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The effect of high frequency electric pulses on muscle contractions and antitumor efficiency in vivo for a potential use in clinical electrochemotherapy

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Abstract

Muscle contractions present the main source of unpleasant sensations for patients undergoing electrochemotherapy. The contractions are a consequence of high voltage pulse delivery. Relatively low repetition frequency of these pulses (1 Hz) results in separate muscle contractions associated with each single pulse that is delivered. It would be possible to reduce the number of unpleasant sensations by increasing the frequency of electric pulses above the frequency of tetanic contraction, provided that the antitumor efficiency of electrochemotherapy remains the same. These assumptions were investigated in the present paper by measuring the muscle torque at different pulse repetition frequencies and at two different pulse amplitudes in rats and studying the antitumor efficiency of electrochemotherapy at different pulse repetition frequencies on tumors in mice. Measurements of muscle torque confirmed that pulse frequencies above the frequency of tetanic contraction (>100 Hz) reduce the number of individual contractions to a single muscle contraction. Regardless of the pulse amplitude, with increasing pulse frequency muscle torque increases up to the frequency of 100 or 200 Hz and then decreases to a value similar to that after application of a 1 Hz pulse train. Electrochemotherapy in vivo with higher repetition frequencies inhibits tumor growth and is efficient at all pulse frequencies examined (1 Hz–5 kHz). These results suggest that there is a considerable potential for clinical use of high frequency pulses in electrochemotherapy.

Keywords: Electrochemotherapy; Pulse frequency; Muscle torque; Pain; Strain gauge

1. Introduction

Electrochemotherapy is an efficient local treatment of tumors, which combines the delivery of nonpermeant, cytotoxic chemotherapeutic agent (e.g. bleomycin, cisplatin) and short, high voltage electric pulses. At the appropriate pulse parameters (amplitude, duration and number of pulses) the permeability of tumor cell membranes increases transiently, thereby allowing the chemotherapeutic agent to enter the cells, and to exert its cytotoxic action.

Electrochemotherapy was found to be efficient in preclinical studies in mice and rats mostly for the treatment of cutaneous and subcutaneous tumors. Due to the promising results, electrochemotherapy has soon found its way from the laboratory to the clinic. The first clinical trial was performed by Mir and coworkers in Villejuif (France) in 1991 [1] and was soon followed by several clinical trials by other research groups, mostly from USA (Tampa [2,3]), France (besides Villejuif also Toulouse and Reims [4]) and Slovenia (Ljubljana [5]). The tumors under investigation

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were head and neck squamous cell carcinoma, basal cell carcinoma, melanoma, and adenocarcinomas. Combined therapy of application of electric pulses to the tumor following intravenous or intratumoral drug injection resulted in objective response rates of the tumors ranging from 62% to 100% [4], while tumors that were exposed only to electric pulses or to a chemotherapeutic drug did not respond. The chemotherapeutic drug administered in the first clinical trials was bleomycin. Bleomycin is a highly cytotoxic drug, which cannot permeate the intact cell membranes, or only in extremely small quantities [6,7]. Concomitant electric pulses that permeabilize tumor cell membranes significantly increase its cytotoxicity. In later clinical trials, another chemotherapeutic drug cisplatin was introduced, which was also demonstrated to be efficient in electrochemotherapy [8–10]. Detailed information on results of clinical application of electrochemotherapy can be found in the latest reviews [11,12].

Despite the success of the therapy, some side effects were reported. They include slight oedema or erythema in the treatment sites, which usually disappeared in less than 24 h after the treatment. In some cases, marks from the electrodes (usually two flat, parallel, stainless steel, plate electrodes with rounded corners) were visible several weeks after treatment, which most probably resulted from the very high local current density that can produce local burns. However, these post-pulse phenomena are not a cause of concern. Most unpleasant or even painful, according to the patients, were the sensations during the pulse delivery, which were mainly attributed to muscle contractions provoked by high amplitude of the pulses. In a great majority of the clinical trials, these pulses were delivered with relatively low pulse repetition frequency (one pulse per second, 1 Hz), which resulted in individual sensations and muscle contractions.

