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Tumor Growth Inhibited by Low-Voltage Amplitude and 5-kHz Frequency Electrochemotherapy

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Abstract The most important unpleasant sensation of electrochemotherapy is muscle contraction. One of the causes of this discomfort is electrochemotherapy in the low-frequency range (1 Hz). To resolve this problem, there are two solutions: first, increasing the repetition frequency of electric pulses above the tetanic frequency and, second, reducing the voltage amplitude. This study examines the antitumor effectiveness of treatment using low electric fields and high frequency in the presence and absence of chemotherapeutic agents. High-voltage amplitude electrochemotherapy was performed by eight pulses, at 1,000 V/cm, of 100-μs duration at 1-Hz and 5-kHz repetition frequency. In the low-voltage amplitude protocol, 4,000 pulses, of 100-μs duration at 5-kHz repetition frequency with 70, 100 and 150 V/cm were delivered to invasive ductal carcinoma tumors after intratumoral injection of bleomycin. Our data demonstrate significant differences in tumor volumes and the curability rate between mice treated by 70 V/cm compared to other groups. Electrochemotherapy, which is specified by a higher repetition frequency of electric pulses (5 kHz) and low voltage, inhibits tumor growth. This protocol has a comparable effect to 1-Hz pulse repetition electric pulses with high voltage. Based on these results, the 4,000 pulses of 70 V/cm with 5-kHz frequency are most effective. This protocol demonstrates inhibition of tumor growth without any need for drug administration.

Keywords Electroporation · Electrochemotherapy · High repetition frequency · Low-voltage amplitude

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Introduction

Electroporation (EP) is a technique that improves the passage of chemical reagents or DNA into cells by the use of high-voltage, short-duration electric pulses (Teissie et al. 1999; Davalos et al. 2003; Kubica 2008). The combination of nonpermanent, cytotoxic chemotherapeutic agents and electric pulses introduced a high-efficiency approach for cancer treatment that became known as electrochemotherapy (ECT) (Sersa et al. 2008; Mir et al. 2006). ECT has been shown to be effective in animal models, mostly for the treatment of cutaneous and subcutaneous tumors, head-and-neck squamous cell carcinoma, basal cell carcinoma, melanoma and adenocarcinomas (Sersa 2000; Semrov and Miklavcic 1998). In preclinical trials, various anticancer drugs have been employed in ECT. The cytotoxic effect of bleomycin has reportedly been more significantly enhanced by EP than other anticancer drugs. Bleomycin is a highly cytotoxic drug, where cells are not able to take up therapeutic amounts or uptake occurs only in extremely small quantities (Mir and Orlowski 2000; Shil et al. 2008).

The standard ECT protocol uses a train of high-amplitude, rectangular pulses with 1-Hz repetition frequency. By this protocol, patients experience an unpleasant sensation and slight edema or erythema. Most unpleasant and painful, according to the patients, are the sensations during the pulse delivery, which are mainly attributed to muscle contractions provoked by high-amplitude and low-frequency pulses. Edema results from high local current density (Sersa et al. 2008; Mir et al. 2006; Mir 2006; Miklavcic et al. 2005; Pucihar et al. 2002). In order to reduce the pain sensation during ECT, application of a high-frequency or low-amplitude electric field has been suggested (Pucihar et al. 2002; Miklavcic et al. 2005; Horiuchi et al. 2000; Miyazaki et al. 2003; Kitamura 2003; Plotnikov et al. 2004; Entin et al. 2003).

In a first approach for pain reduction, it was shown that uptake of a nonpermanent molecule at high repetition frequencies remains at similar levels to that at a 1-Hz treatment, and similar antitumor efficacy was observed for the 5-kHz pulse frequency (Pucihar et al. 2002; Miklavcic et al. 2005). The 5-kHz frequency ECT not only decreased the duration of therapy but also reduced the number of individual muscle contractions from eight contractions to one tetanic contraction (Miklavcic et al. 2005). Recently, clinical experiments did not show any differences in efficacy of ECT delivered by 1-Hz or 5-kHz repetition frequencies of electric pulses (Zupanic et al. 2007; Snoj et al. 2007).

