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Investigation of Effect of Nanosecond Pulsed Electric Field on MCF-7 Breast Cancer Cells

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Abstract

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Pulsed electric field therapy is a novel non-invasive approach for cancer therapy. It serves as a cell permeability enhancing agent for cancer treatment. Nanosecond, high-electrical field pulse power technology is used for delivering variable, controllable, intracellular electrical perturbations in several biological systems. Here, we investigated the effect of nanosecond (ns) electric pulse (nsEP) as a therapeutic tool for cancer. In *in-vitro* study, the breast cancer cells (MCF-7) were exposed with electric field of ~18kV/cm intensity, ~25ns duration, at 1.5Hz in a 2mm electroporation cuvette. Post exposure, observation shows a significant reduction in cell viability. It was evident that after treatment the viability of MCF-7 cancer cells at 630 pulses are remains ~38% only. The optical microscopic analysis of MCF-7 cells shows cell morphology changes after electrical pulse exposure. Moreover, we have also investigated a comparative study of the effect nano-second electrical pulses on MCF-7 cells and Chinese Hamster Ovary (CHO) cell line. The comparative study, demonstrated that the effect of nsEPF on MCF-7 is more destructive than on CHO cell line. The obtained results support that the pulse electrical field of nanosecond (ns) duration therapy would be a potential solution for cancer treatment.

Keywords: Nanosecond Pulse Electric Field; Full Width at Half Maxima; Pulse Forming Line; Pulse Exposure; Viability;

1. INTRODUCTION

In past few decades, treatment of cancer using pulsed electric field has gained extensive attention among scientists because of having virtue of its medical and biological applications, such as gene delivery [1-5], electro-chemotherapy [6-11], and cancer therapy [12-18]. The advantage of pulsed electric field treatment, making it different from conventional and other physical techniques, is the ability to destroy cancer cells in a non-thermal manner [19, 20] by inducing apoptosis. Consequently, pulsed electric field treatment has ability to make it possible to preserve sensitive tissues intact, such as blood vessels and axons [21, 22]. Moreover, this minimally invasive technique allows the possibility of regeneration with healthy cells and tissues in the treatment region and leaves almost no scar [23]. With the aid of ultrasound, CT, MRI etc., pulsed electric field treatment could be monitored in real time, which improves the treatment efficacy immensely [24-26].

Electric field is of having sufficient magnitude causes reversible (microsecond to millisecond and few hundred volts per centimetre) and irreversible changes (microsecond and further short having tenths of kilovolts per centimetre) in cell membranes. The first paper reporting the reversible breakdown of cell membranes when electric fields are applied was published in 1958 [27]. The first report on the increase in permeability of the plasma membrane of a biological cell, an effect that is known as "electroporation" appeared in 1972 [28]. The electric fields that are required to achieve electroporation depend on the duration of the applied pulse. Typical pulses range from tens of milliseconds with amplitudes of several 100s V/cm to pulses of a few microseconds and several kV/cm. Mathematical modelling and analysis shows this result in optimistic manner that microsecond and longer duration pulses causes effect on membrane and shorter pulses from nanosecond affects mostly in intracellular domain pulses of time duration between few nanosecond to microsecond effect on both membrane and intracellular region [29]. For a substantial effect on eukaryotic cells, electric field needs to be 0.5-1V on cell membrane. Activated caspase-3, which is highly required in nanosecond Pulsed Electric Field (nsPEF) treatment, increases 8-fold in Jurkat E6-1 cells and 40% in rat hepatocellular carcinoma and mouse fibrosarcoma cells by 3h post treatment. This increase is non-linear and peaks at 15–20 J/mL for all field strengths [30].