Because a high amplitude of electric pulses is necessary for efficient electropermeabilization of tumor cell membranes it is difficult to eliminate related muscle contractions. On the other hand, it would be possible to at least reduce the number of individual muscle contractions by increasing the frequency of electric pulses above the frequency of tetanic contraction, provided that the efficiency of electrochemotherapy remains the same. In our recent study in vitro we already showed that with increasing pulse repetition frequencies, the uptake of nonpermeant molecule remained at a similar level as at the frequency of 1 Hz [13]. We thus proposed that pulses with higher repetition frequencies could reduce the unpleasant sensations associated with muscle contractions during electrochemotherapy without reducing the efficiency of electrochemotherapy. In addition, higher repetition frequencies would also shorten the duration of the treatment, especially in the case where multiple needle electrodes are used (needle electrodes placed equidistantly in a circle, an additional electrode can be located in the center), where pairs of electrodes are activated sequentially [14,15].

In this paper the assumptions that high frequency pulses would reduce the number of individual muscle contractions and decrease the duration of the treatment while preserving the efficiency of electrochemotherapy were investigated further by performing experiments in vivo. This was done by measuring muscle torque in rats after electric pulse delivery to the *ishiadicus* nerve and by performing electrochemotherapy of a subcutaneous animal tumor model in vivo in mice using different pulse repetition frequencies.

2. Materials and methods

2.1. Measurements of muscle torque in rats

The experiments were performed on 16 male Wistar rats with an average body weight of 250±31 g. The handling of animals was in accordance with the license issued by the Ministry of Agriculture, Republic of Slovenia (license number 326-07-26/98) and consistent with the recommendations of the Panel on Ethics in Biomedical research, Medical Faculty of the University of Ljubljana. Ten minutes before the treatment, the rats were anesthetized with the combination of Rompun, 2% solution (Bayer, Germany) and Ketanest, 10 mg/ml (Parke-Davis, Germany). Anesthesia lasted for 1 h, which proved to be enough for the entire set of measurements. The rats recovered from general anesthesia approximately 30 min after the experiment was finished, which enabled the observation of possible motoric dysfunctions of the stimulated leg. No changes in the motoric function of the leg were observed.

The measurements of muscle response were performed by measuring muscle torque with a strain gauge transducer mounted on a wooden platform [16]. The right hind limb of a rat was stabilized and fastened to aluminium brace equipped with a strain gauge (Fig. 1). The height of the platform was adjustable to keep the leg in plane with the transducer. Muscle contractions rotated the aluminium brace, which induced voltage changes in the strain gauge. Using a BIOPAC MP 100SW data acquisition system (BIOPAC, USA), the analog voltage signal was amplified (BIOPAC, DA 100 amplifier), converted into a digital signal and stored on a computer (Fig. 1). Data acquisition lasted 1 s with a sampling frequency of 1 kHz (i.e. 1000 samples/s).

Electric pulses were generated by an AFG 310 function generator (Sony-Tektronix, Japan). The signal from the function generator was amplified by a power amplifier [17] and delivered to the electrodes (two stainless steel needle electrodes, 0.3 mm in diameter, 10 mm long and 8 mm apart) mounted on a plastic holder to keep the distance constant and reproducible. The electrodes were inserted through the skin into the thigh muscle in the vicinity of the *ishiadicus* nerve branches. Synchronization of electric pulses with data acquisition was ensured by a trigger

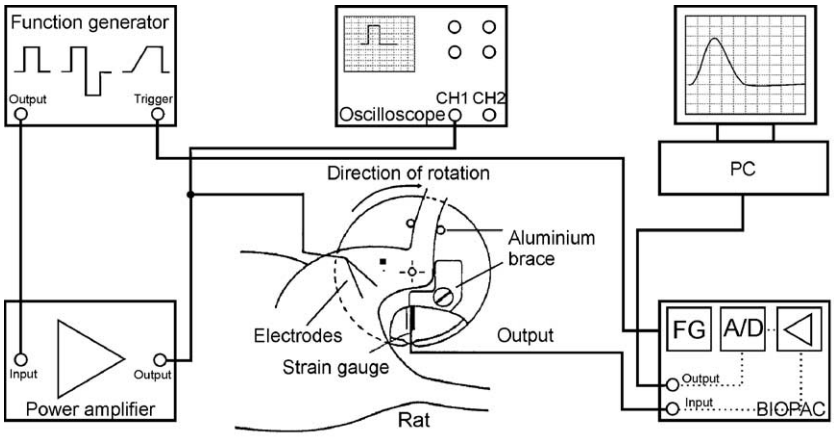

Fig. 1. Setup of the experiment (partly adapted from Ref. [16]). A/D—analog/digital converter, FG—function generator integrated in a BIOPAC system.

pulse from the BIOPAC system to the function generator (Fig. 1).