In another approach, ECT with a low electric field and longer electric pulses (50–100 ms) was examined, and a combination of a low electric field with agents such as bleomycin enhanced chemotherapy (Horiuchi et al. 2000; Miyazaki et al. 2003; Kitamura 2003; Plotnikov et al. 2004; Entin et al. 2003). Matsuki et al. (2008) showed that low-voltage pulses with a voltage lower than the membrane breakdown threshold of human cells could increase the uptake of nonpermanent molecules. Also, researchers demonstrated that the same EP efficiency was obtained using either low values for N (number of pulses) and T (duration of electric pulses) but high E (electric pulse amplitude) or low electric pulses corresponding to high values for N and T (Rols and Teissie 1998), where low electric field by longer pulses was reported to be more advantageous than the short pulses in terms of transfection success (Kubiniec et al. 1988).

These studies led us to suggest a hybrid ECT condition using low electric field and high repetition frequency by increasing the number of electric pulses. In a previous study, we examined a low electric field (100 V/cm), high repetition frequency (5 kHz) and 100-μs duration with varying numbers of pulses (500, 2,000, 4,000 and 5,000). Our results showed that 400 pulses (equal to eight pulses of 50-ms duration) had the best antitumor efficacy (Shankayi et al. 2010).

The aim of the present study was to investigate the effect of using a high repetition frequency electric pulses (5 kHz) with different low amplitudes of ECT (70, 100, 150 V/cm) in the treatment of invasive ductal carcinoma tumors and to examine the possibility of using this suggested protocol compared to currently used standard and clinical protocols (1,000 V/cm, a 1-Hz electric pulse repetition frequency, and 5-kHz electric pulse repetition frequency).

Materials and Methods

Mice and Tumors

In this experiment, inbred female Balb/c mice, aged of 6–8 weeks and weighing 18–20 g, were purchased from the Pasteur Institute (Tehran, Iran). They were maintained at 22°C with a natural day/night light cycle. Before the experiments, mice were subjected to an adaptation period of at least 7 days.

Invasive ductal carcinoma tumor, purchased from the Pasteur Institute, was transplanted by fragments of invasive ductal carcinoma in sizes of 0.5 mm^3 into the flank of mice. About 3 weeks after transplantation, the largest tumor diameter reached about 10 mm; then, mice were randomly divided into 13 treatment groups. Each treatment and sham (without any pulses and drug) group consisted of 10 mice. The Tarbiat Modares University ethics committee approved the present study (letter 52-12503).

Electrochemotherapy

Tumors were treated by a combination of bleomycin and application of electric pulses. Chemotherapy was performed by injecting bleomycin (Nippon Kayaku, Tokyo, Japan) directly into the tumors. Bleomycin was diluted in normal saline (1.5 mg/ml), and 0.016 ml/g of this solution was injected into the tumors. Two minutes after the intratumoral injection of bleomycin, electric pulses were delivered. Mice in the sham group were injected with PBS (pH 7.4) instead of bleomycin. Electric pulses were 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) at 2 min after bleomycin or PBS injection, and two flat, parallel, stainless-steel plate electrodes (width 5 mm, length 15 mm) were placed at the opposite sides of the tumors to deliver the electric pulses. Conductive gel was used to assure good contact between electrodes and skin. Mice were anesthetized by means of intraperitoneal administration of 0.01 ml/g body weight of the following mixture: 0.5 ml of ketamine (100 mg/ml; Virbac, Carros, France), 0.5 ml of xylazine (2%; Bayer Health Care, Leverkusen, Germany) and 4 ml of NaCl.

High-Electric Field Cancer Treatment Protocol

Eight 100-μs square-wave electric pulses of 1,000 V/cm amplitude with repetition frequency of 1 Hz and 5 kHz were delivered to the tumors.

Low-Electric Field Cancer Treatment Protocol

In the first part of the 4,000 pulses, 100-μs square-wave electric pulses of 70, 100 and 150 V/cm amplitude with repetition frequency of 5 kHz were delivered on opposed sides of the tumor.

Tumor Volume Monitoring

Tumor volumes were followed by measuring the diameters along the two largest dimensions with a caliper every 3 days (each diameter was measured three times) and calculated by the formula $V = \pi ab^2/6$, where a is the larger diameter and b the smaller diameter. Tumor growth was normalized by the following: (tumor volume measured at days after treatment [V_n]/tumor volume measured on the treatment day [V_0]) $\times$ 100. The inhibition rates of tumor growth were calculated according to the following formula: inhibition rate (%) = (1 − tumor volume [treatment group]/tumor volume [sham]) $\times$ 100.