Required time to charge the surface membrane is depending upon the electrical parameters of both the cell and the suspension medium. For spherical cell having a surface

membrane that is an ideal dielectric with no leakage currents, the charging time constant is given by [31].

$$\tau_c = (\rho_c + \rho_a/2)C_m \frac{D}{2} \text{...........................} (1)$$

Where C_m is capacitance of the surface membrane per unit area, D the cell diameter, ρ_c the resistivity of the cytoplasm, and ρ_a the resistivity of the medium in which the cell is suspended, for a cell with a diameter of 10 μm, cytoplasm and medium resistivity of 100 Ω-cm, and a membrane capacitance of 1 μF/cm², τ_c is 75ns [32]. It needs to be mentioned that the charging time constant, τ_c, is defined as the time to charge the membrane to 63% of its final value. In order to charge it to 95%, the voltage needs to be applied for a time of $3\tau_c$ (membrane charging time constant). During the charging of the plasma membrane, the nucleus and other organelles are also exposed to the electric field. The smaller the organelle diameter, faster the subcellular membranes will be charged [33]. When the voltage across these membranes reaches critical values, poration is expected. Targeting of subcellular structures with unipolar pulses requires the pulse rise time to be much less than the charging time of the plasma membrane. Moreover, the pulse duration itself should be kept shorter than the time required for the onset of electroporation of the outer membrane. Experiments conducted with HL-60 type cells and Jurkat type cells have proven that, for sub-microsecond pulse durations, the probability of interactions with internal structures increases, whereas, the outer cell membrane stays intact [34].

In the following investigation, pulse generator that produces 3.6 kV, ~25 ns FWHM (Full width at Half Maxima) electric pulses at 1.5 pulses per second. Produced pulsed electric field (~25ns, ~18kV/cm) has been exposed to MCF-7 cancer cells in 2 mm electroporation cuvette. Morphology has been observed before and after pulse exposure and survival rate (viability) is investigated using MTT assay.

2. MATERIAL AND METHOD

2.1. *Pulse Generator*

Tesla transformer driven PFL has been utilized to generate the nanosecond duration pulse. Here, coaxial cable is used as pulse compressor. Figure 1 shows schematic of electrical circuit and OrCad circuit simulation and simulation result is shown in the Figure 2. Capacitor C_1 is initially charged up to 1 kV and discharge through secondary winding of transformer and C_2. To transfer the stored energy from C_1 to C_2 it is required that $C_1 > n^2C_2$, *n* is turns ratio (ratio of secondary to primary turns, here, 90). Semiconductor switch, SW_1 (IGBT, (SKM800GA176D, SEMIKRON) turned on to charge the C_1, C_1 discharges through L_1 and voltage across L_2 developed which charges the C_2 and the cable. Cable compresses the pulse by its delay time as below:

$$\tau = \frac{l}{v} \text{..} (2)$$

And, velocity of wave in cable.

$$v = \frac{c}{\sqrt{\varepsilon_r}} \text{..} (3)$$

Where; *l* is the length of cable and v is the; c is velocity of light and ε_r is relative permittivity of cable dielectric.

