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PRIMARY RESEARCH

Low amplitude pulse electric field for elimination of unpleasant sensation associated with high amplitude electric field for electrochemotherapy

Hassan Buhari Mamman [1], Muhammad Mahadi Abdul Jamil [2*], Mohamad Nazib Adon [3]

[1, 2, 3] Department of Electrical and Electronics Engineering, Universiti Tun Hussein Onn Malaysia, Johor, Malaysia

Index Terms
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***Abstract*—** Electrochemotherapy is a combined use of a chemotherapeutic drug and short intense electric field for cancer treatment. The applied electric field increases the permeability of the cell membrane thereby increasing the free entrance of the drug into the cancer cell for effective treatment at minimal drug dose. However, patients undergoing electrochemotherapy in clinical trial complain of unpleasant sensation due to muscle contraction during the pulse delivery (usually 1000V/cm, 100μs, and 8 numbers of pulses). This unpleasant sensation is caused because of the high amplitude of pulse or due to the low repetition frequency of the pulse (1Hz). Hence, in this paper, a low voltage amplitude (600V/cm) electric pulse at relatively higher pulse durations ranging from 500μs to 20ms was used in electroporating cells in vitro. The percentage of cell permeabilization and viability of the different pulse durations were measured. The result revealed that 500μs duration stimulates the cell proliferation and 20ms result in 90% of cell death. On the other hand 5ms pulse duration resulted in 65% permeabilization and 80% viability. Hence the study suggested that 600V/cm at 5ms duration can be used for electrochemotherapy to potentially eliminate the unpleasant sensation associated with high amplitude pulse.

I. INTRODUCTION

Exposing biological cells to a high electric field of short duration induces an extra potential on the cell membrane, which superimposes on the membrane resting voltage that is continuously under physiological conditions [1]. The resting membrane potential has a value in the range of -40mV to -80mV depending on the cell type, size, and composition [2-4]. However, if the induced potential reaches a threshold value of 0.2-1V, a localized structural rearrangement of lipid bilayer occurrs [5]. This results in formation of nanopores in the cell membrane and hence, increases the membrane permeability and conductivity [6]. Thus, molecules that are otherwise impermeable to membrane can easily enter into the membrane. This process is electropermeabilization or electroporation [7-9]. Ever since its discovery, electroporation has been used effectively for numerous applications in biotechnology and biomedical engineering. These applications include but not limited to gene therapy [10-11], electrochemotherapy (ECT) [12-15], electro-fusion [16-17], electro-sterilization [18] and tumor tissue ablation [19-20]. Among these applications, electrochemotherapy is progressing much more and now it has reached pre-clinical and clinical trials [21].

The use of chemotherapeutic drugs joined together with electroporation is called electrochemotherapy. Electrochemotherapy facilitates the delivery of chemotherapeutic drugs to malignant cell [22]. Many chemotherapeutic drugs cannot cross the cell membrane under normal condition. Therefore, with the help of electrochemotherapy, this can easily be achieved by creating pores in the cell membrane by the use of an electric field [22]. Commonly used drugs for chemotherapy such as bleomycin and cisplatin were found to be much more effective in the electrochemotherapy than in only chemotherapy when applied

*Corresponding author: Muhammad Mahadi Abdul Jamil
†Email: mahadi@uthm.edu.my

to the tumor cell lines both in vitro and in vivo [23-24] with reduction in drug dose and side effect. Despite the increase in the number of preclinical and clinical trials on electrochemotherapy, many questions regarding the therapy are still open. For instance, determination of electric field parameter would guarantee a treatment that is successful with a negligible side effect.

Eight pulses of 100µs duration with a field strength of 1000V/cm at a repetition frequency of 1Hz has been employed as a standard electric field parameter for electrochemotherapy in both pre-clinical and clinical trials [25]. The efficiency of these parameters was first reported by [26] based on the outcome of an in vitro experiment [27]. Same parameters resulted in optimal condition when used in vivo [21, 28]. Since then, these parameters have been employed as the standard parameter for electrochemotherapy. However, patients undergoing electrochemotherapy complain of transient burns in the area that is in contact with electrode [29] plus muscular contraction that are unpleasant [30]. These unpleasant sensations and burns are caused due to the high amplitude electric field or low repetition frequency of the pulse [31].

