, and modulates the diameter of affected blood vessels [2]. Similarly, in tumors, application of EP or ECT increases the permeability of affected blood vessels and causes a vascular lock. In case of normal blood vessels, these effects are short-lived, whereas in case of tumor blood vessels, the effects are long-lasting and resolve more than 72 h after ECT.

In tumors, EP results in an immediate abrogation of blood flow, i.e. vascular lock, which lasts for more than 60 min. Interestingly, the tumor-supplying arterioles respond to EP in the same way as the normal vessels, with rapid vasoconstriction and increased permeability. Thus implying that this is the main cause of the immediate vascular lock observed after EP. EP also results in an increased permeability of tumor blood vessels for macromolecules and a partial long-lasting decrease in perfusion [3]. Thus, EP has a differential effect on normal and tumor blood vessels. Moreover, ECT has a direct cytotoxic effect on tumor endothelial cells, thus it has a vascular disrupting effect. Importantly, this is a differential effect that destroys only tumor blood vessels and retains the functionality of normal blood vessels surrounding the tumor that are also affected (Figure 1) [3].

In summary, ECT has an immediate effect on tumor blood vessels, as well as a differential effect on tumor and normal blood vessels, destroying the first and sparing the latter. This is of a special interest as it implies that ECT is a vascular disrupting therapy with selective action against tumor blood vessels. This shows that ECT can be safely used even in a situation when large blood vessels are close to the tumor and will be exposed to the electric field during the treatment [4].

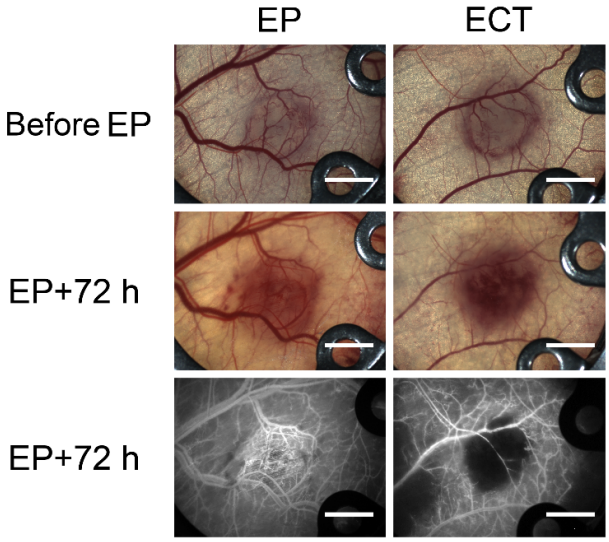

Figure 1: Color images of the dorsal window chamber with a murine SA-1 tumor growing inside taken at a 20x magnification at the designated times after electroporation. Fluorescently labelled 70 kDa dextrans were injected intraorbitally 72h after electroporation and grayscale fluorescent images were acquired at 20x magnification. EP – tumor exposed to electroporation (eight square wave electric pulses, amplitude per distance 1300 V/cm, pulse length 100 µs at repetition frequency 1 Hz) only, ECT – tumor treated with electrochemotherapy (100 µg of bleomycin injected intraorbitally 3 min before electroporation). Scale bar: 2 mm.

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PEF treatment applications in food technology and limitations

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INTRODUCTION

Pulsed Electric Fields (PEF) is an advanced non-thermal processing method for achieving tailored cell disintegration of biological tissues or microbes. Various applications have been identified in several processes of food industry involving improvement of mass transfer phenomena, liquid food preservation and targeted structural modifications. The first industrial commercial applications have been achieved [1]. However, several limitations still remain and have to be addressed prior to the full exploitation of PEF technology in food industry. Most of these limitations are related to the unavoidable occurrence of electrochemical reactions at electrode-food interface of a PEF treatment chamber when typical conditions for PEF processing are applied. The occurrence of these electrode reactions is a very complex phenomena, which is affected by several factors, such as PEF chamber design and electrode material, PEF electrical parameters, as well as composition and chemical-physical properties of the treated products [2]. Electrochemical reactions, especially those involving metal release from the electrodes, may seriously affect food safety and quality as well as electric field distribution and electrode's lifetime, thus affecting the technical feasibility of PEF technology.

In the lecture, the main applications of PEF technology in food industry are briefly described and the main advantages and limitations are highlighted. Then the basic concepts of electrochemistry with specific reference to the phenomena that occur at the electrode-solution interface of a PEF treatment chamber is described. Particular attention is devoted to the main side effects associated to the occurrence of electrochemical reactions and chemical processes. Finally, suggestions on how to limit the extent of electrochemical reactions and their side effects are also reported.

ELECTROCHEMICAL REACTION DURING PEF

A PEF chamber, which consists of two metal electrodes placed in direct contact with an electrolytic solution and electrically connected to a pulse generator, acts as an electrochemical cell. Although electrochemical reactions and corrosion are well known in other fields, the occurrence of these side effects of PEF treatment have been recognized and discussed only since 1990s, when a method to avoid or limit electrochemical reactions and fouling of electrodes in a PEF treatment system was suggested [3]. Since then, most of the attention has been focused on the phenomenon of metal release and electrode corrosion and on how these topics can be predicted (Figure 1) and quantified (Figure 2). However, no or very limited attention has been paid on the effect of electrochemical reactions on chemical and sensorial properties of food treated products and possible toxicity problems. Therefore, additional work is needed to obtain more detailed insights on the influence on these phenomena on the technical feasibility of PEF technology as well as on safety and quality aspects of food products.

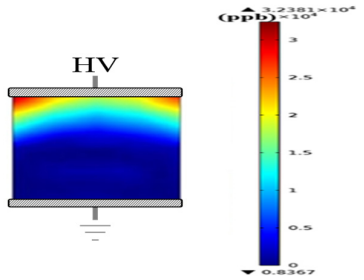

Figure 1: Predicted steady state distribution of iron concentration in the outlet cross section of a parallel plate PEF chamber [3].

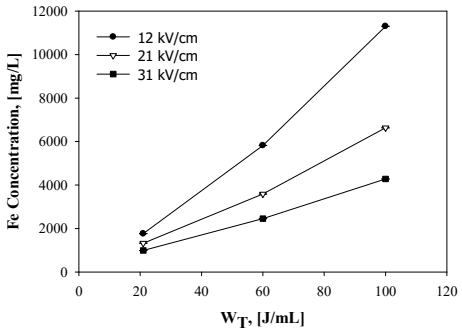

Figure 2: Concentration of Iron in Trizma-HCl buffer solution as a function of total specific energy input and for different field strengths applied [3].

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Electric fields and microfluidics for lab-on-chip applications

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INTRODUCTION

Nowadays the emergence of the field of microfluidics, the handling of small amounts of liquids in fluid channels in the order of the µm, has opened new ways to study the dielectric properties of cells with the development of different strategies using electric field to characterize cells such as electrorotation and impedance spectroscopy. In electrorotation, a rotating electric field leads cells to rotate at different speeds and directions of rotation. In impedance spectroscopy, the electric current flowing between two electrodes is modified by cells located in-between and this modification is frequency dependent. Both the rotation and impedance spectra can be analyzed to characterize single cells and retrieve their dielectric properties since the obtained signals depend on those properties. For example, at low frequency, the cell membrane behaves as an insulator with a capacitive effect which no longer exists as the frequency increases and the interior of the cell becomes accessible to the electric current.

DIELECTROPHORESIS

The use of an electric field on a chip can also be used to interact with the cells. This phenomenon called dielectrophoresis (DEP) relies on the differences in dielectric properties between cells and their suspension liquid [1, 2]. Cells placed in a non-uniform electric field can therefore be either attracted in the high field region (positive dielectrophoresis or pDEP) or repelled in the low field region (negative dielectrophoresis or nDEP) as illustrated in Figure 1.

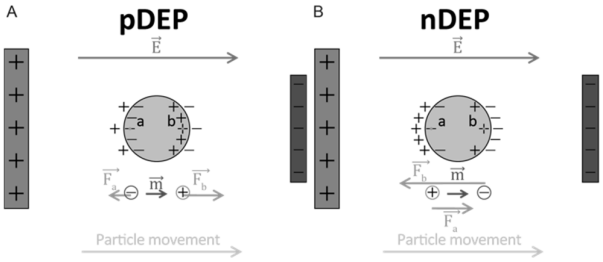

Figure 1: Principle of A) pDEP and B) nDEP. E represents the electric field, m the dipolar moment and $\boldsymbol{F_a}$ and $\boldsymbol{F_b}$ the Coulomb force (F=qE) on each barycenter of the charges on each side of the particle.

The time average expression of the DEP force exerted on a polarizable particle (index p) immersed in a suspending medium (index m) in a non-uniform electric field is expressed in (1):

$$F_{DEP} = 2\pi\varepsilon_0\varepsilon_m r_{ext}^3 Re[CM(f)]\nabla E^2 \quad (1)$$

Where CM(f) is the Clausius-Mossotti factor:

$$CM(f) = \frac{\varepsilon_p^* - \varepsilon_m^*}{\varepsilon_p^* + 2\varepsilon_m^*} \quad (2)$$

And ε_i^* the complex permittivity:

$$\varepsilon_i^* = \varepsilon_i\varepsilon_0 - \frac{j\sigma_i}{\omega} \quad (3)$$

Where ε_i is the relative permittivity, ε_0 the vacuum permittivity, σ_i the electrical conductivity and $\omega = 2\pi f$ with f the frequency.

DEP can therefore be used for various applications such as cell centering, cell separation, cell sorting or cell aggregate creation.

APPLICATION TO CELL AGGREGATES CREATION

Most recently nDEP has been used to create cell aggregates of controlled size in flow conditions as illustrated in Figure 2 [3]. This offers the possibility to create an intermediary model between single cells and cell tissue for various applications such as electroporation. Additionally, the created aggregates can be further studied using impedance spectroscopy

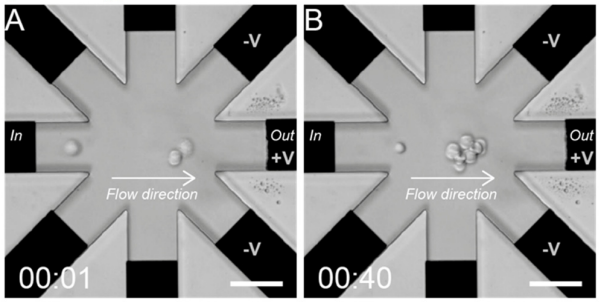

Figure 2: Example of HEK cell aggregation under flow conditions using 3 electrodes (Adapted from [3]).

CONCLUSION

The use of microfluidics and electric field on chip opens the door to a better understanding of the behaviour of cells in an electric field and the possibility to obtain reproducible models for in-vitro studies.

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Treatment of Cardiac Arrhythmias Using Pulsed Electric Field Ablation

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INTRACARDIAC CATHETER ABLATION

Catheter ablation is a technique employed to treat cardiac arrhythmias that result from abnormal or early activation of myocardium through electrical triggers or aberrant conduction patterns in the myocardium [1]. During the procedure, catheters are delivered percutaneously, typically using femoral venous access and fluoroscopic (X-ray) guidance, to allow placement of catheters at key areas of the heart where the problematic myocardial tissue can be ablated. The intracardiac catheters typically have electrodes that sense intracardiac electrogram (EGM) voltage signals that are displayed on an electrophysiology recording system. These electrograms are used to determine the optimal placement of the ablation catheter for elimination of abnormal electrical signals/foci that initiate the arrhythmia. Local EGMs can also be used to evaluate the effectiveness of an ablation, with the goal typically being the annihilation of the local EGM voltage in the tissue targeted for ablation.

Some of the technologies developed over the past three decades for cardiac ablation include radiofrequency (RF) microwave, high intensity focused ultrasound, lasers, and cryogenics [2]. These technologies are often limited in eliminating lesion gaps which can lead to recurrence of the arrhythmia and further complicate treatment strategy. Lesion efficacy is governed by patient-specific tissue anatomy, local blood flow, tissue perfusion, the catheter energy source, and the mechanism of catheter-induced cell death. Without precise knowledge of underlying targeted myocardial thickness and desired ablation lesion depth, the resulting ablation may be far deeper than required for transmural cardiac lesion formation, thus extending into extracardiac structures that may include the esophagus, airways, blood vessels, or phrenic nerves, thereby increasing risk to the patient.