Switch, SW_2 is a spark gap switch to discharge the cable through load.
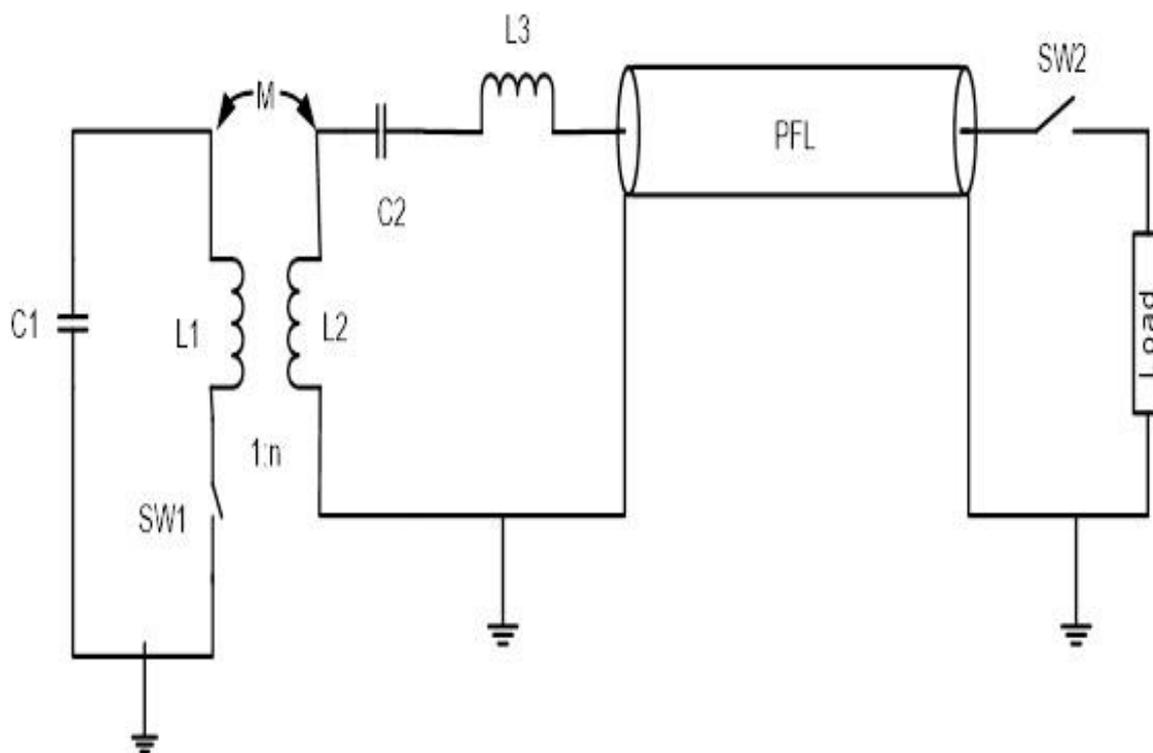

Figure 1: Circuit diagram of Tesla transformer driven pulse forming line (PFL). Inductance of coils of transformer are L1, L2 and L3 is parasitic inductance of line. Spark gap switch SW2, is triggered when cable is charged up to gap breakdown voltage.
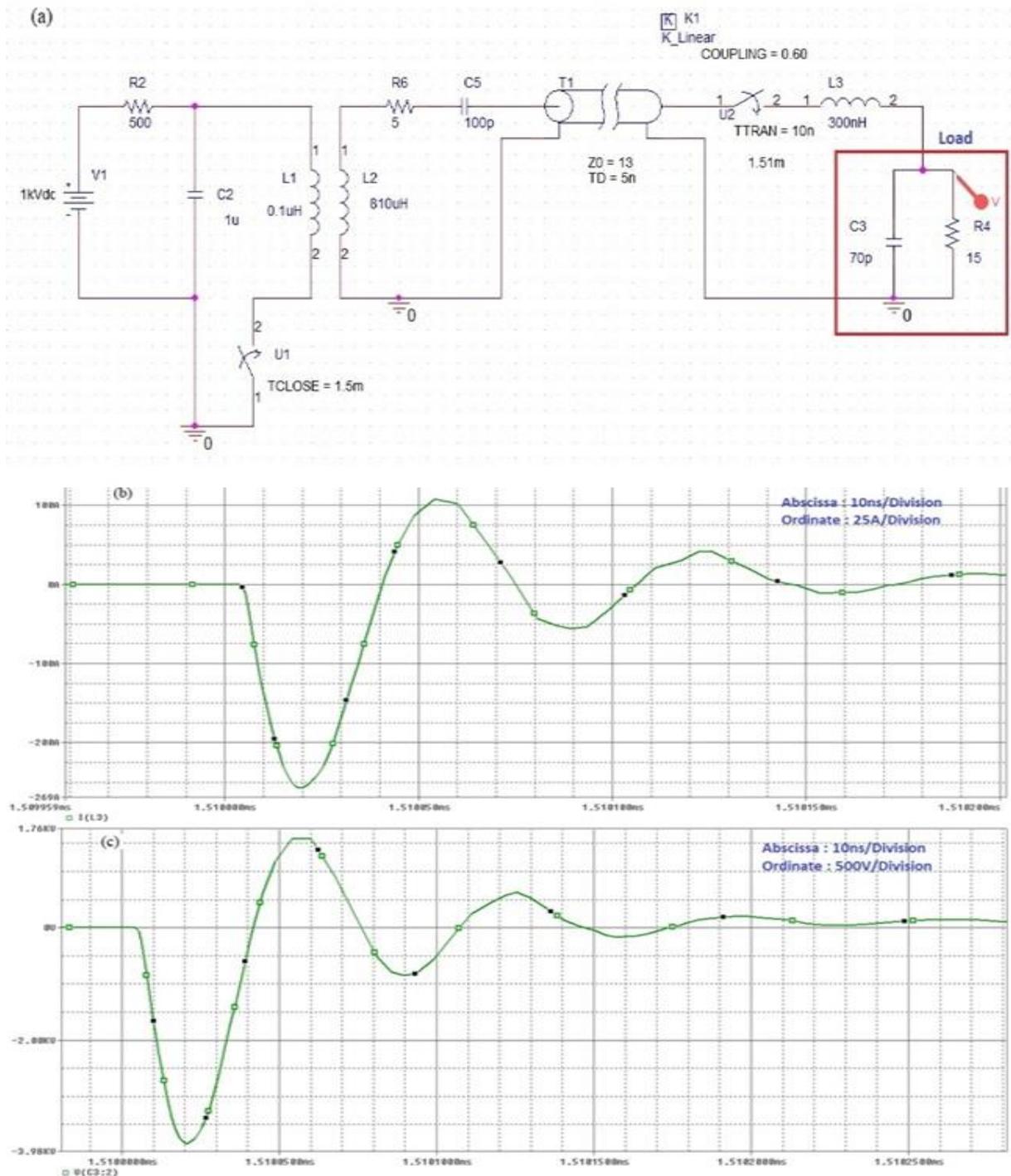
Figure 2: OrCad simulation circuit and simulation results. Circuit diagram (a) and output current (b) output voltage (c) obtained by circuit simulation. Output voltage pulse having full width at half maxima (FWHM) ~25ns and rise time ~10ns, approximately matches with scope output up to a large extent except scope output shows lower damping factor.

