Effect of Irreversible Electroporation on Cell Proliferation in Fibroblasts

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Abstract—**Uncontrolled cell proliferation as in the case of diseases like cancer remain one of the primary concerns in the medical area, more of clinical importance given the significant rise in the cases of cancer diagnosed every year. Current modalities of treatment however are shrouded by the undesired side effects, costs per sitting etc. which has created an awareness in the scientific community to develop effective alternative modes of treatment which could minimize the side effects and prove to be an affordable treatment option to the patients. Irreversible electroporation is a mode wherein electrical voltage pulses are applied in the absence of a chemodrug to kill the cancerous cells. In this study we present our results on the effect of such pulses on the proliferation and viability of fibroblast cells. The electric field applied is varied between 100V/cm to 1500V/cm with a pulse duration ranging from microseconds to milliseconds. Our findings indicate that these fibroblast cells are responding well to the electrical pulses applied and we can achieve proliferation control of these. Our study could be of clinical relevance in understanding the way these pulses affect the proliferation of various cells in the body and then translate these into effective cancer treatment.**

I. Introduction

Cancer is one of the major diseases that leads to deaths in both economically developed and developing countries [1]. Based on GLOBOCAN 2008 estimates about 12.7 million cancer cases and 7.6 million cancer deaths are estimated to have occurred in 2008. Lung Cancer is the most prevalent cancer among males comprising of 17% of the total new cancer cases and 23% of the total cancer deaths [2]. Electrochemotherapy (ECT) has proven to be the most effective treatment among the various cancer therapies [3, 4]. This method involves the application of electric pulses of sufficient intensity and duration on the tumor cells (Electroporation). The application of electric pulses along with the non-permeant chemotherapaeutic drug such as bleomycin, and cisplatin is termed as reversible electroporation. This type of electroporation is advantageous as the drug delivery is targeted and efficiency is higher than conventional methods. However, the disadvantage of using this technique is the side effects associated with usage of drugs like bleomycin, cisplatin etc. The above disadvantage can be overcome by the applica-

tion of high intensity, short duration electrical voltage pulses on tumor cells without using drugs. The intention here is to irreversibly damaging the cell membrane of the cell with the electrical pulses and delivering the apoptotic signal and this strategy is termed as irreversible electroporation. This high electric field can cause the cell membrane to become permanently permeabilized, after which apoptotic signals delivered cause cell death to occur [5]. Recent reviews show that IRE does not cause denaturation of proteins typical to thermal ablation and is not affected by blood flow. IRE is a novel technique that can be used for non-thermal ablation and focused cell necrosis [6, 7].

Irreversible electroporation (IRE) spares connective tissue in blood vessels and other tissue structures [8]. The exact working of the electroporation mechanism is not yet known but it is thought that the electrical field changes the electrochemical potential around a cell membrane and induces instabilities in the polarized cell membrane lipid bilayer. The unstable membrane then alters its shape forming aqueous pathways that possibly are nano-scale pores through the membrane. These are permanent pores in case of IRE and it causes the cell to swell due to osmosis and eventually leads to ablation [9]. Irreversible electroporation is simple and their clinical applications are numerous. The main task is the delivery of appropriate electric fields safely [10].

The main objective of the present work is to study the effects of various pulse parameter on Chick Embryonic Fibroblasts (CEF). Another objective is to study the post proliferative effect of irreversible electroporation on Chick Embryo Fibroblasts. We chose to use the CEFs, because they are used extensively for a variety of research applications, their ease of availability, and their proliferation nature - they are good models for proliferation as they proliferate very fast in the body.

II. Materials and Methods

A. Chick EmbryoFibroblasts

Nine to eleven days old eggs were used. The egg shell surface was wiped with 95% EtOH thoroughly and was cracked at the top using forceps. The embryo was removed and placed in a 100cm^2 sterile petri dish containing sterile Phosphate Buffered Saline (PBS) Solution. The head, internal organs and appendages were removed. Tissues were isolated from the remaining parts under sterile conditions. Isolated tissues were minced and trypsinized using 3ml of 37°C trypsin/EDTA and incubated for 5 min and medium containing FBS was added to stop the action of trypsin. The tissues were removed and the cell solution was centrifuged at 1500rpm for 7 minutes. The cell pellet was rinsed with 2-3 washes with PBS solution. The cells were then cultured in 60mm cell culture dish with DMEM containing 5% FBS and 1% Penicillin-streptomycin-glutamine solution and incubated at 37°C in a 5% CO_2 atmosphere. Fig. 1 shows a view of these cells [11].