Electric pulses were delivered at two different pulse amplitudes. The low pulse amplitude was applied to elicit muscle response locally, while the high pulse amplitude was applied to elicit muscle response at electrochemotherapy conditions. The amplitude of the low voltage pulses was determined before the measurements in the following way. At two repetition frequencies (100 and 200 Hz), where the highest muscle torque was obtained in the preliminary experiments, the pulse amplitude was increased until the plateau of the muscle torque was obtained. At this amplitude the contraction of the hip muscles was not significant. The amplitude which was used for the experiments (70 V) was approximately 20% lower than the amplitude of the plateau. The amplitude of the high voltage pulses was set to a value where the voltage to distance ratio was approximately 1300 V/cm (typical electrochemotherapy value). Because the highest output from our power amplifier was 250 V, the distance between the electrodes was decreased from 8 to 2 mm in order to achieve the appropriate voltage to distance ratio. A numerical calculation of electric field distribution for this experimental application showed that the muscle between the electrodes was exposed to electric fields higher than 1000 V/cm.

The muscle was stimulated with a train of eight 100 μs rectangular pulses. At low pulse amplitudes applied (70 V) the measurements were performed at pulse repetition frequencies of 1, 10, 20, 50, 100, 200, 500, 1000, 2000, and 5000 Hz, while at high pulse amplitudes applied (250 V), the measurements were performed at 1, 100, 500, 1000, and 5000 Hz. A shorter range of pulse frequencies in the latter case was used to prevent muscle fatigue, which would occur especially at low pulse frequencies (10, 20, and 50 Hz), and to reduce muscle and nerve injuries due to high local current density. The frequencies were delivered systematically, for half of the animals in an ascending order, and for the other half in a descending order. The repetition frequency (f_p) was varied by shortening the delay (t_d) between two consecutive pulses while keeping the number of pulses (N=8) and duration of each pulse (t_p=100 μs) constant; $f_p=1/(t_p+t_d)$.

Muscle contractions in response to a 1 Hz pulse frequency were similar on all eight pulses in a train, for both low and high pulse amplitudes, so we stimulated the muscle with only one pulse, which was repeated at the end of the experiment to verify reproducibility with respect to electrode positions, movement artifacts and muscle fatigue. To prevent muscle fatigue, a 2-min delay was taken between each pulse repetition frequency applied.

To determine whether the muscle was stimulated directly or indirectly through its motor nerve, the muscle torque was measured before and after the denervation. The same amplitude of pulses produced similar amplitude of muscle torque before and after denervation, so we concluded that the muscle was to a large extent stimulated directly.

Statistical analysis was performed with one-way repeated measures analysis of variance (ANOVA) test with Dunnett's method for multiple comparisons versus control group (1 Hz) using SigmaStat 2.0 (Jandel Scientific, San Rafael, CA, USA). The difference was considered as statistically significant for $P<0.05$.

2.2. Electrochemotherapy of subcutaneous animal tumor model in mice

In the experiments, the inbred A/J mice of both sexes were used, which were purchased from the Institute of Pathology, University of Ljubljana (Ljubljana, Slovenia). Mice were maintained at 21 °C with natural day/night light cycle in a conventional animal colony. Before an experiment, mice were subjected to an adaptation period for at least 10 days. Mice of both sexes in good condition, weighing 20–22 g, without signs of fungal or other infection, 12–14 weeks old, were included in experiments. Treatment protocols were approved by the Department of Agriculture of the Republic of Slovenia No. 323-02-237/01.

The fibrosarcoma SA-1 tumor model (The Jackson Laboratory, Bar Harbor, USA), syngeneic to A/J mice, was used in the study. Tumor cells were obtained from the ascitic form of tumors in mice, serially transplanted every 7 days. Solid subcutaneous tumors, located dorsolaterally in mice, were initiated by an injection of 5×10^5 SA-1 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%. Six days after transplantation, when the tumors reached approximately 40 mm^3 in volume, the mice were randomly divided into 12 experimental groups, and subjected to a specific experimental protocol on day 0.

