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Performing *in vitro* biological assays to evaluate the impact of electrochemotherapy treatments

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Abstract—Electrochemotherapy (ECT) treatment is now a routine technique for the treatment of cutaneous and subcutaneous tumors. To better understand the underlying mechanisms and effects of electrochemical stimuli on cells, performing biological experiments is particularly important. We propose to evaluate the impact of ECT treatments on cell permeabilization and cell viability. We present here a comparison between two viability assays – crystal violet staining and clonogenicity assays, on mammalian cells submitted to pulsed electric field used along the ECT protocols.

Keywords—*electrochemotherapy, permeabilization, viability, clonogenicity*

I. INTRODUCTION

Electroporation (EP) is a non-viral physical method of vectorization consisting in creating pores in the cell membrane by applying electric fields. This highly facilitates the transfer of compounds into cells or the extraction of intra-cellular molecules.

EP can be reversible or irreversible, depending whether the pores reseal, leading to the recovery of the cell functions, or not, leading to the cell death by apoptosis (see Fig 1).

Electrochemotherapy (ECT) is based on reversible EP technique in order to transiently permeabilize the cell membrane, facilitating the intra-cellular penetration of non-permeant cytotoxic agents [1].

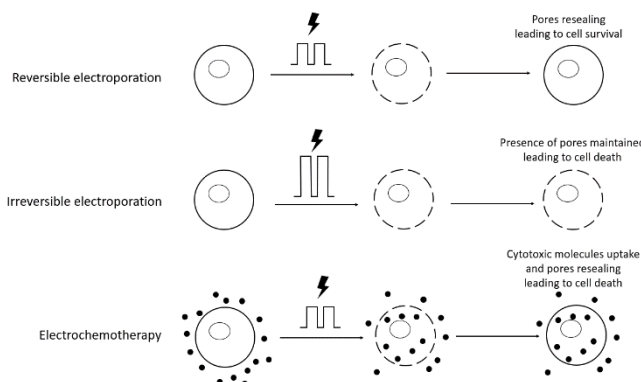


Fig 1 – Principle of reversible and irreversible EP, inspired of [2].

Standardized in 2006 for the treatment of skin metastases and primary skin cancers, ECT relies more commonly on the use of bleomycin and cisplatin as cytotoxic agents. The

electrical treatment generally consists of the application of 8 pulses, of 100 μ s width, 1.3 kV/cm at a frequency of 1 Hz [3]. Pulses intensities vary from 1 to 2 kV/cm.

It is of paramount importance to control the applied parameters, such as the electric field intensity and the cytotoxic compounds concentration, during ECT treatments and to evaluate their effects. Especially, it appears crucial to determine cells permeabilization state as well as the impact of such treatments on cells viability.

Cell permeabilization occurs when its membrane reaches a certain potential difference, the latter being the sum of the resting potential difference and the induced potential difference [4]. As described by Schwan's equation, the applied electric field induces a potential difference that depends on the electric field strength:

$$\Delta\varphi = F g(\lambda) r E \cos\theta$$

With $\Delta\varphi$ the induced potential difference on a cell membrane, F the shape factor of the cell, $g(\lambda)$ a function depending on the conductivities of the cell and the pulsing buffer, r the radius of the cell, E the electric field strength and θ the angle between the direction of the field and the normal to the cell surface.

Determining the intensity of the applied electric field that triggers cell permeabilization, *i.e.*, the permeabilization threshold, E_p , helps to optimize the electrical parameters applied during ECT treatments.

It also appears essential to lead viability assays in order to study whether the treatment induces reversible or irreversible EP. Among viability assays, because of their ease to implement, their low cost and their sensitivity, colorimetric assays are largely employed. Among them, one can encounter MTT, XTT, crystal violet and clonogenicity assays [5].

Here, we propose a comparison between two colorimetric assays after ECT treatments: (i) crystal violet assay, performed 2, 24 or 48 hours after treatment, and (ii) clonogenicity assay, performed 10 days after treatment.

Crystal violet assays on adherent cell lines, developed in 1989, are based on the use of the crystal violet dye, that enters into the cells and targets DNA and proteins. The intensity of crystal violet staining directly depends on the number of cells present on the Petri dish (*i.e.*, that have adhered to the culture support after ECT treatments).

Clonogenicity tests, developed in the 1950s, assess the ability of single cells to form colonies (i.e., able to divide). These tests are known to be particularly representative and precise [6].

II. MATERIAL AND METHODS

A. Cell culture and reagents

The studies carried out are based on HCT-116 cells, that are derived from carcinoma colorectal cancer cells. They are grown in Dulbecco's Modified Eagle Medium, supplemented with 20% fetal calf serum and a mixture of antibiotics 100 U/mL penicillin and 100 μ g/mL streptomycin. Cells are incubated at 37°C under 5% CO₂ for proliferation.