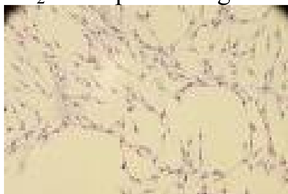


Fig. 1. Chick Embryo Fibroblast cells [11].

B. Irreversible Electroporation

Irreversible electroporation was carried out using 6 different parameters. Both high intensity field, short duration pulses and low intensity field long duration pulses were used. The 6 parameters applied during electroporation are 100V/cm, 24ms; 500V/cm, 24ms; 500V/cm, 1ms; 1200V/cm, 100µs; 1500V/cm, 300µs; 1500V/cm, 500µs. the pulses were given in a single train of 8 pulse with frequency of 1Hz between each pulse. The experiments were conducted in triplicate to avoid experimental error. In addition, cells were also subjected to single pulse at the above conditions and their impact was studied.

A BTX ECM 830, square wave electroporator was used for the experiment. The cuvette size was chosen to be 4mm in order to avoid arcing at high fields. The electroporation buffer used was DMEM 10% FBS. Since no drug is required for IRE 300µl of the cell suspension alone was used for electroporation.

C. Cell viability

The cell viability was determined following electroporation. For cell viability assay, 10µL of the solution containing the cells was transferred under the glass cover of a Neubauer hemocytometer (Nexcelom Bioscience LLC. MA) and the cell imaging and counting were done. Both live and dead cells are counted. By phase contrast technique, the live cell seems to reflect the light and hence it appears bright, whereas the dead cell takes up the light rays from the source filament and appears dark. The cell viability is estimated for each case from the cell count.

A. Colony Forming Unit (CFU) Assay

CFU is a measure of viable colonogenic cell numbers in CFU/ml [12]. A fixed number of isolated chick embryonic fibroblasts were plated in a six well plate along with 2-3 ml of DMEM medium. Then 100 µl of the sample + 700 µl of DMEM medium are taken in a 4-mm gap cuvette and electroporated using various parameters. In this case, only one pulse was applied. After electroporation, the cells were seeded onto tissue culture dishes in DMEM with antibiotics and maintained at 37oC in a 5% CO2 atmosphere. They were undisturbed for 7 days until colonies of cells appear. The dishes were washed with PBS and cells stained with 0.1% Crystal Violet in Methanol and incubated at room temperature for 10 minutes. The staining solution was discarded and the dishes observed under a microscope. A collection of cells of around $100mm^2$ (~ 50 cells) was counted as one colony.

III. Results and Discussion

In these experiments performed, various pulse durations were studied extensively in order to find out their effect in combination with different magnitudes of electric field. It is observed that when the pulse length is too small with a medium value of field, the effect on reducing viability is not significant whereas a higher pulse length with lower field was able to produce the desired results.

A. Cell Viability Analysis

The cell viability in percentage was calculated as a ratio of number of live cells to the total number of cells (sum of the total number of alive and dead cells).

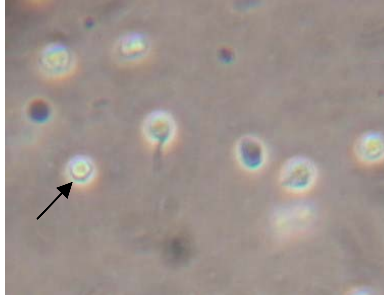


Fig. 2. Cell viability test showing live (indicated by arrow) and dead cells.

Table 1 shows the viabilities obtained for various pulse parameters.

TABLE 1: VIABILITIES FOR VARIOUS PULSE PARAMETERS

Intensity	Duration	Viability	
		8 pulses	1 pulse
100V/cm	24ms	10.19	12.50
500V/cm	24ms	0.00	4.80
500V/cm	1ms	25.56	36.70
1200V/cm	100µs	7.36	12.80
1500V/cm	300µs	7.12	9.60
1500V/cm	500µs	9.07	8.90

Figs. 3 and 4 illustrate the viabilities for the conditions studied. The results show considerable differences in the viabilities influenced by the intensity and duration of the applied pulses. It is seen that 8 pulses at 500V/cm, 24ms, resulted in complete death of all cells (Fig. 4, pulse condition A). For the same field with 1ms pulse length, there is increase in viability. When the pulse length difference is not that significant then there is no observed difference on viability. At 1200V/cm and 1500V/cm, for a difference of 200µs in pulse length the viabilities are 7.36% and 7.12% respectively, showing that a significant change is obtained only when there is considerable difference in pulse length. It was also proved using 300 and 500us pulses at 1500V/cm, that the difference was not much. Statistical analyses of these data indicated no significant difference. This indicates that at 1500V/cm, we can use either 300 or 500µs pulses, and obtain similar cell death magnitudes. These results indicate that with fine-tuning the pulse durations for a given intensity, we can maximize the outcome.