Statistical Analysis

Statistical analyses were performed using 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 analyzed by t-test. $P < 0.05$ was considered significant for rejection of the null hypothesis.

Results

Antitumoral Effect of Different Intensities of the Electric Field on Invasive Ductal Carcinoma

Electrochemotherapy was performed with a combination of bleomycin and application of electric field strengths of 70, 100 and 150 V/cm using 4,000 (Shankayi et al. 2010) electric pulses and 5-kHz repetition frequency to the tumors. The result in terms of tumor growth is shown in Fig. 1. There were significant differences in tumor volumes between mice treated with 100 and 70 V/cm assessed on all days after treatment. Between 150 and 70 V/cm, significant differences were observed after day 12 posttreatment ($P < 0.05$) (Fig. 1).

Comparing Low-Voltage and High-Voltage Protocols

We compared our new ECT protocols using low voltage and standard protocols with eight pulses, 1,000 V/cm electric field strength and 100-μs pulse duration with 1-Hz and 5-kHz pulse repetition frequency. Our protocol inhibited tumor growth significantly more than that observed for two high-strength protocols (Figs. 2, 3), and the curability rate in our protocol increased (Table 1). However, treatment with a low electric field, 70 V/cm, cured completely 20% of the animals; but in the other treatment groups we observed only a partial response.

Antitumor Effect of Low Electric Field Without Cytotoxic Drug

We examined the effect of electric field strength alone (without bleomycin) on tumor cure. Tumor-bearing animals were divided into four groups: (1) nontreated tumoral mice; (2) tumor-bearing mice with an electric field of 70 V/cm, 4,000 pulses and 5-kHz pulse repetition frequency; (3) eight pulses, 1,000 V/cm electric field strength and 1-Hz pulse repetition frequency; and (4) eight pulses, 1,000 V/cm electric field strength and 5-kHz pulse repetition frequency.

Results were plotted and are shown in Fig. 4, demonstrating that the electric field alone could inhibit growth of tumors but in treatment with a low electric field, 70 V/cm, the tumor gradually decreased in size, then started growing after 9 days (Table 2).

We compared the antitumor effect of a low electric field (70 V/cm) using 5-kHz pulse repetition frequency with and without cytotoxic drug and ECT with eight pulses, 1,000 V/cm electric field strength and 1-Hz and 5-kHz pulse repetition frequency (Fig. 5). Our data showed that inhibited tumor growth with the low electric field of 70 V/cm alone was comparable with high-voltage ECT ($P < 0.05$).

Discussion

Electrochemotherapy is used as an efficient local treatment of the cutaneous and subcutaneous nodules in patients. The most unpleasant and painful side effects of ECT reported are the muscle contractions and related sensations during pulse delivery. It would be possible to reduce these painful sensations using pulse repetition frequencies higher than tetanic contractions or lower electric field strength (Mir 2006; Miklavcic et al. 2005; Pucihar et al. 2002; Horiuchi et al. 2000; Miyazaki et al. 2003; Kitamura 2003; Plotnikov et al. 2004; Entin et al. 2003). In vitro studies have demonstrated that chemical (treatment) uptake at the highest repetition frequencies is possible. Also, with increased number or duration of pulses, an additional increase in uptake can be obtained (Pucihar et al. 2002; Lebar et al. 2002a). Miklavcic et al. (2005) showed that pulse frequencies above the frequency of tetanic contractions reduced the number of individual muscle contractions. In in vivo experiments with mice, a similar efficiency of ECT regardless of the pulse frequency examined was shown (Miklavcic et al. 2005). A study on fibrosarcoma tumors transplanted in mice has shown, despite good antitumor results, that ECT was more effective at 1-Hz repetition frequency than at 5 kHz (Sersa et al. 2010). Several articles have reported that a low-voltage electric field could inhibit tumor growth. Exposure of cells to low electric fields

