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Optimisation of Pulse Parameters *In Vitro* for *In Vivo* Electrochemotherapy

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Abstract. *The aim of our study was to optimise electric pulse parameters for electrochemotherapy by sampling the space of pulse parameter variables using systematic in vitro experiments. For this purpose we defined parameters that describe the effectiveness of different sets of electric pulse parameters in vitro and combined them into an objective function that characterises requirements for successful electrochemotherapy. The objective function values were calculated for all the sets of electric pulse parameters included in in vitro experiments. Similar values were grouped together by hierarchical clustering. The 'electrochemotherapeutic' effectiveness of two sets of pulse parameters (8 pulses, 100 µs, 1 Hz and 1 pulse, 1000 µs, 1 Hz), which belong to the most efficient cluster, and one set of pulse parameters (16 pulses, 20 ms, 1 Hz), which belongs to the least efficient cluster, was tested in vivo on a murine tumor model. The sets of pulse parameters from the most efficient cluster had comparable effects in vivo, while the electrochemotherapy with the set of pulse parameters from the least efficient cluster was less effective. Our results demonstrated that electric pulse parameters for effective in vivo application can be determined from in vitro experiments considering application specifications.*

Cell membrane electropermeabilization is a physical method using brief and intense electric field pulses to increase the membrane permeability. Under controlled electric field conditions, the increase in membrane permeability is transient and, as such, has important practical applications. Electropermeabilization is nowadays widely used for the direct transfer of genes (1) and for introduction of other nonpermeant molecules into living cells (2), for biochemical and pharmacological studies (3) and as a method of non-thermal food preservation (4). Clinical applications are in progress, particularly in oncology (5-8). The treatment of solid tumors which combines nonpermeant drugs having high intrinsic cytotoxicity with locally delivered short and intense electric pulses was named electrochemotherapy (9). Clinical trials performed in France, the USA and Slovenia showed excellent results on tumors of various histological origins (5-8). Electrochemotherapy with bleomycin for basal cell carcinomas, for example, resulted in an efficacy close to that of surgery and totally preserved the surrounding tissues (ears, lips, neck, *etc*) (6). Also electrochemotherapy with cisplatin, was well-tolerated by patients and a good cosmetic effect was obtained, with only minimal scarring and a slight depigmentation of the skin (7).

Although the number of preclinical and clinical trials is increasing, several questions concerning the therapy are still open. Among them is the determination of electric pulse parameters to assure successful treatment with minimal possible side- effects. Standard electrical parameters cause transient small burns in the areas which have been in contact with the electrodes (10) plus contractions of surrounding muscles, which are unpleasant (5).

In fact, with the exception of the first articles of Okino's group (11) (who used very high field strength exponentially decaying pulses), eight monophasic square-wave pulses of 100 ms duration at repetition frequency of 1 Hz have been used *in vivo* in the vast majority of the preclinical and clinical trials. Mir *et al.* (9) reported the efficiency of such electrical parameters for the first time. They selected these electrical parameters based on results obtained in experiments on cells in culture (12). 'Optimal conditions' found *in vitro* were applied in the *in vivo* experiments and initial clinical trials, giving excellent results in both cases (5-8). Therefore, the electrical parameters mentioned above became widely used, but have never been objectively subjected to a systematic investigation. Only the effects of changing the applied electric field have been investigated by some authors, either changing the field strength (9, 13, 14), position of the electrodes (15), or electrode design (16). The main target of the studies mentioned was to cover the entire tumor with an electric field strength that was sufficient to cause membrane destabilisation for delivering a drug by electrochemotherapy. Some experimental observations were even supported by numerical calculations of electric field distribution in the tissue (15, 17-19).

