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Effect of Pulse Width and Intensity on Cell Death in Reversible Electroporation of Cancerous Cells

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Abstract
Electroporation (EP) is the process of increasing the permeability of a biological cell or tissue by applying a short-term and sufficient external electric field. The utilization of proper pulse settings is required for EP-based treatments to be successful. Our aim in this study was to examine the effect of different electrical pulse widths and strength on EP efficiency. Human osteosarcoma cells (U2OS) were used in the study. Eight-square-pulses with a frequency of 1Hz at 10µs, 1ms, 5ms, 10ms, and 20ms widths with low electric fields (20-500V/cm) were applied to U2OS cells. 10-15 minutes after the applications, the cells were incubated in 96-well plates with 10 thousand cells in each well for 24 hours. Efficiency of pulses of different intensity and width was evaluated by MTT analysis method. The percent inhibition of U2OS cancer cells elevated as the pulse width increased in almost all electric field values. The highest cell inhibition (%) occurred in pulses with an electric field of 500 V/cm and a width of 20ms (inhibition ratio: 76.25%). No inhibition was observed in the cells at 10µs, 1ms, 5ms, 10ms width pulses with 20 V/cm electric field and 10µs, 1ms width pulses with 50V/cm electric field. In conclusion, our findings show that the electric field intensity and pulse width used in electroporation play an important role in U2OS cancer cell death. According to our results, it may be more appropriate to use high-voltage short-width pulses or low-voltage long-width pulses in reversible EP studies.

1. Introduction

Electroporation (EP) is a biophysical process in which the membrane permeability is elevated by increasing the transmembrane voltage of the cell membrane above the threshold level (0.2-1V) with electrical pulses [1]. Large molecules and ions that unable to pass through the membrane can enter the cell by EP [2]. Tumor treatment techniques include irreversible electroporation (IRE) and chemotherapy+reversible electroporation (electrochemotherapy, ECT). In the IRE technique, high voltage (>600 V/cm) and short duration electrical pulses are used (no anticancer drug administration) to change the membrane potential of the cancer cell, resulting in permanent holes and apoptosis [3]. Since this technique uses larger number of high amplitude pulses, neuromuscular blockade and general anesthesia are required [4]. IRE therapy shows promise for the treatment of malignancies such as cardiac catheter ablation and intra-abdominal cancers. As for the reversible electroporation approach, microelectropores form on the cell membrane during electric field application and these pores close again shortly after the electroporation process. Therefore, this approach allows the cells to live, while IRE destroys the cells directly [5].

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Some cancer medications are designed to cause DNA damage. These drugs can show cytotoxic effects only if they easily pass through the cancer cell membrane and reach their targets inside the cell. Unfortunately, many of the highly cytotoxic anticancer drugs pass little or practically no penetration across the cell membrane [6,7]. Electrochemotherapy (ECT) is a treatment technique in which reversible EP is utilized to enhance the transport of anticancer agents into cancer cells [8]. In ECT, a reversible effect with strong permeabilization is desired. Therefore, cell death and permeability threshold impact, which is controlled by electrical pulse characteristics and type of cell, is necessary [9,10]. It is critical to select the proper amplitude, frequency, and number of pulses for the transitory effect. In many previous studies, the effect of different parameters like the electric field magnitude of the pulse, its frequency, and the number of pulses on the efficiency of electroporation have been investigated [11-13]. For various electroporation applications, a large variety of distinct pulse procedures have been documented to date. Most of the studies for ECT have used 1 or 8 square wave pulse trains with a width of 100 µs and a frequency of 1Hz or 5kHz [4,11,14,15]. While investigating the optimum electroporation conditions, generally keeping these parameters constant, only the electric field was changed [16,17]. In some studies, when the electric field is reduced, the pulse width is increased [18,19]. The appropriate pulse width to be applied in current ECT protocols has not been fully elucidated and there is still some debate about the effectiveness of pulse width and strength in treatment. Osteosarcoma is the most common bone tumor in children and adolescents and is more common in men [20]. In recent years, different modern treatment strategies have been developed for osteosarcoma patients [21]. However, since the negative effects of radio and chemotherapy cause significant morbidities, more studies are needed to eliminate these effects [22]. The aim of the present study was to determine the most suitable electric field and pulse width for human osteosarcoma (U2OS) cells by trying pulses of different widths with different electric fields keeping the pulse frequency (1 Hz) and number (8 pulses) constant.

