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Optimization of Low Voltage and High Frequency in vitro and in vivo for clinical application

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Abstract:

Recently, electrochemotherapy using low voltage and higher repetition frequency (LVHF ECT) has been shown to be effective in inhibiting tumor growth and enhancing cell permeabilization. LVHF ECT was developed and optimized in vitro and in vivo for potential clinical use. For this purpose, we used bleomycin to evaluate the permeabilization of MCF7 cells induced by low voltage (50-150 V/cm) and higher repetition frequency (4-6 kHz) electric pulses. Based on these in vitro results, optimal electrical pulse parameters were identified and then applied to subcutaneous mammary tumor (SMMT) models established in Balb/c mice. The LVHF ECT group was compared to groups receiving electric pulses alone, standard electrochemotherapy protocols from the in vitro study, bleomycin alone, and untreated controls.

In vitro, we demonstrated that reversible cell permeabilization can be achieved using low electric field strengths at higher repetition frequencies. In vivo, our data showed significant differences in tumor volumes and curability rates between mice treated with LVHF ECT compared to the other groups. Following LVHF ECT, the number of TUNEL-positive cells in the tumors increased and the Nottingham histologic grade decreased. In summary, LVHF ECT is an effective anti-tumor technique that reduces muscle contractions and edema associated with standard ECT protocols. Thus, it has potential for clinical translation.

Keywords: Electrochemotherapy, low voltage, higher repetition frequency, permeabilization, Nottingham grade, apoptosis

1. Introduction

Molecules and specifically pharmaceutical compounds and genes, by applying electric pulses. This method is named Electroporation (EP)[1]. EP can disrupt the structural integrity of the cell membrane with the use of high voltage; short-duration electric pulses, and reversibility [2,3]. At this term of electroporation, the membrane recovery occurs and the cell remains viable. But, irreversible EP results if

the exposure is not allowed to permit pore release. The combination of nonpermanent, cytotoxic chemotherapeutic agents and reversible EP introduced a high-efficiency approach to cancer treatment that became known as electrochemotherapy (ECT)[4]. ECT standard protocol has been shown to be effective in animal models, mostly for the treatment of cutaneous and subcutaneous tumors, and head and neck squamous cell carcinoma, basal cell carcinoma, melanoma, and adenocarcinomas[5,6]. The advantages of this therapy are requiring of low drug concentration with good antitumor effectiveness and therefore reducing systemic toxicity [4,6]. But, by this protocol, patients experience an unpleasant sensation and slight edema or erythema[7]. In order to reduce the pain sensation during ECT, the application of high frequency or low amplitude electric field has been suggested[7-9]. Therefore, we used a low electric field pulse with a high repetition frequency for electrochemotherapy and our studies have focused on the optimization of this kind of ECT[10-16].

The aim of the current study was to introduce a LVHF ECT protocols for clinical application. Because electrochemotherapy is based on enhanced uptake of chemotherapeutic drugs into tumor cells with reversible effects on viability, in the first, we examined the effect of different sets of high repetition frequency (4–6 kHz) and low amplitudes (50–150 V/cm) electric pulses on the viability and electropermeabilization of MCF7 cell line. In the second, we treated SMMT mice tumors with the best LVHF ECT protocols which selected from first step. The main aspect of the present study was to show the in vivo potential of low voltage -high frequency ECT to kill tumor cells. As opposed to standard ECT that uses high amplitude (1000 V/cm) at a repetition frequency of 1 Hz, we show that LVHF ECT can be achieved with high repetition frequency and low amplitude.

2.Material and Methods

2-1 Cell works

The human breast adenocarcinoma cell line (MCF7 cell line) was grown in RPMI containing 10 % fetal bovine serum, 160 µg/ml L-glutamin), 100 units/ml penicillin and 16 µg/mg gentamicin, and incubated in 5% CO_2 at 37 °C.