In this paper we present a method for the determination of

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effective electrical parameters for electrochemotherapy from systematic *in vitro* experiments on cells in culture and known requirements for successful electrochemotherapy. In *in vitro* experiments we varied the number of pulses between 1 and 64, pulse duration between 20 ms and 1 ms and pulse amplitude from 40 to 600 V. The influence of different sets of electric pulse parameters on electropermeabilization of the cells to small molecules and cell viability was investigated (20). On the basis of this study, five parameters describing electropermeabilization *in vitro* were defined. The requirements for successful electrochemotherapy were described with these parameters and grouped together into a mathematical form called an objective function. The values of the objective function were calculated for all twenty sets of pulse parameters tested *in vitro*. By hierarchical clustering, we grouped together similar function values according to Euclidean distance. Four groups of the sets of pulse parameters with different 'electrochemotherapeutic' efficiency were obtained. Standard pulse parameters for electrochemotherapy (8 pulses, 100 μs, 1 Hz) belonged to the most efficient group. We chose another set of pulse parameters from this group (1 pulse, 1000 μs, 1 Hz) and one set of pulse parameters from the least efficient group (16 pulses, 20 μs, 1 Hz) for *in vivo* testing on a murine tumor model. Following tumor growth delay and complete responses of the tumors, we monitored the antitumor effectiveness of these sets of pulse parameters. The results obtained confirmed our predictions. Sets of pulse parameters from the most efficient group according to the formulated objective function had comparable effects *in vivo*, while the electrochemotherapy with the set of pulse parameters from the least efficient group was the least effective.

Materials and Methods

Chemicals. Eagle's minimal essential medium (EMEM), trypsin and propidium iodide (PI) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal calf serum (FCS) and L-glutamine were obtained from Gibco BRL (Gaithersburg, MD, USA), penicillin, streptomycin and gentamicin from Lek (Ljubljana, Slovenia) and Crystal violet from Kemika (Zagreb, Croatia). PI was dissolved in sterile H_2O at a concentration of 100 μM. Bleomycin (Mack, Germany) was dissolved in phosphate-buffered saline at a concentration of 3mg/ml.

In vitro experiments. DC3F cells, a line of spontaneously transformed Chinese hamster lung fibroblasts, were grown as a monolayer in EMEM supplemented with 10% CS, 10mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 11 μg/ml gentamicin. The cells were routinely subcultured every 4 days and incubated at 37°C in a humidified atmosphere with 5% CO_2.

The permeabilization of the plasma membrane was measured by means of PI uptake and cell viability after exposure to electric pulses by a colony-forming assay. Cells from the exponential growth phase were trypsinized and centrifuged for 5 minutes at 4°C and 1500 rpm in the culture medium. They were then resuspended in serum-free medium supplemented with 0.5 mM $CaCl_2$ at a density of 2.2 x 10^7 cells/ml. Ninety μl cell suspension was mixed with 10 μl PI for determination of electropermeabilization, or with medium supplemented with 0.5 mM $CaCl_2$ for determination of electropulsed cell viability. A 50 μl droplet of each mixture was placed between two flat, parallel, stainless steel electrodes (length = 6 mm, width = 6mm, interelectrode distance = 2 mm) and subjected to monophasic square-wave electric pulses, with a repetition frequency 1 Hz (Jouan GHT 1287 B, Saint Herblain, France). The number of pulses was varied between 1 and 64, pulse duration between 20 μs and 1 ms and pulse amplitude from 40 to 600 V. Pulse parameters were monitored by an oscilloscope (Hameg HM 205-3, Germany). All experiments were performed under sterile conditions in a laminar flow hood at room temperature.

After the exposure to the electric pulses, the cells were incubated for 5 minutes at room temperature. To measure the PI uptake, 25 μl of pulsed cells were resuspended in 1 ml of 0.01 M phosphate-buffered saline (PBS, pH 7.4) and kept at 4°C till being analysed by flow cytometry (FACSort, Becton Dickinson, CA, USA). Excitation was set at the wavelength 488 nm and emission was detected at 640 nm. Fluorescence was recorded for 5000 particles. Only particles large enough to qualify as cells were taken into consideration. The number of stained cells was determined and normalized to the number of all cells to get the percentage of permeabilized cells.

For the colony-forming assay, the pulsed cells were diluted in the culture medium and seeded in triplicate (300 cells per 60-mm diameter petri dish). After five days colonies were fixed with 96% ethanol, stained with Crystal violet and counted. The survival of cells treated with electric pulses was calculated as the percentage of colonies obtained from the untreated control cells.

