Avoiding neuromuscular stimulation in liver irreversible electroporation using radiofrequency electric fields

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Abstract. Electroporation based treatments typically consist in applying high voltage dc pulses. As an undesired side effect, these dc pulses cause electrical stimulation of excitable tissues such as motor nerves. In the present *in vivo* study, it was explored the use of bursts of sinusoidal voltage in the frequency range from 50 kHz to 2 MHz to induce irreversible electroporation (IRE) whilst avoiding neuromuscular stimulation. Series of 100 dc pulses or sinusoidal bursts, both with an individual duration of 100 μ s, were delivered to rabbit liver through thin needles in a monopolar electrode configuration and thoracic movements were recorded with an accelerometer. Tissue samples were harvested three hours after treatment and later postprocessed to determine the dimensions of the IRE lesions. Thermal damage due to Joule heating was ruled out via computer simulations. Sinusoidal bursts with a frequency equal or above 100 kHz did not cause thoracic movements and induced lesions equivalent to those obtained with conventional dc pulses when the applied voltage amplitude was sufficiently high. IRE efficacy dropped with increasing frequency. For 100 kHz bursts, it was estimated that the electric field threshold for IRE is about 1.4 kV/cm whereas that of dc pulses is about 0.5 kV/cm.

Keywords: Electroporation, Irreversible electroporation, Ablation, Radiofrequency electric fields, Sinusoidal voltages

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Introduction

Electroporation, or electroporabilization, is a biophysical phenomenon in which the cell membrane permeability to ions and molecules is increased when the cell is briefly exposed to high electric fields. This effect can be either transient (reversible electroporation) or, if extreme, can result in cell death (irreversible electroporation).

Electroporation is considered to be a threshold-like phenomenon whose initiation depends on the cell transmembrane voltage (TMV) (Zimmermann *et al* 1974): the phenomenon takes place when the externally applied electric field induces a TMV higher than a certain threshold. This leads to an electric field magnitude threshold to achieve electroporation in tissues (Kotnik *et al* 2010, Ivorra 2010). In electroporation based treatments, treatment planning is performed under the assumption that all cells exposed to an electric field higher than a certain value will experience the desired effect; either a reversible permeabilization level or cell death (Zupanic *et al* 2012). The electric field thresholds are estimated through experimental measurements and depend both on the temporal features of the exposure and on the cells or tissues being treated.

In vivo tissue electroporation is the basis of multiple clinical treatment modalities. Reversible electroporation is the basis of electrochemotherapy, a cancer treatment modality in which electric pulses are applied to enhance cellular uptake of chemotherapeutic agents (Silve and Mir 2011), and it is also the basis of gene electrotransfer, which is used in multiple therapies under clinical trials, including cancer treatments and DNA (Heller and Heller 2015) vaccinations (Irla *et al* 2008). Irreversible electroporation (IRE) is used as a non-thermal ablation method for treatment of solid tumors (Jiang *et al* 2015).

In clinical applications, electroporation treatments usually consist in the delivery, across electrodes, of a series of dc pulses with a single pulse duration in the order of 100 μ s. Unless preventive measures are taken, these pulses are likely to cause acute pain and strong muscle contractions because they stimulate efferent and afferent nerves within, or nearby, the region of treatment. This is particularly relevant when monopolar electrode configurations are attempted (Srimathveeravalli *et al* 2013). Stimulation occurs because, for these dc pulses, the electric field thresholds to trigger action potentials in excitable cells are significantly lower than those for initiating electroporation (Rogers *et al* 2004). The preventive measures typically consist in the administration of local or general anesthesia, in some cases with muscle relaxants for neuromuscular blockade (Ball *et al* 2010). This leads to an increase in the complexity of the whole clinical procedure that may limit the applicability of electroporation based treatments because of the burden to benefit ratio or the risk to benefit ratio.

In a recent numerical modeling study (Mercadal *et al* 2017), we have analyzed the behavior of a relatively novel IRE pulsing protocol consisting of bursts of short (1 μ s to 5 μ s) bipolar square pulses instead of the conventional relatively long (100 μ s) monopolar square pulses (Arena *et al* 2011, Sano *et al* 2014, Siddiqui *et al* 2016, Sweeney *et al* 2016). This protocol, labeled High-Frequency Irreversible Electroporation (H-FIRE), substantially reduces muscle contractions, hence preventing the need for neuromuscular blockade. Our numerical study indeed indicated that, by replacing the conventional pulses by bursts of short bipolar pulses, it will be possible to ablate a large tissue region without triggering action potentials in a nearby nerve.

