Successful tumor Electrochemotherapy using Sine Waves

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***Abstract*— *Objective:* The purpose of the present work is to assess the ability of sine waves to perform Electrochemotherapy (ECT) and to study the dependence of the frequency of the applied sine wave on the treatment efficacy. *Methods:* A subcutaneous tumor model in mice was used and the electric field was delivered in combination with bleomycin. Sinusoidal electric fields of different frequencies, amplitudes and durations were compared to square waves. Computer simulations were additionally performed. *Results:* The results confirmed the ability of a sinusoidal electric field to obtain successful ECT responses. A strong dependence with frequency was obtained. The efficacy of the treatment decreased when the frequency of the sine waves was increased. At low sinusoidal frequency the efficacy of the treatment is very similar to that obtained with a square wave. The collateral effects such as skin burns and muscle contractions decreased with for the highest frequency assayed. *Conclusion:* The use of sine wave burst represent a feasible option for the treatment of cancer by ECT. *Significance:* These results could have important implications for the treatment of cancer in the clinical world where ECT is performed with DC square pulses.**

***Index Terms*—Electroporation, Electrochemotherapy, sine wave, frequency dependence.**

I. Introduction

When a cell is exposed to an external electric field, an induced transmembrane voltage (ITV) is superimposed to its natural resting potential. Under controlled conditions, if the value of this ITV exceeds a certain threshold, the cell membrane increases its permeability to molecular species [1]. This phenomenon is called Electroporation or Electro-permeabilization and can be either reversible or irreversible. Interestingly, although the threshold voltage for electroporation depends on many biological parameters (e.g., cell size and shape, cytoskeleton structure and membrane composition), it is possible to achieve electroporation in vitro and in vivo for every cell or tissue type. This makes electroporation a widespread technique applied in very different applications both in the biotechnological and clinical world [2]–[5].

Of particular interest are the applications that electroporation has in the field of cancer treatment. When the principle of electroporation is combined with certain chemotherapeutic drugs, the cytotoxicity of these drugs is increased by several tens to hundred folds, leading to improved and dramatic responses in the target tumours [6]. This is called Electrochemotherapy (ECT) which is defined as the local potentiation, by means of electric pulses, of the anti-tumour activity of a non-permeant (or a low permeant) anticancer drug possessing a high intrinsic cytotoxicity. Last years, clinical experience with ECT has considerably increased and its effectiveness and safety have brought it into the guidelines for the treatment of different cutaneous and subcutaneous tumours (melanoma, Kaposi's sarcoma, head and neck tumours, etc.) [7]. Additionally, its application in deep-seated solid tumours is nowadays part of several clinical trials in very different cancer types [8]. The second modality of electroporation extensively used specially in cancer treatment, is Irreversible electroporation (IRE). When cells are not able to recover back to its normal state after the electric field exposure, cell death occurs. This phenomenon is used as a non-thermal ablation method for the treatment of solid tumours [9].

Regarding the collateral effects of electroporation, the application of short high-intensity electric pulses to tissue can cause considerable discomfort to patients. This is mainly associated with the appearance of strong muscle contractions during pulse application and pain associated with the electric field applied. In this scenario, it is very usual the use of medication, specifically anesthetics and muscle relaxants. Electrically induced muscle movement is problematic not only because of the discomfort caused to the patient, but also because these movements can imply an incorrect placement of the electrodes during pulse application. In this regard, there is a growing emphasis in the literature on developing novel techniques for performing electroporation that reduce the intensity or extent of muscle contractions [10].

One of the main approaches to reduce de aforementioned issues is to explore the use of novel electric field waveforms. Traditionally, electroporation has made use of short square direct current (DC) electric pulses. The duration of these square pulses can range from nanoseconds to milliseconds, but

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unipolar pulses of 100 µs are the most utilized, especially for clinical applications [11]. Although theoretically described [12]–[14], few experimental reports have studied the ability of AC signals, instead of DC pulses, to cause cell permeabilization [15]–[17]. For example, efficient gene transfer and cell fusion was accomplished by superposition of a radiofrequency sinusoidal field and a DC pulse [18], [19]. Very interesting is the use of high frequency bursts of bipolar square pulses (H-FIRE) that has been deeply studied for tissue ablation by irreversible electroporation [20], [21]. This technique has demonstrated reduced muscle contractions, hence preventing the need for neuromuscular blockade.

When AC fields are used, a new parameter needs to be adjusted: The Frequency. Many theoretical studies have predicted the dependence of electroporation efficacy on the frequency of the AC field applied [22]–[24]. Surprisingly, only few studies could clearly show this dependency experimentally. In vitro in the band from several to hundreds of kHz using sine signals [25], [26] or, recently, one study showed a proof of concept of the successful IRE of liver tissue in a rabbit model using also sinewaves [27]. This study demonstrated that with a careful choice of the sinusoidal frequency it was possible to create IRE ablations avoiding muscle contractions. Apart from the mentioned interest in reducing muscle contractions and associated pain, the use of AC fields is beneficial in the reduction of the electrochemical reactions produced at the electrode interface and that are susceptible of creating undesired effects such as cell death [28].

In the present study, the application of sinusoidal waveforms was studied in the treatment of a subcutaneous tumour model by ECT in mice. As far as the authors know, this is the first time that this type of electric field is studied for ECT.

II. Materials and Methods

A. Sinusoidal generator

High voltage generators for electroporation are usually based on voltage-source inverters, relying in an input capacitor to maintain the desired output voltage [29], [30], and enabling only generating typically unipolar square-wave output voltage. To accomplish this research, a versatile arbitrary waveform generator was developed, enabling the generation of fully configurable sinusoidal waveforms [31].