In vivo experiments. In the experiments, the inbred C57Bl/6 mice of both sexes (Institute of Pathology, University of Ljubljana, Slovenia) were used. They were maintained at a constant room temperature (24°C) with a natural day/night cycle in a conventional animal colony. Before the experiments, the mice were subjected to an adaptation period of at least 10 days. Mice in good condition, without fungal or other infections, weighing 20-22g and 10-12 weeks old were included in the experiments.

The LPB sarcoma cells, syngeneic to C57Bl/6 mice, were obtained from *in vitro* cell culture. The cells were grown in EMEM supplemented with 10% CS, 100 units/ml penicillin, 100 μg/ml streptomycin and 11 μg/ml gentamicin. Solid tumors, located dorsolaterally in the mice, were initiated by an injection of 10^6 cells in 0.1 ml 0.9% NaCl solution. The viability of the cells, as determined by a trypan blue dye exclusion test, was over 95%. Twelve to fourteen days after transplantation when the tumors reached approximately 40 mm^3 in volume, the mice were randomly divided into eight experimental groups, consisting of 7-9 mice each and subjected to a specific experimental protocol on day 0.

Bleomycin, at a dose of 20 μg per animal, was injected intravenously. Electric pulses were delivered by two flat, parallel stainless steel plate electrodes with rounded corners (length = 35 mm, width = 7mm, thickness = 1 mm, interelectrode distance = 8 mm). They were placed at the opposite margins of the tumor in the cranial/caudal direction. Good contact between the electrodes and the skin was assured by means of conductive gel. Monophasic square-wave electric pulses with a pulse amplitude of 1040 V and repetition frequency of 1 Hz, were generated by electropulsator Jouan GHT 1287. Three sets of electrical parameters were used: eight pulses of 100 μs, one pulse of 1000 μs and sixteen pulses of 20 μs.

Treatment with the electric pulses was performed without anaesthesia and was well-tolerated by the mice. In the combined treatment groups, the mice were treated with electric pulses 3-3.5 minutes after BLM injection, which is sufficient for the distribution of BLM. Mice in the control and electric pulses only groups were injected with 0.01 M PBS (pH 7.4) instead of bleomycin. The experiment was repeated three times.

Experimental data analysis and objective function formulation. All *in vitro* experiments were repeated on different days at least three times. The fraction of permeabilized living cells dependent on pulse amplitude was determined for twenty sets of electrical parameters (20). For each experimental point, mean and standard deviation were calculated. Using nonlinear regression, two-parameter sigmoid curves were fitted to cell

viability data and four –parameter sigmoid curves to the data presenting cell permeabilization (21). From the sigmoid curves, we defined five parameters describing electropermeabilization *in vitro*: threshold voltage of reversible and irreversible electropermeabilization, electropermeabilization interval, maximal fraction of permeabilized living cells and voltage at maximal fraction of permeabilized living cells. The threshold voltage of reversible electropermeabilization (U_{re}) was defined as the pulse amplitude leading to permeabilization of 50% of cell population. The threshold voltage of irreversible electropermeabilization (U_{ire}) was defined as the pulse amplitude leading to the death of 50% of the cell population. The difference between these two voltages was defined as the electropermeabilizaton interval (ΔU). The curve representing permeabilized living cells was obtained by multiplying the two sigmoid curves, which presented electropermeabilization and cell viability as a function of pulse amplitude at the same set of electrical parameters. The value of the curve maximum was defined as the maximum fraction of permeabilized living cells (F) and the voltage, at which the maximum is reached, is the voltage at maximum fraction of permeabilized living cells (U_F). With these descriptive parameters formulated for electropermeabilization *in vitro*, we characterised requirements for successful electrochemotherapy.