Cable length chosen ~5 meter to produce pulse duration of $\tau = \sim 25\ ns$. Parasitic electrical parameters of capacitor, cable, connecting wire and spark gap etc affect the pulse shape, duration and output voltage therefore by actual

circuit the obtained pulse shape and duration is different from simulation. Actual arrangement and output pulse shape is shown in Figure 3.
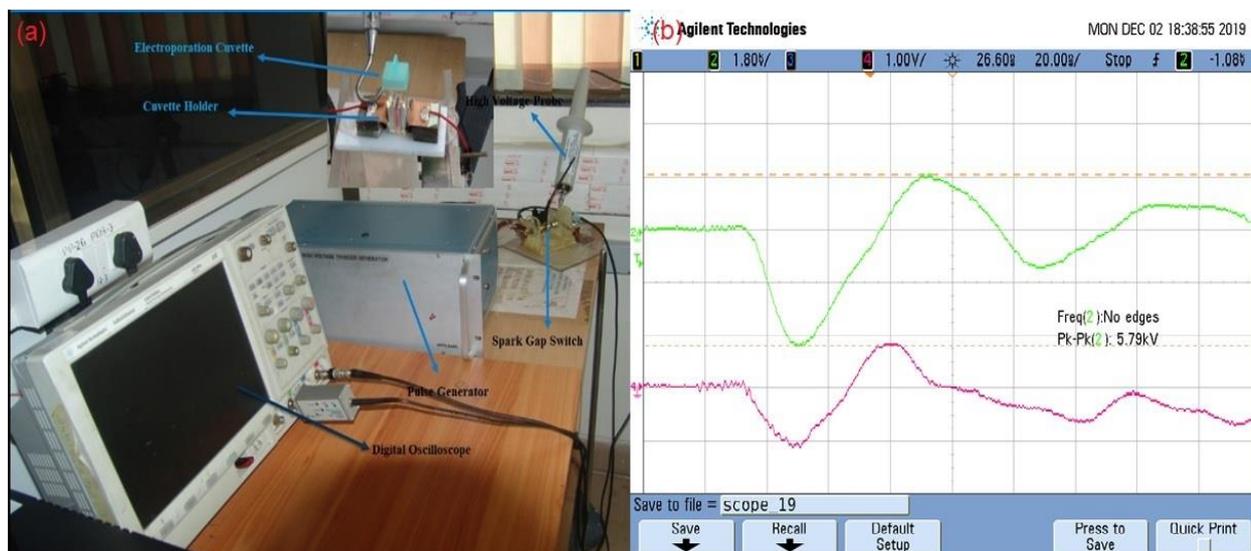
Figure 3: Experimental setup arrangement (a) and Output voltage on digital Oscilloscope (DSO) (b) up to a large extent it matches with the output obtained in the OrCad simulation.

