

## SIGNIFICANCE OF TREATMENT ENERGY IN CELL ELECTROPERMEABILIZATION

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### ABSTRACT

The effect of applied electrical energy on cell survival and permeabilization of the plasma membrane under *in vitro* conditions were examined. Cell viability was measured with a colony-forming assay, permeabilization of the plasma membrane by means of propidium iodide (PI) uptake. Cells were exposed to five regimens of electrical treatment parameters. For a given voltage, applied electrical energy was the same for all regimens. In all applied regimes, cell survival was comparable. The differences in PI uptake between applied regimens were significant. The highest percentage of stained living cells (76%) was observed with four long pulses (200  $\mu$ s) with a repetition frequency of 0.5 Hz at 200 V. At regimens with higher numbers of shorter pulses, the percentage of stained living cells was considerably lower. According to our results, total applied electrical energy is not a determinant parameter for drug uptake and cell survival under *in vitro* conditions.

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## INTRODUCTION

Electrochemotherapy, the use of locally applied electric pulses to potentiate the cytotoxicity of chemotherapeutic agents, was begun in 1987 by Okino and Mohri (1). Since then, a number of *in vitro* and *in vivo* studies of this technique have been conducted (2–4). Promising results have brought electrochemotherapy from the laboratory to the clinic (5–7).

Parameters influencing the protocol for electrochemotherapy *in vivo* are the drug-administration route, delay between drug delivery and application of pulses, electrical treatment parameters, number of treatments, and electrode design (8). Electrical treatment parameters are electric-field strength, pulse duration, pulse shape, number of pulses, and pulse-repetition frequency. The driving force in optimization of this large number of variables is to search for minimal electrical conditions that would lead to a significant antitumor effect. Different shapes of pulses, and a broad range of electric-field strengths and number of pulses (Table 1), have been used in *in vivo* studies. All of the studies mentioned in Table 1 showed considerable antitumor effects of electrochemotherapy. This result hints at the possibility that applied electrical energy may be a determining treatment parameter for successful electrochemotherapy.

*In vitro* experiments are only a rough model for studies under *in vivo* conditions. In the case of electrochemotherapy, such experiments usually result in the evolution of some basic principles dealing with drug uptake and cell survival according to the applied electrical treatment. In the present study we examine the effect of applied electrical energy on cell survival and permeabilization of the plasma membrane.

## MATERIALS AND METHODS

### Chemicals

Eagle's minimal essential medium (EMEM) and propidium iodide (PI) were obtained from Sigma Chemical Co. (St. Louis, MO), fetal calf serum (FCS) from GIBCO (Grand Island, NY), and crystal violet from Kemika (Zagreb, Croatia). PI was dissolved in sterile H<sub>2</sub>O at a concentration of 100  $\mu$ M.

### Cells

DC3F cells (spontaneously transformed hamster lung fibroblasts) were generously provided by Jean Belehradek Jr. of the Institut Gustave Roussy, Villejuif, France. They were grown in monolayer in EMEM supplemented with 10% FCS, 10 mM L-glutamine, 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin, and 11  $\mu$ g/ml gentamicin. The cells were routinely subcultured every 4 days and were incubated at 37°C in humidified air with 5% CO<sub>2</sub>.

### Electropermeabilization Protocol

For each experiment, cells were prepared from the exponential growth phase. They were trypsinized and washed at 4°C in EMEM supplemented with 10% FCS for inactivation of trypsin, and then in serum-free EMEM supplemented with 0.5 mM

**Table 1.** Electrical-Treatment Parameters Used in *In Vivo* Studies as They are Presented in the Literature

First author, year of publication, (reference)	Shape of pulses	Pulse duration	Number of pulses	Voltage amplitude	Electrodes
Okino, 1987, 1990 (1,9)	Exponential	2 ms	1	10 kV	Needle
Kanesada, 1990 (10)	Exponential	4 ms	1	6 kV	Needle
Mir, 1991 (3), Belehradek, 1991 (11)	Rectangular	100 $\mu$ s	8	1.5 kV/cm*	External
Okino, 1992 (12)	Rectangular	2.5 ms– 5.8 ms	1	0 V to 5 kV	Needle
Salford, 1993 (13)	Exponential	325 $\mu$ s <sup>†</sup>	8–12	400 V or 600 V	Needle
Belehradek, 1994 (14)	Rectangular	100 $\mu$ s	8	>1050 V/cm*	External
Sersa, 1995 (4)	Rectangular	100 $\mu$ s	8	1300 V/cm*	External
Heller, 1995 (15)	Rectangular	99 $\mu$ s	8	1.5 kV/cm*	External
Heller, 1997 (16)	Rectangular	99 $\mu$ s	8	1.3 kV/cm*	External
Jarozeski, 1997 (17)	Rectangular	99 $\mu$ s	6	1 kV/cm*	Needle array
Mir, 1997 (18)	Rectangular	100 $\mu$ s	4 + 4 <sup>‡</sup>	0.8 kV/cm*	Needle array

\*Voltage-amplitude-to-electrode-distance ratio.