Hence, in this paper, a low amplitude electric field fixed at an amplitude of 600V/cm and pulse durations equal to or greater than the pulse duration of standard electric parameters for ECT would be employed. This was done to check for a value that results in a similar efficiency of electroporation with the standard electric parameter (8 pulses of 100µs duration at a repetition frequency of 1Hz and field strength of 1000V/cm) in terms of viability and percentage permeabilization, at reduced pulse amplitude. The reduced pulse amplitude, 600V/cm, could, therefore, decrease or eliminate the unpleasant sensation associated with high amplitude pulse.

II. MATERIALS AND METHODS

A. Cell Culture

In this study, the HT29 cell lines were used for the experiments. The colon cell lines were cultured as a monolayer in a 25cm2 culture flask. Complete growth media used were RPMI1640 enriched with 10% fetal bovine serum (FBS) and 1% antibiotic (penicillin and streptomycin) which are all products of Gibco USA. The cells were cultured in a humidified incubator at 37◦ C, containing 5% CO_2 [32-33]. Cells were sub-cultured every 3 to 5 days whenever they reached 80% to 90% confluent [32].

B. Cell Detachment or Cell Trypsinization

For cell trypsinization, the old medium was aspirated and discarded. Afterward, 3ml of phosphate buffer saline (PBS) was added to wash the cells [33]. The added PBS was aspirated and discarded. Subsequently, 2ml of the Tryple Express solution was added in order to disassociate the cells from the substrate [33].

The Flask containing the cells and the Tryple Express was incubated for 5-10 minutes at 5% CO2 at 37^{o}C. This is because the tryple express works well in a warm environment. When the cells were fully detached, an equal volume of complete growth medium was added to stop the effect of the tryple express (neutralization).

C. Electroporation Protocol

After detaching the cells with tryple express and neutralizing with an equal volume of complete growth medium, cell counted with a hemocytometer and resuspended to a concentration of 9.8×10^5 cells/ml. For cell viability measurement, 600µl of cells suspension, at a concentration of 9.8×10^5 cells/ml, were then poured into six (6) 4mm gap electrode cuvette (BTX Harvard Apparatus). A single pulse Electric field with amplitude of 600V/cm (i.e. a voltage 240V for a 4mm gap electrode cuvette) and different pulse duration ranging from 100us to 20ms (that is, 100µs, 500µs, 5ms, 10ms and 20ms) were used in electroporating the cells in five different cuvettes, one duration for each cuvette.

Another cuvette was electroporated with the standard electric field parameter (1000V/cm, 100µs duration, 8 pulses and repetition frequency of 1Hz) as a positive control. Subsequently, a 300µl of cell suspension (representing 294,000 cells) from each cuvette, were then seeded into wells of six-well culture flasks containing 2ml of complete growth medium. The cells were then incubated at 37oC and 5% of CO_2. Similarly, 294,000 cells, from the same initial flask but without electroporation, were seeded in another well containing 2ml of complete growth medium as a negative control. Both flasks were kept under the same condition.

For determination of cell permeability, cells were first diluted with 100µg/ml of propidium iodide (PI) in a ratio of 10:1 (that is 4.5ml of cell suspension to 0.5ml of PI). Thereafter, the same procedure for the determination of cell viability was followed. However, cells were incubated only for 5 minutes at room temperature after the electric treatment and subsequently, images were acquired using fluo-

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rescent microscopy and phase contrast microscopy, from different field view on the day of the experiment.