Electric Pulse Exposure

Electric pulses were applied to 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 [17,15]. The cells suspended were placed between two parallel plate gold electrodes 10 mm apart. Electric pulses applied in our study were as follows: 50-150 V/cm with increment of 10 V/cm in 4, 5,and 6 kHz pulse repetition frequency.

Determination Of Cell Permiabilization

Electro permeabilization were determined as described previously [10,17,11]. Briefly, electro permeabilization of the plasma membrane was measured by means of bleomycin (Nippon Kayaku Co.Ltd.,Tokyo ,Japan) uptake, and cell viability measured by MIT assay. 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 500 rpm and resuspended at a density of 500×10^6 cells/ml in RPMI (Invitrogen, GIBCO, USA). 300 µl of the mixture (containing cell suspension and bleomycin at 1 µM concentration) were immediately deposited between the two electrodes and subjected to the electric treatment. After the delivery of the electric pulses, the cells were kept for 1 min in room temperature and then cells were seeded in 96 well plate and complete cell culture (RPMI containing 20% fetal bovin serum, 320 µg/ml L-glutamine) was added to each well to measure their viability through a MTT assay.

MTT Assay

EP and ECT cytocxity was evaluated by 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 48 hours after the electric field exposure for MCF7 cell line. Then the cells were incubated 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.

2-2 Animal Study

In this experiment, the inbred female Balb/c mice, at the age of 6-8 weeks and weighing 18-20 g were purchased from the Pasteur Institute, Tehran, Iran. They were maintained at 22º C with a natural day/night light cycle. Before the experiments, the mice were subjected to an adaptation period of at least 7 days.SMMT Tumor spontaneously developed in female Balb/cmice. SMMT is an[18]. About 2 weeks after transplantation the largest diameter of tumor reaches about 10 mm, then the mice were randomly divided into **10** treatment and sham groups.

Electrochemotherapy

Tumors were treated by combined treatment of bleomycin, and application of electric pulses. Electric pulses choose from in vitro study. Chemotherapy was performed by injecting bleomycin (Nippon Kayaku Co. Ltd., Tokyo, Japan) directly into the tumors, as described previously[15,16,13]. Briefly, 0.016 ml/g of diluted Bleomycin in normal saline (1.5 mg/ml), were injected into the tumors. Mice in the sham group were injected with PBS (pH 7.4) instead of bleomycin. Electric pulses were chosen from in vitro study, applied to the

tumors 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) at 2 min after bleomycin or PBS injection, pulses were delivered by two parallel stainless-steel electrodes inserted subcutaneously on opposed sides of tumor.

Evaluation of Tumors Response

Tumour Monitoring

Tumor growth was followed by measuring the diameters along the two largest dimensions with digital caliper every 3 days (each diameter was measured three times). Tumor volume was calculated by the formula $V=\pi ab^2/6$ where a is the larger diameter and b longest diameter, perpendicular to a. Each treatment and sham group consisted of 10 mice.

Histology

Nottingham histological grade

At one week after treatment, mice were sacrificed. The tumors were excised and fixed in 4 % paraformaldehyde for 24 hr, then embedded in paraffin. Sections were prepared for hematoxylin and eosin staining. the haematoxylin and eosin slides used for determin the histological grade according to the Nottingham scheme. This histological grading method uses three parameters and a score of 1 to 3 for each parameter as follows: nuclear pleomorphism (none, 1; moderate, 2; pronounced, 3), tubule formation (> 75%, 1; 10–75%, 2; < 10%, 3), and number of mitoses was seen in 10 fields (< 10 mitoses, 1; 10–19 mitoses, 2; ≥ 20 mitoses, 3). Sum of the scores of the three parameters determines the final Nottingham histological grade) NHG(,4 ,3 :or 5 = grade 1; 6 or 7 = grade 2; and 8 or 9 =grade3. All results are given as an average of more than three times[19,20].