Cells permeabilization sensitization as well as cells viability depend on the medium in which they are pulsed [7]. Therefore, we select a pulsation buffer with a low conductivity (1.7 mS), consisting of 10 mM K₂HPO₄/KH₂PO₄, 1 mM MgCl₂, 250 mM of sucrose.

To assess cell permeabilization, we evaluate the entry into the cells of propidium iodide (PI), that is diluted in the cell solution to reach a final concentration of 100 μ M. PI is a non-permeant molecule which binds to double-stranded nucleic acids when the cell membrane is damaged.

The ECT treatments consist in administrating bleomycin to the cell solution, to reach a final concentration of 250 nM. By binding to certain portions of DNA, bleomycin leads to the production of free radicals responsible for DNA strand breaks, resulting in cell death during the G2 phase of the cell cycle.

The colorimetric tests conducted in this study are based on the use of crystal violet (0.1% in aqueous solution).

B. Electroporation set up and procedure

Cells culture medium is removed and cells are washed with phosphate buffer saline (PBS). Trypsin is added and the cells are incubated for 5 minutes at 37°C under 5% CO₂. A centrifugation (1300 rpm, 3 minutes) is then carried out. The cells are suspended in the pulsation buffer to reach a final concentration of 300,000 cells/mL.

Cells in suspension are then placed between flat and parallel stainless steel electrodes, with an inter-electrode distance of 4 mm. The bottom of the Petri dish serves as an electropulsation chamber.

A generator (Jouan GHT 1287) delivers the electric pulses, whose shape are verified by using an oscilloscope (Tektronix TDS 2012C). Electric pulses, comparable to the ones used during ECT treatments (8 pulses, 100 μ s width, 1 Hz), are delivered. Different amplitudes are tested, from 0 to 2.5 kV/cm.



Fig 2 – Test bench for performing EP or ECT treatments on HCT-116 cells in suspension. The test bench includes an electropulsator to which an oscilloscope and electrodes are connected.

C. Biological assays for the evaluation of cells permeabilization and viability

i. Permeabilization assay

To assess cell permeabilization, PI is added prior to the application of electric pulses. After the EP treatment, cells suspensions are kept in ice until performing flow cytometry (BD LSRFortessa X-20) analysis over 20,000 cells. The percentage of permeabilized cells is determined. The obtained results are presented in section III (see Fig 3).

ii. Viability assessment

For cells enumeration assays, cells are seeded in 96-well plates at a final density of 15,000 cells/well. For clonogenicity assays, cells are seeded in 6-well plates at a final density of 300 cells/well. Cells are finally incubated at 37°C under 5% CO₂.

Cells are removed from the incubator after the received treatment: 2, 24 or 48 hours later for cells enumeration assays (i.e., up to 1 or 2 generations time), and 10 days later for clonogenicity assays.

The culture medium is removed and a rinse with phosphate saline buffer containing calcium and magnesium ions is performed. Crystal violet (0.1% in aqueous solution) is added, a 15-minute agitation is carried out and three successive rinses with phosphate saline buffer containing calcium and magnesium ions are done.

After rinsing, depending on the study, the procedure differs: (i) for clonogenicity assays, a count of the number of clones is realized, whereas (ii) for cells enumeration assays, acetic acid is added to lead to cell lysis. A reading of the optical density is then made with a spectrometer (Varioskan Flash) at a 595 nm wavelength.

III. RESULTS

A. Permeabilization assays

Permeabilization percentage among cells is presented in Fig 3.

The uptake of the fluorescent dye into the cells is the signature of membrane electropermeabilization. We submitted the cells to increasing values of E and determine the permeabilization efficiency (i.e., the percentage of cells which become fluorescent). Permeabilization only appears above a threshold value of pulse intensity E_p . For $E < E_p$, which as shown in Fig 3 is comprised between 0.6 and 0.8 kV/cm, no permeabilization occurs. Above E_p , increasing E leads to the progressive permeabilization of the whole cell population, that is obtained at 2.5 kV/cm.

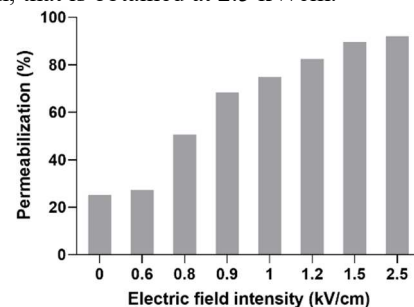


Fig 3 – Permeabilization assay performed on HCT-116 cells in suspension. 100 μ M of propidium iodide is added before applying electric pulses, which intensities vary from 0 to 2.5 kV/cm. For each condition, 20,000 cells are analysed by flow cytometry.