F should be as high as possible, because all clonogenic cells present in the tumor have to be permeabilized in order to obtain complete response. On the other hand, a low threshold voltage of reversible electropermeabilization (U_{re}) is desired from safety aspects and electrochemotherapy device requirements. We combined these two requirements for successful electrochemotherapy into a simple mathematical form called an objective function k:

$$k = \frac{U_{re}}{F}$$

where U_{re} is the threshold voltage of reversible electropermeabilization and F the maximal fraction of permeabilized living cells. The minimal value of this objective function should give the optimal set of electrical parameters for electrochemotherapy, since U_{re} should be low and F high. We grouped together similar function values by hierarchical clustering according to Euclidean distance to obtain the sets of electrical parameters with similar 'electrochemotherapeutic' efficiency. We chose two sets of electrical parameters with similar 'electrochemotherapeutic' efficiency (standard electrical parameters for electrochemotherapy: 8 pulses, 100 μs, 1 Hz and single pulse, 1000 μs, 1 Hz) and one set of electrical parameters which should be less efficient (16 pulses, 20 μs, 1 Hz) for *in vivo* testing.

Measuring three mutually orthogonal tumor diameters (e1, e2 and e3) with a vernier caliper on each consecutive day, we followed tumor growth. Tumor volumes were calculated by the formula $V = \pi \times e_1 \times e_2 \times e_3/6$. From the measurements, the arithmetic mean and standard error of the mean were calculated for each experimental group. If the tumor became unpalpable and did not regrow after 100 days, the therapeutic response was scored as complete response (CR).

Results

In this paper we present a method for the determination of effective electrical parameters for electrochemotherapy from a systematic *in vitro* study performed on cells in culture and the requirements for successful electrochemotherapy formulated by the objective function.

In the *in vitro* study we varied the number of pulses between 1 and 64, pulse duration between 20 μs and 1 ms and pulse amplitude from 40 to 600 V. The influence of twenty different combinations of pulse duration and number of pulses on electropermeabilization of the cells to small molecules and cell viability was monitored as a function of pulse amplitude (20). On the basis of that study, five parameters describing electropermeabilization *in vitro* were defined. Requirements for successful electrochemotherapy were described with these parameters and grouped together into an objective function as specified in the Materials and Methods section. Values of the objective function were calculated for all the sets of electrical parameters tested *in vitro* and are given in Table I. The minimal value was obtained for 64 pulses of 1000 μs. By hierarchical clustering for detecting natural groups in objective function values and Euclidean distance as a distance metric used to compare clusters, we grouped together similar values of objective function to find the sets of electrical parameters with similar 'electrochemotherapeutic' efficiency. The dendrogram and the clusters are presented in Figure 1. Four groups of the sets of electrical parameters with different 'electrochemotherapeutic' efficiency were identified. In the least efficient group are the sets of electrical parameters consisting of 16 pulses of 100 μs and 20 μs. 'Standard' electrical parameters for electrochemotherapy (8 pulses, 100 μs, 1 Hz) belonged to the most efficient group. For *in vivo* testing, we chose 'standard' electrical parameters for electrochemotherapy, another set of electrical parameters from the most efficient group (1 pulse, 1000 μs, 1 Hz) and one set of electrical parameters from the least efficient group (16 pulses, 20 μs, 1 Hz).

Table I. *Values of the objective function k = Ure/F (V/%) at twenty different combinations of pulse duration and number of pulses. Minimal value of the objective function is dashed.*

		Pulse duration			
		20 μs	100 μs	500 μs	1000 μs
Number of pulses	1 pulse	5.11	4.29	3.04	2.05
	4 pulses	4.96	3.31	2.67	1.77
	8 pulses	5.26	2.88	2.35	1.47
	16 pulses	6.02	6.10	1.75	3.00
	64 pulses	3.47	2.53	3.63	1.24

Electrochemotherapy with all three sets of electrical parameters induced an arrest of tumor growth. The reduction in tumor volume was detectable three days after the treatment in all three sets of pulse parameters (Figure 2). Thereafter, the tumors gradually decreased in size. Tumors treated with sixteen pulses of 20 μs and BLM started to regrow seven days after the treatment. Regrowth of the tumors treated with BLM and eight pulses of 100 μs or one