The concept of RF cardiac ablation was first proposed as a potential replacement for DC fulguration in 1987 [3]. Since that time, RF has evolved as the dominant energy source for intracardiac ablation performed by cardiac electrophysiologists worldwide. Despite being most commonly used, issues remain with RF systems for focal cardiac ablation that are commonly designed to deliver RF energy through a single, focal tip electrode. The typical catheter consists of a metal braid reinforced polymer shaft with platinum electrodes mounted near the distal end. All mounted electrodes are capable of recording electrophysiologic signals with the tip electrode also capable of delivering RF energy. Energy is delivered from the tip electrode which is intended to deliver current to the targeted tissue to produce thermal necrosis [4]. The energy return path is provided by one or more large area return electrode patches adhered to the patient's skin. This tip electrode to ground patch energy vectoring is considered to be a "unipolar" configuration. Some devices have been designed to also deliver in a "bipolar" manner, between electrodes of opposite polarities but of similar electrode areas, all located on the same delivery device. Positioning a unipolar focal energy delivery tip electrode in repeated contiguous locations to achieve a continuous ablated line of tissue inside the heart is challenging, despite the use of electromagnetic and electropotential catheter navigation systems. Such "point-by-point" ablations may result in gaps where the lesion is incomplete or sites where over-ablation extends the lesion into extra-cardiac structures, causing collateral injury [5, 6]. To overcome some of these limitations of conventional ablation technologies, a mode of ablation has been sought that minimizes collateral injury while ensuring full thickness continuous lesions in the myocardium. Irreversible electroporation (IRE) is an ablation modality that provides the potential to become that preferred technology for cardiac ablation.

HISTORICAL PERSPECTIVE: CARDIAC IRE

The first cardiac applications of IRE were performed using cardiac defibrillators to deliver single monophasic energy pulses through diagnostic catheter electrodes to the AV Node in an attempt to eliminate A-V conduction in patients with atrial fibrillation [7, 8]. The technical aspects of such energy deliveries and mechanisms of cell death were poorly understood and were considered to be producing fulguration of the targeted tissue. These deliveries were made from the distal electrodes of diagnostic catheters that were not designed for high currents. The effects of a single 200J defibrillation pulse passing from a very small surface area platinum electrode inside the heart were not fully considered. Upon energy delivery, extremely high temperatures were reached, and a vapor cloud formed around the electrode followed by an electrical arc that would pass through the layer of gas, producing a shock wave and associated barotrauma in the surrounding tissues [9]. The tissue was indeed exposed to a high gradient electric field which killed tissue through the mechanism of IRE. However, due to the physical shock wave, barotrauma, and extreme temperatures reached on the electrodes, this technique was abandoned when RF ablation systems designed for cardiac ablation became available. It was not until 2005 that the mechanism of electroporation was recognized as being responsible for defibrillation shock induced cardiac injury [10].

The application of pulsed electric fields was first proposed by Lavee and Rubinsky in 2007 for the surgical epicardial ablation of atrial cardiac tissue by IRE [11]. This preclinical work described the use of bipolar electrode tissue clamps placed over atrial tissue through which high voltage pulses could be delivered through the tissue, creating a transmural lesion. The primary drawback of this technique was the use of long monophasic pulses which were arrhythmogenic, with a risk of induction of ventricular fibrillation. In addition, monophasic deliveries

result in electrical charge deposited in the tissue that requires time to dissipate, rendering local cardiac EGM measurements impossible during the first tens of seconds post-delivery.

Medtronic first reported in 2009 research involving the use of electroporation related to cardiac ablation [12]. This work initially used a research defibrillator to produce lesions using surgical tissue clamps. That research showed that epicardial ablation tissue clamps and bipolar probes were indeed effective in making lesions that were equal to those produced using conventional thermal RF energy deliveries. It became evident during the course of this research that effective and safe pulsed electric fields (PEF) applied to cardiac tissues required the use of a custom pulse generator with the ability to tailor the pulse parameters, including delivery of biphasic pulses for this specialized cardiac application. Figure 1 shows the application of a bipolar IRE tissue ablation clamp on porcine myocardium and a well demarcated chronic lesion with sharp margins which was typical of lesions produced with this system. This initial research additionally provided chronic (longest survival period of 26 weeks) data in sheep indicating transmural fibrotic lesion formation in myocardium with preservation of nerves and blood vessels with no chronic effect on esophageal tissue or coronary arteries.

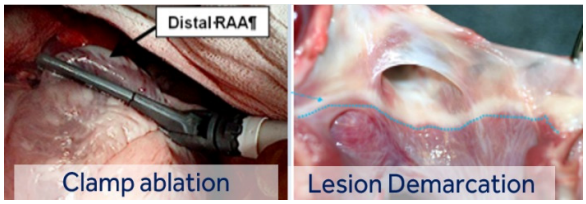

Figure 1: IRE ablation clamp (left) over a porcine right atrial appendage, and chronic lesion example (right).

During the course of surgical research using IRE, a preclinical experiment was performed in 2007 to explore the potential for intracardiac delivery of pulsed electric fields. In this experiment, an Encirclr AL-2 (Medtronic) diagnostic catheter (Figure 2) was placed in the superior vena cava (SVC) of a pig using fluoroscopic guidance. A series of increasing voltage, 200µs pulse width, trains of ten pulse applications at 50, 100, 200, 300, 400, 500, 600, 700, and 780V were delivered in a unipolar mode between a skin patch and all ten Encirclr electrodes connected as one pole. Bipolar EGM signals between each electrode pair were collected after each energy delivery. Figure 2 shows EGM measurements from two electrode pairs at baseline and after the 50, 200, and 700 volt deliveries. Local EGM spike observed on the baseline and post-50V electrograms was substantially eliminated after the 200V delivery train and was completely absent following the 700V train. This experiment was successful in demonstrating the feasibility of eliminating local EGMs using intracardiac pulsed electric fields but a significant drawback of this approach was the generalized muscle stimulation encountered during energy deliveries due to the unipolar vector from catheter to ground patch.

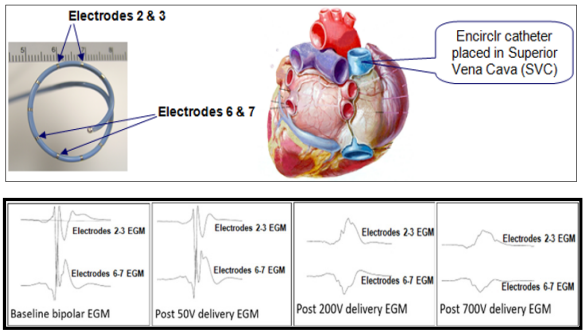

Figure 2: Early intracardiac IRE experimental results using a unipolar delivery to a multi-electrode catheter in a porcine model.

In 2011, the use of a defibrillation pulse applied for ablation therapy to isolate the pulmonary veins was demonstrated preclinically [13]. This was in many ways, a return to the old days of DC fulguration but with a greater understanding of the mechanism of ablation and the need for larger electrode surface areas to avoid the high energy densities that led to hyperthermal events. Several animal studies of these single pulse defibrillation energy deliveries were performed, showing safety in avoiding esophageal injury, coronary artery occlusion, phrenic nerve injury, and pulmonary vein stenosis. While feasibility and safety of cardiac ablation was shown through these early preclinical studies, there remained a number of issues that limited the interest in pursuing this method of delivering monophasic defibrillation pulses through the body to a return electrode patch. The unipolar delivery of direct current through the body caused a large skeletal muscle response which would require general anaesthesia and paralytic agents if used clinically. Deliveries needed to be gated to the cardiac cycle to occur during the ventricular refractory period to avoid arrhythmia induction. In addition, such high energy monophasic pulses produced large volumes of gas bubbles that embolized into the systemic arterial circulation.

CONSIDERATIONS FOR INTRACARDIAC IRE

Before considering the safety aspects of intracardiac IRE, the clinical reports of intraprocedural complications due to IRE deliveries around tumors located near the heart need to be understood. One systematic review of safety looked at cardiac related events. The findings included induction of atrial fibrillation, sinus tachycardias, and hypotension. These early reports were reporting on cardiac effects of tumor ablations that were in close proximity to the heart. A more recent study looked at the cardiac effects of such tumor ablations by monitoring for arrhythmias and measuring for high sensitivity troponins that detect myocardial injury. They determined that IRE delivered near the heart was associated with sub-clinical myocardial injury and non-sustained and non-lethal

cardiac arrhythmias. What has not been understood by the tumor ablation technical community, is that the PEF parameters used in commercial IRE systems deliver pulses that are inherently arrhythmogenic. Relatively long, 100µs pulse widths are capable of inducing action potentials in cardiomyocytes, such that they can easily induce a ventricular arrhythmia if delivered during the vulnerable period of ventricular repolarization (seen as the T-wave on a surface electrocardiogram (EKG)). A similar effect can occur in the atria during atrial repolarization where atrial fibrillation may be induced. Compounding this issue is that these IRE systems deliver monophasic pulses which increases the chance that they will induce an arrhythmia.

When delivering high voltage PEF within the heart for the purpose of ablating myocardial tissue, there are unique factors that must be considered. Most important for intracardiac ablation is the fact that energy is often delivered within the left chambers of the heart which are the source of systemic arterial blood circulation. As such, any embolic particles or gas bubbles produced as a result of catheter manipulations or energy deliveries within the left heart could travel to the brain and other critical end organs. This is a critical consideraton for cardiac ablation which other medical applications of PEF do not have to deal with. Avoidance of the creation of both bubbles and particles requires careful attention to the pulse parameters for any intracardiac energy delivery system. Energy or current densities must not result in heat generation that is sufficient to denature blood proteins. Such denatured blood proteins are known to occur with RF ablations when the tissue interface temperatures range between 50 and 80º Celcius, with denaturation happening rapidly at 80ºC while more time is required as the temperature is reduced [14]. Proteins that collect on hot electrodes may embolize and result in ischemic injury to the brain. It is imperative that heating to those levels is avoided during PEF deliveries within the left chambers of the heart. Unlike soft thrombus produced in the blood through the clotting cascade, thermally denatured proteins known as ”coagulum” will not be broken down by natural fibrinolytic enzymes so thermally generated emboli will persist with a high risk of embolic ischemic injury to end organs. This distinction between a naturally occuring ”thrombus” and thermally generated ”coagulum” must be understood.

Arrhythmogenicity of deliveries is the most apparent concern for cardiac PEF delivereis. As described previously, the pulse parameters and delivery timing used in or near the heart are critical to avoiding induction of cardiac arrhythmias. The use of biphasic pulses is an important way to minimize arrhythmogenicity. Figure 3 provides an example of ventricular fibrillation (VF) induction due to an intentional delivery of a monophasic pulse train during the T-wave in this porcine ventricle.

Deliveries of pulse trains in or near the ventricles that are timed to coincide with the ventricular repolarization phase are prone to arrhythmia induction. For this reason, gating of the PEF deliveries to occur within the ventricular refractory period as shown in Figure 4 is normal practice to minimize the potential of inducing arrhythmias.

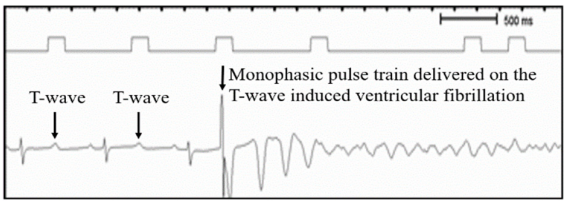

Figure 3: Delivery of a monophasic pulse train from electrodes located within the ventricle during the repolarization phase (T-wave timing) of the ventricle can induce ventricular fibrillation (porcine experiment).

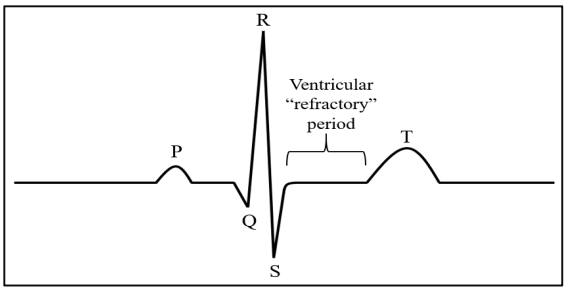

Figure 4: Ventricular refractory period noted in the EKG where gated PEF deliveries are least arrhythomgenic.

PRECLINICAL SAFETY AND EFFICACY TESTING

There are a number of important complications that occur at some frequency in RF ablation cases. The major issues include atrial-esophageal fistulas, vagus nerve injury and gastric hypomotility, pulmonary vein stenosis, phrenic nerve paralysis or palsy, hyperthermal tissue steam pops and perforations leading to cardiac tamponade, thermal injury and occlusion of coronary arteries, and cerebral embolic events. These potential safety issues have been examined with regard to cardiac PEF by preclinical research groups as summarized in a comprehensive review by Sugrue *et al.* [15]. Others, including the groups at the University of Utrecht and the Mayo Clinic have investigated the application of high voltage pulses applied to the major safety issues of concern in cardiac ablation procedures [16,17,18,19]. The objective of these studies was to demonstrate in animal models that delivery of PEF into or adjacent to the tissues known to be vulnerable to thermal injuries will not result in those injuries due to PEF. Indeed, the preclinical studies have uniformly found that PEF does not produce the injuries known to occur in patients from RF ablations. The limitation is that these studies have been performed on large animals that may have tissue responses that differ from those of humans.