Apoptosis detection

Assessment for apoptosis was performed by the TUNEL (transferase-mediated dUTP nick end-labeling) method. The formalin-fixed paraffin-embedded tissues were sectioned at 3 μm and stained according to a standard procedure using a commercially available kit (in situ cell death detection kit, POD; Roche Diagnostics, Germany). Negative and positive controls were used for the determination of TUNEL-positive cells. The number of TUNEL-positive cells was counted on 10 randomly selected ×200 fields for each section by use of a light microscope. All results are given as an average of more than three times.

Statistical Analysis

SPSS for windows 16.0. (SPSS, Inc., Chicago, IL). All data were tested for normality. One-way ANOVA, followed by LSD, was performed; after that, statistical differences were analyzedby t-test. P values of less than 0.05 was considered significant for rejection of the null hypothesis.

3.Results

Cell Permiabilization

In the present study we varied the amplitude of pulses between 50 to 150 V/cm increment of 10 V/cm in 4,5 and 6 kHz repetition frequency. The MTT test was carried out to determine the sensitivity of MCF7 cells to thirty three electric pulses alone and combine with chemotherapy drug (Fig 1, 2). Figure 1; shows the viability of the MCF7 cells at 48 h after the LVHF ECT. The cytotoxic effect in the group with the LVHF ECT in the presence of the BLM was significantly higher than that in the group with BLM alone. Comparisons were also performed between the ECT group and the group of electric pulse alone (figure 2).

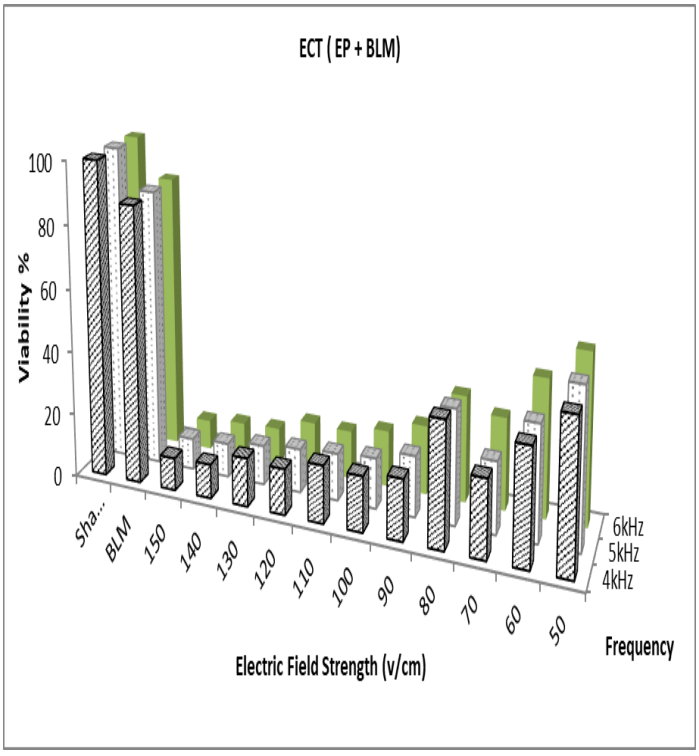


Figure 1. Mcf7 cell viability after exposure to LVHF ECT. Cells were grown and exposed to one set of LVHF electric pulses, and 48 h later, MTT assay was used to evaluate the viability of the tumor cells. The data are reported as mean ± SD of at least 3 separate experiments.