2.2. Cell Culture Preparation

MCF-7 (Breast Cancer) cell line, were used in the present study. Cells were harvested in tissue culture flask ($25cm^2$, HiMedia), in Dulbecco's Modified Eagle Medium (DMEM, HiMedia) at 37ºC in humidified, 5% CO_2 incubator. DMEM contains 10% fetal bovine serum (FBS, GIBCO) and 1% antibiotics Penicillin-Streptomycin-Amphotericin (PSA) which support the cell growth. After achieving 90% confluency, cultured cells adhering to the bottom surface of the flask were washed twice with phosphate buffered saline (PBS, Wako) and detached from the flask bottom using PBS based 0.025% trypsin and ethylenediaminetetraacetic acid (EDTA, HiMedia). The cell suspension was centrifuged at 1200 rpm for 5 min and re-suspended with fresh DMEM medium to form a required cell concentration of 1 Million/ml.

2.3. Pulse Exposure

MCF-7 cancer cells in suspension are exposed to 3.6kV, 315-630 pulses with increment of 45 pulses at 1.5Hz (1.5 pulse per second), in a 2 mm electroporation cuvette (BioRad, Inc., Hercules, CA). Electroporation cuvette has embedded aluminium electrodes with a gap of 2 mm, which produce field of ~18 kV/cm at 3.6kV. Current is measured using current shunt (R=0.005056 Ω). Peak current calculated as ~178A. Therefore, Energy = VIt , (~16.2 mJ) in single pulse is deposited in volume of 400μl (Volume of cuvette), and energy density would be 40.5mJ/ml.

2.4. MTT Assay

Post exposure of electrical pulses cells were seeded in 96 well plate at concentration of $5x10^4$ cells per well. Total volume of well was made 120 μl and cells were incubated for 24 hours in 5% CO_2 incubator at 37^0 C. After 24h incubation media was removed carefully from 96 well plate. MTT solution in DMEM (0.5 mg/ml) was added in each well and again incubated for 4h. After 4h MTT solution was removed

and 100μl of DMSO+1% Glacial Acetic acid was added in each well. The plate was shaken for 10min by orbital shaker. It is ready for quantitative analysis under 96 well plate spectrophotometer (micro-plate reader, Synergy H1, BioTek) at 570-nm wavelength. Experiment is performed in triplicate.

$$\text{Viability (\%)} = \frac{\text{Average OD at 570nm of treated group}}{\text{Average OD at 570nm of the untreated group}} \times 100$$

2.5. Observation under Optical Microscope

Morphology of untreated and treated cells were observed under microscope (RTC7, Radical), before pulse exposure, immediately after pulse exposure and post 24h of incubation of both treated and untreated cells.

3. RESULTS AND DISCUSSION

Results

3.1 *Pulse treatment does not induce necrosis immediately after treatment:* Cell growth inhibitory effect was examined by viability assay and morphological observation under optical microscope. Qualitative observation of control and treated cells as shown in Figure 4 shows there is no morphological difference between the control and treated cells immediately after treatment. This shows there is almost no necrosis post exposure of electrical pulses.