Medtronic has performed similar tests evaluating the full range of potential complications from energy deliveries. In an early ovine study, differential effects on cell types within the esophagus were observed where a bipolar clamp was used to deliver PEF entirely through the esophagus, resulting in injury to the muscularis but

having no effect on the submucosal lining within the esophagus [12]. The lesions were restricted only to the muscle layer. The epithelial layer lining the esophagus and the delicate Lamina muscularis mucosae –a small rim of smooth muscle cells beneath the epithelial basal layer were observed to be without pathological changes. In contrast, the RF clamp ablations compressed the esophageal wall and destroyed the epithelial and muscular layers (Figure 5, D*), and the adventitia, see Figure 5 for the contrasting gross and histopathology images of RF and IRE lesions.

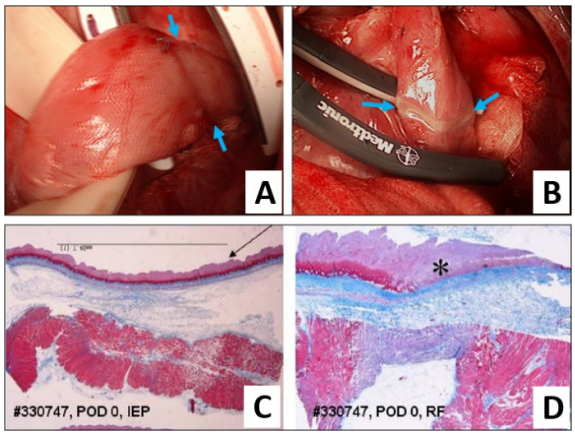

Figure 5: Gross views of the sheep esophagus immediately post-ablation using bipolar tissue clamps delivering PEF (A) and RF (B). Histology sections of these acute lesions, PEF (C) and RF (D).

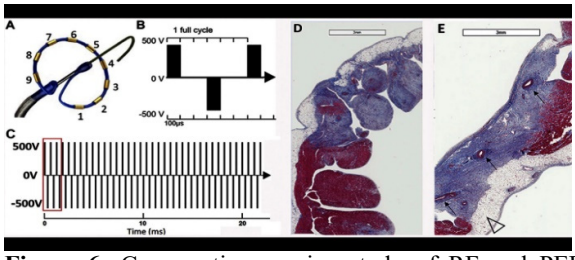

Figure 6: Comparative porcine study of RF and PEF delivery from an intracardiac 9-electrode circular catheter (A). Pulse profile and train (B, C) and histopathology images of an RF lesion (D) and PEF lesion (E).

Beyond the surgical clamp evaluations, research was performed to compare the delivery of PEF to duty-cycled RF, both delivered from the circular catheter. This porcine study was performed to directly compared intracardiac delivery of PEF to an existing duty-cycled RF technology to create myocardial lesions [20,21,22]. The intracardiac PEF deliveries were made in a purely bipolar manner from the 9-electrode circular electrode array using alternating electrode polarities (Figure 6). PEF produced targeted cardiomyocyte death, reduced electrogram amplitude, and resulted in lasting atrial lesions when delivered from the multi-electrode circular array catheter. Compared to duty cycled RF ablations, healing characteristics of PFA lesions were devoid of a thermal signature, had an absence of lingering "sequestered" cardiomyocyte groups, had more uniform replacement fibrosis, showed significantly reduced epicardial fat inflammation, resulted in less intralesional blood vessel remodeling, while both PFA and RFA deliveries were devoid of collateral damage.

HUMAN CLINICAL EXPERIENCE

Beyond the early experience with fulguration in the 1980's, the first reported human use of PEF for the treatment of cardiac arrhythmias came in 2018 [23]. The authors reported acute results for both surgical and intracardiac catheter-based deliveries of PEF to treat atrial fibrillation. Successful isolation of the pulmonary veins was achieved in 6/7 surgical patients and 15/15 intracardiac catheter ablated patients during the procedure. Surgical patients received ablations of 2100-2400 V PEF delivered systematically between specific pairs of electrodes on a 30-electrode surgical catheter. The intracardiac patients received ablation deliveries of 900-1000 V from a five-arm catheter that was placed in each of the pulmonary vein ostia to produce electrical isolation of the veins. Succinylcholine was administered during the intracardiac catheter procedures to supress skeletal muscle stimulation.

One year later, further results were presented that indicated a lack of long-term PV isolation (18% isolated chronically) in the initial intracardiac catheter ablated patients [24]. Those patients with limited procedural success had received Protocol-A that consisted of monophasic 900 V PEF deliveries. Additional results were presented on clinical testing of three additional pulse trains on patients using Protocols B, C, and D. These 1800 or 2000 V deliveries used biphasic waveforms, with differences in the way the catheter electrode arms were deployed and positioned. Protocol-D produced 100% PV isolation acutely that was verified in remap studies after three months.

The group at the University of Utrecht reported on their first ten atrial fibrillation patients treated by delivery of intracardiac defibrillation shocks [25]. The procedure involved placement of a 14-electrode circular array catheter in each of the pulmonary vein ostia, where an average of 2.4 defibrillation pulses (6ms duration) were delivered per vein to achieve isolation. The shocks averaged 2154 ±59 peak voltage with mean current of 33.9 ±1.6 A with current passing from the catheter electrodes to a dispersive energy return patch on the patient. No acute recurrences of conduction were observed during the procedures but in two patients, ST-segment elevation was observed immediately after pulses. This transient cardiac ischemia was possibly caused by embolic microbubbles from the monophasic energy deliveries.

CONCLUSION

Preclinical studies and the limited human clinical experiences all indicate that pulsed electric fields have the potential to eliminate thermally mediated complications while improving the efficacy of intracardiac ablations of cardiac arrhythmias. The modes of delivering IRE using

PEF vary considerably in the published reports but all show similar promise of improved safety and efficacy.

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NOTES

NOTES

SHORT PRESENTATIONS

Determining the initial degree of anisotropy of porcine skeletal muscle tissue using the dynamic model of electroporation *ex vivo*

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INTRODUCTION

Skeletal muscles are the most widely used tissue for gene electrotransfer due to their large size and ease of access [1]. Electrical conductivity of skeletal muscles exhibits a high degree of anisotropy, i.e. the electric conductivity in the direction of the muscle fibres is considerably higher than the electric conductivity in the direction perpendicular to the muscle fibres [2]. For successful numerical modelling that can be used for optimisation of electric pulse parameters and the geometry of the electrodes, electric properties of the skeletal muscles have to be known.

In the present study, an attempt was made to determine the degree of initial (i.e. pre-treatment) anisotropy of porcine skeletal muscle tissue (*Longissimus dorsi*) by using *ex vivo* measurements of the electric voltage and current in the aforementioned tissue, combined with numerical modelling.

MATERIALS AND METHODS

Measurements of electric voltage and current during electroporation pulse delivery were performed *ex vivo* in the porcine back muscle (*Longissimus dorsi*). Ten pairs of measurements were performed with applied voltages between 60 V and 225 V. One pair of measurements consists of two measurements with the same applied voltage and different orientations of the electrodes with respect to the muscle fibres: the first orientation, whereby the electrodes are inserted in such a way that the direction of the applied electric field is the same as the direction of the muscle fibres, and the second, whereby the direction of the applied electric field is perpendicular to the direction of the muscle fibres. For each measurement we delivered a 100 µs long pulse and recorded electric voltage and current. We used two parallel needle electrodes with an active length 20 mm and diameter 0.7 mm. Distance between the electrodes was 4 mm.

For numerical modelling we used the recently developed dynamic finite-element model that can be used for efficient modelling of time evolution of electric current during delivery of electroporation pulses [3]. We defined and used the parameter AR_{xy}^0 as a measure of anisotropy in the tissue, it is defined as:

$$AR_{xy}^0 = \sigma_{xx}^0/\sigma_{yy}^0. \quad (1)$$

AR_{xy}^0 is the ratio of anisotropy of the initial electrical conductivity of non-electroporated tissue, σ_{xx}^0 and σ_{yy}^0 are the initial values of conductivity in the direction of muscle fibres, and in the direction perpendicular to the direction of muscle fibres (and also perpendicular to the direction of the electrodes in the tissue), respectively. With model simulation we calculated curves relating the normalized expected electrical current to the electrode-to-muscle fibre orientation for different values of the ratio of anisotropy, and for different applied voltages. We then used these curves in relation with the performed *ex vivo* measurements in order to assess the value of AR_{xy}^0 for each pair of measurements.

RESULTS AND DISCUSSION

Values of the parameter AR_{xy}^0 as determined using the procedure described above are shown in Figure 1 for each pair of measurements. The geometric mean of all determined values for the parameter AR_{xy}^0 equals 1.15, which indicates that, on average, the initial conductivity in the direction of the muscle fibres is indeed higher than in the direction perpendicular to the muscle fibres, as was expected, but the dispersion of the values is too high for the mean to be accepted as reliable.

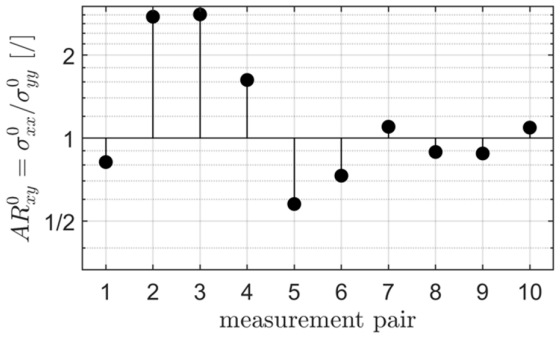

Figure 1: Simulation-based estimates for the parameter AR_{xy}^0 for ten pairs of *ex vivo* measurements.

For more reliable and repeatable results it would be necessary to ensure a high accuracy of electrode insertion into the tissue, then attempt to measure the actual orientation of electrodes with respect to the muscle fibres accurately, and, if possible, perform the measurements *in vivo*.

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Numerical investigation of electroporation induced by a He and He-O_2 (1000 ppm) plasma jets on healthy and cancerous skin tissues

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INTRODUCTION

Electroporation is a widely used method in applications such as electrochemotherapy, gene therapy, DNA transfer etc. Electroporation is typically achieved through conductive wires. In this abstract we describe an alternative and non-contact method of achieving electroporation, i.e. through the use of atmospheric pressure plasma jets (APPJs). APPJs have gained tremendous attention in recent years due to low production costs, easy implementation and handling and applications ranging from surface modification, plasma medicine, sterilization etc. In a recent study of Helium APPJ it was found that the addition of oxygen admixtures increased its effectiveness in cancer treatment. This effectiveness was attributed to reactive oxygen and nitrogen species (RONS) produced by the APPJ. However, a recent simulation study from our team showed that the oxygen admixtures increase the strength of the induced electric field (IEF) on the treated surface [1]. We propose that this IEF increases the cell membrane permeability, and thus helps molecules penetrate into the cells. So, it can be inferred that the effectiveness of APPJs lies in the combination of the two effects (high amount of RONS and high IEFs on the treated surface). In this abstract we will study the distribution of IEF from both pure He and He-O_2 APPJ in healthy and cancerous skin. We will show how APPJ can cause electroporation to living tissue in a non-contact method and will show how the addition of oxygen admixtures can increase electroporation.

THEORY AND METHODOLOGY

In this study the interaction of a He and He-O_2 (1000 ppm) APPJ with healthy and cancerous skin tissues placed normal to the jet axis are investigated. The geometry and operational parameters of the plasma jet device and of the simulation model are described in detail in [1]. The healthy and cancerous skin tissue considered here (geometry and electrical characteristics) is similar to those described in [2], [3]. Specifically, the healthy tissue is composed of the stratum corneum, epidermis, dermis, adipose tissue, muscle and subcutaneous tissue. When the tissue is affected by cancer, a tumour is placed in the layer of dermis. The numerical simulations for determining the electric field distribution inside the target tissue are done in two steps. In the first step the plasma model is used to determine the voltage on the surface of the tissue. In the second step that voltage is used to determine the electric field distribution inside the tissue (solving eq. 1 from [2]).

RESULTS AND FIGURES

The induced voltage on the surface of the stratum corneum layer for He-O_2 (1000 ppm) plasma jet is presented in Figure . This is the first step from our methodology. In the second step we will determine the field distribution across the various skin layers. We can assume that in the regions where the field is higher than 400 V/cm there will be electroporation.