<sup>†</sup>Time constant.

<sup>‡</sup>Four pulses of each polarity.

CaCl<sub>2</sub>. Suspensions of  $2.2 \times 10^7$  cells/ml were prepared and kept at 4°C during experimentation.

The permeabilization of the plasma membrane was measured by means of PI uptake. For this purpose, 60  $\mu$ l of the cell suspension were mixed with 6.6  $\mu$ l PI. Following this, 50  $\mu$ l of the cell suspension (i.e.,  $10^6$  cells) were placed between two flat, parallel stainless steel electrodes (length = 2 cm; width = 1 cm; interelectrode distance = 2 mm). The electric field was generated as a high-voltage square-wave applied between the electrodes, with electronically selectable duration and repetition frequency (Electropulsator Jouan GHT 1287, Saint Herblain, France). The number of pulses was counted with a HM8021-3 Universalzahler (Hameg, Germany). Pulses were monitored on line with an oscilloscope (Hameg Storage Scope HM 205-3). After exposure of cells to electric pulses, the cells were incubated for 5 min at room temperature (24°C). Aliquots of 25  $\mu$ l of cells were resuspended in 1 ml of 0.01 M PBS (pH 7.4) and analyzed with flow cytometry (Becton Dickinson, Mountain View, CA). The percentage of stained cells was determined in comparison with control cells that were not subjected to electric pulses.

Cell viability was measured with a colony-forming assay. For the assay, 60  $\mu$ l of cell suspension were mixed with 6.6  $\mu$ l EMEM supplemented with 0.5 mM CaCl<sub>2</sub>. Following this, 50  $\mu$ l of the mixture (i.e.,  $10^6$  cells) were exposed to electric pulses. Aliquots of 5  $\mu$ l of cells exposed to electric pulses were diluted 500 times and plated

in 60-mm Petri dishes (200 cells/dish) in triplicate. After 6 days, colonies were fixed, stained with crystal violet, and counted. The results were expressed as the percentage of colonies obtained with respect to the untreated control cells. The plating efficiency of control cells was close to 100%.

### Experiments

Five different regimens of electrical treatment parameters were chosen (Table in Figure 1). The electrical energy ( $E$ ) applied in the experimental regimen was calculated as follows:

$$E = \int_0^{\tau} U(t)I(t)dt = \int_0^{\tau} U(t) \frac{U(t)}{Z(\omega, t)} dt = \frac{1}{Z} \int_0^{\tau} U(t)^2 dt \quad , \quad (1)$$

where  $U(t)$  was applied voltage and  $\tau$  was treatment duration. During experiments, electric current  $I(t)$  followed  $U(t)$  exactly, and impedance of the load  $Z(\omega, t)$  was therefore assumed to be ohmic and constant ( $Z$ ) during the treatment.  $U(t)$  was constant during each pulse:

$$E = \frac{U^2}{Z} \sum_{i=1}^N T_i = \frac{1}{Z} U^2 \tau \quad . \quad (2)$$

where  $T_i$  was the duration of the pulse during the treatment,  $N$  was the number of pulses, and  $U$  was the amplitude of the voltage.

For a given voltage, applied electrical energy was the same for all regimens. PI uptake was measured for 160 V, 200 V, and 240 V. Cell viability was determined for 160 V, 200 V, 240 V, and 280 V. Three experiments (PI uptake + cell viability) were done for all regimens.

### RESULTS

As described in the Materials and Methods, permeabilization was measured by PI staining of cells, and cell viability was measured with a colony-forming assay.