3.2 *Pulse treatment reduces viability of treated cells post 24 hrs:* As per microscopic observation after 24h, it can be observed that the morphology of treated cells has been changed significantly (Figure 4). Viability test conducted to control and treated cells shows treated cells viability is reduced from ~100% to ~38%, at 630 pulses Figure 5 (a). This observation infers that most of the cell death is probably apoptotic rather than necrosis. Although further analysis is required to confirm this.
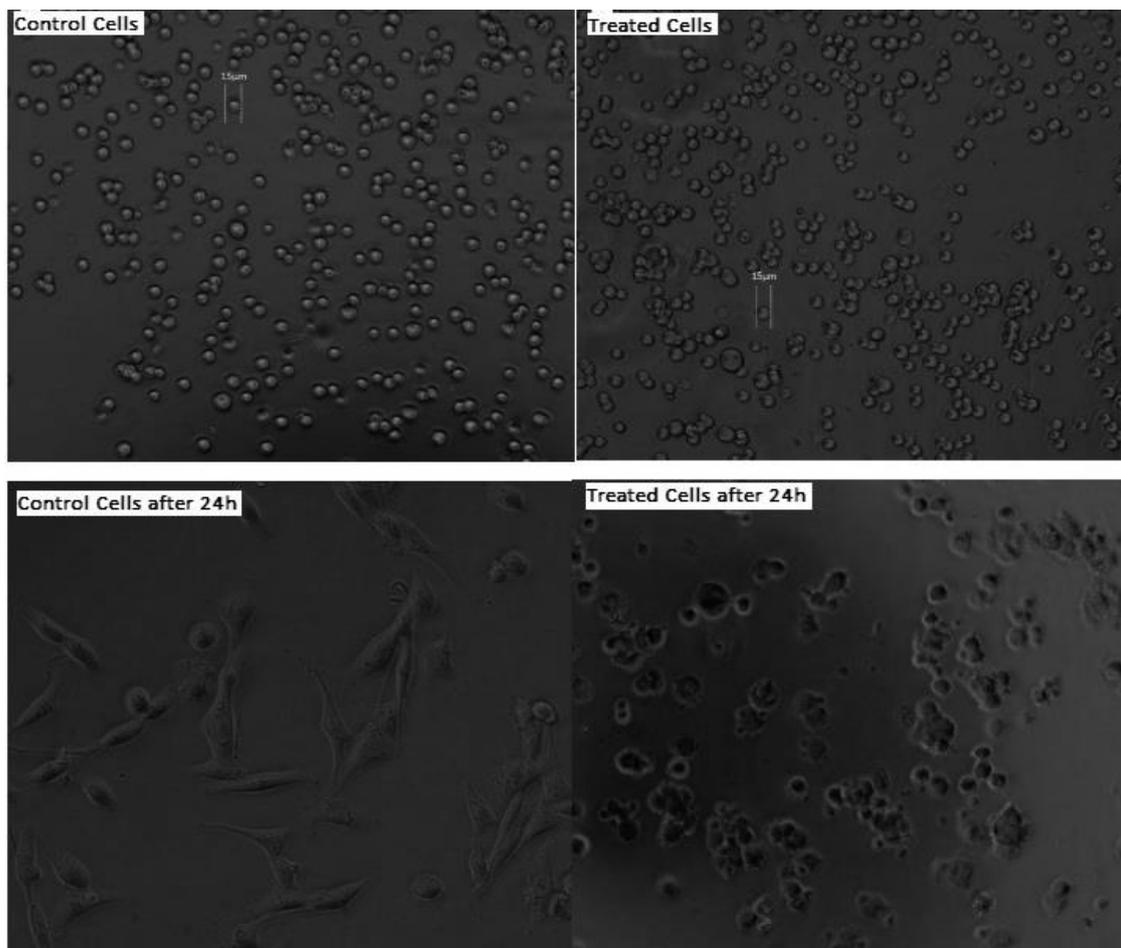
Figure 4: Morphology of control cells, treated cells, Control cells after 24h and treated cells after 24h. This shows that there is no morphological difference between control and immediately after treatment but post 24h there is cell shrinkage is observed in treated cells.