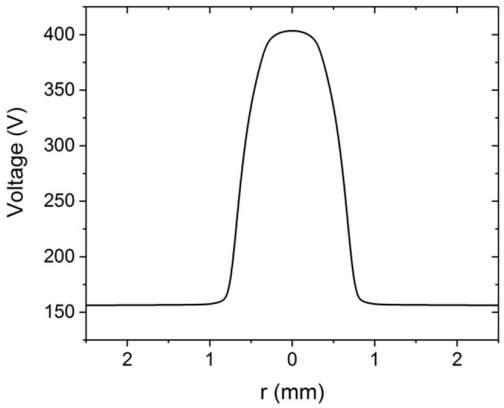

Figure 1: Induced voltage on a healthy skin tissue by a He-O2 (1000 ppm) plasma jet device.

ACKNOWLEDGMENT

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Development of flexible organic microelectrodes for the detection and treatment of brain cancer - Calibration with an optogenetic engineered cell line

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INTRODUCTION

Glioblastoma is one of the most resistant tumor to therapies and leaves patients with a poor prognosis. Use of new emerging technologies based on bioelectronics seems to be a promising way for the detection and treatment of cancer. Therefore, we want to investigate whether we can use home-made, flexible and biocompatible organic microelectrodes for the detection of electrophysiological activity in brain tumors, since they are known to express voltage-gated ion channels and exhibit electrical activity [1].

In this work, we set up a cellular model of glioblastoma based on the avian chorioallantoic membrane (CAM) model [2]. In the same time, we developed and characterized electrical biosensors to detect weak electrophysiological signals on this model.

IN OVO CELLULAR MODEL OF GLIOBLASTOMA

We set up an *in ovo* model of glioblastoma based on the CAM model. The avian CAM is a highly vascularized and transparent extra-embryonic membrane, which performs multiple functions during embryonic development including gas exchange.

We grew Mouse glioma 261 (Gl261) cells in three dimensions to form spheroids. This model exhibits many features of an avascular tumor, especially the existence of a hypoxic and necrotic core due to the limited diffusion of oxygen to the inner part. The grafting of the spheroid in shell-less quail egg CAM induces angiogenesis, which corresponds to the growth of new blood vessels from the existing vasculature of the embryo. Neovessels supply the spheroid, which leads to the disappearance of the hypoxic core and the growth of the tumor. We obtained a relevant *intravital* model of glioblastoma, which is more robust and complex than *in vitro* model.

BIOSENSORS

In the same time, we developed and characterized flexible and biocompatible Multi Electrodes Arrays (MEAs) and Organic ElectroChemical Transistors (OECTS) for the detection of weak electrophysiological signals around the tumors [3]. In order to test the sensing devices, we used Optopatch Spiking HEK cells (OS-HEK) [4]. These genetically modified cells produce an action potential triggered by blue light. We characterized the electrical properties of the cells by patch clamp measurements and ensured that we are able to induce action potentials in a reproducible and reliable way with the exposure of blue light (Figure 1).

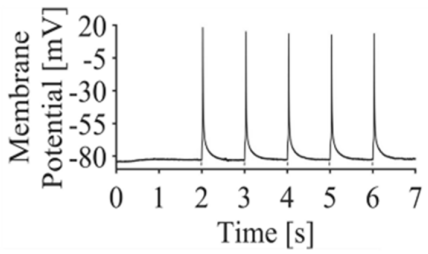

Figure 1: Patch clamp measurements of action potentials. Repeatability of action potentials: 5 pulses of 1 ms at λ_{exc} = 500 nm with a frequency of 1 Hz.

PERSPECTIVES

In the future, we want to investigate whether we can measure electrophysiological signals of brain tumor using the *in ovo* model we developed. First, the OS-HEK cells will be used on MEAs and OECTSs to assess the ability of those devices to detect weak electrophysiological signals. Then, the sensing devices will be applied on our *intravital* model of glioblastoma before going towards *in vivo* experiments.

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Effect of calcium ions on liposomes as cell membrane analogues

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INTRODUCTION

Calcium electroporation is a new experimental anti-cancer treatment, internalizing calcium into cells by application of short, high voltage pulses. The anti-tumour effectiveness of this approach has been demonstrated *in vitro*, *in vivo* and in a clinical study [1]. However, effects of calcium ions on biological cells have not been fully elucidated.

Physical processes and molecular-scale mechanisms involved in addition of cations, such as calcium ions, to the cell membrane can be studied using liposomes. Liposomes are spherical vesicles consisting of lipid molecules. Its properties, such as size, differ with the lipid composition and the method of preparation. Furthermore, chemical modifications can occur that induce changes in the liposome particle size.

Aim of the study was to determine the effects of calcium ion addition on structural rearrangements and size changes of liposomes as cell membrane analogues and gain better insight on how calcium electroporation increases the anti-tumour effectiveness of the treatment.

MATERIALS AND METHODS

The lipids L-α-phosphatidylcholine, hydrogenated (Soy) (HSPC),1,2-dioctadecanoyl-sn-glycero-3-phospho-ethanolamine (18:0 PE, DSPE) and cholesterol were from Avanti Polar Lipids (Alabaster, AL, USA). Liposomes from HSPC:DSPE:cholesterol (4:3:3 molar ratio) were prepared from dried thin lipid films via hydration using ammonium acetate (NH_4OAc), 40 mM, pH 8.4.

With a nano-electrospray gas phase molecular mobility analyser (nES GEMMA), we are able to obtain a size-distribution analysis and determination of particle-number concentrations Gas-phase electrophoresis [2] was carried out on a TSI Inc. instrument (Shoreview, MN, USA): A nES aerosol generator equipped with a ^{210}Po α-particle source, a nano differential mobility analyser and a butanol-based ultrafine condensation particle counter were applied.

Analysis of obtained number based particle concentrations was carried out using Matlab 2017b. Obtained particle counts were filtered using a zero phase Butterworth filter to ensure a smooth graph, which would enable us to determine the peak apex of the particle size distribution.

RESULTS

Liposomes with a diameter of approximately 76.6 nm were prepared. Calcium chloride (Sigma Aldrich) in four different concentrations (0 mM, 10 mM, 20 mM and 40 mM) was added to the prepared liposome solution; consequently, calcium ions affected the external environment of the prepared liposomes. With the addition of calcium ions, the size of the liposomes did shift to lower diameter values as can be seen on the figure below.

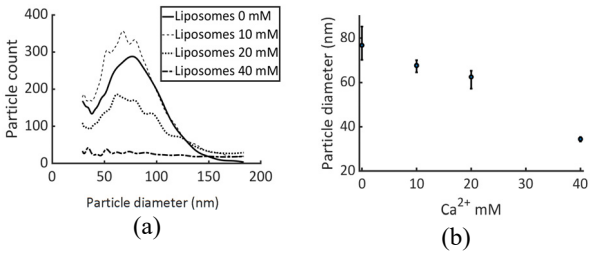

Figure 1: (a) Sample particle size distribution with the graph apex representing liposome peaks. (b) Decrease in liposome particle diameter with respect to concentration of calcium solution added to the liposome sample.

The apex of the liposome particle peak presented on the figure (1a) above shifts to lower particle diameter values with increasing calcium concentration. Figure (1b) represents the liposome particle size decrease in dependence of increasing calcium concentration. Therefore, calcium cations induce changes to the lipid molecules causing the liposome particles to decrease in size. With higher calcium concentrations, the lipid vesicles ruptured, as evidenced by the lack of liposome peak (figure 1a).

CONCLUSION

In our study, we were able to observe and quantify the changes occurring in the lipid structures, such as liposomes, induced by the addition of calcium ions. We were able to demonstrate that with an increasing concentration of calcium ions the liposome vesicles decrease in size.

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Comparison of Electroporation Threshold between KcsA Membrane Protein System and Bare Lipid Bilayer with Applied Picosecond Pulsed Electric Field

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INTRODUCTION

Electroporation is a process in which permeabilizing structures appear in cell membrane due to applied electric fields. In recent years, it was discovered that the subnanosecond and picosecond pulsed electric field (psPEF) could affect the mitochondria and induce cell apoptosis based on the electroporation. The psPEF may thus become a new tool in the field of tumour therapy.

The membrane is a complex system of lipid molecules and proteins. Protein is an important component of cell membrane. Among them, KcsA protein is a very common and critical protein and belongs to the potassium channel family, which is of great research significance.

In this article, the electroporation thresholds of the KcsA system and bare system with applied psPEF are calculated. Additionally, the results of large system and small one is also compared. Our findings may provide the theoretical guidance for the application of psPEF in the tumour therapy.

SIMULATION MODEL AND PARAMETERS

The large KcsA membrane protein system and the large bare system are shown in Fig. 1. The two large models are 14.3 nm × 14.3 nm × 8.9 nm in size. And the size of the two small models is 7.7 nm × 7.7 nm × 8.9 nm. The waveform of psPEF is shown in Fig. 2.

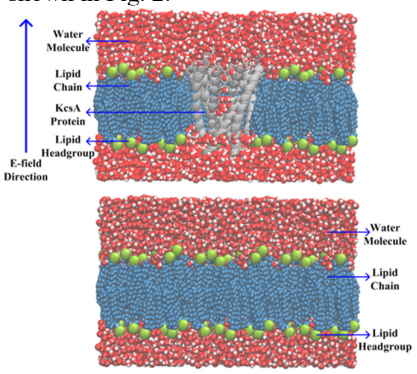

Figure 1: The large KcsA membrane protein system (figure above) and large bare lipid bilayer system (figure below)

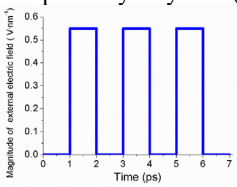

Figure 2: The waveform of 0.55 V/nm picosecond pulsed electric field with the repetition frequency 0.5 THz

RESULTS AND DISCUSSIONS

It is shown from Fig. 3 that the electroporation threshold of the large KcsA system is larger than that of the bare one with the applied psPEF, which is consistent with the result of constant electric field. While on the contrary, electroporation threshold of the small bare system is larger than that of the small KcsA one with the applied psPEF.

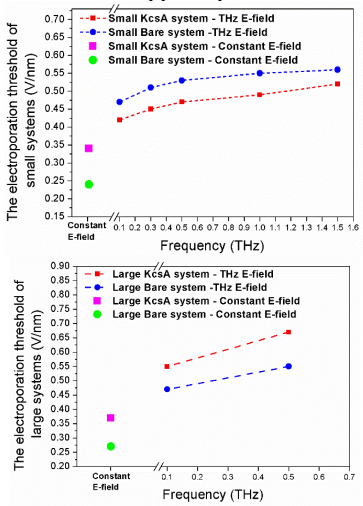

Figure 3: The correlation between the psPEF repetition frequency and the electroporation threshold. The above one is small system and below one corresponds to large system

This phenomenon may be related to the protein fluctuation with the applied psPEF. It has been found that, the protein fluctuation of small system increases a lot with the applied psPEF. However, when the model size increases the model is more reliable and the protein becomes more stable. Then the result of psPEF is consistent with that of constant electric field.

The unstable protein of small system results in the perturbation of lipid molecule and interfacial water with the applied psPEF, and then affects the water protrusion appearance time. Accordingly, the electroporation threshold of the small KcsA one becomes smaller than that of small bare one with applied psPEF.

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Using electroporation to influence cellular processes through calcium oscillation modulation in Mesenchymal Stem Cells

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INTRODUCTION

Cytosolic calcium concentration is known to regulate a high number of cellular processes such as apoptosis, cell proliferation and differentiation [1].Through the action of different cellular effectors, cytosolic calcium concentration can vary in the form of oscillations which can exhibit different amplitudes, durations and frequencies. These parameters of the calcium signal encode important information for cells [2]. Calcium oscillations naturally occur in Mesenchymal Stem Cells (MSCs) [3], which have high potential in regenerative therapies due to their ability to differentiate into an important number of cell types [4]. The frequency of these oscillations can vary over the course of various cellular processes. Furthermore, *de Menorval et al*. showed that calcium oscillations similar to the natural ones in shape and duration can be reproduced in MSCs through electroporation using µsPEFs (microsecond pulsed electric fields) or nsPEFs (nanosecond pulsed electric field) [5]. *Hanna et al.* showed that these PEFs (Pulsed electric fields) are able to permeabilize both outer and inner cell membranes and subsequently release calcium to the cytosol from both extracellular and intracellular storages [6,7].

