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The effect of low voltage high frequency electric pulses on the extracellular conductivity, cell permeability, and time-depended manner of MCF7 cell line

Zeinab Shankayi[1,2], S. Mohammad Firoozabadi[1*], Mahmoud Ashouri Mendi[3]

[1]Department of Medical Physics, Tarbiat Modares University, Tehran, Iran.
[2]Neuroscience Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran.
[3]Students' Research Committee, Baqiyatallah University of Medical Sciences, Tehran, Iran.

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ABSTRACT

Background: Low voltage, high frequency electrochemotherapy (LVHF ECT) has recently been explored as a method to enhance the permeability of cell membranes to non-permanent chemotherapeutic agents.

Objective: Despite recent advances, it remains unclear whether classical ECT and LVHF ECT (using 50–150 V/cm at pulse frequencies of 4–6 kHz) affect the cell membrane through similar mechanisms.

Materials and methods: We investigated the efficiency of reversible membrane permeabilization in the MCF7 cell line induced by LVHF electric pulses. Specifically, we examined changes in extracellular conductivity, the time-dependent nature of permeabilization, and the effects of this protocol on commonly used permeabilization markers.

Results: LVHF ECT protocols significantly increased the conductivity of the extracellular medium, indicating enhanced membrane permeability in MCF7 cells. This increased permeability was closely associated with elevated membrane conductivity. Notably, most of the membrane permeabilization occurred during pulse application and subsided within one minute after the delivery of LVHF pulses. Experimental data indicate that these electric pulses induce the formation of short-lived pores in the membrane. Furthermore, LVHF pulses did not alter the cytotoxicity of bleomycin; however, this protocol resulted in the quenching of Lucifer yellow fluorescence, a classical marker for membrane permeabilization. These findings suggest that bleomycin is a reliable marker for cell electro-permeabilization under LVHF ECT conditions.

Conclusion: Our results demonstrate that LVHF ECT induces transient, short-lived pores in the cell membrane and increases membrane permeability without affecting bleomycin cytotoxicity. Bleomycin appears to be a suitable marker for assessing electro-permeabilization in this context.

Introduction

Cell poration (CP) relates to the electrical increases of membrane permeability of target cells to the molecules, specifically pharmaceutical compounds and genes.[1-3] This technique, called Electroporation (EP), demonstrates that the theory of the electrical breakdown of cell membrane and pore formation is the most widely accepted.[4,5]

The reversible phenomenon permits the increase of cell membrane permeability and cell suspension conductivity for a limited time.[6,7] During this time of increased membrane permeability, if the high toxicity and non-permeable chemotherapy drug – such as bleomycin– is achievable, then it can enter the cell cytoplasm and

* **Corresponding contributor.**
Author's Address: *Department of Medical Physics, Tarbiat Modares University, Tehran, Iran.*
E-mail address: smohammadfiroozabadi24@chmail.ir
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target the intracellular components. This physical process is commonly referred to as Electrochemotherapy (ECT).[7-10] Classical ECT is obtained with a train of eight pulses of 1 Hz or 5 kHz frequency and 1300V/cm amplitude. Such pulses have been successfully applied to treatments of superficial tumors in animals and humans.[8-10] Recently, we focused on the effect of much smaller amplitude pulses. Such protocols are referred to as Low Voltage and High Frequency ECT (LVHF ECT). Permeabilization of the cell membrane and treatment of animal nodules that were induced by LVHF pulses have been reported.[11-15] Whether classical ECT and LVHF ECT act on the membrane in a similar way is unclear. To clarify this ambiguity, we consider the effect of Low Voltage and High Frequency (LVHF) electric pulses on the extra-cellular conductivity, cell permeability, and role of time on cell permeabilization.

The disruption of the cell membrane and its permeability can be indirectly demonstrated using different types of markers added to the cell culture mediums. Many of these markers – such as fluorescence, radiolabels, and bleomycin – have been used in permeabilization research.[13,14,16-18] Bleomycin is a non-permeant molecule which possesses a very high intrinsic cytotoxicity. In vitro studies indicate that a low therapeutic dose of bleomycin is sufficient to generate the breakage of the DNA double-strand and kill the cell by a mitotic and apoptotic cell death process.[17,19,20] Due to previous studies revealing that bleomycin molecules are very sensitive cells to use as a permeabilization marker, we exhibited bleomycin's cytotoxicity effects on MCF7 cells exposed to LVHF electric pulses, and suggested the best LVHF ECT protocol.