2. Material and Method

2.1. Cell culture

In this study, U2OS were used as a model. The cell line was provided by Muş Alparslan University Application and Research Center. U2OS cells were seeded in culture flasks with a surface area of 75 cm2 in Dulbecco's Modified Eagle Medium (DMEM) (Gibco™, USA) with 1% Pen-Strep and 10% FBS, then left to incubate at 37 °C, 75–85% humid and 5% CO_2 environment. They were fed 3 times a week until the cells reached sufficient numbers.

2.2. Electroporation Applications

When U2OS cells were approximately 70% confluent, they were taken out using trypsin and then centrifuged for 5 minutes at 1300 rpm. The cells were re-suspended at a density of $1x10^6$ cells/l in fresh media. After 400 µl of cell solution was placed in each electroporation cuvette (4mm gap), the cuvettes were put in the electroporation and currents were applied. ECT applications were performed using Gene Pulser Xcell TM (Bio-Rad, Hercules, CA, USA) [23]. In the electroporation application, pulses with different electric field intensities of 20 V/cm, 50 V/cm, 250 V/cm and 500 V/cm were used while keeping the 1 Hz frequency and eight square wave parameters constant. Five different pulses with pulse widths of 10µs, 1ms, 5ms, 10ms and 20ms were used in each electric field application. No electric field was applied to the control cells placed in 4 mm EP cuvettes. After the applications, the cells were kept at room environment for 12-15 minutes, then placed into 96-well plates with 10 thousand cells/well and allowed to incubate for one day. The MTT assay was conducted to measure cell viability. Ethics committee approval is not required as the study was performed on a commercially purchased cell line.

2.3. MTT assay

U2OS cells viability was determined by 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test after exposure to different electric fields. Cells that were left to incubate after electric field application were removed from the incubator after 24 hours, and 20 µl of MTT solution was applied to the wells. The cells were then incubated for another 4 hours at 37°C. After which, the liquids in the wells were emptied, 100 µl of DMSO was put instead and mixed. After 15 minutes, the optical density formed in the wells was measured at 540 nm by a Multiscan ELISA reader (Labsystems Multiscan MS, U.K.).

2.4. Statistical analysis

The data presented in the study were expressed as averages with standard error of the mean (mean±SEM), and the graphs presented in the study were created using these values. Tests were performed in at least three replicates.

3. Results and Discussion

The Pulses with different electric fields and widths were applied to U2OS cancer cells. In EP applications, 8 square wave electrical pulses with a frequency of 1 Hz were used. The effect of different pulses applied in EP on cell viability was determined and was given in Table 1.

The % inhibition values caused by electric field pulses with different electric field intensities (20V/cm, 50V/cm, 250V/cm and 500V/cm) and widths (10 µs, 1 ms, 5 ms, 10 ms and 20 ms) in U2OS cancer cells were given in Figure 1.

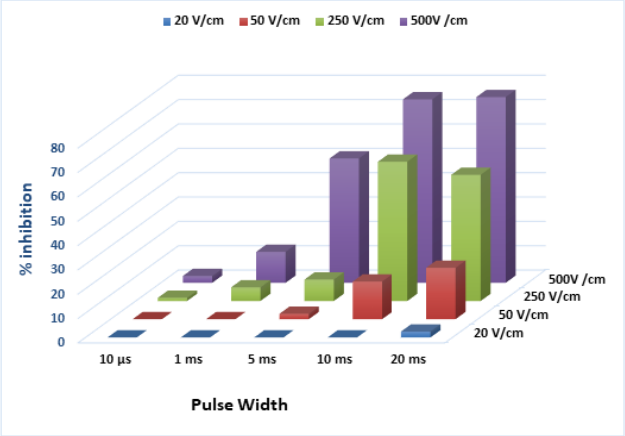

Figure 1. The inhibition (%) of U2OS cancer cells 24 hours after EP.