3.3 _Pulse treatment reduces viability of MCF7 more than CHO cells:_ Viability test on CHO cell line shows that in comparison to MCF-7, viability of CHO is more compared to MCF-7 at same number of pulse exposure (Figure 5(a)). Compared to MCF-7, CHO cells are small in size and at this pulse parameter, pulses are not fast enough to charge the internal organelle membrane (eq. 1) and not able to expose a threshold voltage on intracellular organelles membrane to

trigger apoptosis that causes significant cell death. Although some death in those cells is may be because thermal effect as exposure high number of electrical pulses. Since energy per pulse is only 16mJ therefore thermal effect should not be significant as pulse duration is too short. At 315 pulses viability of MCF-7 is ~80% shows no significant cell death but further viability is decreasing.
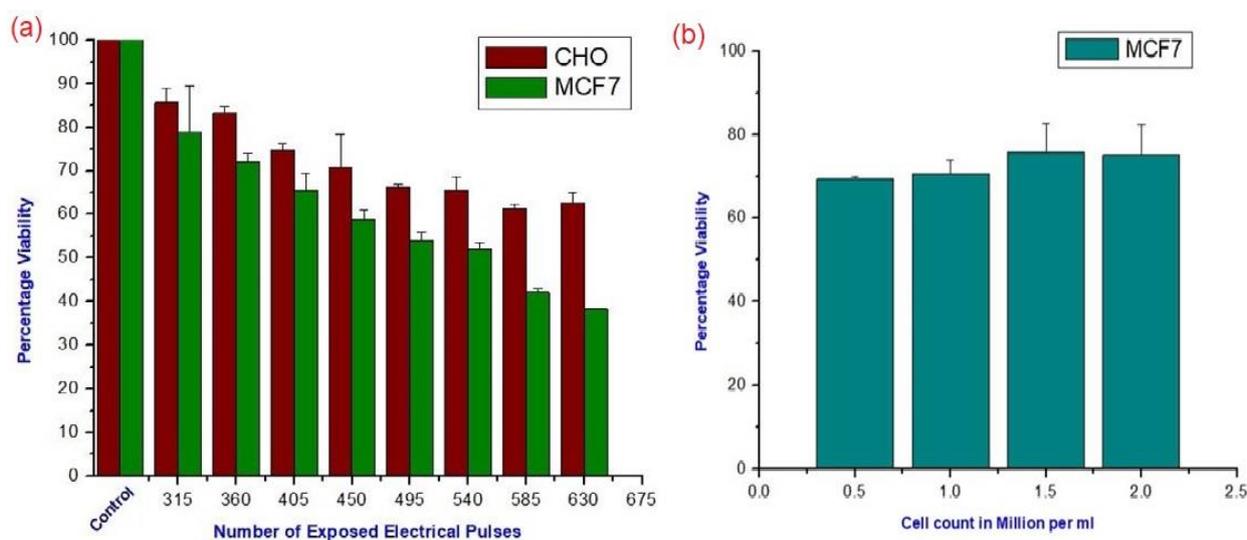

Figure 5: Number of applied pulses v/s viability graph. Viability of MCF-7 and CHO cell line vs pulse number (a) Comparing to CHO, MCF-7 cell line is more affected by electrical pulses. Viability of MCF-7 vs Cell count at 450 pulses (b).

3.4   *Cell count effects the viability in pulse electric field:* The effect of cell count per ml v/s percentage of viable cells with respect to control at 450 pulses is shown in Figure 5 (b). Application of electric field on 0.5 Million/ml is quite significant while for other cell count effect is not substantial. This could be because of variation of permittivity of the cells culture with respect to cell count. It can be inferred with this observation that number of cells in suspension increases permittivity of suspension and electric field exposed in the suspension decreased which further reduces detrimental effect to the cells, as per eq. (4).

$$E_{\varepsilon_r} = \frac{E_0}{\varepsilon_r} \dots\dots\dots\dots\dots\dots\dots(4)$$

E_0 is electric field in air and E_{ε_r} is electric field when permittivity of medium is ε_r.