In addition, some researchers have studied the changes in the electrical properties of cell suspensions due to EP.[6,7,21,22] These studies concluded that cell suspension conductivity increased, an effect that was observed only for above-the-threshold electric fields.[7,21] Recently, it was suggested that measurements of conductivity could enable observations of cell electroporation.[6,21,23,24] In the current study, we will present results based on the degree of reversible membrane permeabilization as a function of the conductivity and composition of the external medium over a wide range of treatment parameters.

Materials and methods

Cell Line

The adenocarcinoma cell line (MCF7 cell line), found in human breasts, was grown in RPMI containing 10% fetal bovine serum, 160 µg/ml L-glutamine (all from Invitrogen, GIBCO, USA), 100 units/mL penicillin, and 16 µg/mg gentamicin, incubated in 5% CO2 at 37 °C.

Electric pulse exposure

The process of directing electric pulses towards the cells using an ECT-SBDC (designed and made in the Small Business Development Center and Electromagnetic Laboratory of the Medical Physics Department of Tarbiat Modares University, Tehran, Iran) has been described in detail in previous articles.[13,15] The suspended cells were placed between two parallel gold-plated electrodes and LV-HF pulses were applied. 4, 5, and 6 kHz with 4000 electric pulses for 100-µs durations and the high voltage electric pulses were 1000 V/cm in repeated pulse frequencies of 0.001 and 5 with 8 electric pulses for 100-µs durations.

Medium conductivity measurement

After trypsinization and inactivation of trypsin (Bio Idea Group, Tehran, Iran) by the serum factors of the complete medium, the cells were centrifuged for 5 minutes at 500 rpm and resuspended at a density of 500×10^6 cells/mL in RPMI (Invitrogen, GIBCO, USA). 300 µL of the mixture was deposited between the two electrodes and subjected to the selected electric treatment (1: 60 v/cm and 5 kHz repetition frequency, 2: 70 v/cm and 4 kHz repetition frequency, 3: 70 v/cm and 5 kHz repetition frequency, 6:70 v/cm and 6 kHz repetition frequency). A conductometer (CyberScan CON 6000, EUTECH Instruments) was used to measure the conductivity of the cell media. The mean value for all the parameters was calculated from at least three of the measurements.

Time effect

To study the extent of LVHF ECT-induced uptake of bleomycin as a function of time interval after or before exposure of cells to LVHF electric pulses, the chemotherapy drug at 0.1 µM and 1 µM was either present during the electric treatment or added to the cell suspension at various times after the treatment from 0-32 minutes. For determination of cell permeability, the cells were incubated for 48 hours using the same protocols as described before.[25]

The effect of LVHF electric pulses on the permeabilization marker

In the current study, two markers were used. The first marker used bleomycin, the electric pulses (4000 pulses with 70 V/cm and 5 kHz frequency) was applied to bleomycin and immediately this suspension was added to MCF7 cells. The second marker used was Lucifer yellow (Sigma-Aldrich Life Science, USA) which was diluted in phosphate-buffered saline (PBS) with a concentration of 500 µM. The fluorescence emission was measured offline in arbitrary units on a spectrofluorometer (Shimadzu RF-5000, Japan) 40 minutes after the exposure of the LY to the electric pulses. The excitation and emission wavelengths were set at 418 nm and 525 nm, respectively.

Statistical analysis

All results are given as an average of more than three times and are represented in bar graphs. Vertical bars represent the standard deviation of the mean. Statistical analyses were performed using SPSS for windows 16.0 (SPSS Inc., Polar Engineering and Consulting). All data were tested for normality. One-way ANOVA, followed by LSD, was performed and statistical difference analysis was accomplished by a test. The p,0.05 were considered significant for rejection of the null hypothesis.

Results

Medium conductivity

The conductivity changes after low voltage and high frequency for selected protocols compare to standard protocols are displayed in Figure 1. The media conductivity increased considerably for LVHF ECT with 70 V/cm at 4 and 5 kHz frequency. However, based on the synergistic effect and cell suspension connectivity, we found the 70 V/cm amplitude and 5 kHz frequency with 4000 square wave electric pulses to be the best LVHF ECT protocol.