The proposed converter is based on an isolated multi-level approach, enabling to generate the desired output waveform by combining the output voltage of each level cell (Fig. 1b). In order to achieve the desired high-frequency performance, state-of-the-art gallium nitride LM5200 (Texas Instruments Inc., Dallas, Texas, USA) power devices were used. As a result, the proposed generator achieves 2400 Vpp with 60 V resolution and switching frequency up to 1 MHz. In the present study, sinusoidal bursts of different frequencies between 10 and 100 kHz were studied. An example of the used sinusoidal waveforms is shown in Fig. 1b. Other parameters such as duration, number of bursts and field intensity were varied depending on the experiment.

B. Square waves

For comparison, a classical ECT protocol using 8 square unipolar pulses of 100 µs duration and 1 Hz repetition frequency was applied. These short square-wave electric pulses were generated by an electroporation power supply (CliniporatorTM, Igea, Carpi, Italy). The output voltage of the generator was fixed to produce electric fields (calculated as the voltage to distance ratio E=V/d) of 1300 V/cm (see Fig. 1c). This parameter was fixed based on previous publications [32].

Finally, bipolar square pulses with the same duration than the sinusoidal bursts were also used in this study for comparison. These pulses were generated using the high power generator described in detail in [29]. It is important to mention that in some cases due to the long duration of these pulses, the system, that was designed for applying pulses in the microsecond range, was not able to maintain a stable flat level during the whole exposure duration. Instead, a decaying amplitude was observed (see Fig. 1d). For this reason, the initial output voltage of the generator was adjusted to be slightly higher than the desired level in order to apply, during the entire pulse, an electric field average amplitude corresponding to the target amplitude.

C. Animal model

Tumor cells from the LPB cell line, a methylcholanthrene-induced C57 Bl/6 mouse sarcoma cell line (Belehradek et al 1972), were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Life Technologies) supplemented with 1 % Penicillin/Streptomycin (Sarbach, France) and 10% heat-inactivated Fetal Bovine Serum (30 min at 56 °C, Gibco). Cells were routinely passed every two days for at least two weeks before inoculation.

The day of inoculation, the cells were detached with TrypLE Express (Gibco, Life Technologies) and resuspended at a final concentration of 5.106 cells/ml in PBS. Subsequently, C57 Bl/6 female mice, 8-9 weeks old (ENVIGO), were inoculated subcutaneously in the left flank with 100 µl per animal (5.105 cells), producing tumors of mean volume, after randomization of groups, of 60 mm3 after 10-12 days. Animals were housed and handled according to recommended guidelines (UKCCCR). All the procedures described were approved by the French Ministry of research after ethical evaluation by the CEEA 26 committee (project number 2018_014_13409).

D. Experimental procedure

Prior to treatment, mice were anesthetized with 2% isoflurane/oxygen mixture gas anesthesia in an induction chamber. When necessary, 20 µg/animal of bleomycin (Roger Bellon S A, Neuilly-Sur-Seine, France) were intravenously injected in the retro-orbital sinus 4 min before the delivery of the electric pulses. Controls administering only bleomycin were not performed for two reasons: 1) because we have published many times that under the experimental conditions followed during the present study, there is no antitumor effect of the bleomycin alone [33]–[35] ; 2) to reduce the number of animals used according to the ethics 3Rs' rule. Exposure to the electroporation electric fields was performed using two parallel stainless steel plates (1 cm width, 1 mm thick and 4 cm long).

The edges of the electrodes were placed in direct contact with the skin surface at both sides of the subcutaneous tumor (see Fig. 1a). The distance between the electrodes was 5 or 4 mm in all treatments. To ensure a correct electric field distribution around the tumor volume, conductive gel (Asept Uni'Gel US, Aspet Inmed, France) was used to fill the space between electrodes and tumors [36]. Immediately after the electric field exposure, anesthesia was stopped and, after their complete recovery, mice were placed back in their cages.

Tumor volume was assessed every 2-3 days measuring the longest (a) and the orthogonal second largest diameter (b) with a precision caliper. Tumors were approximated as ellipsoids and their volume was calculated following the formula $V = ab^2 \pi/6$. Mice were euthanized when the limit value of 1500 mm^3 in tumor volume was reached.

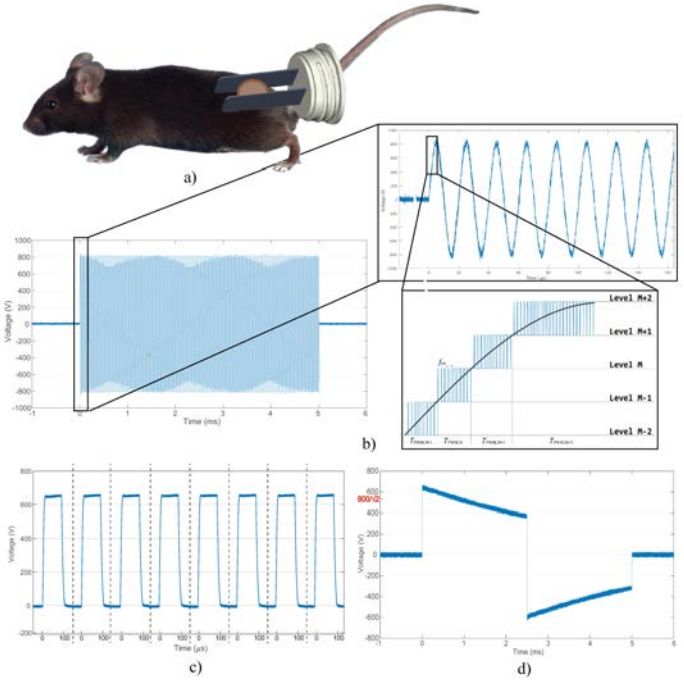

Fig. 1. a) Schematic representation of the experimental model used. b) Example of an oscilloscope recording of a 5 ms, 50 kHz sinusoidal burst. The detailed inset depicts the multi-level approach used in the waveform generation. c) Oscilloscope recording of the classical 100 µs pulses. Notice that the horizontal axis is truncated and the real delay between consecutive pulses was 1 second. d) Oscilloscope recording of a typical single 5 ms bipolar square pulse used for the comparison. Notice that the amplitude was reduced by a √2 factor compared to the sinusoidal burst.