That study has prompted us to hypothesize that, by using sinusoidal bursts instead of the H-FIRE bursts of bipolar pulses, a similar behavior would be obtained. Sinusoidal bursts preserve bipolarity and may be advantageous because of at least two reasons. First, high-voltage sinusoidal bursts may be easier to generate than high-voltage bipolar square pulses thanks to resonant circuit topologies (Dieckerhoff *et al* 1999, Yong-Nong and Chih-Ming 2013). Second, sinusoidal bursts are less likely to cause electromagnetic compatibility issues because of their limited frequency spectrum (Paperman *et al* 2009). Considering these two potential advantages, we decided to perform the present experimental study.

To the best of our knowledge, intentional use of radiofrequency (RF) electric fields for *in vivo* irreversible electroporation is unprecedented. However, it must be noted that the use of RF fields has been explored in the field of non-thermal liquid pasteurization for the inactivation of microorganisms by irreversible electroporation (Geveke and Brunkhorst 2004, Bai-Lin Qin *et al* 1994, González-Sosa *et al* 2014).

The main objective of the present study was to experimentally confirm the hypothesis that sinusoidal bursts may produce non-thermal lesions equivalent to those produced by conventional IRE pulses whilst avoiding or minimizing neuromuscular stimulation. A secondary objective was to examine the dependency of IRE electric field thresholds on the frequency of the sinusoidal bursts.

Methods

In vivo Experiments

Animal model

All aspects of this study were conducted according to the guidelines approved by the Government of Catalonia's Animal Care Committee (authorization number CEEAH 3336 and DAAM 8904) following the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

Three New Zealand White rabbits (Granja San Bernardo S.L., Spain) were acquired and maintained under standard conditions with a laboratory diet and water *ad libitum*. The animals were premedicated with ketamine 10 mg/kg IM (Imalgene, Merial Laboratorios, Spain) + midazolam 3 mg/kg IM (Midazolam Normon®; Laboratorios Normon, Spain) + buprenorphine 0.02 mg/kg IM (Buprecare, Animalcare Limited, UK) and they were anesthetized with propofol 4 mg/kg IV (Propofol-Lipuro, B.Braun Vetcare, Spain) during the induction phase and maintained with isoflurane (Isovet, Piramal Healthcare, UK) from 1.5 to 2% and oxygen 100% mixture once the animal was endotracheally intubated. Heart rate, respiratory rate, pulse oximetry and capnography were monitored using a multiparametric monitor (Vetcare, B.Braun Vetcare, Spain).

Treatment setup

By midline laparotomy, a hepatic lobe of the animal was exposed and laid over a 10 mm thick piece of polyethylene foam. Using a paper template, the treatment electrodes – stainless steel acupuncture needles (Brand: acimut, distributor: Novasan, S.A., Spain) with a diameter of 260 μm – were inserted throughout the liver reaching the foam. The orientation of the needles was perpendicular to the foam. Once inserted, the exposed upper part of the needle was covered with a piece of silicone tubing for preventing accidental electrical contact with other needles. Left lateral lobe, left medial lobe and right medial lobe were used, and a total of ten needles separated by 5 mm (distributed in two rows of five needles) were inserted in each of the lobe (Figure 1). For the application of the treatments, the protective tubing of the target needle was removed and the needle was electrically connected to the positive terminal of the generator by means of small metallic tweezers while the negative terminal was connected to the ground electrode pad placed at the back of the animal. After the treatment, the protective tubing was brought again around the needle.

Figure 1 – Electrode placement in one of the liver lobes of an anesthetized New Zealand White rabbit.

A total of 90 treatments were carried out, ten in each of the three largest hepatic lobes of each animal (see Table 1). Total time for each lobe (10 treatments) was about half an hour. All the treatments were performed using 100 pulses or bursts with a duration of 100 μs per pulse or burst and delivered at a rate of one per second.

Table 1 - Treatments performed during the animal experiments.