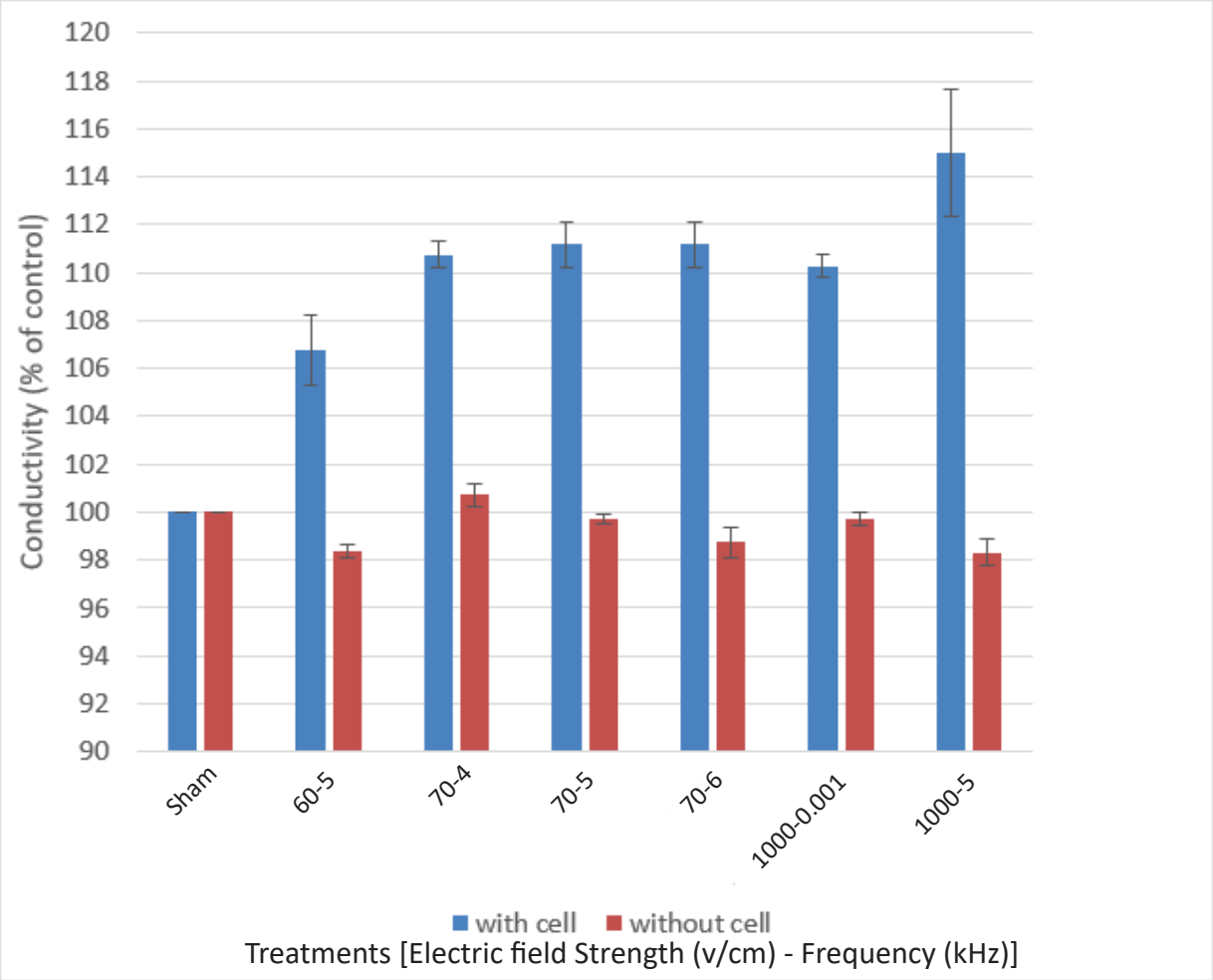

Figure 1*. Conductivity of the cell media changes of attached MCF7 cells to 4 different electric pulse protocols exposures. 1: 60 V/cm with 5 kHz repeated frequency (6-5), 2: 70 V/cm with 4 kHz repeated frequency (70-4), 3: 70 V/cm with 5 kHz repeated frequency (70-5), 4: 70 V/cm with 6 kHz repeated frequency (70-6). The Results are presented as mean±SD. Attributed number to the control group was chosen as 100 and the connectivity of other groups was computed as the percentage of control connectivity.*

Time Effect

To assess the possible reversible pore induction during the pulsation, bleomycin was added to the cell suspension at different times either before or after pulsing. Figure 2 shows that higher uptakes resulted when the chemotherapy drug was added to the suspension before or during the pulsation time. This suggests that a permeabilization occurred during the pulse delivery and the effect disappeared 1 minute after the end of the electric pulse application.