AIM OF THE FUTURE WORK

Since calcium oscillations similar to the natural ones occurring in MSCs can be reproduced using µsPEFs or nsPEFs, our aim would be to exploit the potential of using these PEFs in mild conditions as a tool to manipulate calcium oscillations, using non-lethal concentrations of external calcium, to try to influence various cellular processes occurring in MSCs.

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Influence of pulsed electric field (PEF) and moderate electric field (MEF) protocols on the reversible permeabilization of Thai basil leaves.

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INTRODUCTION

Reversible electroporation of guard cells could provide benefits for the drying of plant leaves. Electroporated guard cells provoke stomata to remain open during the drying process resulting in faster drying rate and better dried product quality such as colour, aroma, and rehydration capacity [1]. There is the need for establishing electroporation protocols that would provoke permeabilization of the leaves and at the same time specifically target the guard cells. In this study, different protocols for two electroporation techniques, pulse electric field (PEF) and moderate electric field (MEF) were tested for the reversible electroporation of Thai basil leaves with guard cells permeabilization.

TREATMENTS AND ANALYSIS

PEF (squared pulse) parameters tested including voltage (100-650 V/cm), pulse width (50-1000 µs), pulse space (380-1520 µs) and number of pulses (0-1500 pulses). MEF (sinusoidal AC) parameters tested including voltage (100-600 V/cm), frequency (60-1200 Hz) and treatment time (0-13000 ms). The decrease of bulk electric resistance of the leaves after electroporation was used to determine the efficiency of the protocols for the electroporation of the bulk of the tissues in the leaves. The electroporation of epidermal cells and guard cells on the leaves surface was investigated under the microscope using propidium iodide (PI) as permeabilization indicator. The viability of the tissues was observed using fluorescein diacetate (FDA) as vital staining [2].

RESULTS

As the electroporation of the bulk of the tissues in the leaves progressed, electroporation of the epidermal cells increased by increasing the voltage, pulse width and number of pulses of the PEF treatment and the voltage and treatment time of the MEF treatment. The electroporation of the guard cells required higher PEF voltage, pulse width, and number of pulses compared to the electroporation of epidermal cells. Most of the guard cells were electroporated with different combinations of PEF parameters. Two PEF protocols were found electroporated most guard cells on Thai basil leaves (**PEF1** and **PEF2, Table 1**).# he stablished PEF protocols in this study can be used to electroporate the guard cells of Thai basil leaves while maintaining the viability of the leaves.

MEF protocols showed to electroporate the bulk of the tissues in the leaves and complete permeabilization of the epidermal cells, however, no MEF protocol was found to electroporate the guard cells of stomata.#

Table 1: Electroporation parameters of pre-drying electroporation protocols

Protocols	V/cm	Pulse width (µs)	Pulse space (µs)	Number of pulses	Frequency (Hz)	Treatment time (ms)
PEF1	650	50	760	300	-	-
PEF2	650	175	760	125	-	-
PEF3	650	50	760	150	-	-
MEF	100	-	-	-	1200	1200

The drying time of the guard cells electroporated samples decreased by approximately 60% (**Figure 1**) compared to untreated and the samples with only reversible epidermal cells electroporation (**PEF3** and **MEF, Table 1**).

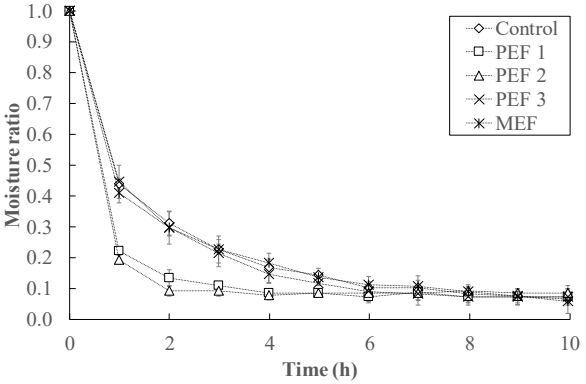

Figure 1: Drying characteristic curves of Thai basil leaves samples treated with different PEF and MEF protocols.

CONCLUSIONS

PEF and MEF protocols could be used to electroporate the epidermal cells on the surface of Thai basil leaves homogeneously. Two PEF protocols stablished in this paper could be used to electroporate the guard cells of Thai basil leaves. Compared to epidermal cells, the electroporation of guard cells required higher voltage, pulse width, and number of pulses. No guard cells electroporation was found in the samples using MEF protocols. Guard cells electroporation could be used to decrease the drying time of Thai basil leaves.

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The relationship between cell viability and the extraction of intracellular molecules after electroporation

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INTRODUCTION

The temporary increase of cell membrane permeability, termed electroporation, has been investigated for several decades [1]. One of the most recent applications of electroporation is irreversible electroporation, a method of non-thermal tumour ablation that is becoming increasingly popular, especially for the treatment of tumours located near major veins or ducts [2]. However, this treatment method is restricted by the lack of specific knowledge about the mode and dynamics of cell death and its dependence on the parameters of electric pulses used.

One of the factors that may play a role in cell death post electroporation treatment is the loss of intracellular compounds due to electroporation induced nonspecific leakiness of the membrane. One of these compounds is ATP, a small molecule which is involved in a large number of metabolic and signalling pathways [3]. Therefore, we investigated the ATP loss from electroporated cells and the cell viability in a number of different electric pulse combinations in attempt to elucidate the role of intracellular ATP decrease on the survivability of the cells.

MATERIALS AND METHODS

Experiments were performed using CHO cells. The cells were loaded with calcein by 1 h incubation with calcein-AM. Cells were electroporated with BTX T820 electroporator. 1, 5 or 9 HV pulses (0.6–4.2 kV/cm, 100 μs, 1 Hz) were delivered. The calcein leakage from the cells after electroporation were determined using flow cytometry (BD Accuri C6), and the ATP in supernatant was measured using a commercial kit. The cell viability was measured 6 days after treatment by clonogenic assay. Results are presented as average ± SEM. Statistical significance was tested by Student's t-test for independent samples.

RESULTS AND DISCUSSION

To investigate the dependence of intracellular molecule loss from electroporated cells, we decided to measure the leakage of calcein from calcein-AM loaded cells as the model of artificially introduced small molecule, and the leakage of ATP as the model of a small molecule naturally occurring in the cell. The dependence of the release of both these molecules was similar, increasing with both the higher number of electric pulses and higher electric field strength.

The next step was to investigate the relationship between the electric field parameters and the cell viability after the treatment with the same electric pulses as the ones used in our extraction experiments. The results for these experiments are presented in Figure 1. They show that increasing the number and/or the strength of the electric pulses has a negative impact on cell viability. 5 pulses with 3.0 kV/cm strength or 9 pulses with 2.4 kV/cm strength were enough to kill all the cells in the sample.

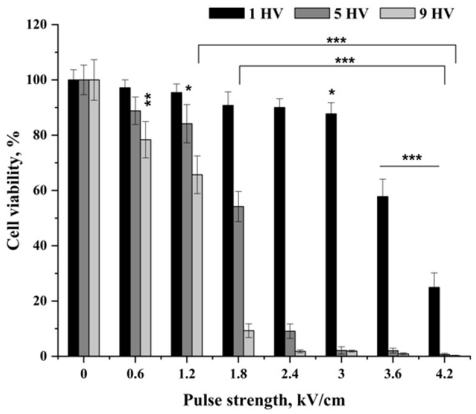

Figure 1: The dependence of the cell viability on the number and strength of electric pulses. * note statistically significant differences from the control (0 kV/cm). * means two-tailed $p < 0.05$, ** – $p < 0.01$, *** – $p < 0.001$.

Our results also show that the same amount of ATP release can be achieved with different electric pulse combinations that lead to different cell viability, although the conditions that cause a higher ATP release also cause a stronger decrease in cell viability.

CONCLUSIONS

Our results show no direct correlation between the ATP leakage from the electroporated cells and the loss of cell viability in the range of tested electric field parameters.

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Pulsed Electric Fields (PEF) to enhance the desalting of cod

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INTRODUCTION

Salted cod is a highly appreciated product, traditionally imported by Mediterranean countries and commercialised with different moisture content depending on the extension of the drying process. Prior to consumption, the fish must be desalted, and this step could last up to 5 days. This process is usually carried out immersing the product in stagnant water, resulting not only in the loss of salt but also in sample rehydration [1].

Market trends evolve towards ready-to-use products; thus, the cod industry should adapt to consumer requirements including the desalting step among the industrial operations. However, the industrial-scale cod desalting presents many problems mainly linked to long processing times and the quality of the final product. For this reason, many researchers have focused on finding new desalting methods to improve the mass transfer processes, such as the use of vacuum pulses [2], high pressures [3] or high-intensity ultrasounds [4].

The application of pulsed electric fields (PEF) has been proposed as an alternative method to enhance the mass transfer phenomena in many food processes. However, there is no previous literature on the use of PEF to improve the desalting of foods. Therefore, the aim of this work was to evaluate the possibility of applying PEF treatment in cod desalting from the study of mass transport kinetics.

MATERIALS AND METHODS

Salted cod (*G. morhua*) fillets were supplied by a local importer, and prior to desalting experiments, they were manually cut in cubic-shape pieces (2x2x2cm), obtained from the upper part of the fillet, and kept refrigerated at 4 ± 1 °C.

PEF pre-treatments of cod samples were performed using a lab-scale PEF unit (Mod. SP7500, Alintel, Italy), and providing monopolar rectangular-shape pulses at two different current intensities of 10A ($PEF_{(1)}$) and 20A ($PEF_{(2)}$).

The desalting process was carried out in cold tap water (5 ± 0.5 °C) using a ratio of cod:water of 1:10 (w/v).

Weight gain, water activity†, NaCl and water content† were determined at 0, 4, 6, 24, 48, 72, 96,120 and 144 h of the desalting process. Five cod samples were used at each sampling time. The changes in the water and cod electrical conductivity† were also tested throughout the desalting experiments. Changes in water electrical conductivity were registered using a conductimeter (Mod. Basic 30, Crison, Spain), while the electrical conductivity of samples was determined using a precision impedance meter (Mod. LCR-8105G, GW Instek, Taiwan) connected to a needle-probe with a 2 mm gap between electrodes.

RESULTS AND FIGURES

Total weight changes (ΔM^o_t) determined as shown in Eq. 1 (being M^o_t and M^o_0 the cod weight at the sampling time t and 0, respectively) can be observed in Fig. 1.

$$\Delta M^o_t = (M^o_t - M^o_0) / M^o_0 \quad (1)$$

These results show that the application of PEF significantly accelerated the desalting kinetic of samples, although no differences appeared between the two PEF intensities applied.

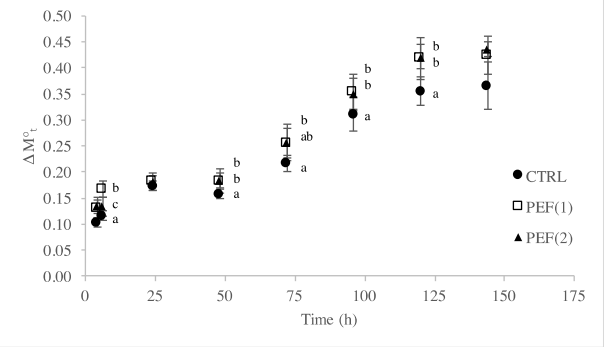

Figure 1: Total weight changes (ΔM^0_t) of untreated (CTRL) and treated samples ($PEF_{(1)}$-$PEF_{(2)}$) throughout the desalting process. Different letters indicate significant differences between the groups ($p<0.05$) (one-way ANOVA).

This preliminary study showed that the application of pulsed electric fields should be investigated further as an alternative method in cod desalting process to decrease this time-intensive industrial operation.

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Application of Electroporation in Plant Biotechnology

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INTRODUCTION

Electroporation is the increase of cell membrane permeability due to externally applied pulsed electric fields. Although observation of the effects of pulsed electric fields on biological material dates back more than 250 years, only in the past two decades have practical applications of electroporation emerged in food processing, pharmaceutics, and medicine [1]. In plant biotechnology, it can be used for electrostimulation or for electrotransformation. Gene electrotransfer, or electroporation-based DNA transfer, is an efficient and simple means for transfecting plant tissues [2]. Applying an electric field to cells induce temporary pores in the cell membrane, allowing the cell to take up DNA, RNA, drugs or other chemicals [3]. Since electrotransfection has proved its suitability for transferring DNA, RNA and proteins, it is potentially useful for the uptake of ribonucleoproteins complexes (RNPs). Electroporation is also being applied in plant biotechnology for the uptake of plant viruses and reporter dyes, and the successful electroporation of intact cells suggests a possible approach for the transformation of species that are difficult to regenerate from protoplasts and microspores [4]. Transforming plants by microspore electroporation could bypass some of the problems associated with current procedures. Upon release from the tetrad, the microspore is uninucleate and thin walled. A microspore is, at this stage, potentially more amenable to transformation than other plant cells. In addition, microspore development can be altered *in vitro* to produce either haploid embryos or embryogenic callus that can be regenerated into plants. Thus, transformed microspores could be regenerated directly into haploid plants or doubled haploid fertile plants upon chromosome doubling [5].