Bleomycin at a dose of 50 μg per animal was injected intravenously. This dose was selected because it usually does not result in complete responses in order to observe only the tumor growth retardation. Electric pulses were delivered by two flat, parallel stainless steel plate electrodes with rounded corners (length=35 mm, width=7 mm, thickness=1 mm, interelectrode distance=8 mm). They were placed at the opposite margins of the tumor in cranial/caudal direction. Good electrical conductance between the electrodes and the skin was assured by applying a conductive gel to the skin. Eight 100 μs rectangular electric pulses with a pulse amplitude of 1040 V and repetition frequencies 1 Hz, 10 Hz, 100 Hz, 1 kHz and 5 kHz were generated by an electropulsator, developed for this purpose at the Faculty of Electrical Engineering, University of Ljubljana [18]. In the combined treatment groups, mice were treated with electric pulses 3 min after bleomycin injection, which was sufficient for the distribution of bleomycin. Mice in the control group and the group subjected to electric pulses only were injected with 0.01 M PBS (pH 7.4) instead of bleomycin. Each treatment group consisted of 6 to 10 mice and the experiment was repeated twice.

Tumor growth was followed by measuring three mutually orthogonal tumor diameters (e_1, e_2 and e_3) with a caliper on each consecutive day following the day the treatment was performed. Tumor volumes were calculated from the equation $V=\pi\times e_1\times e_2\times e_3/6$. From the measurements, the arithmetic mean and standard error of the mean (SE) were calculated for each experimental group. If the tumor became unpalpable and did not regrow after 100 days, the therapeutic response was classified as a complete response (CR).

3. Results

3.1. Measurements of muscle torque in rats

A typical muscle response to low pulse amplitudes (70 V) and different pulse repetition frequencies ranging from 1 Hz to 5 kHz is shown in Fig. 2A. Eight pulses of 100 μs duration were applied at all repetition frequencies except for 1 Hz where only one pulse was applied (see Materials and methods). The repetition frequency of the pulses was adjusted by changing the delay between two consecutive pulses in a train. The average muscle torque as a function of pulse frequency is shown in Fig. 2B. With increasing