E. Computer simulations

A numerical model was built in order to calculate the electric field distribution and the temperature increase during the delivery of the treatments. This was done using the finite elements method (FEM) software COMSOL Multiphysics 4.4 (Stockholm, Sweden). The geometry of the model is shown in Fig. 2. The model consisted of two parallel plate electrodes, a block between them corresponding to the conductive gel, a spherical tumor, a skin layer and a block representing the muscle. The tumor center was located slightly above the plane defined by the skin layer to simulate the worst case scenario where the superficial tumor has a certain penetration in the muscle region. Based on this geometry, a free tetrahedral mesh consisting on 7884219 elements was automatically generated by COMSOL.

In order to calculate the electric field distribution for each frequency, the electric potential distribution (V) under a voltage difference between the electrodes corresponding to the peak amplitude of the sine waves was obtained by solving the Laplace equation in our geometry:

$$\vec{\nabla} \cdot (\sigma \vec{\nabla} V) = 0 \quad (1)$$

where the conductivity σ of the biological tissues was defined as a function of the local electric field, E, in order to account for the conductivity changes due to electroporation:

$$\sigma\left(\left|\vec{E}\right|\right) = \sigma_0 + \Delta_\sigma e^{-e^{-\left[b\left(\left|\vec{E}\right|-E_0\right)\right]}} \quad (2)$$

where σ_0 is the initial conductivity of the non-electroporated tissues, Δ_σ is the maximum conductivity variation between a non-electroporated and fully-electroporated tissue and E_0 is the electric field threshold for electroporation. Values for σ_0 were taken from the IT'IS database for each sinusoidal frequency analyzed [37]. However, due to the lack of data in the literature regarding the conductivity changes due to electroporation for the frequencies used in this study, the rest of the parameters were arbitrarily defined. Plausible values of the other parameters in (2) were chosen based on data obtained with monopolar pulses found in the literature [36] [38]. Briefly, we assumed that at 10 kHz the conductivity has the same behavior as when applying monopolar square pulses. Then, using those values as a reference, the parameters for the remaining frequencies were defined based on two premises: first, Δ_σ must be lower as the frequency increases (conductivity spectrum flattens when tissues are electroporated) and second, E_0 depends on the induced transmembrane voltage and therefore increases with the frequency (more details can be found in Supplementary material). The values assigned to these parameters as well as the electrical properties assigned to the non-biological materials can be found in Table I.

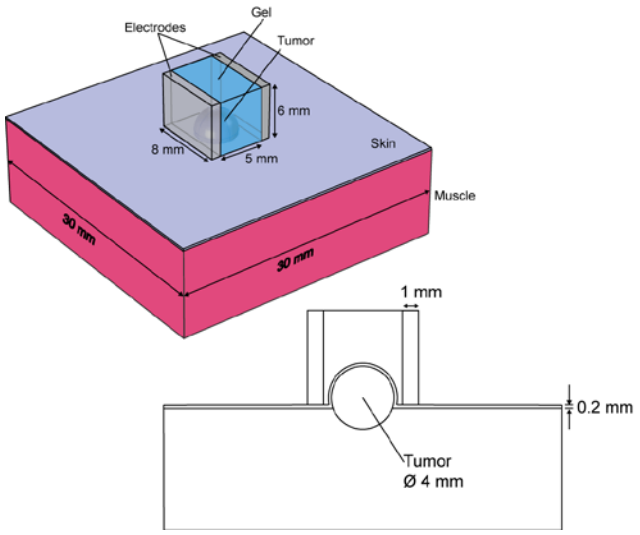

Fig. 2. Geometry used in the FEM simulations in COMSOL.

The steady state solution of the problem described above was determined through the electric currents mode in the AC/DC

module of COMSOL using a stationary solver. Once the electric field distribution was obtained, the induced transmembrane voltage (ITV) in the tumor cells was approximated from the local electric field by a modified version of the Schwann equation [22]. In particular, the 3/2 factor in the equation was reduced to 1 in order to account for the cell packing density in tissues [39]:

$$ITV = \frac{\left|\vec{E}\right| \cdot R}{\sqrt{1+(2\pi f\tau)^2}} \quad (3)$$

where R corresponds to the cell radius, f is the frequency of the applied sine signal and the time constant τ can be calculated from the membrane capacitance (C_m) and the resistivity of the intracellular (ρ_i) and extracellular (ρ_e) compartments:

$$\tau = RC_m(\rho_i + \rho_e/2) \quad (4)$$

The values assigned to these parameters were: R=11 μm, C_m =0.08 F/m, ρ_i =2 Ω·m, ρ_e =0.5 Ω·m.

Finally, thermal simulations were also performed in the FEM model to calculate the possible thermal damage due to Joule heating (more details can be found in Supplementary material).