Assuming no significant thermal effect, the reduction in cells viability is because of pulse exposure. Viability assay shows toxicity in the cell is due to pulse exposure. Since NAD(P)H-dependent oxyidoreductase is released by mitochondria in live cells only, which forms formazan when reacts with MTT. MTT assay analysis and morphology infer that viability reduction could be because of induction of apoptotic cell death. Electrical pulses of nanosecond duration put stress on mitochondria, which further releases cytochrome-C and in response apoptotic pathway begins. Energy delivered per pulse was ~16.2mJ, which limits the temperature rise of suspension.

3.5 *Viability of cancer cells decreases with increase in exposure time of pulse treatment:* When cells were exposed to electric field ~25ns, ~18kV/cm of for 10min, 20min, 30min and 40min at 1.5 pulses per second rate.   The observed cell death is high as the increasing exposure time increases temperature of medium is and hence cells may undergo necrosis (Figure 6).
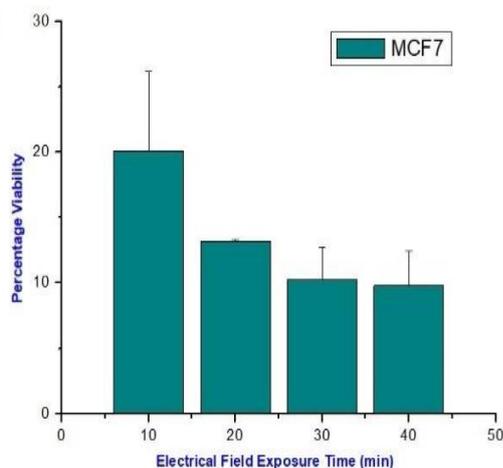

Figure 6: Cell viability v/s pulse exposure time. This shows death of cells when electrical pulses are exposed from 10min-40min at 2pulse per second. Higher electrical pulses cause necrosis and death is because of thermal effect.

Discussion

Study investigates the effect of electric field exposure to cancer cells. Our findings show cell death ~62% because of pulsed electric field. Exposure of pulsed electric fields (50 pulses) of 100 μs duration to MCF-7 at repetition rate of 1 pulse per second, 280-640 V at 40V step, in 4-mm electrode spacing, cell's death was obtained 40% (maximum) using MTT assay [35]. Although in this report western blot and flow-cytometric analysis was performed to confirm the apoptosis. Also, it was shown in this report that combination of

Photodynamic therapy and irreversible electroporation in synergy essentially shows higher cell death rate compare to electric field alone. In our experiment only electric field of nanosecond duration is used. Pulse repetition rate is kept 1.5 pulses per second to avoid any death by thermal effect. Combination of gemcitabine and electrical pulses cell death increases effectively [36]. Investigations with low voltage (200-500 V/cm), milliseconds duration and high voltage (1200 V/cm), microseconds duration, pulses at 1 Hz were used in electro-chemotherapy (ECT). Efficacy in ECT was high because of direct involvement of toxic drugs. Combination of Paclitaxel and 200μs 1200V/cm electric field reduces the viability of cells to ~20% [37]. Although, combination of EP+IR (100μs+Ionizing radiation) shows lowest viability ~65.1% [38].

4. CONCLUSION

Present study investigated the effect of nsPEF, in vitro experiment on MCF-7 breast cancer cells and morphology observation and viability assay confirms reduction in viability of cancer cells. Reduction in viability is significant due to pulse exposure. Nanosecond pulse exposure for cancer treatment will require only electrical field without any anticancer drug in additional that makes it free of side effect. However, more studies for different cell lines and in vivo experiments are required to reach to certain conclusion for the use of EP in clinics. We seek ways to improve our delivery system for the use of medical purposes, to eliminate working boundaries and to achieve our expectations for the biological experiments.

Acknowledgement

Conflict of Interest

None Declared

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