Figure 1 provides regimen parameters (table), viability of cells, and the percentage of fluorescent cells in the population for all five regimens (Graphs A, B, C, D, and E). Viability of the pulsed cells was the same as that of control cells for all five regimens, at least at voltages up to 200 V. Viability was in the range of 97.5%–98.5% for Regimen A, 85%–91% for Regimen B, 86%–91.5% for Regimen C, around 98% for Regimen D, and around 93% for Regimen E. Viability of the cells submitted to higher voltages (240 V, 280 V) decreased. For shorter electrical pulses, when the applied frequency and the number of pulses was higher, (Regimens D and E), the decrease in cell viability was less dramatic. With Regimen E it fell only to 52% at 280 V.

The percentage of stained cells in the population increased with increasing voltage (applied energy). The longer the pulses (applied frequency was lower, as well as the number of pulses), the more the cells were permeabilized. For all applied electrical energies, cells pulsed with four long pulses (Regimen A, Graph A) exhibited

Regime	$n$	$T_1$ ( $\mu\text{s}$ )	$f$ (Hz)	$\tau$ (s)
A	4	200	0.5	8
B	8	100	1	8
C	16	50	2	8
D	40	20	5	8
E	80	10	10	8

Table

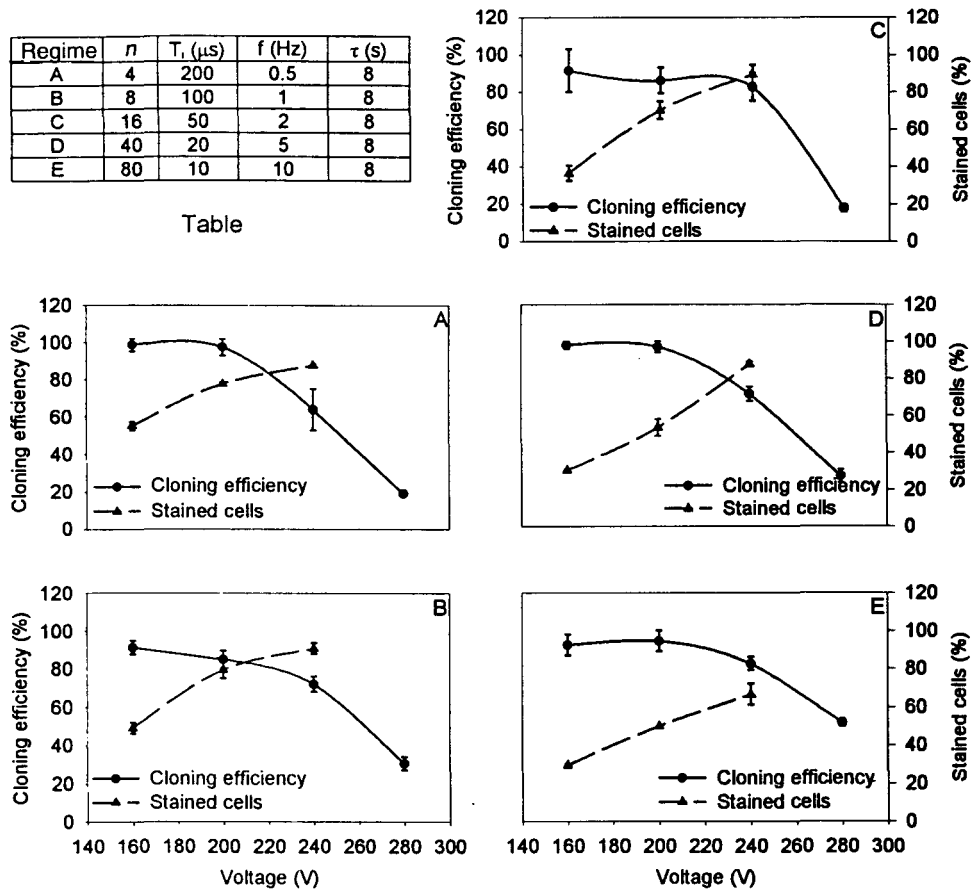


FIGURE 1. Response of DC3F cells to different regimens of electropermeabilization. Regimens and their parameters are listed in table;  $n$  is number of square-wave pulses;  $T_1$  is duration of each pulse,  $f$  is repetition frequency, and  $\tau$  is duration of the treatment. Graphs show cloning efficiency and percentage of stained cells as a function of applied voltage for regimen A (A), regimen B (B), regimen C (C), regimen D (D), and regimen E (E).

significantly higher percentages of stained cells (78% at 200 V) than did cells pulsed with 80 shorter pulses (Regimen E, Graph E; 50% at 200 V).