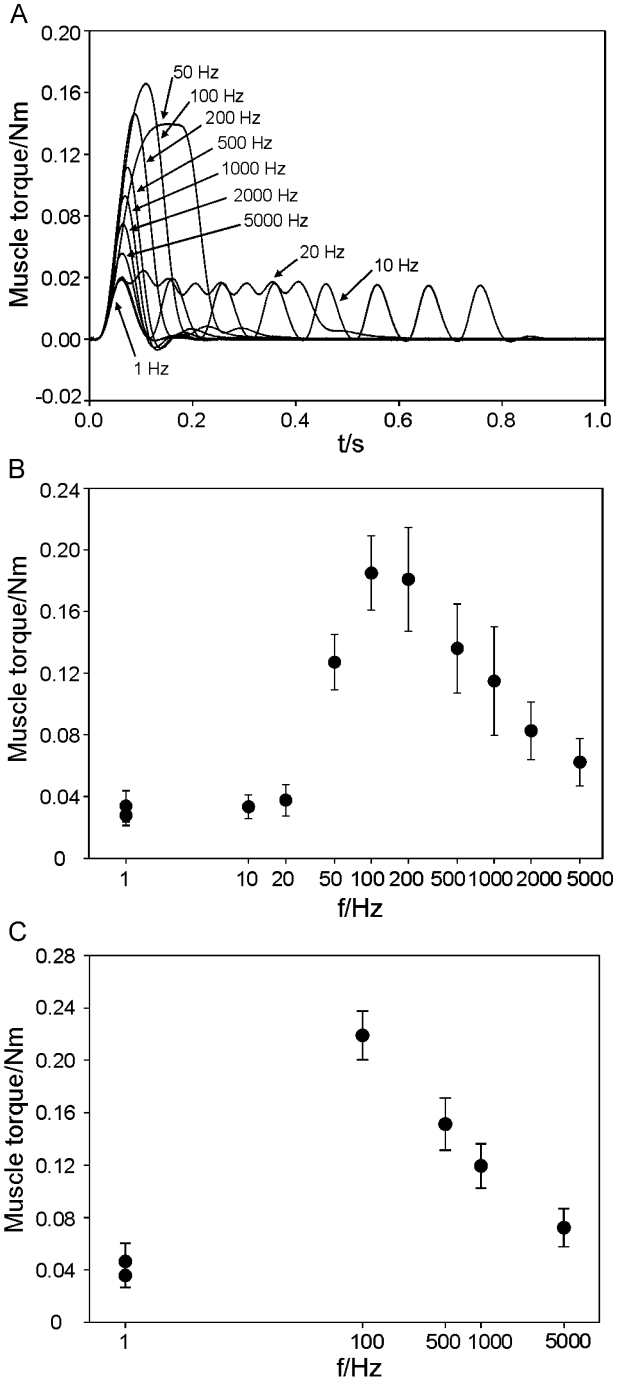

Fig. 2. (A) Time course of a typical muscle response to different pulse repetition frequencies at low pulse amplitudes (70 V, interelectrode distance 8 mm). (B) Muscle torque as a function of pulse repetition frequency at low pulse amplitudes (70 V, interelectrode distance 8 mm) and (C) high pulse amplitudes (250 V, interelectrode distance 2 mm). Each point represents the mean of eight experiments (rats)±S.D. A train of eight pulses was used at all frequencies examined except at 1 Hz where only one pulse was applied.

Table 1
Pulse parameters of muscle response to low pulse amplitudes and different pulse frequencies in rats

f/Hz	t_{max}±S.D.	t_{delay}±S.D.	t_{rise}±S.D.	$t_{50\%}$±S.D.
1	56.4±1.1	23.1±0.8	25.3±1.7	44.4±1.8
50	117.3±23.3	35.4±3.2	52.9±7.2	143.6±5.1
100	104.1±4.1	35.6±2.2	48.3±2.3	79.5±4.1
200	84.3±3.3	32.9±1.9	39.1±1.5	59.1±3.3
500	70.8±1.9	28.9±2.2	32.6±1.4	49.6±1.4
1000	65.6±2.6	26.4±2.9	30.3±2.2	46.9±1.5
2000	61.9±2.2	24.8±2.9[a]	28.5±1.9	45.8±1.3[a]
5000	60.9±2.9	23.3±3.7[a]	28.8±2.7	46.0±1.3[a]

The values are given as the mean of eight measurements±S.D. Parameters were determined only at pulse frequencies where tetanic contraction was obtained. t_{max}—time at maximum muscle response, t_{delay}—time at which 10% of the maximum response is obtained, t_{rise}—time between 10% and 90% of the response, $t_{50\%}$—time in between 50% of the response. Time is in ms.

[a] No statistically significant difference compared to 1 Hz.

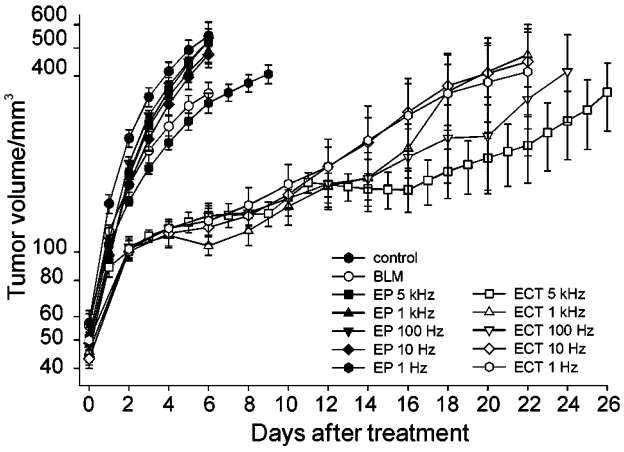

Fig. 3. Electrochemotherapy of tumors in mice with different pulse repetition frequencies. The results are presented as mean±S.E. of the mean. BLM—bleomycin only, EP—electric pulses only, ECT—electrochemotherapy (BLM+EP).

frequency of the pulses, muscle torque increases, reaches a maximum value between 100 and 200 Hz, and then decreases. At frequencies of 50 Hz or higher, muscle response becomes smooth (tetanic contraction). Instead of eight consecutive muscle contractions, only one, tetanic contraction is obtained. At 5 kHz, muscle torque is approximately twice as high as the muscle torque at 1 Hz. Higher repetition frequencies also shorten the duration of the muscle response, at frequencies above 2 kHz the muscle response to a pulse train is similar to the response to a single pulse (Table 1).