A comparison was made between the smaller and larger doses of the bleomycin. Figure 2 demonstrates that the concentration correlates with the bleomycin uptake only if the bleomycin was added to the cell suspension before or during the pulsation.

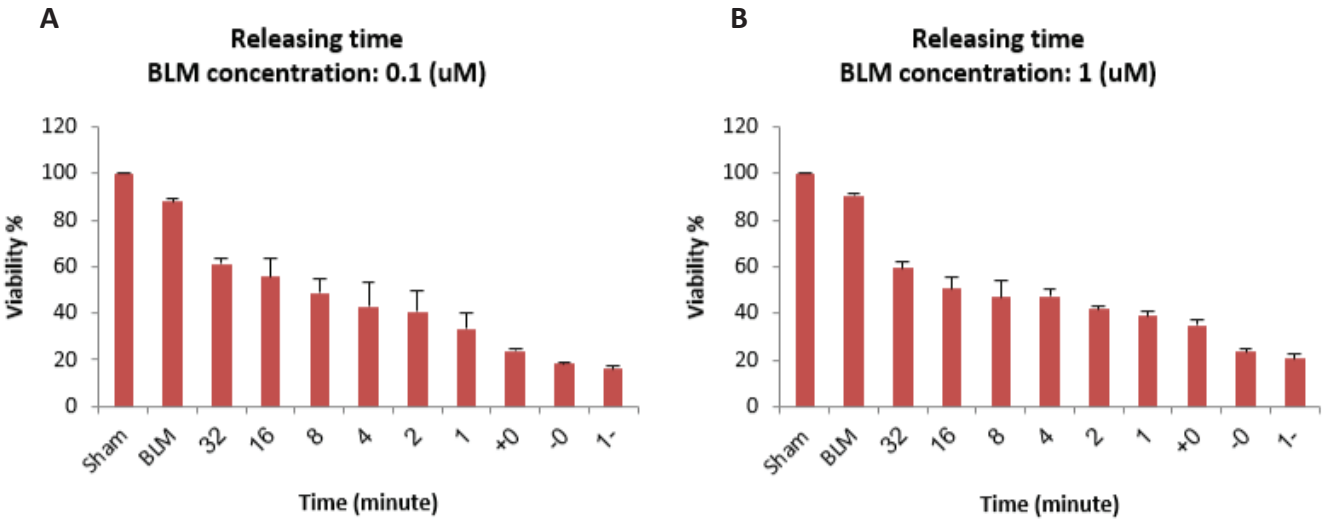

Figure 2*. The temporal decay kinetics of low-voltage, high-frequency electric field-induced bleomycin permeabilization were analyzed. Data are expressed as mean±SD, with control group viability normalized to 100%; all other groups were calculated as a percentage of this baseline. Experimental Groups consist of: Groups 1-32: Bleomycin administration occurred 1-32 minutes post-pulsing after the final electric pulse. Groups 0^- and 0^+: Bleomycin was added immediately before (0^-) or after (0^+) electric pulse delivery. Group -1: Bleomycin was introduced to cell suspensions 1 minute before electroporation.*

The effect of LVHF electric pulses on the permeabilization marker

Our results from the previous part of this research lead us to assume that marker-property changes occurred with electric pulse exposure during the pulsation. Therefore, we set a different experiment. This time, we applied the electric pulses directly to the markers and measured their cytotoxicity and fluorescence intensity.

The changes were not observed between the cytotoxicity of normal bleomycin and that of the bleomycin exposed to the electrical pulses (Figure 3A). Figure 3B indicates the fluorescence intensity for best LVHF ECT protocols (70 V/cm amplitude, 5 kHz frequency with 4000 square wave electric pulses). The comparison between the electric-field-induced fluorescence intensity and the control group is indicative of an enhanced quenching of the fluorescence.

