The Endothelial Permeability Increased by Low Voltage and High Frequency Electroporation

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ABSTRACT

Propose: Standard electroporation and electrochemotherapy caused the endothelial cell permeability and reduction in tumor blood flow. The effects of low voltage and high frequency electroporation on the endothelial cells permeability and viability were expected. Therefore, the propose of present study was to evaluate the effect of electroporation with bleomycin or alone on viability and permeabilization of human Embryo microvascular endothelial (HUVEC) cell line.

Material and Methods: The HUVEC cells were exposed to 4000 electric pulsed with 100µs duration, 50-150 v/cm with increment of 10 v/cm in 4, 5 and 6 kHz frequency (33 experimental groups) and then uptake and viability reduction was measured in each group.

Results: The results of this study demonstrated that electric pulses alone reduce the cell viability and with bleomycin significantly increases the toxicity to endothelial cells.

Conclusion: Our data indicated that low voltage, high frequency ECT is highly cytotoxic for a HUVEC cell offers a possible effect of antivascular actions of this kind of electrochemotherapy.

Keywords

Electrochemotherapy, Electroporation, Endothelia cell, Low voltage, High frequency

Introduction

The technique of electrochemotherapy (ECT) facilitates cellular chemotherapy drug delivery for drugs which initially are nonpermanent in to the cell [1-3]. Standard electroporation involves the application of high voltage with low frequency electric pulses to cells that cause the permeability of the plasma membrane [1-4]. Newly, electrochemotherapy is used for treatment of superficial and solid tumors such as melanoma and breast tumors [4-5]. Two major reasons for the high antitumor effectiveness of ECT are assumed. The first is due to increased uptake and accumulation of nonpermanent chemotherapeutic drugs, especially bleomycin and cisplatin, into the tumor cells [3, 6]. The second reason involved endothelial damage and a reduction in tumor perfusion. The vascular disrupting by isolation the tumor cells of oxygen and nutrients cause a tumor cell death. In standard electroporation protocols (1000 v/cm, 1 Hz frequency), researchers have shown that damage to the electroporated endothelial cells, leads to the anti-

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vascular action and reduction of tumour blood flow, which participate in the antitumour effectiveness of electrochemotherapy[6-10].

By standard protocol, patients experience an unpleasant sensation and slight edema or erythema. Most unpleasant and painful, according to the patients, are mainly attributed to muscle contractions provoked by high-amplitude and low-frequency pulses [11-16]. Edema results from high local current density [12]. Recently, we used new ECT protocols using low voltage with high frequency (LVHF ECT) in treatment of tumors and similar antitumor efficacy was observed for the 70 v/cm pulse amplitude with 5 kHz frequency [13-16]. This ECT not only decreased the duration of therapy but also reduced the number and intensity of individual muscle contractions [12-16]. We hypothesize that the high effectiveness of LVHF electrochemotherapy, such as standard protocols, may be partly due to its endothelial cells targeting. It is our hypothesis that damages to the endothelial cells are a voltage and frequency dependent which previous studies with standard protocol showed this.

The aim of our study was to investigate the effect of LVHF electrochemotherapy on the endothelial cells viability in the antivascular action of electrochemotherapy. The sensitivity of human Embryo microvascular endothelial cells, to electroporation alone and combined with bleomycin was determined.

Materials and Methods

Cell line

The human Embryo microvascular endothelial (HUVEC) cell line was grown in DMEM containing 15% fetal bovin serum, 160 µg/ml L-glutamine (all from Invitrogen, GIBCO, USA), 100 units/ml penicillin and 16 µg/mg gentamicin and incubated in 5% CO2 at 37°C.

Electric pulse exposure

Electric pulses were applied to the cells by 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). The cells suspended in DMEM placed between two parallel plate gold electrodes and HUVEC cells exposed to 4000 electric pulsed with 100µs duration in 33 different electric intensity and frequency for 3 times. Electric pulses applied in our study were: 50-150 v/cm with increment of 10 v/cm in 4, 5 and 6 kHz frequency.

Determination of cell permiabilization

To determine the uptake of molecules into the permeabilized cells bleomycin were added to cell suspension before pulsing. In present experiments, anticancer drug bleomycin (Nippon Kayaku Co. Ltd., Tokyo, Japan) at 1 µM concentration was used.