Pulse or burst type	Pulses/Bursts	Duration	Frequency	Magnitude	n
CONTROL	100	100 μs	dc	0 V	14
dc pulses	100	100 μs	dc	75 V	2
dc pulses	100	100 μs	dc	90 V	2
dc pulses	100	100 μs	dc	100 V	11
dc pulses	100	100 μs	dc	115 V	2
dc pulses	100	100 μs	dc	150 V	8
dc pulses	100	100 μs	dc	200 V	5
dc pulses	100	100 μs	dc	300 V	1
dc pulses	100	100 μs	dc	400 V	1
RF bursts	100	100 μs	50 kHz	250 V	4
RF bursts	100	100 μs	100 kHz	250 V	4

RF bursts	100	100 μ s	250 kHz	250 V	10
RF bursts	100	100 μ s	500 kHz	250 V	10
RF bursts	100	100 μ s	1000 kHz	250 V	5
RF bursts	100	100 μ s	2000 kHz	250 V	2
RF bursts	100	100 μ s	500 kHz	600 V	8
RF bursts	100	100 μ s	500 kHz	1000 V	1

Muscle contractions during treatment were monitored in one of the three animals by means of a 3-axis digital accelerometer (ADXL345, Analog Devices, Inc., USA) mounted on a printed circuit board (2 cm $\times$ 2 cm) which was sutured to the animal skin in the abdomen, approximately 6 cm below the liver.

In addition to monitoring contractions during the above referred treatments, with the purpose of gathering more information on neuromuscular stimulation, additional assays were performed in which the frequency of 200 μ s sinusoidal bursts was swept from 20 kHz to 100 kHz. These assays were performed using an additional needle placed in the proximal part of the lobe far from the treatment area.

Three hours after the treatments, the animals were euthanized using an intravenous overdose of sodium pentobarbital (Dolethal, Vetoquinol, Spain). Following this, the hepatic lobes were routinely processed for histological study. Briefly, lobes were placed perpendicular to the needle insertion in cassettes, fixed in 10% formalin during 48 hours, processed in an automatic tissue processor and finally paraffin embedded. Four micrometer sections were made and stained with Hematoxylin and Eosin (H&E). Color images of each slice were digitalized using NanoZoomer Digital Pathology System (Hamamatsu Photonics K.K., Japan) for histological and morphometric study.

The conventional dc pulses were generated using a custom developed generator (Figure 2(a)). Briefly, it consists of a commercial high voltage dc source (EP005127p2450CLD, iseg Spezialelektronik GmbH, Germany) and a 1 mF capacitor able to guaranty a constant dc voltage magnitude. The pulses were delivered by switching the transistors (STGW30NC120HD, STMicroelectronics, Switzerland) of an h-bridge topology which allows to generate both monopolar and bipolar pulses.

Most of the radiofrequency bursts were generated by means of a function generator (AFG 3022B, Tektronix Inc., USA) followed by a high voltage amplifier (1040L, Electronics & Innovation Ltd., USA) allowing to generate arbitrary radiofrequency up to 250 V of amplitude.

In order to generate sinusoidal bursts of higher magnitude, it was designed a LCC resonant tank at 500 kHz (Figure 2(b)). This resonant tank was connected at the output of the pulse generator (Figure 2(a)). The LCC tank consisted of a custom made inductor of 13 μ H and two capacitors, C_1 and C_2 , of 6.4 nF and 1.6 nF respectively. When excited with a bipolar square voltage of 500 kHz, the LCC tank provided a sinusoidal output five times higher than the input.

Figure 2 – (a) Schematic representation of the generator of dc pulses used in the present study and a representative output waveform captured during the experiments. (b) Resonant tank employed to generate sinusoidal bursts of 500 kHz with a voltage magnitude of up to 1000 V and a representative output waveform captured during the experiments.

Numerical study

A numerical study was conducted with two objectives. First, to check whether thermal damage due to Joule heating can be present in the applied treatments. Second, to obtain the electric field distribution in tissue. This modeled electric field distribution was later compared to the experimentally observed damage for determining the IRE electric field thresholds.

The numerical study was based on simultaneously solving the electric potential and the temperature distributions in the treatment region. A similar approach has been previously employed in order to simulate this type of treatments (Arena *et al* 2011, Garcia *et al* 2011). Both the thermal problem and the electrical problem were solved numerically using the finite element method (FEM) by means of COMSOL Multiphysics 4.4 (COMSOL Inc., Sweden). The adopted monopolar treatment setup shows axial symmetry (Figure 3).

Figure 3 – Geometry of the model used in the numerical assessment of electric field and temperature.

Starting from a temperature of 37°C, the temperature (T) at time (t) due to applied heat (Q) was obtained using the *Pennes Bioheat Equation* (1) (Pennes 1998). According to the defined density (ρ), heat capacity (c), thermal conductivity (k) and blood perfusion (ω_b), the heat dissipated by blood circulation depends on its density (ρ_b), heat capacity (c_b) and the difference between the liver and the arterial blood (T_b) temperature:

$$\rho c \frac{\partial T}{\partial t} = \nabla(k \nabla T) - \rho_b \omega_b c_b (T - T_b) + Q_m + Q \quad (1)$$

The *Pennes Bioheat Equation* also takes into account the heat produced by the tissue metabolism (Q_m), a small contribution which was not considered. The same equation, under the assumption of no blood perfusion ($\omega_b = 0$) was employed for the metallic electrode.