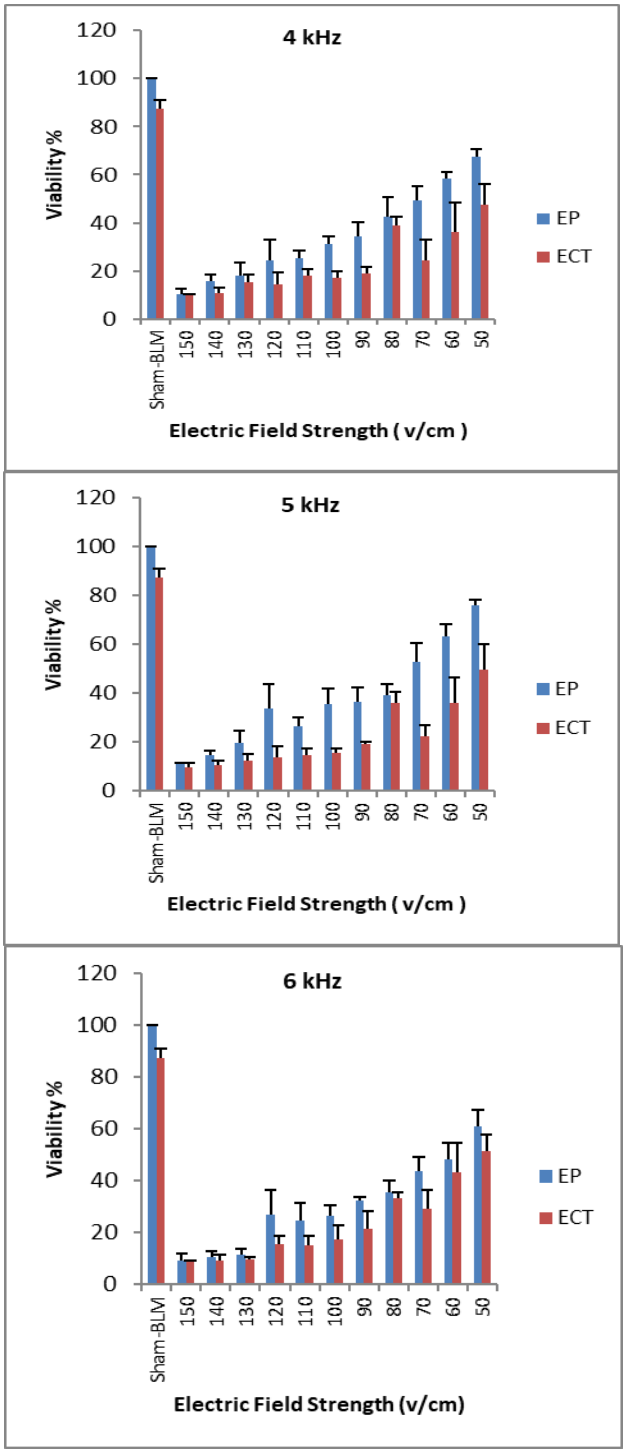


Figure 2. Comparing the viability of the MCf7 cells at 48 h after the LVHF ECT and EP. Results are presented as mean ± SD.

In vivo Tumors Treatment

Tumor monitoring

Electrochemotherapy was performed with a combination of bleomycin and application of selected electric pulses. Electric pulses protocols are as follows: strengths of 70 V/cm, 4, 5 and 6 kHz repetition frequency and 60 V/cm, 5 kHz repetition frequency using 4,000 electric pulses [16] with 100 µs duration. The result in terms of tumor growth is shown in Fig. 3 and 4. There were significant differences in tumor volumes between mice treated with LVHF ECT and other treated groups assessed on all days after treatment ($P < 0.05$). In control group, tumor volume reached to 11 times the initial volume after 24 day. But, in ECT groups, this ratio is less than 2 and in EP groups is less than 5 (Fig. 3, 4).