D. Determination of Percentage Cell Permeabilization

After exposing the mixture of cells and the PI to the different electric field parameters, the cells were incubated at room temperature for five minutes. The cells were then transferred to a top stage of an inverted microscope for imaging. Images were taken using both fluorescent microscopy using Nikon Ti-series inverted microscope. Additionally, a corresponding phase contrast image of the same field of view was acquired at the same time. Each experiment was repeated three times. Percentage permeability was measured by quantifying the penetration of PI into the cells. Cell percentage permeabilization was calculated by taking the ratio of a total number of permeabilized cells in a region of interest to the total number of cells in that field of view times 100%.

E. Determination of Percentage Cell Viability

Cell viability was computed using trypan blue exclusion test with aid hemocytometer. After the electric treatment, cells were incubated at 37^{o}C and 5% of CO_2 for 24 hours. Afterward, cells were trypsinized and counted for viability. Percentage viability was calculated as a total number of viable cells in a region of interest, divided by the total number of cells (live plus a death in that region of interest) time 100

III. RESULTS AND DISCUSSION

Cell percentage permeabilizations were measured by counting the number of cells successfully penetrated by PI in a region of interest to the total number of cells in that region with the help of fluorescent microscopy. Fluorescent images and their corresponding phase contrast images were acquired using a 20X objective microscope. The photomicrograph of images was shown in figure 1. The mean percentage permeabilization with standard deviation (SD) for three replicate experiments was given in Table 1 and figure 2. At a fixed pulse duration of 600V/cm with single pulse, 100µs, 500µs, 5ms, 10ms and 20ms revealed 5.2% ±2.2SD, 36.3% ±4.7SD, 70.3% ±2.3SD, 78.0% ±2.4SD and 88.7% ±5.7SD percentage permeability respectively. The negative control group revealed a 1% ±0.1SD permeability while the positive control revealed 80.2% ±3.2SD permeability. Percentage permeability was found to increase with an increase in pulse duration from 100µs to 20ms at constant pulse amplitude (600V/cm) and one pulse (1).