The trend of our results correlates with the those obtained by Rubinsky's team [6]. Figs. 5 a and b show the result obtained by them using HepG2 (primary human hapatocarcinoma) cells using a single pulse at 1ms, 1.5ms, 3ms, 6ms and 24ms durations for the intensities of 2500V/cm, 2000V/cm, 1500V/cm, 100V/cm, and 500V/cm respectively. It can be seen that 1000V/cm, 6ms has reduced viability than 1500V/cm, 3ms pulses, pos-

sibly due to the reduced intensity, despite twice the duration. The difference between our results and theirs could be attributed to the difference in cell type.

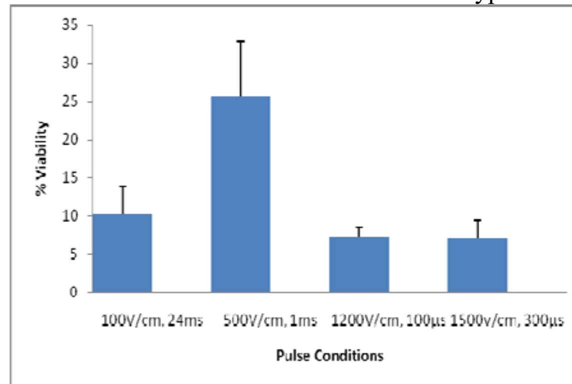


Fig. 3: Viability of of Chicken Embryo Fibroblasts after Irreversible Electroporation at 100V/cm, 24ms; 500V/cm, 1ms, 1200V/cm, 100µs, and 1500V/cm, 300µs pulses. Error bars indicate std deviation/sqrt(3).

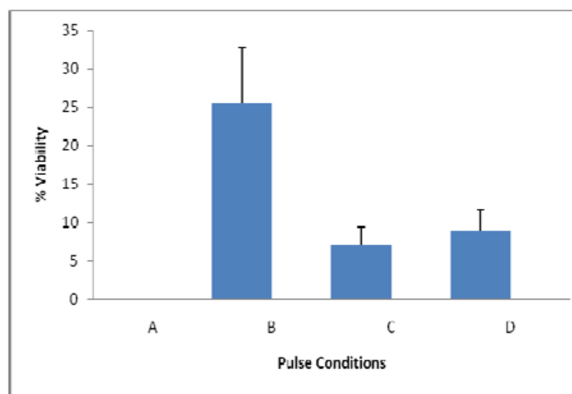
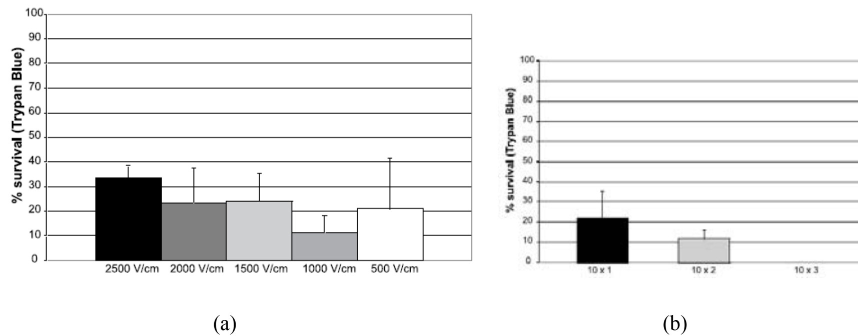


Fig. 4: Viability of of Chicken Embryo Fibroblasts after Irreversible Electroporation at various pulse parameters: A–500V/cm, 24ms (zero viability), B-500V/cm, 1ms, C-1500V/cm, 300µs, and D-1500V/cm, 300µs. Error bars indicate std deviation/sqrt(3).