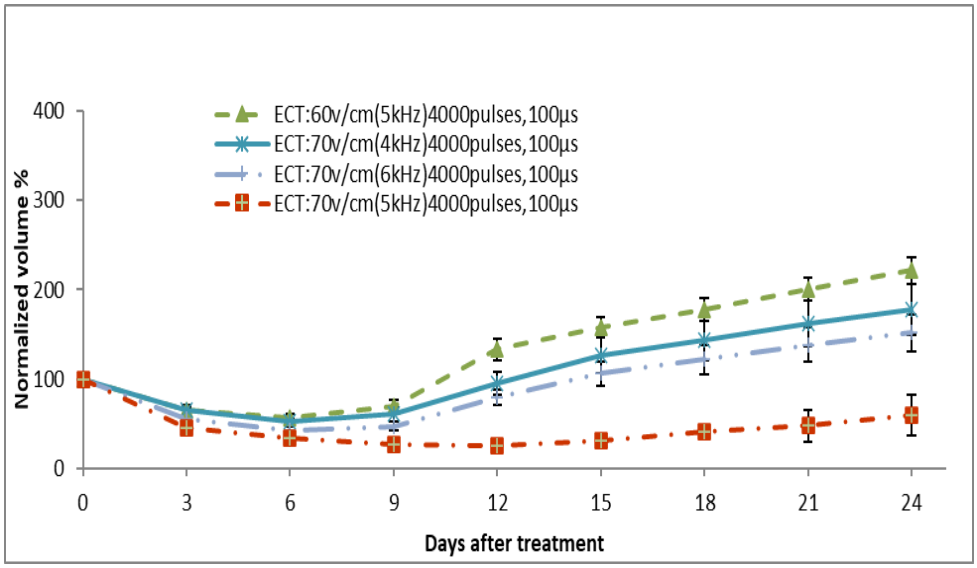


Figure 3: Electrochemotherapy of tumors in mice with 4 different protocols. Results are presented as mean ± SE. ECT: electro chemotherapy.

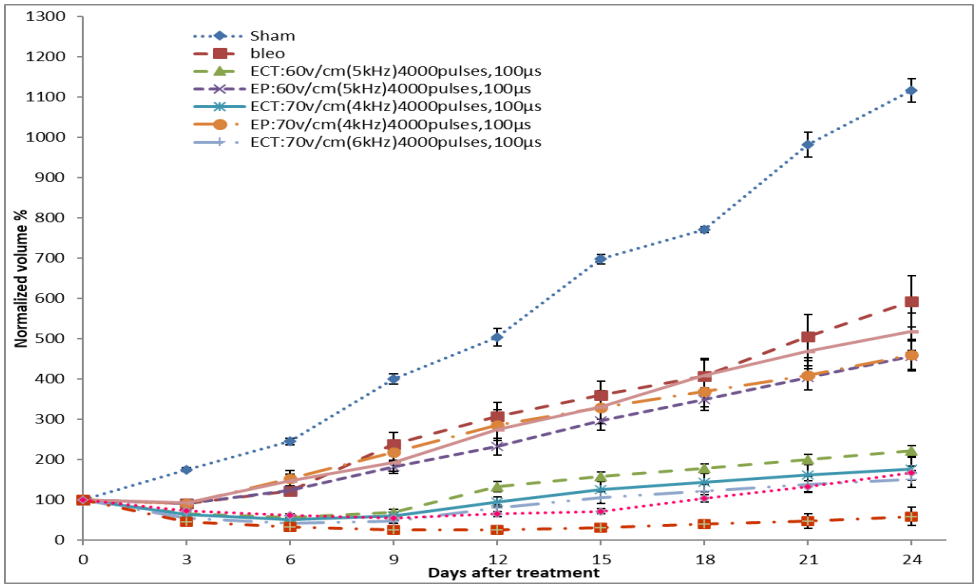


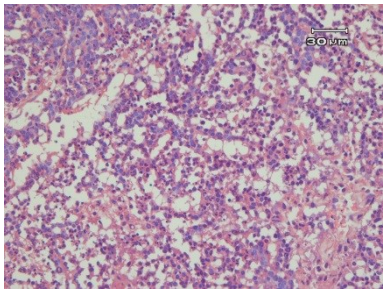
Figure 4: Electrochemotherapy of tumors in mice with 3 different protocols. Results are presented as mean ± SE. BLM: bleomycin only, EP: electric pulses only, ECT: electrochemotherapy

Nottingham histological grade

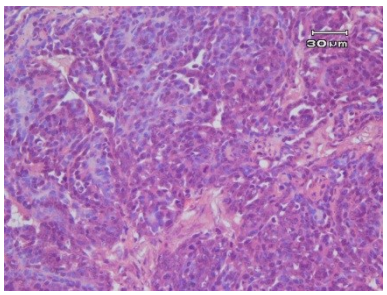
Table 1 shows the distribution of grade and scores for control and treatment groups. The result showed that electric pulse alone and bleomycin alone reduced the score of tubule formation and nuclear pleomorphism and had no effect on mitotic activity. In all ECT groups, The NGH grade was equal to grade one and in ECT which treated using 70 V/cm amplitude, mitotic activity and tubule formation scores get score one.