After trypsinization and inactivation of trypsin (Bio Idea Group, Tehran Iran) by the serum factors of the complete medium, cells were centrifuged for 5 min at 1000 rpm and resuspended at a density of 1×10^6 cells/ml in DMEM (Invitrogen, GIBCO, USA) eventually containing bleomycin at 1 µM. 300 µl of the mixture were immediately deposited between the two electrodes and subjected to the electric treatment. After the delivery of the electric pulses, cells were kept for 1 min in room temperature and then cells were seeded in 96 well plate and compelet cell culthure (DMEM containing 30% fetal bovin serum, 320 µg/ml L-glutamine) added to each well to measure their viability through a MTT assay.

EP and ECT cytocxity was evaluated with MTT assay. The viability of the cells after electric field exposure was tested by the 3(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Invitrogen, GIBCO, USA). 20¬µl of MTT solution (5mgMTT/ ml in PBS) was added to the wells at 24hours after the electric field exposure for K562cell line and 72 hours for Mia Paca-2 cell line. Then the cells incubate at 37°C for 4 hours. 100µl DMSO was added to the well and mixed. After

15 min optical density was measured in a Multiscan MS ELISA reader (Labsystems Multiscan MS, U.K.), with a 540-nm filter.

Statistical analysis

All results are given as average of more than 3time repetitions and are presented in bar graphs. Vertical bars represent 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 after that, statistical differences analysis was accomplished by t test. The P values of less than 0.05 were considered significant for rejection of the null hypothesis.

Result

Electroporation of human Embryo microvascular endothelial cell

In order to determine the cytotoxicity of low voltage high frequency electroporation, the cell viability was measured at different electric field intensities and frequency which allow isolating the reversible and non-reversible LVHF EP protocols (figure 1). Viability of HUVEC cells, at higher electric field intensities of 80 v/cm, reduced to less than 50% but, at the lower intensity of 80 v/cm less than 50% of cell were killed by electric field at all three frequency.

Electrochemotherapy of human Embryo microvascular endothelial cell

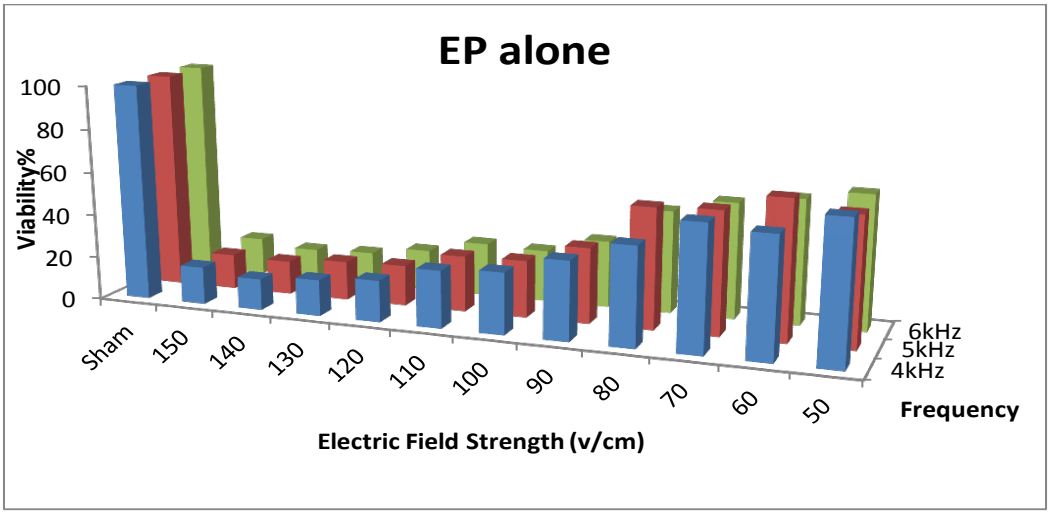

Figure 1: Viability of the HUVEC cells at 72 h after the EP.

To determine an appropriate pulse field strength and frequency for ECT, we applied a train of 400 pulses with different pulses strength (50- 150 v/cm) and three frequencies (4-5 kHz) in present of bleomycin and cell viability measured with MTT assay. Bleomycin is a nonpermeant with a high cytotoxicity chemotherapy drug. Therefore, the detection of cell death indicates plasma membrane permeabilization. It is interesting to note that the best result, based on reversible effect of EP with high permeability, obtained at protocol which using 60 v/cm and 5 kHz frequency (figure 2, 3).