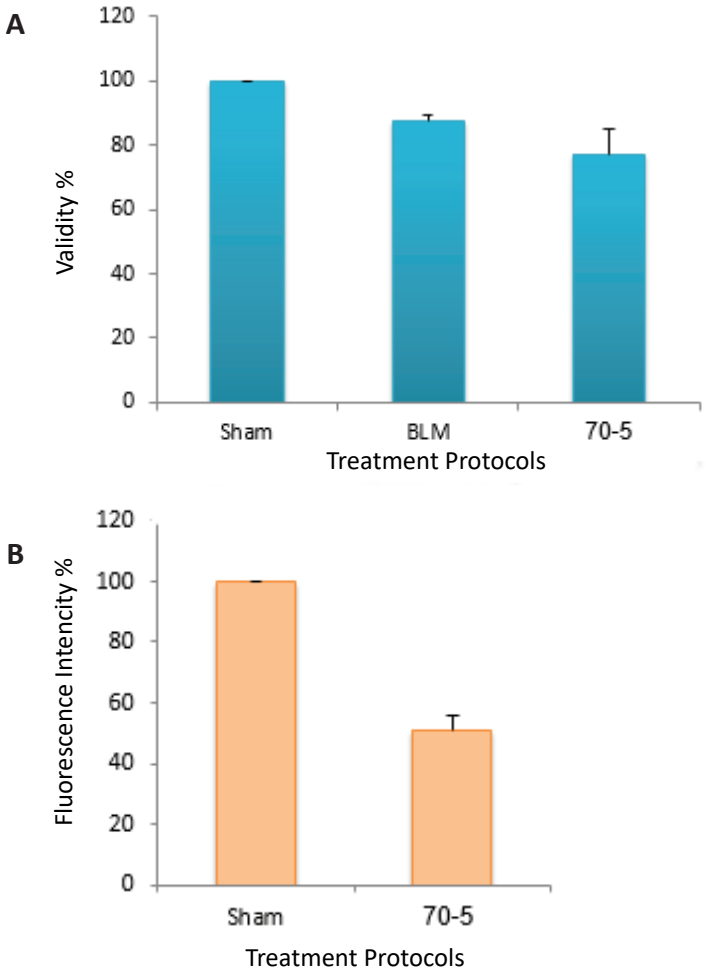

Figure 3. *The effect of pulsed electric field.* ***A:*** *the cytotoxicity of bleomycin. the electric pulses (4000 pulses with 70 V/cm and 5 kHz frequency) were applied to bleomycin and immediately this suspension was added to MCF7 cells and viability was evaluated by an MTT assay,* ***B:*** *Fluorescence intensity of Lucifer Yellow (LY) versus 70 v/cm and 5 kHz electric pulses. The fluorescence emission was measured offline in arbitrary units on a spectrofluorometric 40 minutes after the exposure of the LY to the electric pulses. Results are presented as mean±SD.*

Discussion

As shown in previous studies, electrochemotherapy using low intensity and high frequency increases cell permeability and cures tumors.[13,25,26] Also, in previous studies, we suggested LVHF ECT to reduce the side effect of classical ECT. Using low voltage electric field and high frequencies decrease the number of muscle contractions and reduce the patient's pain during the treatment session.[11-15] This kind of ECT has been shown to be efficient in treatment of animal and human tumors.[11,15,25,26] However, its mechanism is unclear. Theoretical study predicted that cell membrane electro-permeabilization occurred when strong enough electric pulses (more than 300-400 V/cm) are applied and the transmembrane voltage reached to an above threshold transmembrane membrane potential (between 0.2-1.0 V).[6,27] This high electric pulse induced transient aqueous pores, thus enabling the transfer of molecules into cells.[23,28] But, given the results of the in vitro uptake experiments, the membrane permeabilization threshold of cells was less than critical electric pulses amplitude.