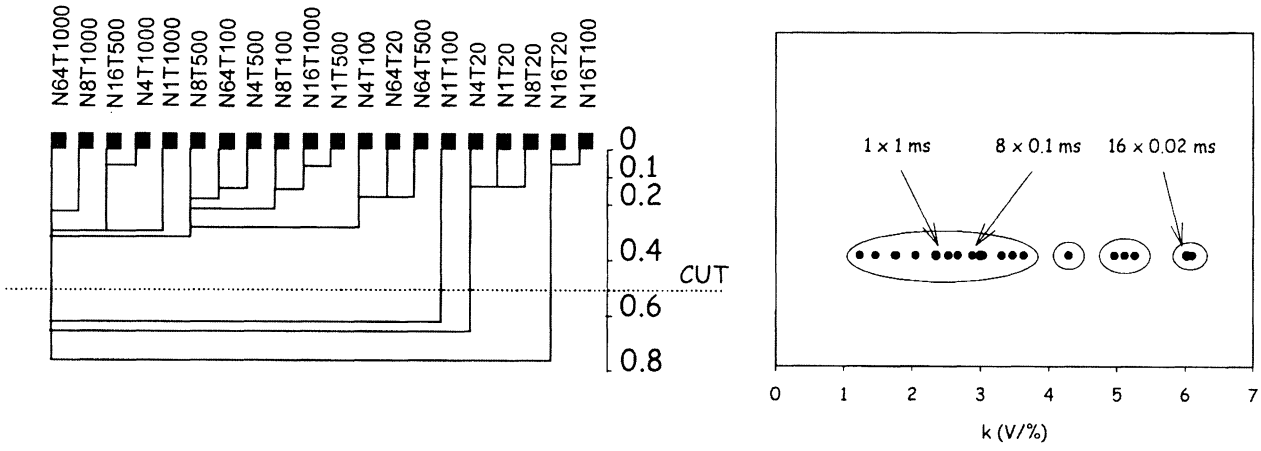

Figure 1. *Dendrogram (left) and the clusters (right) obtained by hierarchical clustering according to Euclidean distance. (left) The lengths of the branches connecting the objective function values in the dendrogram represent distances between these values. (right) Black points represent objective function values. Four clusters are encircled. The arrows mark objective function values at sets of electrical parameters that are selected for in vivo testing.*

1000 ms pulse was observed from the ninth day after the treatment. It can be seen that electrochemotherapy with one 1000 μs pulse was similarly effective electrochemotherapy with eight pulses of 100 μs, while electrochemotherapy with sixteen pulses of 20 μs was less effective.

The difference in antitumor effectiveness was also shown in the percentage of CR (Figure 3). The highest number of complete responses (4, *i.e.* 4 out of 9) was observed for the set of electrical parameters consisting of one pulse of 1000 μs. Electrochemotherapy with eight pulses of 100 μs resulted in 2 (2 out of 9) complete responses and they occurred 13 days later than in the case of electrochemotherapy with one 1000 μs pulse. Only one complete response (1 out of 9) was obtained in the case of electrochemotherapy with 16 pulses of 20 μs.

Discussion

This study confirms the possibility that effective electrical parameters for electrochemotherapy can be determined from *in vitro* experiments. The proposed method is based on representation of the results obtained from *in vitro* experiments by descriptive parameters and formulation of requirements for successful electrochemotherapy with the same descriptive parameters. By an objective function formulation and calculation of its values at different sets of electrical parameters, we predicted the relative efficacy of the electrochemotherapy performed *in vivo* by these sets of electrical parameters. The lower the value of the objective function, the more efficient the set of electrical parameters. However, the values of an objective function were calculated from the experimental data, which were loaded with an experimental error. This is the reason why, probably, the calculated optimal set of electrical parameters did not give better results than the sets of electrical parameters with the objective function values close to the local minimum. Therefore we grouped together similar values of objective function to find the sets of electrical parameters with similar 'electrochemotherapeutic' efficiency. The results obtained in *in vivo* study confirmed our predictions. Sets of electrical parameters from the most efficient group according to the objective function formulated also had comparable effects *in vivo*, while electrochemotherapy with the set of electrical parameters from the least efficient group was the least effective. The drug dose selected for *in vivo* experiments was extremely low (20 μg per animal) in order to observe predominantly the electrochemotherapeutic effect on tumor growth delay. With a higher drug concentration and/or changing electrode orientation, up to 80 % of CR can be reached (15).