AIM OF THE STUDY

The aim of the research is to develop a method for the uptake of ribonucleoproteins (RNPs) complexes, composed of sgRNAs and Cas9 proteins, in microspores (immature pollen) of *Brassica napus* L. by electroporation. This will enable targeted mutagenesis without introducing foreign DNA into the plant genome.

MATERIAL AND METHODS

The first steps of the experiment are to establish optimal conditions for the cultivation of rapeseed plants, to optimize the isolation of microspores and regeneration of (doubled) haploid embryos and plants. For the method of RNPs introduction with electroporation, it is crucial to determine the optimal electroporation parameters i. e. number and type of pulses, pulses strengths and duration. In the study, the electroporator Gene Pulser Xcell™ Electroporation System (Bio Rad) will be used. We will target the vernalization determinant FRIGIDA (FRI) gene. Many plants, which are grown in temperate climates, require vernalization and must experience a period of low winter temperature to initiate or accelerate the flowering process. This ensures that reproductive development and seed production occurs in spring and winters. If the vernalization determined gene is removed, it would be possible to produce more plants in a short period (e. g. two cycles in one year).

EXPECTED RESULTS

Using optimized electroporation conditions, we will be able to introduce different RNPs to induce target mutations in different exons of the FRI gene in oilseed rape microspores. The presence of the mutation will be confirmed by next generation sequencing and by analyzing the sequences with CRISPR RGEN Tools Cas-Analyzer software.

We believe that by optimizing the electroporation parameters, we will manage to introduce RNPs into rapeseed microspores and to regenerate mutated doubled haploid plants.

ACKNOWLEDGEMENTS

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Investigation of promoter methylation pattern following cell electroporation and calcium influence on efficiency of nucleic acids electrotransfer

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INTRODUCTION

Genome methylation is known as one of epigenetic factors that can influence gene expression. For example, methylation of promoter sequences as a rule downregulates gene transcription. Whether, electroporation, an efficient method for intracellular delivery of molecules that are cell membrane impermeant due to their size or charge [1] can modify genome methylation is largely unknown. Therefore, we plan to evaluate genome methylation after cell electroporation. Some studies, already have shown that promoters DNA methylation can have significant influence on transformation efficiency [2].

Other studies have also shown tendency of increased electrotransfer to the cells when nucleic acids (DNA, miRNA, sgRNA) are present in external medium [3]. Similarly, our group has recently shown that presence of extracellular DNA can enhance bidirectional membrane transport of small impermeant molecules [4]. This effect is an object of further investigations.

PROMOTER METHYLATION ROLE

Methylation has a critical role in transcriptional regulation by directly affecting gene's activity (Fig. 1A-B).

To our knowledge, there is no research that would show promoter methylation status pattern after the cell/tissue is electroporated. Studies revealed that the stressful event evoked the demethylation of distinct CpGs within the promoter [5]. Considering electroporation as a stressful event, hypothesized effect of electroporation on promoter methylation status provided in Figure 1C.

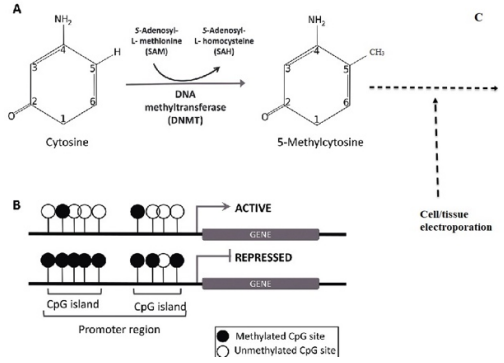

Figure 1. Promoter methylation status regulation. (**A)** Process is catalyzed by DNMTS that results in formation of 5mC. (**B)** 5mC is transcriptionally inactive and promotes suppression of gene function. (**C)** Cell stress induced by electroporation could lead to aberrant demethylation.

NUCLEIC ACIDS ELECTROPORATION

Due to the many effects of calcium, the intracellular calcium concentration is tightly regulated. Drastically increased ion's concentration can lead to cell death by induction of protease and lipase activity and reduction of functional activity of mitochondria. What is more, it was shown that calcium concentration in the cell effects DNA and RNA electrotransfer to the cell [6]. However, this connection is not fully understood yet and further analysis is crucial. In addition to that, our preliminary studies have shown that presence of calcium ions decrease efficiency of electrotransfer of small molecules, like propidium iodide. Therefore, study is planned to investigate the role of calcium ions on efficiency of nucleic acid electrotransfer.

MATERIAL AND METHODS

CHO and cancer line cells will be used in experiments. Cell treatment by electric pulses will be performed in HEPES based media with different CaCl2 concentrations. Transfection efficiency will be evaluated by flow cytometry. Promoter methylation studies workflow provided below.

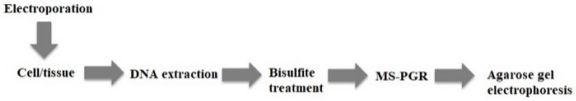

Figure 2. Schematic course of promoter methylation study.

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Electroporation does not affect directly human dermal fibroblasts proliferation and migration properties but indirectly

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INTRODUCTION

When cell is exposed to an external pulsed electric field, plasma membrane transiently becomes permeable for ions, drugs and molecules as large as plasmid DNA, otherwise impermeable. This physical phenomenon of cell electropermeabilization named "electroporation" is localized in space and time [1]. Electroporation is used in medicine to introduce therapeutic plasmid in gene electrotransfer (GET) and to deliver antitumor drug by electrochemotherapy (ECT). Interestingly, clinicians and patients commonly observed aesthetic and functional wound healing of the tumor sites treated with ECT [2]. It has to be known that in an intact epidermis, the active segregation of Na+ ions creates a sodium gradient from the basal layer of the epidermis to the upper layer : the transepithelial potential (TEP) [3]. Depending on the body part, this TEP is estimated between 10 to 50 mV. After skin damaging the break of the TEP leads to the creation of an endogenous electrical field at the margins of the wound [4]. This endogenous electric field seems to display some major effects on cells *in vitro* and *in vivo* [5]. Electrostimulation is an emerging technology inspired by the body's natural electrical healing process that directs cell migration and re-epithelialization. In this study, we hypothesized that ECT and GET electric parameters could stimulate cutaneous cell migration and proliferation and thus improve the quality of healing after ECT of GET treatment.

METHODS

Primary human dermal fibroblasts were grown in monolayer and submitted to ECT (8 pulses of 100 µs, 1 Hz, 200 to 800 V/cm) and GET (10 pulses of 5 ms, 1 Hz, 50 to 300 V/cm) electric field. Videomicroscopy approach was used to monitor and quantify cell survival, proliferation and migration in calibrated scratch wound assay.

RESULTS

Neither galvanotaxis nor statistical modification of fibroblasts migration were observed in calibrated scratch wound assay after ECT and GET application (Figure 1). Proliferation properties were not modified by almost all the ECT and GET electric parameters conditions, except for the GET strongest one which drastically reduced fibroblasts cell number through induction of mitochondrial stress and apoptosis. Finally, we found out that 24h-conditioned media from fibroblasts submitted to GET electrical parameters (200 V/cm) tended to increase migration properties of cells unexposed to electric field, probably through modulation of growth factors secretion.

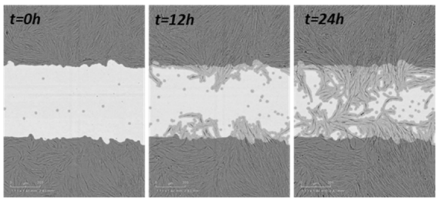

Figure 1: Human dermal fibroblasts migrations properties was assessed by videomicroscopy in calibrated scratch wound assay after ECT and GET electric parameters application

PERSPECTIVES

Concerning growth factor synthesis, further experiments would be needed to elucidate what kind of factors are exactly secreted and/or released, including experiments at protein level such as ELISA

To go further, the effect of electric fields will be studied in a 3D tissue models produced by tissue engineering, as the dermal cell sheets. Using these models rich in endogenous extracellular matrix will help for example to further study extracellular matrix remodelling after ECT and GET application, thanks to physico-chemical approaches such as vibrational analysis by Fourier transform infrared spectroscopy/ attenuated total reflectance (FTIR/ATR) and differential scanning calorimetry analysis.

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Gene electrotransfer of plasmids encoding chemokines CCL5 and CCL17 to tumours results in their overexpression and modified cytokine expression profile

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INTRODUCTION

Chemokines, known as modulators of leukocyte trafficking, have over the years showed promising results regarding enhanced infiltration of immune cells in tumour tissue, pointing to their potential use in the field of cancer immunotherapy [1]. Among them, two proinflammatory CC-type chemokines, CCL5 and CCL17, were both associated with reduced tumour growth and T lymphocyte infiltration [2]. Herein, we investigated effects after delivery of plasmid DNA encoding for either CCL5 or CCL17 using lipofection in tumour cells *in vitro* and gene electrotransfer (GET) in tumours *in vivo*.

METHODS

For *in vitro* study two murine breast cancer cell lines, 4T1 and E0771, and two murine colon cancer cell lines, CT26 and MC38, were transfected with plasmid encoding either murine or human CCL5 or CCL17 by using Lipofectamine 2000 reagent in order to exclude the effects of electroporation. Presto Blue viability assay and qRT-PCR were carried out on untreated cells and 48h after transfection.

The antitumour effect of each chemokine was evaluated by determining the tumour growth delay of murine colorectal carcinoma CT26 tumours in BALB/c mice after GET (animal licence: U34401-1/2015/7). Tumours were induced by a subcutaneous injection of $2x10^5$ cells on the right side of the mouse. When tumours reached a volume of 50 mm^3 a 50 µg of plasmid DNA encoding murine CCL5, CCL17 or control plasmid pDNA Ctrl was injected intratumourally. Immediately after, tumours were subjected to high voltage (HV) pulses or combination of high voltage and low voltage (HV-LV) pulses using non-invasive 6 mm plate electrode (Table 1). Good contact between the electrodes and the tumour was assured by means of an ultrasound gel. Tumour growth was monitored three times a week by measuring the tumours using a Vernier calliper until the tumour volume reached 500 mm^3. Expression of murine cytokines and chemokines was determined on day 3 and 7 after therapy by performing qRT-PCR.

Table 1: Electric pulses used in GET therapy.

Type of pulses	HV	HV-LV
Number of pulses	8	4 x (1HV + 1LV)
Voltage/distance [V/cm]	1300	HV: 1300 LV: 150
Duration [ms]	0,1	HV: 0,1 LV: 20
Frequency [Hz]	1	1
Time between HV and LV [ms]	/	50
Time between (1HV + 1LV) [s]	/	1

RESULTS

Viability assay of 4T1, CT26 and MC38 showed survival rate 48h after transfection to be above 90%, while viability of E0771 was around 80%. Expression analysis showed significantly increased expression of CCL5 and CCL17 in the surviving cells after transfection. Concurrent expression analysis of 9 other murine cytokines revealed slightly increased levels of IL-6 and CXCL10 in all cell lines, except after CCL17 transfection in MC38 and E0771 cell lines. GET of plasmids encoding murine CCL5 or CCL17 to tumours resulted in minor growth delay of the treated tumours (Figure 1). Moreover, expression analysis of treated tumours showed increased expression of CCL17 and CCL5 at both time points after therapy. Expression of CXCL10, IL-6 and other cytokines after GET therapy was mainly unaltered.

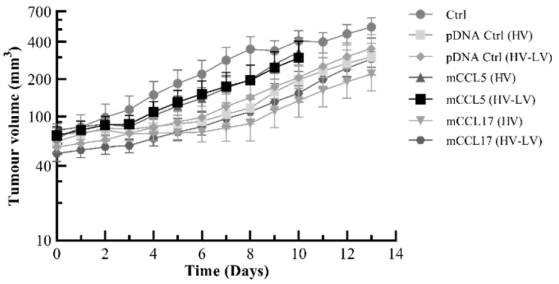

Figure 1: Tumour growth curves of CT26 tumours after GET therapy. Data of all groups presented as mean ± SEM.

CONCLUSIONS

Transfection of four cancer cell lines resulted in increased expression of two proinflammatory chemokines CCL5 and CCL17. Although both chemokines after GET therapy were associated with minor tumour growth delay, future experiments will be directed towards elucidating the infiltrated immune cells in treated tumours.