TABLE I
ELECTRICAL PROPERTIES OF THE DIFFERENT TISSUES

Tissue/ Material	Frequency (kHz)	σ_0 (S/m)	Δ_σ(S/m)	b	E_0(V/cm)	Ref
Skin	10	$2.04 \cdot 10^{-4}$	0.4	0.005	833	[37] [36]
	50	$2.73 \cdot 10^{-4}$	0.4	0.005	842	[37] [36]
	100	$4.51 \cdot 10^{-4}$	0.4	0.005	947	[37] [36]
Muscle	10	0.341	0.2	0.01	440	[37] [36]
	50	0.352	0.19	0.01	480	[37] [36]
	100	0.362	0.185	0.01	780	[37] [36]
Tumor	10	0.15	0.1	0.003	600	[36]
	50	0.16	0.095	0.003	630	[36]
	100	0.18	0.08	0.003	830	[36]
Gel	-	0.1	-	-	-	[36]
Electrodes	-	$1.5 \cdot 10^6$	-	-	-	Stainless steel

Table I. Electrical properties of the different materials introduced in the FEM model for the different sinusoidal frequencies studied.

III. RESULTS AND DISCUSSION

A. Simulations

In Fig. 3a the resulting electric field intensity distribution from the 3D FEM simulations is shown for three different sinusoidal frequencies (10, 50 and 100 kHz) when an external electric field intensity E_{peak}=1600 V/cm was applied. The values are shown in the middle plane perpendicular to the electrodes. Besides the differences in the initial conductivities of the tissues introduced in the model at the different frequencies (see Table I), the simulations show that the resulting electric field distribution inside the tumor is very similar in all cases, with values between 1000 and 1400 V/cm. This similarity is explained by the fact that upon the application of the external electric field, the differences in the initial conductivity are significantly reduced as a result of electroporation. In fact, a previous study showed that, at sufficiently high frequency, the impedance changes produced by electroporation could be considered negligible, simplifying the models and improving the accuracy of the predictions [21]. For 100 kHz, the simulation results show that there is a slight difference in the field distribution, with a deeper penetration of the electric field in the tumor tissue likely due to the low impedance of the skin at this frequency.

However, although the model shows similar values of electric field at the different frequencies, this does not involve that the tissue will be equally electroporated for these frequencies. In Fig. 3b the resulting ITV inside the tumor calculated with (3) for the steady state situation depicted in Fig. 3a is shown for the three analyzed frequencies. The lower insert displays an example of the dependence of the ITV with frequency calculated with (3) for a maximum Epeak of 1300 V/cm. Considering that the transmembrane voltage threshold necessary for electroporation is between 0.8-1.2 V, the simulations show how the resulting ITV for 10 and 50 kHz is mostly above this values with a slightly different distribution between them, meaning that most of the tissue will be electroporated at these frequencies. On the contrary, at 100 kHz most of the tumor tissue remains below or near the threshold of electroporation predicting much less efficacy of this electric field.

The thermal calculations done for the worst case scenario (lowest frequency and highest electric field) showed a temperature increase inside the tumor lower than 4 °C (see Supplementary material).

B. Proof of concept

The first part of this study was intended to assess the treatment efficacy of a sinusoidal wave in comparison to a classical Electrochemotherapy (ECT) protocol using 100 μs duration square pulses. In order to make the most accurate comparison between sine waves and square pulses we decided to apply the same number of pulses/bursts (8 consecutive pulses) repeated every second in both cases. However, as demonstrated in our previous in vitro study [26], amplitude and/or duration must be adjusted to deliver an equivalent power in a Root Mean Square (RMS) sense between both waveform types. To apply an RMS equivalent power with a sine wave compared to a square wave, it is possible increase either the peak amplitude by a √2 factor (5) or increase the duration of the burst by a factor of 2 (notice that in (6) the integral goes from 0 to 2T). In the present case, based on previous experiences [34], [35], [43], we chose a set of known parameters for the 100 μs square pulses and applied sine-wave bursts with a duration twice the duration of the square pulses (200 μs) and with the same E_{peak}=1300 V/cm.

$$V_{RMS\_square} = \sqrt{\int_0^T \frac{1}{T} V_{\sin}^2 \sin^2(\omega t)dt} = \frac{V_{\sin}}{\sqrt{2}} \quad (5)$$

$$V_{RMS\_square} = \sqrt{\int_0^{2T} \frac{1}{T} V_{\sin}^2 \sin^2(\omega t)dt} = V_{\sin} \quad (6)$$