The percentage of stained living cells in the population as a function of applied voltage is shown in Figure 2. Considerably different percentages of stained living cells could be observed with various chosen regimens. When the applied voltage was 200 V, the highest percentage of stained living cells (76%) was observed with Regimen A ( $r$  pulses, duration of each pulse 200  $\mu\text{s}$ , and repetition frequency 0.5 Hz). A high percentage of stained living cells (74%) could also be observed with Regimen C (16 pulses, duration of each pulse 50  $\mu\text{s}$ , and repetition frequency 2 Hz), but in this case the applied voltage was higher (240 V). With other regimens and applied voltages, the percentage of stained living cells was below 70%.

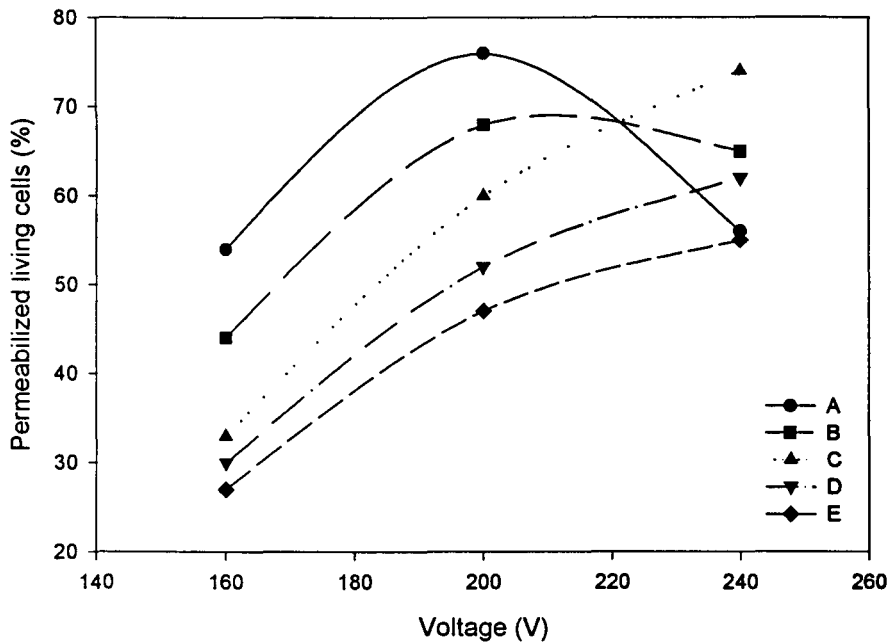


FIGURE 2. Percentage of stained living cells in the population as a function of applied voltage. (A) Repetition frequency ( $f$ ) = 0.5 Hz, number of pulses ( $n$ ) = 4, duration of each pulse ( $T_i$ ) = 200  $\mu$ s; (B)  $f$  = 1 Hz,  $n$  = 8,  $T_i$  = 100  $\mu$ s; (C)  $f$  = 2 Hz,  $n$  = 16,  $T_i$  = 50  $\mu$ s; (D)  $f$  = 5 Hz,  $n$  = 40,  $T_i$  = 20  $\mu$ s; (E)  $f$  = 10 Hz,  $n$  = 80,  $T_i$  = 10  $\mu$ s.

## DISCUSSION

The purpose of this work was to investigate the role of applied electrical energy in *in vitro* electroporation. We found that applied electrical energy was not a determinant parameter for drug uptake and cell survival under *in vitro* conditions.

Our results support the observations made in other *in vitro* studies (19–22), but they also give these results a new dimension. Many authors have studied the dependence of *in vitro* electroporation on electrical variables. The main conclusion related to pulse duration has been that the permeabilization of cells increases with pulse duration for a given number of pulses and field strength (19,21,23). Our results indicate that for a given field strength, permeabilization of the cells increases with pulse duration even when a smaller number of longer pulses is used and applied electrical energy is kept constant.

Efficient electroporation should satisfy two conditions: (1) the viability of the pulsed cells should be more or less the same as the viability of control (unpulsed) cells; and (2) all live cells should be permeabilized. Our results show (Figure 2) that for a given applied electrical energy, the percentage of permeabilized living cells is significantly higher for regimens with longer electrical pulses, although the number of pulses is smaller.

Although there is some evidence that energy may be a reasonable predictor of successful electrochemotherapy *in vivo* (8,12), our results do not show such a rule under *in vitro* conditions.

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