ACKNOWLEDGEMENTS

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Macromolecule transdermal drug delivery: design of carbon nanotubes-based hydrogel devices for electroporation

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INTRODUCTION

The transdermal route for drug delivery is fast and allows better preservation of the drug integrity compared to other alternative routes but is invasive and as such provokes pain and discomfort. To improve the quality of life of patients in need of regular injections, efficient non-invasive transdermal drug delivery methods are required. The main challenge for this kind of delivery is the crossing of the *stratum corneum*, the most external layer of the epidermis, which is hydrophobic and impermeable to macromolecules. In this work, we rely on the electroporation method to temporary permeabilize the skin and allow the macromolecules delivery, with limited degradation of its barrier properties. To ensure the drug to be close to the permeabilized skin, we developed a composite device which combines an hydrophilic drug reservoir and an electrode for electroporation. Those functions are provided by an agarose (AGR) hydrogel matrix where Double-Walled Carbon Nanotubes (DWCNT) are entrapped, to enhance both mechanical and electrical properties [1]. The proof of concept for macromolecules transdermal delivery using such device combined with electroporation as well as its electrical characterisations were the subject of previous works [2, 3]. We present here our latest results on skin penetration obtained by fluorescence microscopy.

MATERIAL AND METHODS

Device processing

Hydrogel was prepared with a concentration of AGR of 25g/L and DWCNT of 1 wt. % (*to AGR*). AGR solution was prepared by dissolving dry AGR (Sigma-Aldrich) in water (*115-120°C*) under stirring at 700-800 rpm for 20-30 min. Suspension of DWCNT in deionized water was obtained using 1h probe sonication, then added to AGR solution and processed with Ultra Turrax (*20 min, 80°C, 8000 rpm*). The homogeneous suspension was then poured into silicone molds and let cooled at room temperature. We obtained cylindrical gel pellets (diameter 10 mm, thickness 2 mm).

Drug loading

The hydrogel nanocomposites were dried overnight and swollen 24h in a buffer solution containing the molecule of interest. In order to monitor the penetration of macromolecules inside the skin we used fluorescein isothiocyanate functionalized dextran (FD4) (*3-5 kDa)* from Sigma-Aldrich at 1mM. Propidium Iodide (PI) from Sigma-Aldrich at 100µM was also added as a cell permeabilization marker.

Electroporation of the skin

The nanocomposite hydrogels were placed on freshly explanted hairless mice skin and covered with a copper electrode to ensure the electrical contact with the generator. Electrical parameters were set at *300V/cm (between electrodes), 20ms, 1Hz,* with 8 repetitions (square waves). Electroporated skin were left to incubate for 30 min, then rinsed with PBS and observed by fluorescence macroscopy.

RESULTS

In the Figure 1**,** fluorescence intensity yields measured on hairless mice skin electroporated with and without the presence of CNT inside the hydrogel are displayed. We observed a non-significant but clear improvement of the FD4 labelling at the cathode side. A more obvious improvement of the PI labelling occurred at the anode side. This strong PI labelling proves that the cells of the skin have been permeabilized using this method. Enhancement of the markers staining at both electrodes demonstrated the interest of the presence of carbon nanotubes inside the device.

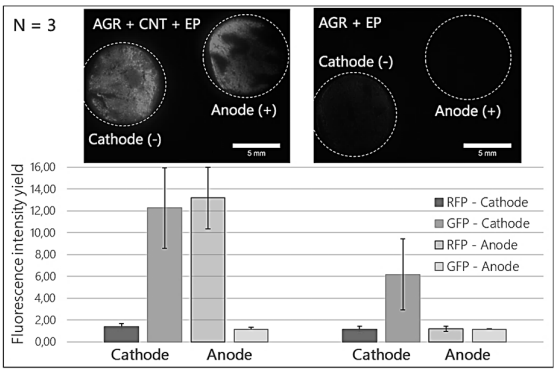

Figure 1: Fluorescence macroscopy and intensity yields of electroporated hairless mice skin with and without CNT in the device.

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Changes in the electrochemotherapy-treated patients' quality of life – prospective analysis

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INTRODUCTION

Electrochemotherapy is an effective method for treating cutaneous tumors. Nowadays this treatment is mainly performed in a palliative intent in advanced or numerous tumours often with bleeding, odour, or suppuration. The aim of this study was to evaluate the changes of quality of life, health status and pain in ECT treated patients with cutaneous tumors of different histotypes.

METHODS

ECT treatment was performed according to the ESOPE criteria [1,2]. Before and after treatment prospective data collection was carried out at each visit. The first visit was at 3 weeks, the most recent visit was at 2-25 months after the ECT session. Quality of life was assessed using the EuroQol-5 Dimension (EQ-5D) questionnaire, including mobility, self-care, usual activities, pain/discomfort, anxiety/depression, where each dimension had 3 levels. Health status was evaluated on a scale between 0-100, and pain was measured according to the Visual Analog Scale (VAS) (0-10). All standardized questionnaires were completed by the patients at each visit. IBM SPSS 24. Software was used for the statistical analysis.

RESULTS

Forty-five patients were treated with electrochemotherapy. Out of the 197 treated lesions, 44% of them were BCC, 25% were cutaneous metastases of malignant melanoma, 11% were Kaposi sarcoma, 9% were cutaneous metastases of SCC, 5% were angiosarcoma, 2% were cutaneous metastases of breast cancer, 2% were malignant Schwannoma, and 2% were carcinoma basosquamosum. The average age was 67 (12-86 years). Eight patients had irradiation on the treated area before ECT, 20 of the had at least one lesion bigger than 3 cm.

Table 1: Histotypes of the treated tumours.

	N^{01}=197
BCC	44%
Cut. met. of MM	25%
Kaposi sarcoma	11%
Cut. met. of SCC	9%
Angiosarcoma	5%
Cut. met. of breast adenocarcinoma	2%
Malignant Schwannoma	2%
Cc. basosquamosum	2%

In our case series, we found a significant improvement in the health status comparing its value before ECT treatment and the most recent visit (p=0,001).

There was also a significant increase between the first and the most recent health status (p=0,004).

Amongst the assessed quality of life dimensions, a significant decrease was found in anxiety/ depression between the visit before ECT and the last visit (p=0,007), and between the visit before ECT and the first visit after it (p=0,012), whilst regarding the other 4 dimensions, significant changes were not found.

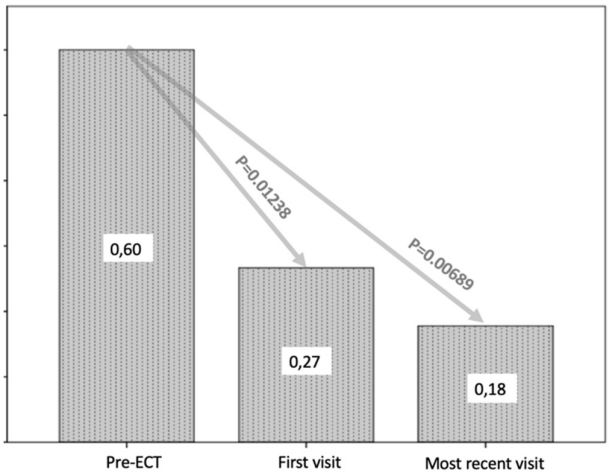

Figure 1: Decrease of anxiety values.

The measured pain was significantly higher at the first visit, than the initial pain values (p=0,0314). Pain was significantly higher at the first and the last visit in patients who had previous irradiation in comparison with the non-irradiated patients.

CONCLUSIONS

Our results are in correspondence with the published results of the literature, namely that electrochemotherapy has a positive effect on quality of life, health status and pain of the treated patients.

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Evolution of vitamin complex C K_3: ab initio study

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INTRODUCTION

Ascorbate (vitamin C) and menadione (vitamin K_3) mixture is known as powerful antioxidant with strong expressed cytotoxic effects for malignant cancer cells therapeutic effect of such mixture is known (ends apoptosis but not necrosis), however the mechanism of action is still not fully understood. It has been shown that a three- step reaction of reagents C and K_3 yields significant amount of H_2O_2 (hydrogen peroxide) that is involved in cancer treatment procedures.

In this work we show the complex evolution analysis, when in vitro complex represents the associate of C and K_3 in aqua surrounding (pH <7) and with different ions. Intermolecular charge transfer was modelled according to quantum chemistry simulations.

QUANTUM CHEMISTRY SIMULATIONS

Ground state complex geometry optimization was done by using B3LYP/6-31++G(d,p) method in Gaussian09 package. Effects of aqueous solution (surrounding properties) were estimated by using SCRF=(solvent=Water) routine. Electronic excitation and following intermolecular charge transfer (CT) were calculated by using Time-Dependent Density Functional Theory (TD-DFT). Figure 1 represent the electronic charge redistribution for protonated complex of vitamin C an K_3 in water. Intermolecular charge transfers from vitamin C to K_3 and from H_2O to K_3 respectively are significant initiators for the following formation of ascorbyl. Presented sandwich-type complexes differ from previous, described in Ref. [1] (the same complex without surrounding effect). Vitamin C is well known as a proton donor and the profiles such reaction was studied before [2- 3].

Geometry of vitamins C and K_3 complex structure with proton was studied and presence of proton in vitamin C area was estimated as the significant for the charge transfer reaction. Localization of additional proton in K_3 area was estimated as negligible for CT.

Presence of proton in vitamin complex in water solution is more energetically favorable situation for ascorbyl formation in comparison to non- protonated complex.

Figure 2 represented Complex of vitamin C an K_3 in aqua with Mg^{2+}. Magnesium ion binds between vitamin C and K_3 form an open L - type structure. The magnesium ion act the intermolecular bridge between the two molecules.

CONCLUSIONS

Presence of proton in vitamin complex with water surroundings show more energetically favorable situation for ascorbyl formation in comparison to non- protonated complex.

Incorporation of Mg^{2+} ion into the vitamin C and K_3 complex in water solution result as a highly efficient bridge for intermolecular electron transport

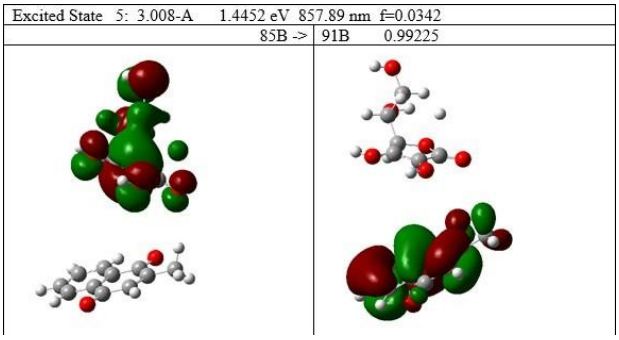

Figure 1: Protonated complex of vitamin C an K_3 in water. Intermolecular charge transfer from C to K_3.

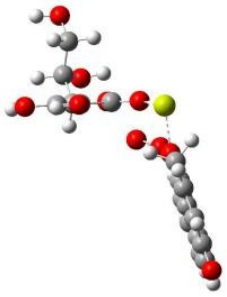

Figure 2: Complex of vitamin C an K3 in water and Mg^{2+}.

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Pilot Study on the Enhancement of Pharmacological Effect of Paclitaxel with Electrochemotherapy for Breast Cancer Treatment

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INTRODUCTION

Invasive ductal breast carcinoma is the most common cancer type in México. Based on the poor outcome to conventional therapies and the successful clinical response to electrochemotherapy (ECT) of different cancer types [1], ECT optimization for breast cancer is encouraged [2]. Hence, the determination of cell-specific electroporation (EP) thresholds for an efficient permeabilization in adhered cells, and the results obtained for ECT *in vitro* are presented in this work.

METHODOLOGY

In silico prediction of the electric field distribution due to the application of EP was carried out through the finite element method (FEM). Subsequently, the outcomes were experimentally validated taking into account cell viability and external molecule uptake to establish the most efficient EP protocol. Cell viability was measured by the MTT assay, and molecular uptake was monitored through epifluorescence microscopy.

ECT was performed using the selected protocol along with the taxane Paclitaxel in two breast cancer cell lines, BT-20 and MCF-7. Cell death was determined to assess drug effectiveness improvement. The pharmacological effect of paclitaxel was evaluated through the Annexin V and the Ethidium Homodimer III (EthD-III) recognition.

Table 1: Electroporation protocols

Cell line	Pulse amplitude [V]	Electric field [V/cm] *In silico*	*In vitro*	Cell viability [%]
BT-20	160	415	332	50
MCF-7	200	415	415	100

RESULTS

The optimal electric field for the permeabilization of MCF-7 cells, was in agreement with simulations. Conversely, BT-20 cells were permeabilized with a lower electric field than the simulated [Table 1]. The molecule uptake is shown in Figure 1.