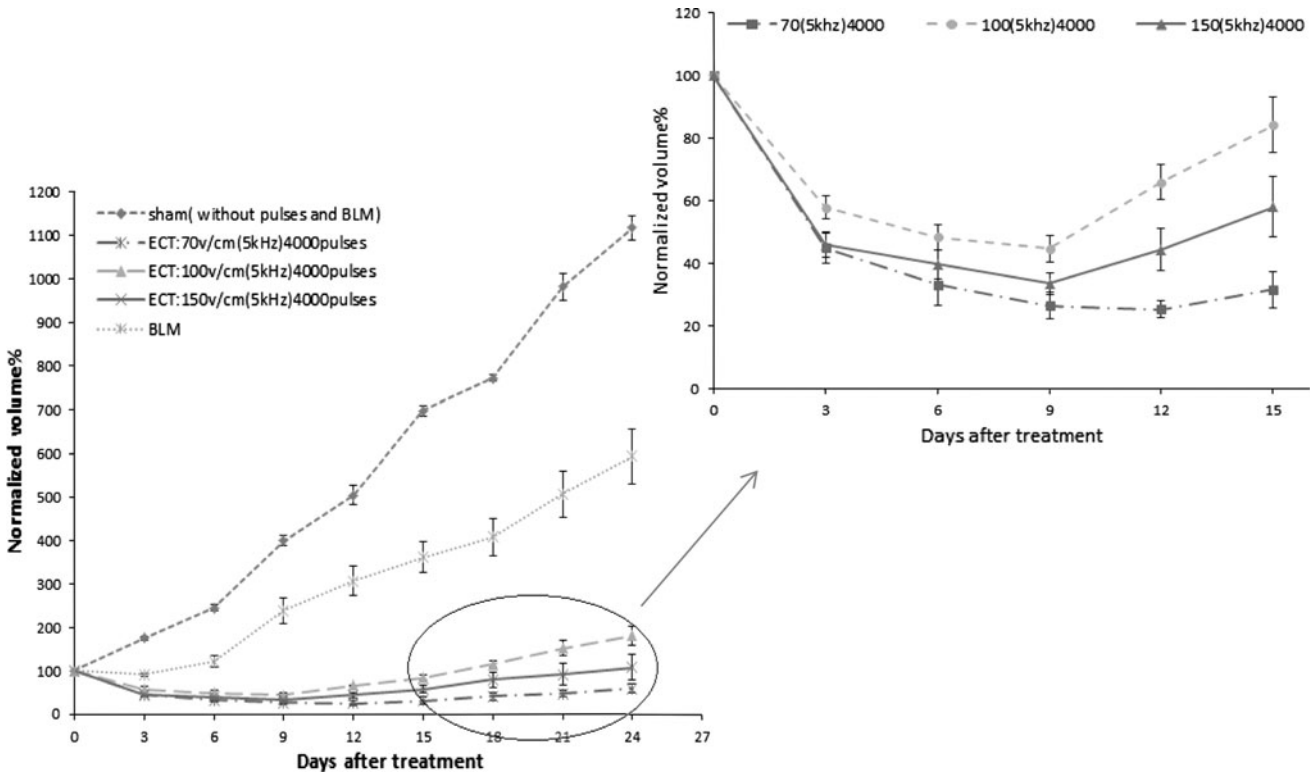

Fig. 1 ECT with 4,000 pulses of 70-, 100- and 150-V/cm electric field amplitude and 5-kHz frequency. Results are presented as mean ± SEM