Although the results obtained are promising, we have to explain a few facts that could be a cause for scepticism. First, a small, low-molecular-weight test molecule PI (mol wt 660 Da) was used in the *in vitro* experiments as a marker of cell permeabilization, while the *in vivo* experiments were performed with the anticancer drug bleomycin. This has bigger molecules and a higher molecular weight (1500 Da). In one of our previous studies we showed, that electropermeabilization curves obtained by PI for a given set of electrical parameters, were comparable with the electropermeabilization curves obtained using bleomycin (14). So we are certain that objective function values are also valid for bleomycin. Second, the antitumor effectiveness of electrochemotherapy is dependent on the amplitude of the electrical pulses (9, 22). For *in vivo* testing we chose three sets of electrical parameters: standard electrical parameters for electrochemotherapy (8 pulses, 100 μs, 1 Hz), another set of electrical parameters from the most efficient group (1 pulse,

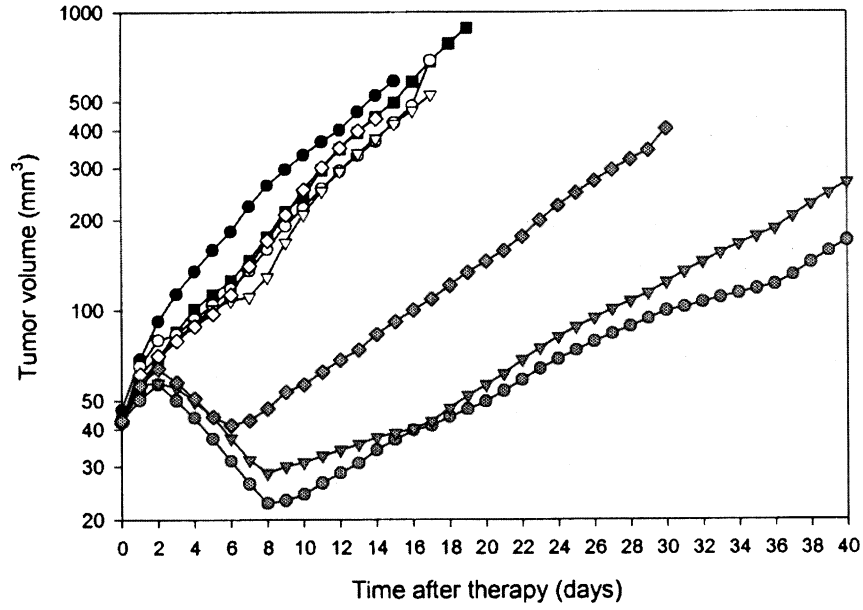

Figure 2. *The anti-tumor effectiveness of electrochemotherapy with different sets of electrical parameters on sarcoma LPB subcutaneous tumors. Mice (at least 7 per group) were treated with bleomycin intravenously and/or with electric pulses 3 – 3.5 minutes after bleomycin injection. The symbols denote the arithmetic mean of the tumor volumes measured every day:* ● *control,* ■ *bleomycin,* ○ *electric pulses 8 x 100 µs,* ▽ *electric pulses 1 x 1000 µs,* ◇ *electric pulses 16 x 20 µs,* ◍ *blemycin and electric pulses 8 x 100 µs,* ▼ *bleomycin and electric pulses 1 x 1000 µs,* ◈ *bleomycin and electric pulses 16 x 20 µs.*