MCF-7 cells showed an increased response to the ECT with a higher cell death through apoptosis than chemotherapy alone. In contrast, BT-20 cells showed a poor response to ECT only exhibiting a faster triggering of apoptosis with a similar cell death compared to chemotherapy.

CONCLUSION

Based on the results, EP may potentiate paclitaxel efficacy in hormonal cancer cells (MCF-7) resulting in an adjuvant therapy. Regarding triple negative cells (BT-20), the low cell viability due to EP suggests that irreversible electroporation could be a more effective treatment without the need to use an antineoplastic drug.

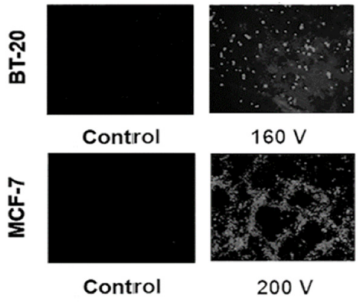

Figure 1: Cell-specific electroporation protocol based on external molecule uptake.

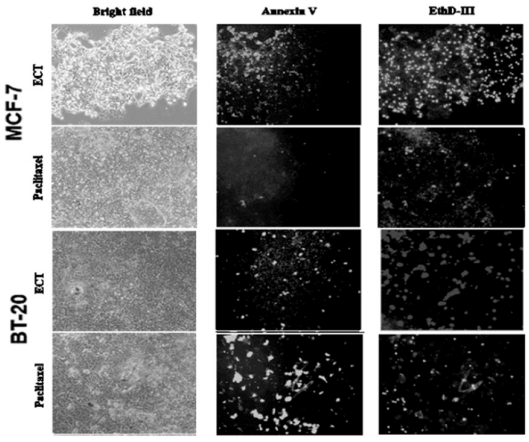

Figure 2: Recognition of apoptotic cells after ECT by Annexin V and EthD-III.

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The use of high-frequency short bipolar pulses in cisplatin electrochemotherapy in vitro on skin melanoma cells

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INTRODUCTION

In electrochemotherapy chemotherapeutic drugs cisplatin and bleomycin are used in combination with the application of high voltage 100 µs long monopolar pulses to the patient's tumor area.

Drawbacks of the ECT treatments are pain and muscle contractions, connected with the application of longer monopolar pulses. Thus, it is necessary to use muscle relaxants, general anesthesia and to synchronize pulses with the heart rhythm of the patient which complicates and prolongs the electrochemotherapy treatment. These problems can be overcome by delivering bursts of short high-frequency bipolar pulses, i.e. the so-called high-frequency irreversible electroporation (H-FIRE) pulses used for irreversible electroporation [1].

The aim of this work was to determine if it is possible to use H-FIRE pulses in electrochemotherapy (reversible electroporation) which we call high-frequency electroporation (HF-EP) pulses.

METHODS

We performed *in vitro* experiments on mouse skin melanoma cells (B16-F1). First, we determined the optimal electric field strength (highest permeability and highest cell survival) for both types of pulses. Then, we added 100 µM cisplatin (CDDP) and delivered long monopolar and high-frequency electroporation (HF-EP) pulses (Figure 1).

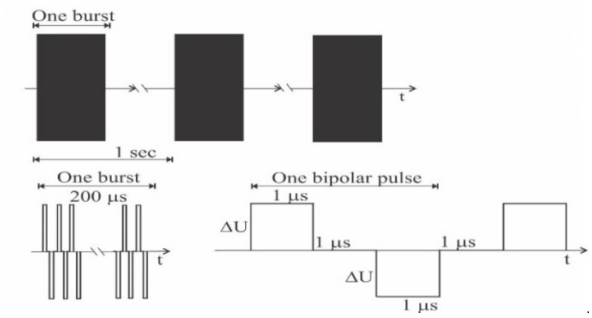

Figure 1: Short bipolar pulses (HF-EP). Above: 8 bursts were applied with a repetition frequency of 1 Hz. Down left: One burst was 200 µs long and consisted of 50 bipolar pulses. Below right: One bipolar pulse of amplitude ΔU consisted of 1 µs long positive pulse, 1 µs long negative pulse with a 1 µs long delay between pulses**.**

We used 2 mm parallel plate stainless steel electrodes to deliver pulses to cells in suspension. We determined cell permeability using propidium iodide and flow cytometry and cell survival using metabolic MTS assay, 72 h after the treatment.

RESULTS

We determined the optimal electric field to be 1.2 kV/cm for the monopolar pulses and 3 kV/cm for the HF-EP pulses. We measured cisplatin cytotoxicity with electroporation at different electric fields with monopolar (Figure 2A) and HF-EP (Figure 2B) pulses. Both figures show that the combination of electric pulses and CDDP is more efficient in achieving cell death than applying only electric pulses. In addition, cell death increases with increasing electric field. Figure 2B shows that a comparable cell death can be achieved with HF-EP pulses as with monopolar pulses, however higher electric field needs to be applied with HF-EP pulses.

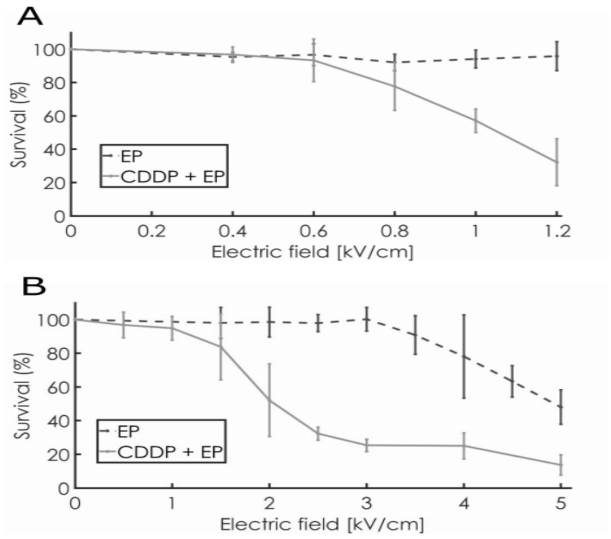

Figure 2: Cytotoxicity of 100 µM of CDDP in combination with electric pulses, delivering A) long monopolar and B) HF-EP pulses.

CONCLUSIONS

The results obtained show that it is possible to use HF-EP for electrochemotherapy, but applying a higher electric field than for monopolar pulses is needed [2].

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Effect of electroporation and electrochemotherapy on expression of pattern recognition receptors on lung cancer cells and immune cells – *in vitro* study design

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INTRODUCTION

Electrochemotherapy (ECT) is an established cancer treatment that uses electroporation (EP) in combination with cytotoxic drugs. It is mainly used for skin-based tumour treatment due to the easy accessibility of the tumours to the treatment. In recent years, more and more tumour types are treated with ECT as more advanced devices became available to also treat internal tumours[1]. Despite the great effort in improving the effectiveness of ECT, ECT is still only offered as a palliative treatment. ECT has shown great effectiveness in reducing the local tumour burden and is well tolerated by most patients, but ECT lacks a systemic response as it does not induce a sufficient immune activation[1], [2].

Sufficient and long-lasting induction of the immune system is necessary to create a system-wide response, which potentially can eradicate metastasis elsewhere in the body, but also generate immunological memory to prevent reoccurrence.

Several studies have shown that ECT induces immunogenic cell death and in doing so, creates activation signals for the immune cells and also makes tumour-associated antigens available to antigen-presenting cells[3], [4]. This modulation should induce immune system activation, but little is known about the immune cells in the context of ECT, to determine, why immune activation does not happen.

In this abstract we present the design of an *in vitro* study of lung cancer cell lines and primary immune cells to evaluate the effect of ECT on their immune profile.

AIM

The main aim of our proposed *in vitro* study is to assess the influence of EP and ECT on immune cells and their activation status. We will assess the survival rate for immune cells and also the expression of Pattern-recognition receptors.

Furthermore, we will assess if the activation status of the immune cells changes after EP or ECT. If possible, we will use the gained knowledge in specifically targeting immune cell activation in combination with ECT.

We will use a range of field strengths to determine with which setting, we get optimal immune cell survival and immune activation. We will also compare those settings with the optimal settings for ECT in several cancer cell lines to create a more holistic approach to choosing the right ECT settings.

The final aim of our research is to develop a combination treatment for lung cancer patients with a focus on the immune control of lung cancer.

METHOD

The proposed study will be carried out in a panel of lung cancer cell lines and primary immune cells from healthy human donors.

1. Cells will be electroporated using a variety of field strengths to determine optimal settings for the various cell types.
2. ECT will be performed with cisplatin and oxaliplatin as bleomycin is known to cause lung problems and the optimal field strength determined in 1.
3. Cell permeability and cell viability after treatment will be analysed with PI staining on flow cytometry.
4. Expression of intracellular and extracellular Pattern-recognition receptors will be done by flow cytometry.
5. Cell survival (for the cancer cell lines) will be measured using clonogenic assay.

EXPECTED RESULTS AND FURTHER WORK

We expect to see an upregulation of TLR9 and TLR2 after ECT and maybe even after EP alone.

Depending on the observed influence of ECT on the immune status of cancer cells and immune cells, we will design an immunological co-treatment for ECT, which will either inhibit pro-tumorigenic signalling cascades in cancer and/or immune cells and/or stimulate immune cells to create a system-wide immune response.

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Simultaneous detection of Ca^{2+} signaling and ATP release in the developing cochlea

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INTRODUCTION

During pre-hearing stages of development in mice, periodic transient elevations of cytosolic free Ca^{2+} concentration occur spontaneously in the greater epithelial ridge (GER) and propagate as intercellular Ca^{2+} waves invading variable portions of the GER. Prior work by our and other groups indicates that intercellular Ca^{2+} waves in the GER rely on the interplay between IP3, generated intracellularly, and ATP, released at the apical surface of cochlear non-sensory cells. A vast body of data supports the hypothesis that, in the developing cochlea, ATP is released through connexin hemichannels [1], however a direct proof is lacking.

To test this hypothesis, we designed and built a closed microfluidic chamber (10 µl max. volume) in which the transparent roof was covered with plated HEK23T cells (facing the fluid interior of the chamber, stably expressing P2Y2 purinergic receptors and sensitive to ATP in the nM range). These biosensor cells sitted at <100 µm from the surface of an organotypic cochlear culture plated on the chamber bottom.

RESULTS

After loading both biosensor cells and cochlea with Ca^{2+} -selective dyes, or by using genetically encoded GCaMP6s Ca^{2+} sensors, this architecture allowed us to monitor Ca^{2+} responses in HEK293T biosensor during propagation of Ca^{2+} waves in the GER of the cochlea underneath. To image Ca^{2+} dynamics while discriminating optical signals originating from the two focal planes, we stepped up and down, rapidly and repeatedly, the objective of a custom-made multi-photon microscope. For these experiments, the saline solution trapped within the chamber contained an endolymphatic Ca^{2+} concentration (20 µM) and ARL67156 (100 µM), an inhibitor of ectonucleotidases. Ca^{2+} signals disappeared upon replacing ARL67156 with apyrase (40 U/ml, an enzyme that catalyzes the sequential hydrolysis of ATP) in the extracellular medium, confirming that ATP mediated both Ca^{2+} wave propagation in the GER and the ensuing Ca^{2+} responses in the HEK293T biosensors.

To determine the source of the released ATP, we tested cochlear organotypic cultures from mutant mice with global deletion of pannexin 1 ($Panx1^{-/-}$), and another strain with global deletion of connexin 30 ($Cx30^{-/-}$) that cause a reduction of the expression of Cx26 in the organ of Corti [2]. Using the microfluidic chamber, we determined that Ca^{2+} signals in the ATP biosensor cells were the same irrespective of whether they faced $Panx1^{-/-}$ or age-matched $Panx1^{+/+}$ or $Cx30^{+/+}$ cochlear cultures. In contrast, Ca^{2+} signals strongly depressed in the presence of $Cx30^{-/-}$ cultures. Furthermore, Ca^{2+} waves in the GER were reversibly inhibited by flufenamic acid (50 M) and the anti-connexin antibody abEC1.1 (952 nM) [3], both of which are not effective on pannexin 1 channels [4]. Together, these results validate our working hypothesis and confirm that pannexin 1 channels are not involved in the ATP release process that mediates Ca^{2+} wave propagation in the GER.

ACKNOWLEDGMENTS

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NOTES

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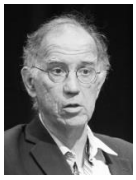

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