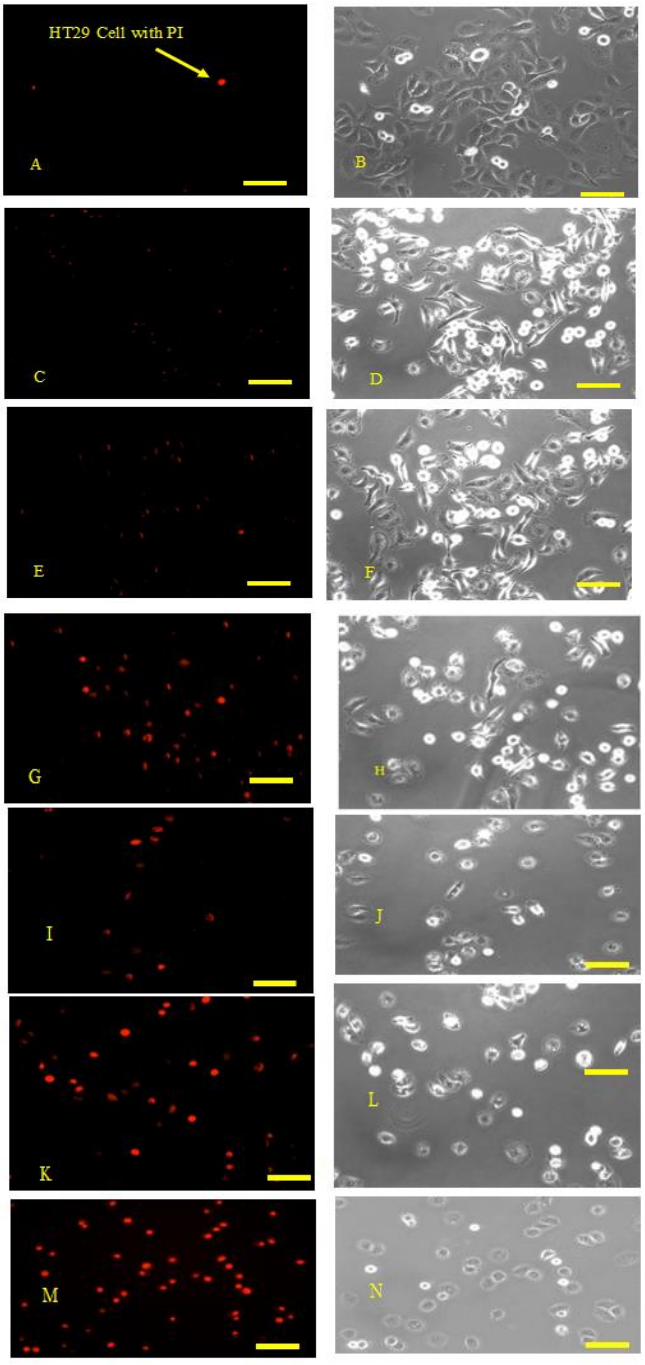

Fig. 1 . (A - M): Photomicrograph of fluorescent and phase contrast images of HT29 cell line after electroporation; (A-B) negative control (0V/cm); (C-D) 100us; (E-F) 500µs; (G-H) 5ms; (I-J)10ms; (K-L)20ms (M-N) standard parameter for ECT.

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TABLE 1
MEAN PERCENTAGE PERMEABILITY OF HT29 CELL LINE AFTER ELECTROPORATION WITH DIFFERENT ELECTRIC FIELD PARAMETERS (N=3)

Electric Field Parameter	Percentage Permeability (%) ±SD
Control (0V/cm)	1±0.1
100µs, 600V/cm	5.2±2.2
500µs, 600V/cm	36.3±4.7
5ms, 600V/cm	70.3±2.3
10ms, 600V/cm	78.0±2.4
20ms, 600V/cm	88.7±5.7
Standard parameter for ECT	80.2±3.2

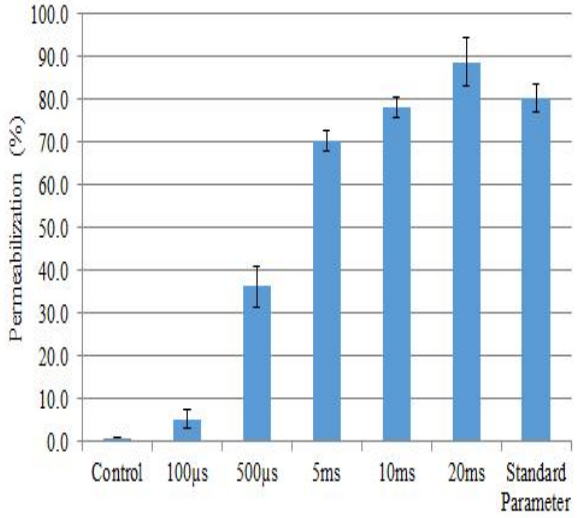

Fig. 2 . Mean Percentage Permeability of HT29 Cell Line after Electroporation with different Electric Field Parameters. (N=3). Standard deviation is used as error bars (n=3)

Cell viability was counted using trypan blue exclusion test with aid of a hemocytometer 24 hours after the electric treatment (electroporation). Figure 3 shows a photomicrograph from one field of view, one for each treatment from several acquired fields of view during viability counting. The mean percentage viability with standard deviation (SD) for three replicate experiments was given in Table 2 and figure 4. At a fixed pulse duration of 600V/cm with single pulse, 100µs, 500µs, 5ms, 10ms and 20ms revealed 86.1% ±2.2SD, 94.3% ±2.6SD, 80.4% ±3.0SD, 55.0% ±4.0SD and 23.0% ±3.4SD viability respectively. The negative control group revealed a 90.3% ±1.8SD viability while the positive control revealed 91.5% ±5.1SD viability. Percentage viability was found to decrease with increase in pulse duration from 500µs to 20ms at constant pulse amplitude (600V/cm) and one pulse (1).