Discussion

In this study, HUVEC cell line was electroporated using a range of voltages pulse and different frequencies. The results of this study

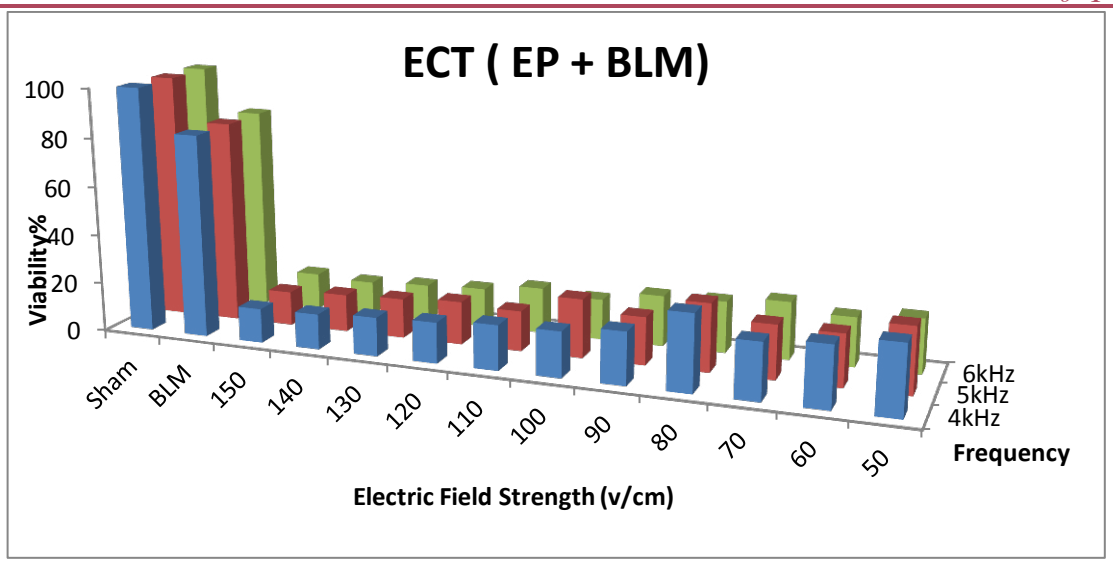

Figure 2: Viability of the HUVEC cells at 72 h after the ECT.