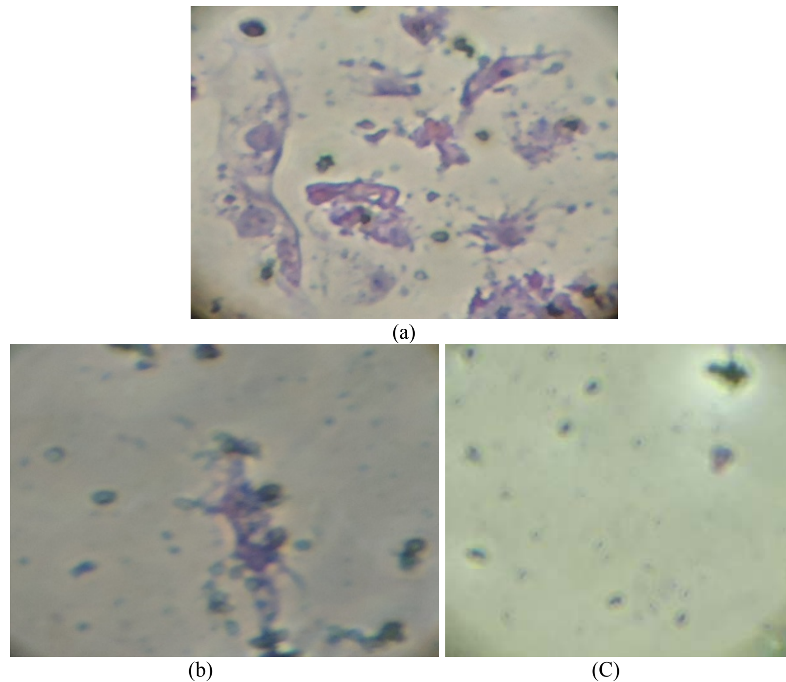


(a) (b)

Fig. 5. Viability of cells after Irreversible Electroporation – a) for various pulse parameters using single pulse, b) due to a number of pulses for 1500V/cm, 300µs pulses [6].

B. CFU Assay

Figs. 6a-c show the colonies formed by the various samples. Fig. 6a show the large number of colonies formed in the control sample (no pulses applied). In this case, the number of colonies was found to be 86CFU/ml whereas after electroporation, the cell multiplication recedes leading to less number of colony forming units. The number of colonies at 1500V/cm, 200μs was 2 CFU/ml. At 250μs, only a few cells were observed (Fig. 6c) and no colony forming units were found. This could be due to the due to slightly increased pulse length, indicating the sensitivity of the column forming units in this case.



(a)

(b) (C)

Fig. 6. Colonies after the CFU assay. (a) Colonies formed without any electroporation, (b) colonies formed after electroporating at 1500V/cm, 200μs, and (c) colonies formed after electroporating at 1500V/cm, 250μs, one pulse in each case, b and c.

IV. CONCLUSIONS

Irreversible electrochemotherapy is an extremely effective technique for ablation of cells using electrical pulses alone. The results of our study and previous studies indicate that electrical pulses can be used alone in order to provide a considerable reduction in viability of cells. This reduces the cost of chemo drugs which vary from a few hundred dollars per month to several thousands per month, depending upon the type of drug. In case of irreversible electroporation this drug cost is completely eliminated.

Irreversible electroporation could be considered to be more effective than reversible because IRE can cause cell death in the absence of any drug. In contrast to thermal tissue

ablation, IRE is truly unique due to its advantage in ablating tissues without being affected by a "heat-sink" effect. One major problem with thermal ablation is the heat sink effect, which describes the ablative thermal heat dissipated by adjacent blood flow. Therefore, thermal ablation on tissues near blood vessels is limited and ineffective since these tissues are exposed to suboptimal thermal energy. But IRE does not show any joule heating effect as mentioned above. Another significant advantage of IRE is the ability to create a distinct margin separating ablated tissues and normal tissues i.e., there is clear demarcation of IRE induced cell death from normal cells [13]. Several preliminary studies have shown that IRE may create cell death via combination of both necrotic and apoptotic pathways.

The experiments performed show that a longer pulse duration (millisecond) combined with a low intensity field gives a reduced viability. Low intensity fields are desirable due to reduced voltage levels and hence increased safety and reduced cost of operation. It can be seen that as low a magnitude of 100V/cm at 24ms length, could elicit irrversible electroporation effect with appropriate number of pulses. Thus our study is distinct from others in the use of low voltage magnitudes of the pulses.

When this long duration pulse is combined with a low/high intensity field, depending upon the number of pulses and the interval between them, complete cell ablation occurs even in the absence of drugs. This can be extended to cancer cells and studies can be performed using the effect of IRE on tumor cells which can be used for novel cancer treatment modalities.

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