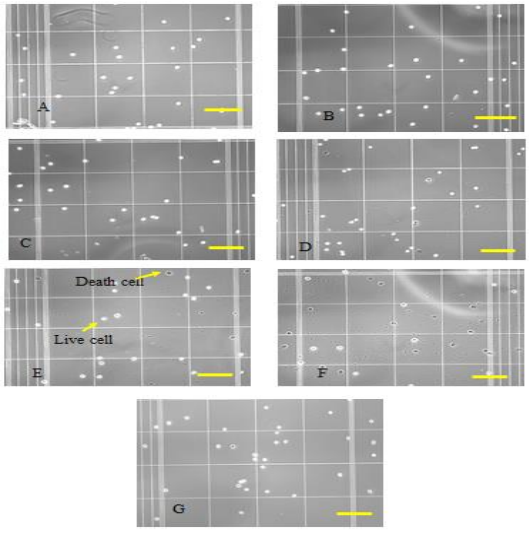

Fig. 3 . Mean Percentage Permeability of HT29 Cell Line after Electroporation with different Electric Field Parameters. (N=3). Standard deviation is used as error bars (n=3)

TABLE 2
MEAN PERCENTAGE VIABILITY OF HT29 CELL LINE, 24 HOURS AFTER ELECTROPORATION WITH DIFFERENT ELECTRIC FIELD PARAMETERS (N=3)

Electric field parameter	Percentage viability (%) ±SD
Control (0V/cm)	90.3±1.8
100µs, 600V/cm	86.1±2.2
500µs, 600V/cm	94.3±2.6
5ms, 600V/cm	80.4±3.0
10ms, 600V/cm	55.0±4.0
20ms, 600V/cm	23.0±3.4
Standard parameter for ECT	91.5±5.1

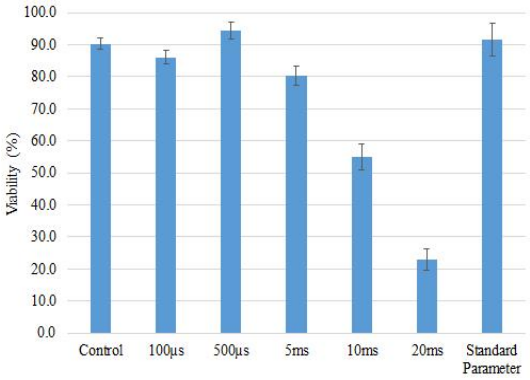

Fig. 4 . Mean Percentage Viability of HT29 Cell Line, 24 hours after Electroporation with different Electric Field Parameters. (N=3). Standard deviation is used as error bars (N=3)

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In this paper, attention was given to the effect of pulse duration on cell viability and permeability of the cell membrane. Pulse amplitude was fixed at 600V/cm and the number of the pulses was fixed to one. The standard electric parameter for electrochemotherapy was used as positive control while non-electroporated cells were used as negative control.

The cell's viability and permeability dependence on the pulse duration were measured using five pulses durations (that is 100us, 500us, 5ms, 10ms, and 20ms). Cell's viability was found to decrease with increase in pulse duration at constant pulse amplitude [34]. Whereas, permeability was found to be proportional to pulse duration (that is, permeability increases with an increase in pulse duration from 100us to 20ms), with constant pulse amplitude and the number of a pulse [35, 36].

This result of this study is in agreement with that of [6, 25 26]. Among the parameters used, only 5ms at a pulse amplitude of 600V/cm reveals relatively similar permeability and viability with Standard electric parameter. Hence, this low pulse amplitude could completely eradicate the muscular contraction and unpleasant sensation associated with high amplitude pulse during electrochemotherapy in vivo with the same efficiency.

IV. CONCLUSION

The result in this paper revealed that pulse amplitude of 600V/cm with duration of 500μs stimulated cellular proliferation while 20ms duration resulted in more than 80% cell death. On the other hand, 5ms pulse duration resulted to similar percentage of permeability and viability with the standard electric field parameter for electrochemotherapy. Hence, we can conclude that 600V/cm and 5ms duration can be used to eliminate the muscular sensation felt during electrochemotherapy (using standard electric field parameters).

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