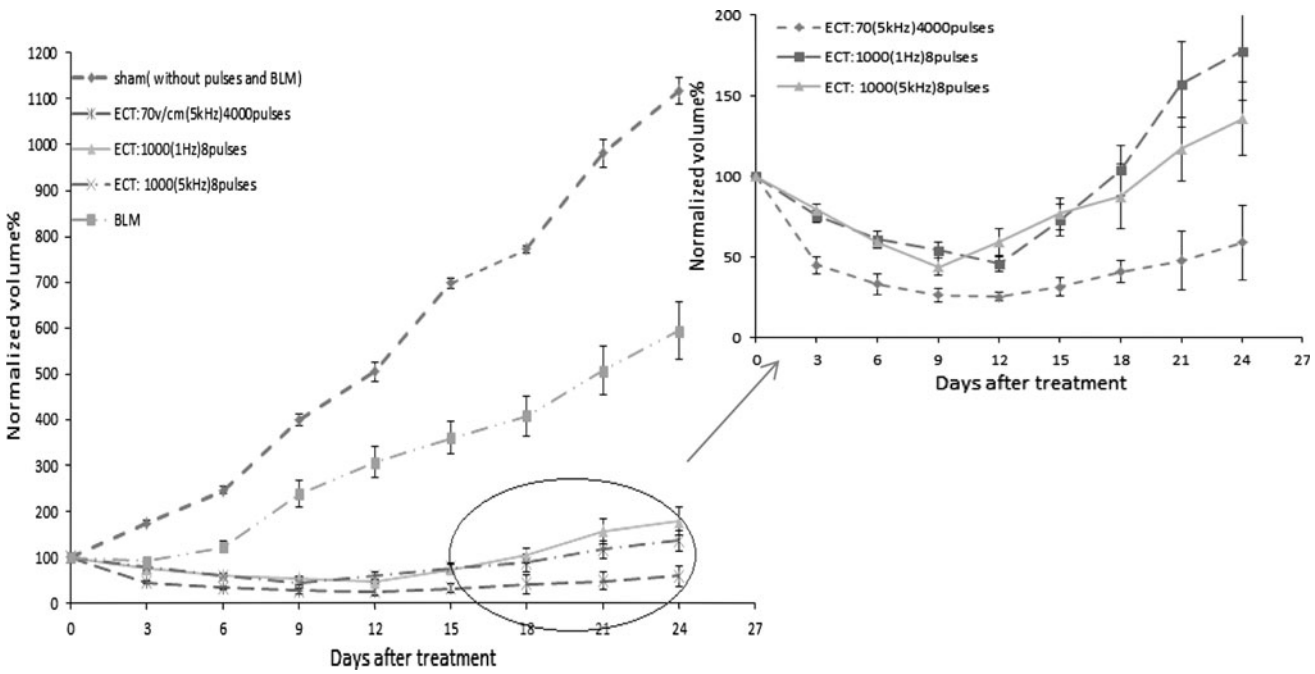

Fig. 2 ECT with 4,000 pulses of 70 V/cm and 5-kHz frequency and standard protocols. Results are presented as mean ± SEM

resulted in an effective uptake of molecules via endocytotic-like processes. Therefore, with this mechanism low electric fields can increase the delivery of chemotherapeutic drugs directly into the cytoplasm of cancer cells and, thus, increase the effectiveness of the drugs against solid tumors (Horiuchi et al. 2000; Miyazaki

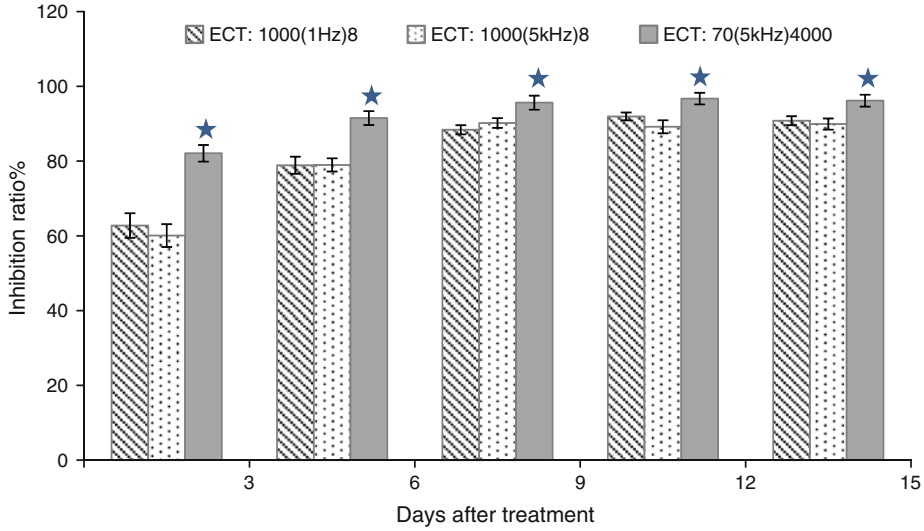

Fig. 3 ECT with 4,000 pulses of 70 V/cm and 5-kHz frequency and standard protocols. Results are presented as mean ± SEM (*$P < 0.05$)

Table 1 Antitumor efficiency of electrochemotherapy with different frequencies and amplitude

ECT protocol	Number	Partial response (%)	Complete response (%)	Objective response (%)
70 V/cm, 5 kHz, 4,000	10	80	20	100
100 V/cm, 5 kHz, 4,000	10	70	0	70
150 V/cm, 5 kHz, 4,000	10	100	0	100
1,000 V/cm, 1 Hz, 8	10	60	0	60
1,000 V/cm, 5 kHz, 8	10	60	0	60