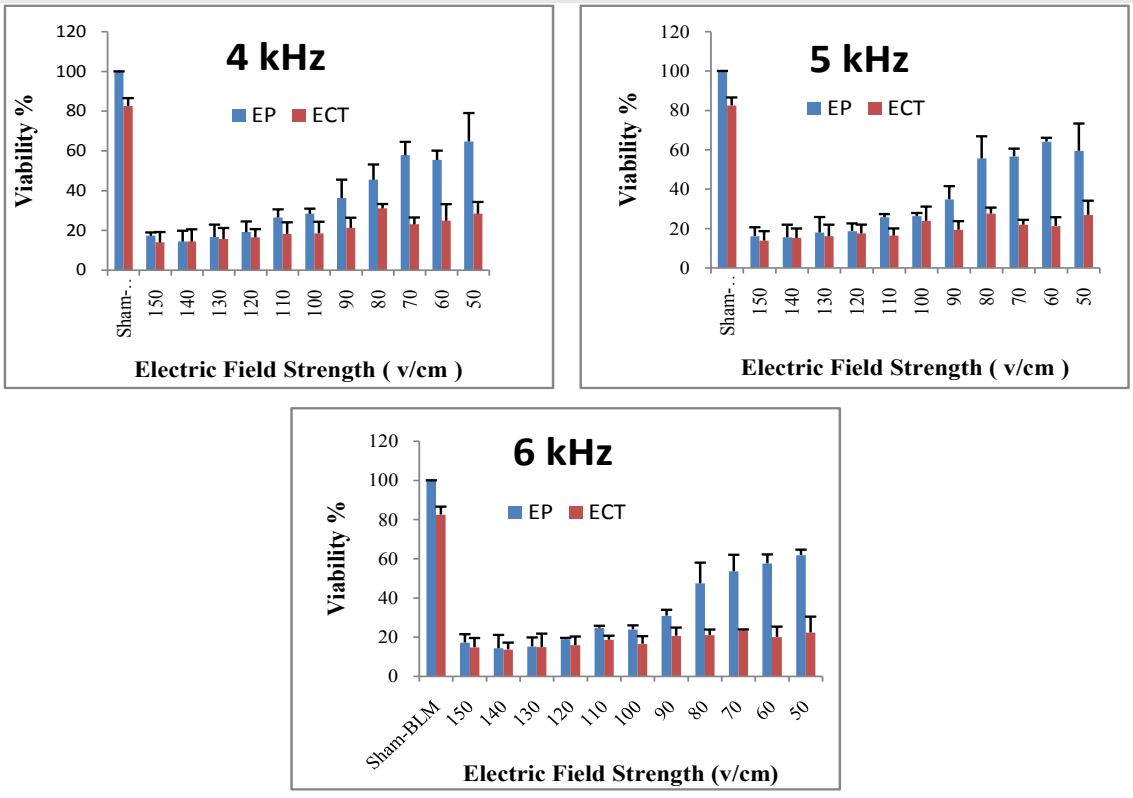

Figure 3: Viability of the HUVEC cells treated with ECT and EP at 72 h after the treatment. Vertical bars represent standard deviation of the mean.

demonstrated that electroporation reduce the cell viability and with bleomycin significantly increases the toxicity to endothelial cells. Our data showed that the viability decrease with increasing voltage other than the electric pulse alone using 60 v/cm with 5 kHz frequency which viability increase significantly . It is likely that in vivo LVHF ECT may damage the endothelium and subsequently, the vascular action.

Despite the advantages of ECT with standard protocols (a train of eight, high-amplitude, rectangular pulses with 1-Hz frequency), some side effects were reported. The most unpleasant and painful side effects of ECT reported are the muscle contractions which related sensations during pulse delivery; the others are edema and erythema [5, 12]. It would be possible to reduce these side effects using high pulse frequencies or lower electric field strength [5, 11-13]. Therefore, recently we used low voltage high frequency electric field for treatment of mice tumors. At this approach, we combined electric pulses with 5 kHz frequency and low voltage amplitude (70, 100 and 150 V/cm) with 500, 2000, 4000 and 5000 number of pulses and the most effective protocol was that utilizing 70-V/cm amplitude, 4,000 pulses of 100-µs duration and 5-kHz frequency [14-16]. An vascular disrupting action of standard ECT is now well established with the result of studies that showed an increase of vascular permeability and a decrease of blood flow in tumor was occurred in response to standard ECT which this mechanism increases the efficiency of treatment [6-10].

To clarify the possible effect of the vascular disrupting actions of low voltage with high frequency electrochemotherapy, we studied the viability and permeability response of the endothelial cells to LVHF electric pulses with increasing voltage (50–150 V) and frequency (4-6 kHz). Our result demonstrated that LVHF electric pulses reduce the HUVEC viability. Similar to electric pulses alone, LVEHF ECT cause a decrease in endothelial cell viability which represents an increase in tumor vascular permeability to bleomycin; these results are an evidence for an anti-vascular mechanism of electrochemotherapy.

In recent study, electric pulses were used to deliver bleomycine inside the cells as a drug delivery method which bleomycin is a nonpermeant chemotherapy drug with a high cytotoxicity [17-18]. In previous studies, an increased drug delivery and following of that BLM cytotoxicity enhancement of standard ECT has been reported in vitro for endothelial and cancerous cell line [7, 17-18]. Cemazer and et al for the first time demonstrated a high sensitivity of human endothelial cells to ECT which they used MTT assay for show this sensitivity such as present study [7]. In continue, they assumed that this cytotoxicity can decrease tumor perfusion and induced the vascular disrupting action at standard ECT [7].

Interestingly, unlike the linear cell viability reduction after electric pulse induction, the cell permeability is increased at lower voltage, specifically in ECT using 60 v/cm with 5 kHz. Therefore, the choice of a treatment protocols will depend on the reversible and irreversible for which is being used. Reversible application will require keeping the cells survival with high permeabilization [3, 20]. In such cases, the lower voltage pulses (less than 90V/cm) should be most effective. If irreversible is considered, then much stronger electroporation conditions could be used.

In conclusion, our data demonstrated that LVHF EP combined with bleomycin is highly cytotoxic for a human Embryo microvascular endothelial cell, offers a possible effect of antivascular actions of LVHF electrochemotherapy. But further experiment needs to identify the determinants of endothelial response and vascular disruption of this new ECT.

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Conflict of Interests

None declared.

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