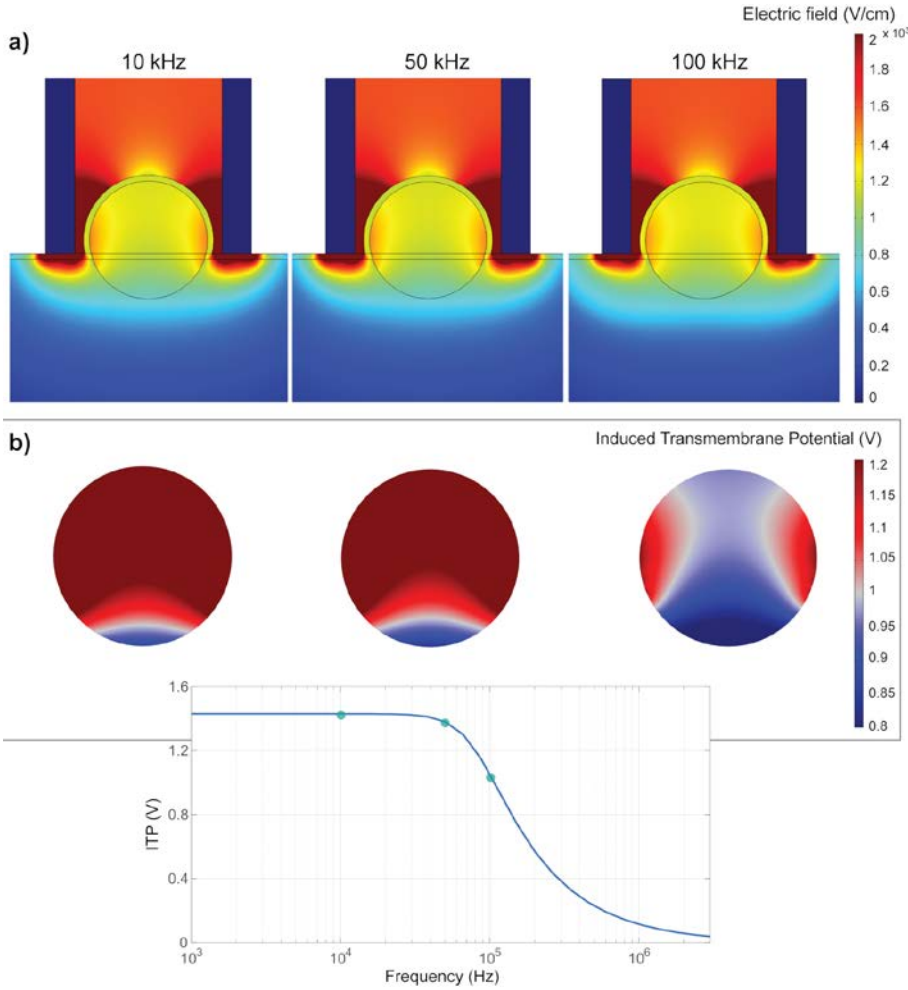

Fig. 3. Results of FEM simulations. a) Resulting electric field intensity distribution in a middle plane perpendicular to the electrodes for three different sinusoidal frequencies (10, 50 and 100 kHz). b) Resulting ITV distribution calculated using (3) for simulations shown in a). Lower insert depicts the ITV dependence with frequency calculated with (3) for an Epeak of 1300 V/cm and the parameters shown in Table I.

The choice of the frequency of the sine wave used in this test (30 kHz) was based on theoretical predictions and also in the observations made in our previous study in vitro [26]. At this frequency, the ITV should still be maximum according to (3) (see Fig. 3 lower insert). Additionally, a third group was treated with the same number of sine-wave bursts, with the same frequency and duration, but increasing the amplitude to Epeak=1600 V/cm.

Fig. 4a shows the results for the mean tumor volume evolution for the three treated groups and the control. As shown, there is a clear difference between the control and the groups subjected to the ECT treatment. For the sinewave burst sequence with an Epeak=1300 V/cm, there was a complete stop of tumor growth up to 10-12 days after the treatment followed by a significant regrowth of tumors afterwards. For the classical square wave protocol and for the sinewave bursts with Epeak=1600 V/cm the evolution was very similar, almost equivalent: interestingly, there was a complete stop of tumor growth or even a slight regression in average volume up to 16-18 days after treatment followed by a complete disappearance of some tumors and the regrowth of others afterwards. Considering the results shown in Fig. 4b, the application of a sinewave with Epeak=1300 V/cm did not result in the complete regression of tumors in any treated mouse. However, for the other two groups, the percentage of mice displaying complete tumor regression was 33 % (square waves) and 42 % (sinewave-1600 V/cm) at the end of the 3 weeks follow up (Fig. 4b).

These first results confirm the feasibility of using sinewaves to perform ECT in a safe and efficient way. However, in terms of equivalence between a classical square wave and a sinewave burst, it was necessary to increase both duration and amplitude of the sinewave to produce results comparable to those with the square waves. This observation suggests that the equivalence in duration for delivering the same RMS power was not correct under the present experimental conditions. According to our previous study in vitro, the RMS equivalence in terms of amplitude was confirmed comparing sinewaves and bipolar square pulses. In this case, with unipolar square pulses, it seems that the increase in the duration is not valid for achieving the same electroporation efficiency.

C. Parameter optimization of single burst treatment

Because one of the potential advantages of using sinewaves is their reduced spectral content (narrow band spectrum), and taking into consideration that when a pulsed signal is

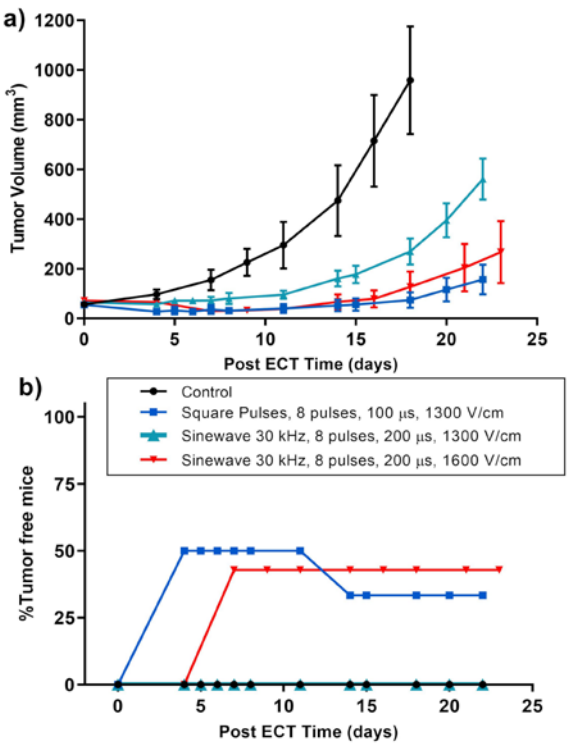

Fig. 4. a): Mean tumor volume (±SE) evolution after ECT treatment. Mice were treated at day = 0. Control group received no treatment. The tumor evolution is shown until the first animal of each group was sacrificed or the end of the experiment. b): Evolution in the percentage of tumor free mice for the different treatment groups. Groups size was n=6-7.

periodically repeated, new spectral content is introduced at low frequency, our goal was to explore the use of one single long sinusoidal burst instead of applying repetitive shorter bursts.