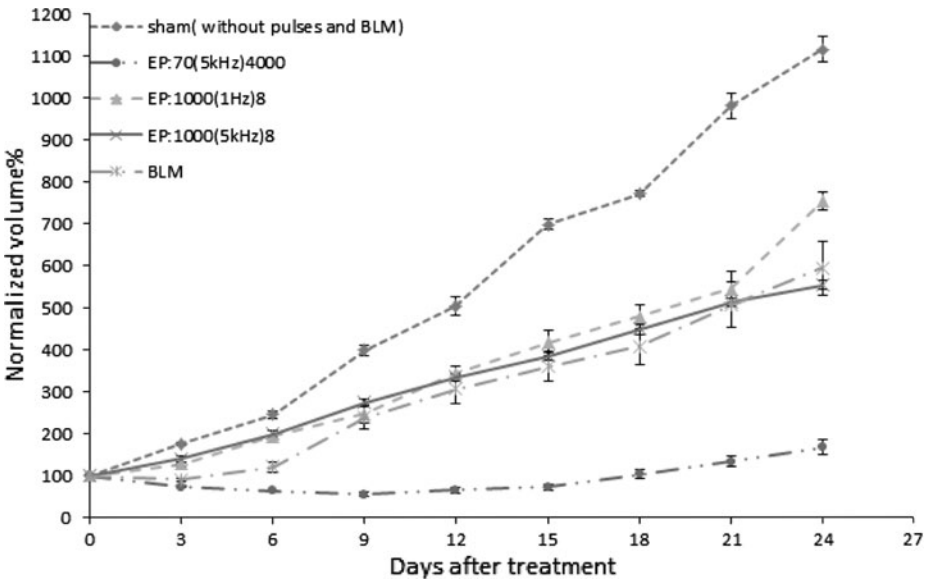

Fig. 4 EP with 4,000 pulses of 70 V/cm and 5-kHz frequency and standard ECT protocols. Results are presented as mean ± SEM

et al. 2003; Kitamura 2003; Plotnikov et al. 2004; Entin et al. 2003).

According to a previous study, 150, 100 and 60–70 V/cm introduce the best low electric field strength (Miyazaki et al. 2003). However, applying a high frequency or low electric field strength has been examined separately. In a hybrid approach, we combined these optimal conditions (electric pulses using 5-kHz frequency and 100 V/cm amplitude) and tried to find the effect of number of electric pulses on the treatment of invasive ductal carcinoma tumors (Shankayi

Table 2 Normalized volume of tumors in treatment groups with different frequencies and amplitude: mean ± SD

Treatment groups	Day 3	Day 6	Day 9	Day 12	Day 15	Day 18	Day 21	Day 24
ECT: 70 V/cm, 5 kHz, 4,000	44.93 ± 16.01	33.21 ± 20.81	26.47 ± 13.75	25.27 ± 8.51	31.57 ± 18.27	41.08 ± 22.64	47.65 ± 57.30	58.93 ± 72.44
ECT: 100 V/cm, 5 kHz, 4,000	57.8 ± 11.83	48.35 ± 12.84	44.64 ± 13.06	65.92 ± 17.23	84.11 ± 28.14	114.98 ± 54.29	151.7 ± 79.91	181.03 ± 92.63
ECT: 150 V/cm, 5 kHz, 4,000	45.89 ± 12.24	39.67 ± 14.67	33.49 ± 10.69	44.36 ± 20.93	58.00 ± 30.39	79.43 ± 31.31	91.50 ± 26.02	107.54 ± 31.62
ECT: 1,000 V/cm, 1 Hz, 8	75.42 ± 11.48	60.88 ± 15.91	54.04 ± 15.68	45.71 ± 15.05	72.9 ± 32.06	103.64 ± 49.04	157.15 ± 84.37	177.83 ± 97.81
ECT: 1,000 V/cm, 5 kHz, 8	79.04 ± 11.51	59.23 ± 12.62	44.06 ± 17.29	59.34 ± 26.09	77.1 ± 31.15	87.4 ± 63.56	117.02 ± 62.01	135.62 ± 71.63
EP: 70 V/cm, 5 kHz, 4,000	73.58 ± 10.37	62.93 ± 10.94	54.59 ± 16.69	64.99 ± 18.05	72.10 ± 17.89	103.84 ± 30.26	133.90 ± 43.10	142.93 ± 55.28