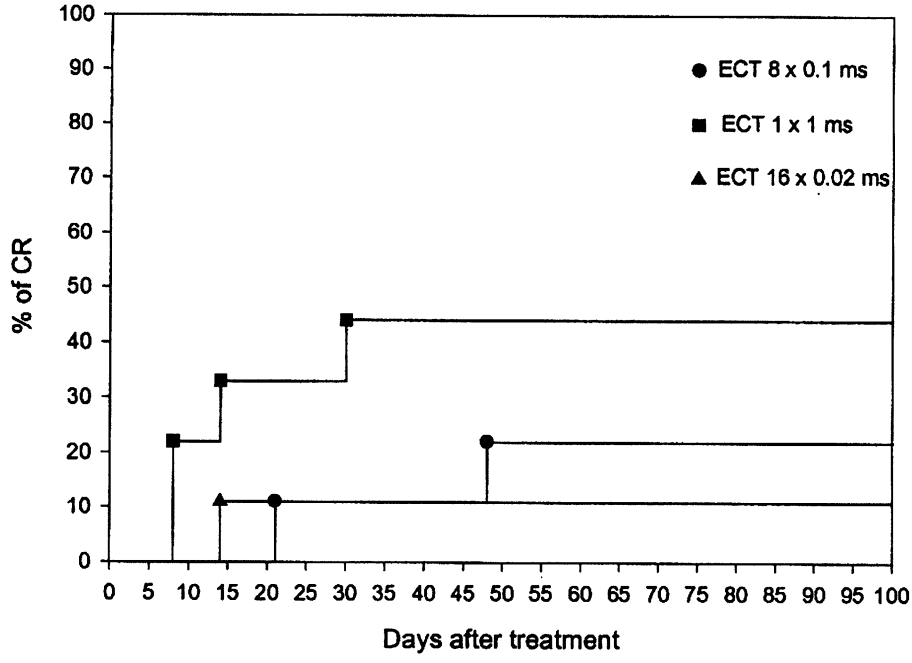

Figure 3. *Percentage of complete responses (CR) in LPB - bearing mice after electrochemotherapy (ECT). Mice bearing approximately 40 mm^3 tumors were treated with bleomycin intravenously and with (●) 8 electric pulses of 100 ms, (■) 1 pulse of 1000 ms or (▲) 16 pulses of 20 ms (1040 V, 1 Hz) 3 – 3.5 minutes after bleomycin injection.*

1000 μs, 1 Hz) and one set of electrical parameters from the least efficient group (16 pulses, 20 μs, 1 Hz). In all three cases the same pulse amplitude of 1040 V was used. This voltage was determined experimentally for standard electrical parameters (9). Our *in vitro* study showed that the maximal fraction of permeabilized living cells is obtained at 205 V for 8 pulses of 100 μs, at 211 V for 1 pulse of 1000 μs and at 274 V for 16 pulses of 20 μs, respectively. Voltages at maximal fraction of permeabilized living cells (U_F) for 8 pulses of 100 μs and for 1 pulse of 1000 ms are comparable, while U_F for the set of electrical parameters consisting of 16 pulses of 20 μs was higher. Probably, with the higher amplitude of electrical pulses, better antitumor effects could be achieved even with 16 pulses of 20 μs. However, we have to emphasise that comparison of different electrical parameters with respect to objective function means that all the other parameters, which are included in the objective function formulation, remain constant. So, if the low voltage used in experiments *in vivo* is the reason for lower efficiency of the last set of electrical parameters, it is already included in the objective function values. In other words, the value of the objective function at 16 pulse of 20 μs is high, also because the voltage at the maximal fraction of permeabilized living cells is high. Third, if only one electric pulse is used for electrochemotherapy, the anticancer effect is proportional to the duration of the pulse (23). The cumulative duration of electrical treatment during electrochemotherapy with 8 pulses of 100 μs is 800 μs, which is (again) comparable with the duration of one 1000 μs pulse. The cumulative duration of electrical treatment during electrochemotherapy with 16 pulses of 20 μs is 320 μs, which is less than half of the cumulative duration of the previous two sets. But again, if a shorter cumulative duration of electrical treatment during electrochemotherapy is the reason for a lower efficiency of the last set of electrical parameters, it is already included in the objective function values.

This study is the first attempt to optimise pulse parameters for *in vivo* application of electropermeabilization by parameter optimisation *in vitro*. If the method becomes reliable, it will be the cheapest and the easiest way for optimisation of electric pulse parameters *"in vivo"*.

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