The average muscle response to pulses with high amplitudes (250 V) and different pulse repetition frequencies is shown in Fig. 2C. Similar to low amplitude pulses (70 V), with increasing pulse repetition frequency muscle torque first increases, reaches a maximum value at 100 Hz, where a tetanic contraction is obtained, and then decreases. At the highest pulse frequency examined (5 kHz) muscle torque is approximately twice as high as the muscle torque at 1 Hz, but instead of eight consecutive muscle contractions only one muscle contraction is obtained.

The amplitude of muscle torque to a single pulse (1 Hz) before and after the measurements showed no muscle fatigue (Fig. 2B and C).

3.2. Electrochemotherapy of subcutaneous tumors in mice

As Fig. 3 shows, electrochemotherapy with bleomycin inhibits tumor growth at all five repetition frequencies of electric pulses investigated in this study (1 Hz, 10 Hz, 100 Hz, 1 kHz, and 5 kHz) (Fig. 3). The antitumor efficiency of electrochemotherapy with different pulse repetition frequencies is also evident in the percentage of complete responses (CR) of the tumors (Table 2). The highest percentage of CR (36.8%) was observed for the "standard" repetition frequency of 1 Hz, and the lowest (9.1%) for repetition frequency 1 kHz. Neither electric pulses alone nor bleomycin alone significantly inhibit tumor growth.

4. Discussion

Electrochemotherapy is used as an efficient local treatment of cutaneous and subcutaneous tumors in patients [1,4,11,12,19]. The most unpleasant side effects of electrochemotherapy reported so far are the muscle contractions and related sensations during pulse delivery [4,11,19,20]. Although they subside immediately after each pulse, patients find them uncomfortable and sometimes painful. To some extent, it would be possible to reduce these unpleasant sensations by using pulse repetition frequencies higher than tetanic, as an alternative to the 'standard' pulse frequency of 1 Hz. However, the efficiency of electrochemotherapy with such pulse frequencies should be preserved. We thus examined the effect of different pulse frequencies on muscle response and the efficiency of electrochemotherapy in vivo.

According to our results, the muscle torque increases with an increase of the repetition frequency of the pulses, reaches a plateau between 100 and 200 Hz and then decreases. At the highest frequency examined (5 kHz) the muscle torque was two times higher than at 1 Hz (Fig. 2B). This bell-shaped dependence of muscle torque on pulse frequency can be explained as follows. At pulse repetition frequencies lower than tetanic, each pulse in the train of pulses provokes an isolated muscle contraction. With increasing frequency of these pulses, consecutive muscle contractions eventually fuse, thereby increasing the muscle torque and reaching a tetanic contraction. In the case of an infinite number of pulses, further increase in pulse frequency eventually results in the same maximum muscle torque regardless of the pulse frequency applied (in time, muscle torque would decrease due to muscle fatigue).

Table 2
Antitumor efficiency of electrochemotherapy with different pulse repetition frequencies

Therapy	Control	BLM	EP 1 Hz	EP 10 Hz	EP 100 Hz	EP 1 kHz	EP 5 kHz	ECT 1 Hz	ECT 10 Hz	ECT 100 Hz	ECT 1 kHz	ECT 5 kHz
N	16	12	10	13	12	7	13	19	18	17	11	18
CR	0	0	0	0	0	0	0	7	2	5	1	4
(%CR)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(36.8)	(11.1)	(29.4)	(9.1)	(22.2)

BLM—bleomycin only, EP—electric pulses only, ECT—electrochemotherapy (BLM+EP); *N*—number of animals in each experimental group; CR—number of complete responses (absence of any trace of tumor at day 100 post-treatment).

However, in the case of a low number of pulses in a train, as in the case of electrochemotherapy (e.g. eight pulses), a further increase in pulse frequency (above 200 Hz) actually decreases muscle torque. This occurs because of a progressively shorter duration of the pulse train compared to the duration of a typical muscle response. For example, at a pulse frequency of 1 kHz, the duration of a train of eight 100 μs pulses is 8 ms, while the response of a skeletal muscle, necessary to develop its maximum force of contraction, typically lasts from 40 to 100 ms. Also, at higher pulse repetition frequencies, an increasing number of pulses from the pulse train fails to provoke a muscle contraction because of the latent period of the muscle (during this period the muscle cannot contract and is completely refractory). If, for example, the repetition frequency of the pulses is 1 kHz and the latent period of the muscle is 2 ms, only three out of eight pulses applied will induce muscle contractions, and the cumulative amplitude of muscle response will consequently decrease.