et al. 2010). This study showed that ECT with low electric field amplitude and a 5-kHz pulse repetition frequency is an effective treatment. According to our results, the number of pulses, 2,000 (equivalent to four pulses of 50-ms duration) or 4,000 (equivalent to eight pulses of 50-ms duration), with 5-kHz repetition frequency and 100-μs duration, is not the crucial parameter provided that bleomycin was in excess in the treated tissue; however, better antitumor effects were obtained with 4,000 pulses. Furthermore, with more than 4,000 electric field pulses, the inhibition of tumor was comparable with these data. Mir and Orlowski (1999), in a treatment using a standard protocol, discovered that the effective number of pulses with injected bleomycin is eight. The roles of the ECT parameters have been examined by other authors. Rols and Teissie (1998) reported that increasing the number of pulses (N) enhanced the rate of permeabilization. Also, the same permeabilization efficiency was obtained at a low value for E and high values for N and T. Lebar et al. (2002b) experimented on lipid bilayer membranes and demonstrated interpulse intervals shorter than 250 ms; the second pulse resulted in an immediate jump in membrane conductance, while for longer interpulse intervals, the conductance behavior was similar to that of the first pulse. Prior to our study, Matsuki et al. (2008) demonstrated in vitro that consecutive low-voltage pulses (75 V/cm), with a voltage lower than the membrane breakdown threshold of human cells, could increase the membrane potential of the threshold required to induce EP.

The aim of this study was to evaluate the effect of pulse amplitude. Therefore, we examined different voltages using 4,000 pulses with 100-μs duration and 5-kHz pulse repetition in the treatment of invasive ductal carcinoma in female Balb/c mice. Our data indicated that treatment with high-frequency, 70 V/cm, electric field strength had the best effect on the inhibition of tumor growth and cures (Figs. 3, 5). Then, we compared our protocols with the standard protocols using 1,000 V/cm and eight pulses, 100-μs duration with 1-Hz and 5-kHz frequency repetition. We found that treatment with our protocol increased the ability of ECT and decreased tumor volumes to about 25% of primary volumes. The other advantage of the 70-V/cm electric pulse strength with high frequency was the ability to reduce the tumor volume with electric pulse alone, which was comparable with standard protocols.

In conclusion, we observed that in vivo low electric field and high-frequency EP by bleomycin for reducing and eliminating invasive ductal carcinoma transplanted into Balb/c mice is more effective and considerable. In the conditions that we examined, the best protocol for treatment and malignancy reduction was that utilizing 70-V/cm amplitude, 4,000 pulses of 100-μs duration and 5-kHz repetition frequency. However, further animal and clinical studies are needed to confirm our findings.

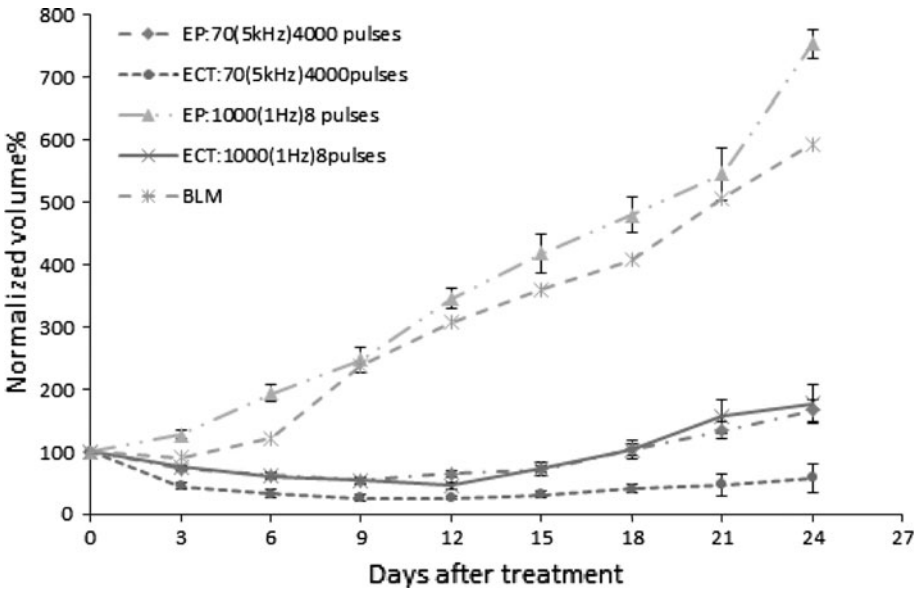

Fig. 5 EP and ECT with 4,000 pulses of 70 V/cm and 5-kHz frequency and standard protocol. Results are presented as mean ± SEM

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