In this section, tumors treated with ECT were exposed to a single sinusoidal burst (30 kHz) of different durations or amplitudes. The goal in this series of experiments was to find the optimal conditions for obtaining an equivalent effect of a classical square pulse protocol. Fig. 5 displays the evolution in tumor volume (Fig. 5a) and the evolution in the percentage of tumor free mice (Fig. 5b) for the different conditions tested. Additionally, the results corresponding to the application of repetitive sinusoidal bursts of 30 kHz (8 pulses of 200 µs duration and 1600 V/cm) from the previous experiment are represented for comparison.

As observed, there was a dependent tumor response both with the amplitude and duration of the applied sine burst. At 2 ms and 1300 V/cm there was a slight delay in the tumor growth, compared to control, with only 1 out of 6 complete tumor regression (corresponding to a small initial tumor). When amplitude was increased to 1600 V/cm, there was a considerable increase in the ECT effect with a complete stop of tumor progression for 13 days after the treatment and 33 % of tumor-free mice at the end of the 3 weeks follow up. Interestingly, there is a good agreement between these last results and the ones obtained in the first trial for the same field intensity and frequency but using a sequence of short pulses (grey dashed line in the figure). This suggests that, under these conditions, the outcome obtained with a single long pulse (2 ms) or 8 short 200 µs pulses separated by 1 second (cumulative

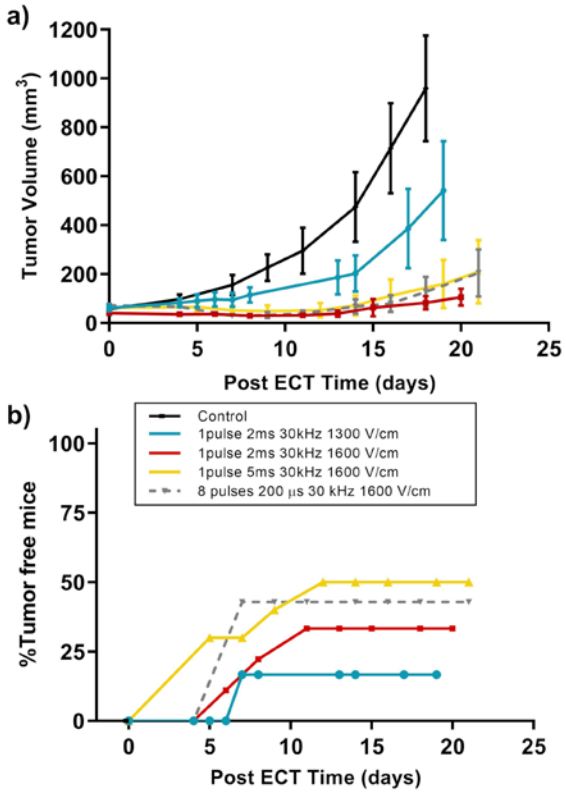

Fig. 5. a): Mean tumor volume (±SE) evolution after ECT treatment. Mice were treated at day = 0. Control group received no treatment. The tumor evolution is shown until the first animal of each group was sacrificed or the end of the experiment b): Evolution in the percentage of tumor free mice for the different treatment groups. Groups size was between n=6 (controls) and 9 (most of the treated groups). Grey dashed lines correspond to a group from the previous section where 8 pulses of 200 µs were applied.

treatment duration:1,6 ms) is equivalent.

Finally, when both the amplitude and duration were increased (1600 V/cm and 5 ms), the number of animals displaying a complete tumor regression increased up to 50 % with no significant difference in the evolution of the average tumor volume compared to the previous condition (2 ms, 1600 V/cm). Under this condition, no adverse effect was observed in any mice.

These results demonstrate the safety and efficacy of applying a single sine wave burst with duration in the millisecond range instead of sequential repetitive short (microsecond range) bursts. In clinics, the most common approach is to use a sequence of 8, 100 µs square pulses with an interpulse frequency of 1 Hz or 5 kHz [44]. The higher frequency of 5 kHz is presented as an alternative for both reducing the treatment time and reducing the number of contractions to one [45]. Similarly, in this sense, the application of a single sinusoidal burst also involved a reduction in the treatment time and under the conditions tested, only one contraction during pulse application was observed.

D. Sinewave frequency analysis

1) ECT effectiveness

Once the duration and amplitude of a single sinusoidal burst at a fixed frequency of 30 kHz were determined for obtaining

an equivalent effect compared to a classical ECT protocol, the next goal was to study the dependence on the frequency of the sinusoidal burst on the efficiency of the treatment. This comparison was performed at a fixed single burst duration of 5 ms, two different electric field intensities Epeak=1300 V/cm and Epeak= 1600 V/cm and three AC frequencies (10, 50 and 100 kHz). Additionally, a group where a single bipolar square pulse of 5 ms duration and a RMS equivalent amplitude (Vpeak/√2) was also included for comparison. In these experiments, the follow up duration was extended to 2 months in order to assess the complete disappearance of the tumors that regressed.