We also examined how the muscle would respond to pulse amplitudes that produce voltage to distance ratios similar to those used in electrochemotherapy (1300 V/cm). Due to limitations of our voltage amplifier, we decreased the interelectrode distance to achieve the appropriate voltage to distance ratio (see Materials and methods). However, different interelectrode distances and consequently different portions of the muscle between the electrodes made quantitative comparison of the amplitude of the muscle response impossible. A numerical calculation of electric field distribution for the case of high pulse amplitudes showed that the muscle between the electrodes was exposed to electric fields higher than 1000 V/cm. Although this value exceeds the reported thresholds for reversible or irreversible electropermeabilization [21–25], the dependence of the muscle response to different pulse repetition frequencies still remains qualitatively similar to the dependence of the muscle response at low pulse amplitudes (Fig. 2B and C). Though we would expect muscle electropermeabilization to have an influence on the muscle response, we did not observe such an influence. This is most probably because at such pulse amplitudes the surrounding muscle groups, which contribute to the muscle response, were excited but were not electropermeabilized.

Despite the fact that at the highest pulse frequencies examined muscle torque remains at values above those at 1 Hz, the number of individual sensations reduces from eight to a single sensation, which is a significant advantage over the 1 Hz pulse application. These measurements are in agreement with the hypothesis presented in our previous paper [13].

It is reasonable to assume that the dependence of muscle torque on pulse frequency in humans would be similar to that observed in rats. Indeed, we have performed measurements of muscle torque in healthy volunteers and the results, although obtained at low pulse amplitudes, substantiate our assumptions (data not shown). This suggests that pulses with repetition frequencies higher than the frequency of tetanic contraction (approx. 40 Hz for humans) would considerably reduce the number of individual sensations for the patients during electrochemotherapy. Also, pulse frequencies higher than tetanic would eventually decrease the muscle response and consequently the intensity of sensation to a value similar to that after application of a single pulse.

It was shown by several authors that pain sensation depends on pulse parameters such as pulse amplitude, number, duration, frequency, and shape of the pulses [26–31]. With respect to pulse frequency, the authors [29,31] concluded that with increasing pulse frequency the pain sensation decreases and electrical excitation becomes more tolerable. Although sinusoidal pulses were examined in these two studies, the conclusions are in agreement with our results.

Another important benefit from the use of high frequency pulses is a shorter duration of the therapy in case of multiple arrays of electrodes. Usually six to nine electrodes are used in such experiments, and pairs of electrodes are activated sequentially [14,15,19,32,33]. Since more than one pulse can be applied on each pair of electrodes [14,15] important improvement of using high frequency pulses is a significant reduction of the total duration of the treatment.

While we presented the benefits of using higher pulse frequencies, it was also important to determine the antitumor efficiency of electrochemotherapy with each of these frequencies. In experiments in vitro it was already shown that the uptake of molecules is comparable for repetition frequencies ranging from 1 Hz up to 8.3 kHz [13]. A step forward was made in the present study, where we demonstrated that electrochemotherapy with higher pulse frequencies can also be successfully performed in vivo. According to the results of electrochemotherapy on our

tumor model in mice, electrochemotherapy is efficient regardless of the pulse repetition frequency applied (at least in the investigated range). We should note that the bleomycin dose used in our experiments usually does not result in complete responses. Despite low bleomycin dose, some tumors responded completely to electrochemotherapy at all pulse repetition frequencies examined (Table 2).

In a clinical environment, the efficiency of electrochemotherapy with high frequency pulses still needs to be investigated. Daskalov et al. [34] have demonstrated the antitumor efficiency of electrochemotherapy in patients, but they have only compared pulse frequencies of 1 Hz and 1 kHz. At these two pulse frequencies, they did not observe any difference between tumor responses.

In summary, we demonstrated that pulse frequencies above the frequency of tetanic contraction (above 100 Hz) gradually reduce the number of individual muscle contractions. At pulse frequencies higher than 2000 Hz the muscle torque is similar to that after application of a 1 Hz pulse train (a typical electrochemotherapy protocol), but with an advantage of only one sensation instead of eight. Experiments in vivo in mice demonstrated similar efficiency of electrochemotherapy regardless of the pulse frequency examined. These results suggest that there is a considerable potential for clinical use of high frequency pulses in electrochemotherapy.

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