Table 1: Histological grade of SMMT cancer as assessed by the Nottingham Grading system

Treatment groups	Tubular Differentiation	Nuclear Pleomorphism	Mitotic Count	sum of the scores	Overall Grade
Sham	3	2	3	8	3
Bleomycin	2	1	3	6	2
60 V/cm, 5 kHz: EP	2	1	3	6	2
60 V/cm, 5 kHz: **ECT**	2	1	2	5	1
70 V/cm, 4 kHz: EP	2	1	3	6	2
70 V/cm, 4 kHz: **ECT**	2	1	1	4	1
70 V/cm, 5 kHz: EP	2	1	3	6	2
70 V/cm, 5 kHz: **ECT**	2	1	1	4	1
70 V/cm, 6 kHz: EP	2	1	3	6	2
70 V/cm, 6 kHz: **ECT**	2	1	1	4	1



A: control group



B: ECT Group

Figure 5: Histological grade of SMMT cancer as assessed by the Nottingham Grading system.

Apoptosis detection

TUNEL staining for apoptosis in all treated tumors was positive (Fig. 6, Table 2). There was no evidence of cell apoptosis in the control tumors. The number of TUNEL -positive cells was significantly greater in the treated tumors with ECT than in the control group, electric pulses alon and bleomycin alone, treated tumors.

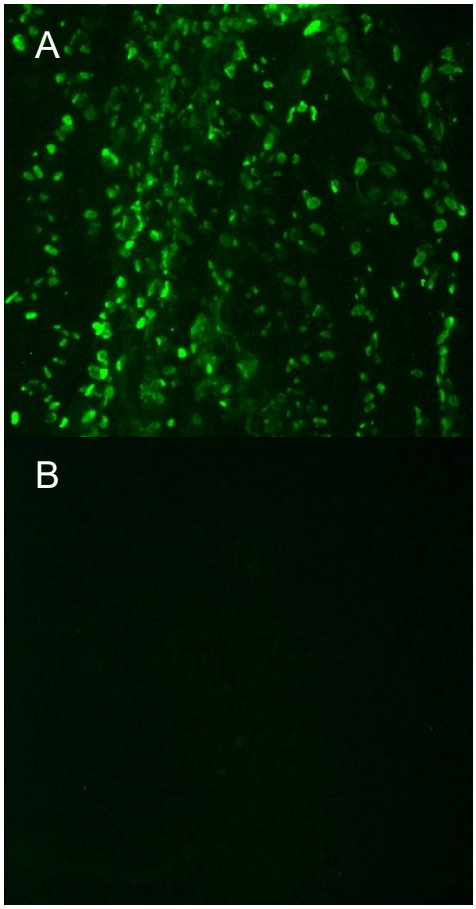


Figure 6: TUNEL staining of tumor one week after treatment with LVHF ECT.In fig A, the luminescent cells are apoptotic. No apoptosis is detected in untreated control tumor (B).

Discussion

In the present study, we show by in vitro and in vivo experiments that low voltage and high repetition frequency electrochemotherapy with bleomycin is a potent anticancer modality. After LVHF ECT, the number of TUNEL-positive cells in the tumor increased and NHG grade of tumors decreased. These results are consistent with previous investigations that have used LVHF ECT for cell permeabilisation and tumors treatment [10-15].

Electro cell poration occurs when the difference in electrical potential across the membrane (transmembrane potential) reaches threshold values. When the applied pulse achieves a critical voltage, the cell membrane becomes permeable and nonpermanent molecules allow passage through the membrane [21-23]. The most important parameters for electroporation are the electric field amplitude and pulse duration. If the low electric field is used, the breakdown transmembrane potential is achieved with longer pulses and vice versa [21-23].