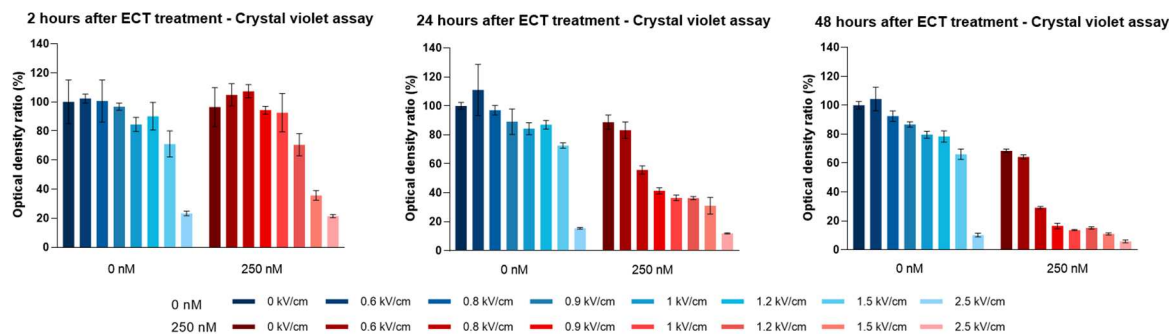


Fig 4 – Cell viability percentage obtained 2, 24 and 48 hours after performing ECT treatments on HCT-116 cells in suspension. Electric field strengths from 0 to 2.5 kV/cm are applied. The effect of the electric field alone is studied, as well as the cumulative effect of the electric field with the administration of bleomycin. This study is representative of 3 independent experiments.

B. Short term viability assessments: cells enumeration assays

The next step is the determination of the cell viability in order to assess whether the intensities that lead to cells permeabilization whether induce reversible or irreversible EP.

The 2h, 24h and 48h viability studies show similar viability trends (see Fig 4). As expected, the effect of bleomycin on cells is much more pronounced at 48h, as the drug acts during the G2 phase of the cell cycle.

Bleomycin alone (250 nM) does not induce significant mortality (about 17% of cell death 48 hours after treatment). In agreement with the studies that can be found in the literature, the effect of the treatment is potentiated when an electric field is applied [8]. For example, 48 hours after combining bleomycin to electric pulses at 1 kV/cm, cell death is about 79%.

As the electric field intensity is increased, cell mortality rate increases. For field values higher than 1.5 kV/cm, viability is affected (more than 30% mortality). One can notice that at 2.5 kV/cm, the electric field effect alone is sufficient to induce a high mortality rate (more than 80% mortality).

Once obtained, such a kind of results easily allows to define the best conditions for ECT treatment (high membrane permeabilization with low effect on cell survival). In our present work, the electric field values that can be used range from 0.8 to 1 kV/cm.

C. Long term viability assessments: clonogenicity assay

The results of the clonogenicity tests performed 10 days after ECT treatments confirm the short-term viability assay trend: an increase of cell death is induced when the electric field intensity is increased (see Fig 5). When bleomycin alone induces 70% mortality, electric fields significantly increase the mortality rate (only 5% of cell survival at 1 kV/cm).

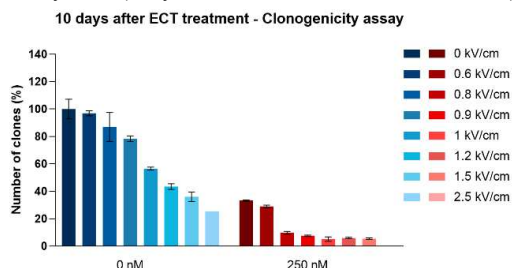


Fig 5 – Percentage of clones numbers obtained 10 days after EP and ECT treatments on HCT-116 cells in suspension. For all conditions, two batches are studied.

IV. CONCLUSIONS

In the present study, we present biological assays to evaluate the effects of ECT treatments on cells permeabilization and viability. Permeabilization assay allows us to define the ranges of electric field intensity that induce permeabilization ($E > 0.6$ kV/cm). Viability assays allow to highlight the effectiveness of ECT treatments in comparison with the application of electric fields alone or the use of bleomycin alone. We evaluate the impact of these treatments at different temporal scales and evidence their effectiveness as early as 2h post-treatment in the case of crystal violet assays. As a comparison, clonogenicity, which is the gold standard method usually applied due to its high reliability, is much more time-consuming (several days) and requests high quantities of bio-reagents.

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