In Fig. 6a and 6b the evolution in the average tumor volume is shown for 1300 V/cm and 1600 V/cm, respectively. Although the experiment extended during 60 days, volume data are only shown until the first animal of each treated group was euthanized. A clear dependence with frequency was observed in both cases, with a loss of efficacy with the increase in the frequency. For 1300 V/cm there was a clear difference between the results at 10 kHz and the other two frequencies; the differences between 50 and 100 kHz were less noticeable. Differently, for 1600 V/cm the results for 10 and 50 kHz were quite similar, at least until day 15, while at 100 kHz the behavior was very different. The same conclusions can be extracted from the percentage of tumor-free mice displayed in Fig. 6c. These results confirm that for the two electric field intensities, the frequencies of 10 kHz and 100 kHz result in clearly differentiated ranges of effectiveness, while the frequency of 50 kHz is in the transition zone where a small modification in the intensity of the electric field is translated in a large change in the treatment efficacy. This observation perfectly matches with the theoretical calculated dependence of the ITV with frequency.

These results reinforce the charge-dependent nature of the electroporation phenomenon, where the cell membrane must be charged at a minimum ITV for obtaining an observable effect [46]–[48]. Under the present experimental conditions, the high frequency electric field was not able of inducing a sufficient level of transmembrane voltage for electropermeabilization. According to [25] it should be possible to obtain an equivalent effect at 100 kHz compared to the one obtained at 10 kHz by increasing the intensity of the electric field. According to that in vitro study, the increase factor could be roughly between 1.5 and 4 depending on the conductivity of the external buffer. In the present case, using (3), the external electric field at 100 kHz should by theoretically increased by 1.35 times for reaching a similar ITV value to the one at 10 kHz. For a higher frequency range, another theoretical study calculated that the increase factor for inducing an equivalent ITV between a sine signal of 100 kHz and 1 MHz was between 3 and 5, for high and low conductivity buffers, respectively [49]. These relations are highly dependent on the frequencies compared and are no longer valid for frequencies above 1 MHz, where a negligible net ionic charge will accumulate at both sides of the membrane

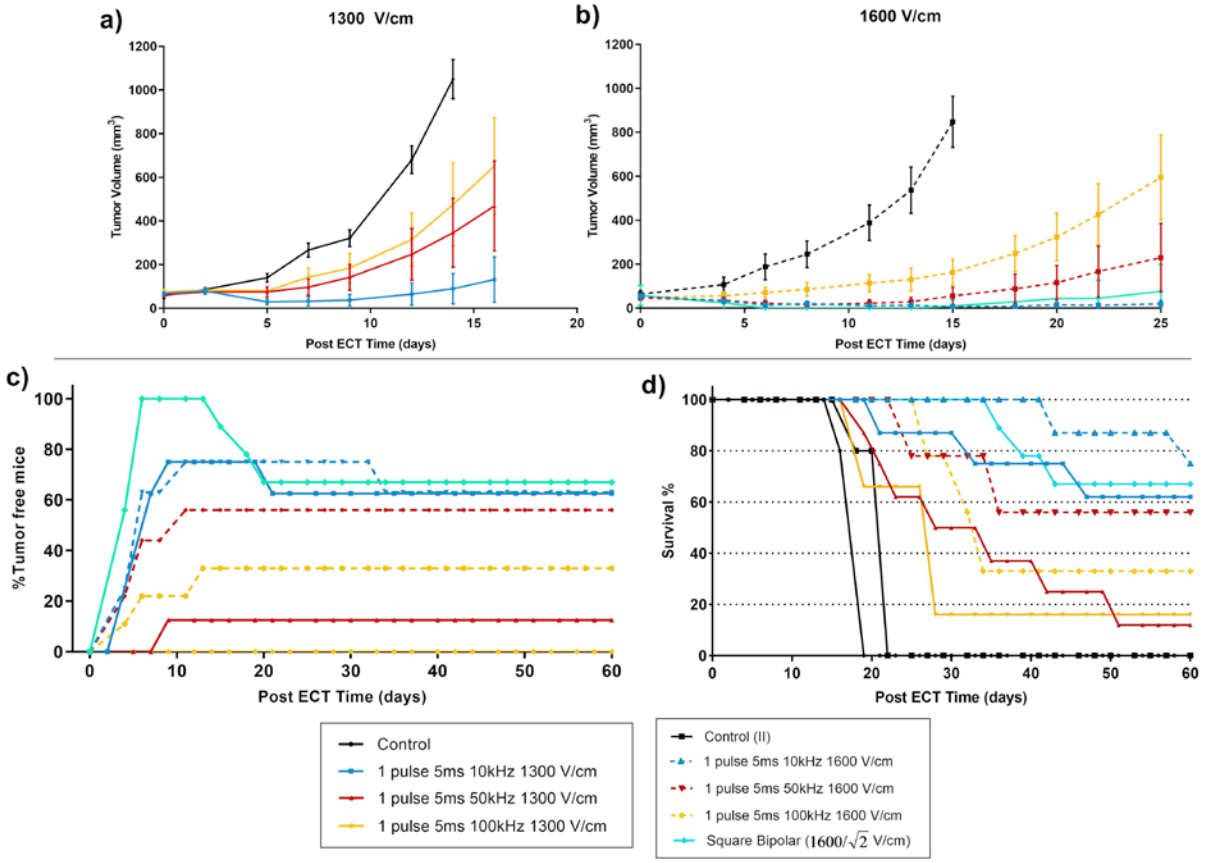

Fig. 6. Results of ECT treatment with a single sinusoidal burst of 5 ms duration and different frequencies (10, 50 and 100 kHz). Mice were treated at day = 0. Control groups received no treatment at all. a) and b): Mean tumor volume (±SE) evolution after ECT treatment with an Epeak intensity of 1300 and 1600 V/cm, respectively. The tumor evolution is shown until the first animal of the treated groups was sacrificed. c): Evolution in the percentage of tumor free mice for the different treatment groups. d) Survival rates results. Groups size was between n=6 (controls) and 9 (most of the treated groups).

leading to a situation where the charge-dependent nature of the electroporation is controversial [46]. Additionally, depending on the intrinsic parameters of a specific biological system, such as the extracellular and intracellular conductivities, membrane capacitance, cell radius, etc., the charging time and thus the frequency response of the cells can be different [50].