Typically, electric fields of 1000 V/cm and durations of 100 μs are optimal parameters for the drugs delivery, while for the genes delivery lower electric amplitude and longer pulses (In the millisecond range) are suggested[21,24,23]. When electric pulse of 100 μs is used for in vivo ECT application, the threshold value of electric field amplitude is about 300 V/cm in tumors [25,22]. But, we show that cell reversible permeabilization using low electric field strength with 100 μs is achievable. However, this is dependent on the pulse number and repetition frequency [17,16,10]. Transient permeabilization is important because electrochemotherapy is introduced as an application of reversible electroporation. Therefore, to reach this optimization level, we investi-

uptake of bleomycin into electropermeabilized cells. In vitro experiments allow us to omitted irreversible protocols and choose the best reversible protocols based on maximum cell permeabilization and minimum cell death.

In ECT, high permeablization is desirable. Fluorescence markers are the most widely used in cell membrane permeability studies. The restriction on the use of these markers is that fluorescence markers interact with the cell inside components, like DNA. As a result, fluorescence intensity of these classic markers will change [26]. Between these fluorescent molecules, Lucifer Yellow (LY) does not interact with cell components[26]. Nevertheless, in previous study, we reported a fluorescence quenching of Lucifer Yellow in the exposure of the electric field. Owing to the quenching effects of electric field on LY, the use of LY as a marker in electric permeabilization is questionable[27]. According to these points, we investigated the effect of LVHF ECT on the uptake of bleomycin molecule into electropermeabilized cells. In this method, the detection of cell death indicates that at least 500 molecules have been able to cross the plasma[26].

Standard electrochemotherapy is used as an efficient local treatment of the superficial and solid nodules in patients. The standard ECT protocol uses a train of high -amplitude (1300 V/cm), rectangular pulses with 1-Hz repetition frequency [5,4]. One challenge associated with standard ECT that further distinguishes them from LVHF ECT is that by this protocol, patients experience muscle contractions which is an unpleasant sensation during pulse delivery and edema that results from high local current density[7,28,29]. In order to reduce these unpleasant sensations, application of a high repetition frequency or low amplitude electric field has been suggested [8,9,7]. Optimal parameters was achieved at an applied voltage of 70 V/cm and with 5 kHz frequency, delivered in 4000 pulses each of 100 microseconds. Therefore, the optimum electrical pulse parameters used in this study for electropermeabilization of SMMT tumors has been found to be comfortable.

Induction of tumor cells apoptosis is the other advantage of the LVHF ECT. Morphological manifestation of programmed cell death is apoptosis. It can be considered as the cell suicide. In contrast to necrosis, which induces inflammatory reactions, apoptosis causes death of cells that can quickly be removed by phagocytosis before the contents of the cell can spill out onto surrounding cells and cause damage. Therefore, apoptosis is considered as a suitable method for cancer therapy [12,30]. Mansourian [12] have reported that cell apoptosis could be induced by LVHF ECT in vitro. Furthermore, this research demonstrated leukemia cancer cells have a higher apoptosis percentage than lymphocytes normal cells when exposed to the same ECT protocols

[12]. This result was consistent with other study [31,22]. Effectiveness of electric pulses on inhibiting cancer cells and tissues with apoptosis induction has been proved by presented research. Therefore, this result is great potential as an anticancer treatment method.

Conclusion

This study was performed to show the potential of low voltage, high frequency ECT to treatment tumors and to reduce the unpleasant sensation of muscle contractions seen in standard ECT. In vitro examination demonstrated that low voltage and high frequency can reversibility increase membrane permeabilization electroporation. In tumor treatment, LVHF ECT performed with 4 – 6 kHz repetition frequencies and electric field amplitude less than 70 V/cm. these protocols induced apoptosis, reduce histological tumors grade and had an effective effect on tumor reduction volume. Therefore, LVHF ECT has the potential to be used clinically without the administration of muscle contractions and any edema.

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