Cell suspension conductivity is a physical permeability marker which we confirmed in the current study. We found that our four selected protocols increased media conductivity. These results are in accordance with some previous research results indicating that the cell membrane permeabilization efficiency positively correlated with external conductivity during the application of high-voltage pulses.[28, 29] This increased permeability of a cell membrane was accompanied by the increased membrane conductivity. When the conductivity increased due to electric pulse applying procedure, the membrane partially discharged through the pores as a conductive pathway. This increased membrane conductivity consequently reduced the transmembrane voltage at the regions where electroporation occurred and was accompanied by permeability of a cell membrane. Therefore, our results implied that LVHF electric pulses may extend cell permeabilization immediately after exposure.[6,7,28,29] According to our data, high-intensity ECT was more effective for conductivity than low-intensity ECT, whereas protocols with the same intensity and different frequency revealed

that the medium connectivity may be dependent on cell viability. Dependence of the cell suspension conductivity on the electric field intensity is in accordance with some of the results obtained by previous groups.[23,29] Moreover, results of various studies suggest that concentrations of viable or dead cells after pulsing can influence medium conductivity. Therefore, our results are in agreement with the conclusions of prior literatures.[7,21]

As we noted earlier, measurement of cell medium conductivity is an indirect and convenient method to show cell membrane pore formation.[7,21] Theoretical description predicts that when the pores form, conductivity increased.[21,23,30] Based on this theory and our findings in the previous section, we evaluated the effect of time on cell electro-permeabilization. Pore dynamic theory of electroporation explained that the pore formed in a microsecond, but complete resealing of transient pores occurs in the range of minutes, depending on the electrical parameters used.[31-34] Our observations revealed that the majority of cell membrane permeability occurred during electric pulse application but ended within 1 minute after the delivery of LVHF electric pulses. These results agree with pore dynamic theory.[31-33] Indeed, there are two possible ways of describing the LVHF ECT electro-permeabilization. One way is by the pores formation, which our results support this way very well. The LVHF electric pulses formed a pore with a short life span in the cell membrane which allowed cellular uptake during the electric pulse application. However, pore dynamic theory predicts that small pores grow and expand during pulsation and can survive for a longer period of time after the electroporation.[32,35] If only small pores formed, then the addition of different doses of bleomycin behaved as a function of time, demonstrating that smaller doses killed fewer cells and larger doses killed more cells. Interestingly, between 0.1 µM and 1. 0µM bleomycin, there was a significant difference just when the chemotherapy drug was added to the suspension before or during the pulsation. Therefore, we think this result will require another mechanism. The second way which researcher introduced for electro-permeabilization, is by electro-endocytosis hypothesis. Electro-endocytosis is an endocytosis-like process which induces the cells via low-voltage electric pulses. This process is much longer than the electroporation process and describes a mechanism for uptake of macromolecules or DNA into the cells.[13,24,32,36] Previous studies have reported that plasmid must be added to cells before the electric pulse induction.[32,37] But further studies are needed to maintain the role of electro-endocytosis on the long time electro-permeabilization.

In the current study, we tried to determine the most probable mechanism of LVHF ECT electro-permeabilization. Hence, we examined the effect of LVHF electric pulses on the bleomycin cytotoxicity because the highest number of dead cells was obtained when the bleomycin was added to the cell suspension during or before pulsation. This effect may be due to increased cellular uptake of BLM by electro-permeabilization, or bleomycin cytotoxicity changed during the pulsation. As previously mentioned, the fluorescence spectrum used as a cell membrane permeabilization marker (LY) decreased with the application of an external electric field.[16] Therefore, to clarify the mechanism of LVHF electro chemotherapy, we applied the electric pulses directly to the permeability markers, LY and bleomycin. Our experimental data showed that the fluorescence spectra were reduced after the exposure of the LVHF electric field. This finding is in line with our recent report demonstrating that fluorescence spectra are quenched by electric fields.[16] This is because the electric pulses created oxygen ion radicals which acted as collisional-quencher candidates. Finally, experimental data shown in Figure 3 demonstrated that the changes were not observed between the cytotoxicity of normal bleomycin and that of the bleomycin exposed to the LVHF electric pulses. This result indicated that the electric field has no effect on BLM cytotoxicity and that LVHF electric pulses can induce desirable cell electro-permeabilization.

Conclusion

In the current study, we concluded that LVHF electric pulses could induce electro-permeabilization. The second conclusion that we reached was that the influence of medium conductivity was detected. By measuring electric conductivity and time effect to transport, we suggested that pores with a short life span expanded during the LVHF electric pulse application. Our experimental results exhibit those additional mechanisms, such as the electro-endocytosis process, could be applied to increase molecular uptake during the pulsation.

Ethical approval

The study is approved by ethics committee of Baqiyatallah University of Medical Sciences (IR.BMSU.REC.1402.043).

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