In Fig. 7 the survival rates during the two months follow up period are shown for all the previously discussed treatment conditions. A similar dependence with frequency and intensity of the electric field to that previously discussed is observed. Up to a 75 % of survival was achieved for the group treated with a sine wave of 10 kHz and 1600 V/cm.

Regarding the results obtained with a single bipolar square pulse (5 ms total duration and an amplitude of $1600/\sqrt{2}$ V/cm), the final outcome is very similar to the results obtained for 10 kHz and 1600 V/cm. This observation demonstrates that the membrane response to a sine wave of sufficiently low frequency is similar to a direct current (DC) bipolar pulse and that in this case the RMS equivalence is valid. Surprisingly, from day 5 to 15 a transitory 100 % of complete response was observed with the square bipolar pulse and not with the sine waves.

Finally, an additional experimental group exposed to sinusoidal bursts of 5 ms, 10 kHz with an intensity of 1600 V/cm but that did not receive bleomycin was included in this study. The response of this group was exactly as the control groups (data not shown) demonstrating that the observed effect is a consequence of reversible electroporation of the tumor cells in combination with the cytotoxic activity of bleomycin and not to other collateral effects like irreversible electroporation or thermal damage (as confirmed by the FEM calculations).

2) Additional observations

One of the potential advantages of using sine waves instead of DC pulses is the possibility of reducing the extent or the intensity of muscle contractions during an ECT treatment. Similar to the recently proposed method for irreversible electroporation using bursts of high frequency square waves (H-FIRE) [20] or sinusoidal waves [27], if the frequency of the sinusoidal burst was correctly adjusted it would be possible to perform tissue reversible electroporation with a reduced possibility of undesired muscle contractions. In the present study, contractions were observed in the leg proximal to the tumor site for all the exposure conditions. Among others, this could be due to the size of the application electrodes and their proximity to the leg nerves. However, from a qualitative visual evaluation it was noticed that the intensity of the contractions at 100 kHz was considerably lower than the contractions observed at 10 kHz or with the single bipolar square pulse.

It must be mentioned that in the group treated with a sine burst of 10 kHz and 1600 V/cm, three out of 9 mice displayed abnormal mobility and/or reduced force in the leg near the treated tumor. It is plausible that, due to the vicinity of the sciatic nerve to the exposed area, some kind of nerve injury was produced. No similar effect was observed in the other groups.

Regarding the common marks in the regions of the skin in contact with the electrodes during the electric field exposure, a dependence on the frequency of the applied electric field was observed. These marks, usually attributed to local thermal damage or irreversible electroporation, were negligible at high frequency and more visible at low frequency. This suggests that the high frequency field is less concentrated across the epithelial cells, thus reducing the damage to the skin. For the group treated with a single bipolar square pulse, these marks were even more pronounced and some mice displayed areas with small wounds in the skin that healed in a few days. This observation supports the idea that at sufficiently high frequencies it would be possible to have access to deeper tissues without affecting the skin barrier as it was proposed in [51].

All the previous observations demonstrate that the undesired collateral effects produced during, or as a consequence, of the electric field exposure (undesired contractions and skin damage) are reduced with high frequencies. However, as shown in previous sections, the efficacy of electroporation was also reduced with the increase of the frequency for a constant electric field intensity. Theoretically, in the frequency range studied, it should be possible to obtain an effect by only increasing the intensity of the applied electric field for the higher frequencies. In [27], the authors suggested that unlike for the case of nerve stimulation, the relation between electroporation thresholds and frequency is not directly proportional. Interestingly, also theoretical predictions suggested that there could be a difference between the dependence of electroporation and nerve stimulation thresholds on the frequency [52].

IV. Conclusions

In this study we demonstrate that AC sinusoidal fields can be applied to achieve convenient reversible electroporation in vivo and that efficient electrochemotherapy of subcutaneous tumors can be achieved. The results demonstrated the safety of the treatment when single sine wave burst with duration in the millisecond range was applied instead of sequential repetitive short (microsecond range) bursts. An equivalent efficacy was found between a single burst exposure and a repetitive sequence.

The response to the ECT treatment displayed a strong dependence with frequency with a clear loss of efficacy for the highest frequency of 100 kHz. The comparison between a single sinusoidal burst and a single bipolar square pulse of the same duration showed a similar response for equivalent RMS field amplitudes.

The undesired effects of the electric field exposure such as muscle contractions and skin damage were significantly lower when a high frequency wave was applied in comparison to the low frequency and the square bipolar pulse. According to our results, a tradeoff between the frequency and field amplitude should be accomplished to reduce the undesired effects of pulse application while still obtaining the expected response.

These results could have important applications in the clinical practice where sinusoidal bursts could be applied in the current ECT treatments. However, there is a need for the appropriate equipment for exposing large tumors to the electric pulses. In the future, the challenge will be to study if the exposure parameters can be optimized to obtain a complete tumor response avoiding muscle contraction. Additionally, the novel use of waveforms with a very narrow spectral content could represent an improvement for targeting specific tissues.

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