Table 1. Cell viability (%) of U2OS cancer cells 24 hours after applying pulses with different electric fields and widths

Electric field strength	Pulse width 10 µs	Pulse width 1 ms	Pulse width 5 ms	Pulse width 10 ms	Pulse width 20 ms
20 V/cm	100±0.5	100±0.7	100±1.62	100±2.72	97.59±2.75
50 V/cm	100±1.20	100±2.35	97.76±2.54	84.54±3.23	78.92±1.52
250 V/cm	98.97±2.35	94.4±3.45	91.2±1.45	42.83±1.95	48.19±1.42
500V /cm	97.2±1.33	87.3±1.23	48.95±2.43	24.67±2.43	23.75±2.44

The inhibition (%) of U20S cancer cells increased as the pulse width increased in almost all electric field values (Figure 1, Table 1). The increase of the electric field also increased the cell inhibition percentage in a similar way. The highest cell inhibition (%) occurred in pulses with an electric field of 500 V/cm and a width of 20 ms (inhibition ratio: 76.25 %). No inhibition was observed in the cells at 10 µs, 1 ms, 5 ms, 10 ms width pulses with 20 V/cm electric field and 10 µs, 1 ms width pulses with 50 V/cm electric field. Our data show that the pulse width should be reduced at high electric field pulses and increased at low electric field pulses.

Membrane permeability is increased transiently when cells are subjected to high-intensity electrical impulses via electroporation (EP) [24]. The use of EP in medicine and biotechnology has resulted in new therapeutic techniques for drug delivery, gene therapy and cancer treatment [22]. ECT (anticancer agent+EP) is a therapy approach that uses reversible EP to help anticancer medicines enter cancer cells [25,26]. Much research has been undertaken to determine the efficacy of EP in cancer treatment. Bicek et al. [27] reported that New Anti-Tumor Metastasis Inhibitor-A (NAMI-A) treatment decreased the cell viability by 10 % when used alone and by 90 % when used in combination with EP in the mouse melanoma (B16F1) cells. Mali et al. [28] conducted an investigation on the findings of the previous studies that examined the effectiveness of ECT application with various drugs in treating head/neck tumors, malignant melanoma, and breast cancer. They discovered that in all tumor types, ECT exhibited an objective response percentage of 84.1% and an entire remission rate of 59.4%. In addition, ECT contributes to the efficiency of therapy with its vascular lock, that is, keeping the high drug concentrations near the cancer cell for many hours [29,30]. Unlike the chemo- and radiotherapy, ECT is highly selective and has less negative side effects [30]. Being effective for subcutaneous and cutaneous tumors in humans [31], ECT has recently been used for the therapy of tumors in the internal organs and brain [32].

In ECT treatment technique, EP is expected to induce a strong permeability while causing minimal cell death. Therefore, it is very important to properly select parameters such as the width, amplitude, repetition frequency, waveforms and number of pulses of the electric field. Because these parameters have a significant effect on the efficiency of EP [33]. In the current research, we investigated the

toxicity of the electric pulse in U2OS cancer cells as a function of electric field strength and pulse width. As seen in Figure 1, the inhibition (%) of U2OS cancer cells increased as the pulse width increased in almost all electric field values. Cell inhibition was maximized when pulses with a high electric field (500 V/cm) and long pulse width (20 ms) were used in EP. No inhibition was observed in the cells at 10 µs, 1 ms, 5 ms, 10 ms width pulses with 20 V/cm electric field and 10 µs, 1 ms width pulses with 50 V/cm electric field. Our data suggest that high-voltage short-width pulses or low-voltage long-width pulses should be used in reversible electroporation studies [34]. The electric field strength and pulse width used in electroporation play an important role in pore formation. Sulaeman and Widita [35] showed that high electric field density and long-width pulses would yield high pore intensity in the cell membrane. Increasing the electric field intensity with a long pulse width can cause a condition in which the pores can coalesce, resulting in cell death [36]. Some studies have suggested that EP plays a major role in transporting molecules across the cell membrane and that the pulse duration long enough for adequate uptake is crucial [37]. Our findings show that a change in pulse amplitude used in EP can be compensated for by another parameter such as pulse duration. Also, cell death is maximized when high electric field (500 V/cm) and long width (20 ms) pulses are used in EP.

4. Conclusion and Suggestions

In conclusion, our findings show that the electric field intensity and pulse width used in electroporation play an important role in U2OS cancer cell death. According to our results, it may be more appropriate to use high-voltage short-width pulses or low-voltage long-width pulses in reversible EP studies. More research is needed with different EP parameters to determine the width and strength of electric field pulses to be used in EP for different cancer cell lines.

Contributions of the authors

M.E.A and Y.A designed the study, performed the experiments and wrote the article. E.E performed the calculations, checked the language and contributed to the writing of the manuscript.

Conflict of Interest Statement

There is no conflict of interest between the authors.

Statement of Research and Publication Ethics

The study is complied with research and publication ethics.

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