The induced heat is due to Joule heating. In accordance to the frequencies employed in the study, the magnetic induction contribution can be neglected (Berjano 2006), therefore, the induced heat depends on the local electric field magnitude and the electric conductivity of the material (σ):

$$Q = \sigma |E|^2 \quad (2)$$

The two types of treatment, conventional IRE square pulses and pulsed radiofrequency bursts, were numerical assessed. Since the electrical conductivity in biological tissues is frequency dependent, this value differs between both treatments. In case of conventional IRE treatments the conductivity is commonly modeled as a sigmoid function dependent on the electric field magnitude (3) (Sel *et al* 2005).

$$\sigma_{IRE} = \left(\sigma_0 + \frac{(\sigma_1 - \sigma_0)}{1 + 10 \cdot e^{-\frac{(E-58000)}{3000}}} \right) \cdot 1.02^{T-37^\circ\text{C}} \quad (3)$$

On the other hand the radiofrequency signals are less affected by the electroporation phenomenon as the currents are relatively free to flow towards the cell membrane. In this study it was defined the maximum conductivity corresponding to the null

interaction with the membrane. This can be considered the worst scenario in terms of thermal damage induction.

$$\sigma_{RF} = \sigma_1 \cdot 1.02^{T-37^\circ\text{C}} \quad (4)$$

The conductivity of ionic solutions increase about 2% per each temperature degree, therefore, an additional term was included in both conductivity expressions (3-4) (Castellví *et al* 2017).

The electric field distribution resulting from applying a voltage difference between the needle electrode and the dispersive ground pad was obtained by solving the electric potential (φ) that satisfies the Laplace equation (5) when a voltage difference ($\Delta\varphi$) is applied between the needle surface and the ground electrode.

$$\nabla \cdot (\sigma \nabla \varphi) = 0 \quad (5)$$

The method employed to simulate the whole temporal solutions consist in concatenate different temporal studies where the applied voltage was considered as null during the periods outside the pulse duration. In order to save computational cost, the sinusoidal signals were replaced by a constant value with equal energy ($V_p \cdot \sqrt{2}$).

The amount of thermal damage resulting from every treatment was evaluated according to the computed temperature distribution. In case were low temperature increases are expected during a large period of time, the cumulative equivalent minutes at 43°C (CEM₄₃) is widely used as a metric for thermal damage (He *et al* 2009).

$$\text{CEM}_{43} = \int R^{43^\circ\text{C}-T(t)} dt \quad (6)$$

For most biological tissues, the value of R is between 0.25 and 0.5, we used this last value as conservative measure. In liver tissue, the critical dose for considering the tissue as thermally damaged was set to 340 minutes (Graham *et al* 1999).

The electric and thermal properties of the tow materials included in the model (i.e. the metallic electrode and hepatic tissue) are listed in Table 2.

Table 2 - Materials properties used in the numerical model

Tissue/material	Description	Param.	Value	Reference
Stainless steel	Electric conductivity	σ	$7.4 \times 10^6 \text{ S m}^{-1}$	(Berjano <i>et al</i> 2006)
Stainless steel	Thermal conductivity	k	$15 \text{ W m}^{-1} \text{ K}^{-1}$	(Berjano <i>et al</i> 2006)
Stainless steel	Density	ρ	8000 kg m^{-3}	(Berjano <i>et al</i> 2006)
Stainless steel	Heat capacity	C_p	$480 \text{ J kg}^{-1} \text{ K}^{-1}$	(Berjano <i>et al</i> 2006)
Liver	Electric conductivity	σ_0	0.08 S m^{-1}	(Ivorra and Rubinsky 2007)
Liver	Electric conductivity	σ_1	0.31 S m^{-1}	(Ivorra and Rubinsky 2007)
Liver	Thermal conductivity	k	$0.502 \text{ W m}^{-1} \text{ K}^{-1}$	(Trujillo <i>et al</i> 2013)
Liver	Density	ρ	1080 kg m^{-3}	(Trujillo <i>et al</i> 2013)
Liver	Heat capacity	C_p	$3455 \text{ J kg}^{-1} \text{ K}^{-1}$	(Trujillo <i>et al</i> 2013)
Liver	Blood temperature	T_b	37°C	(Trujillo <i>et al</i> 2013)
Liver	Blood heat capacity	C_b	$4180 \text{ J kg}^{-1} \text{ K}^{-1}$	(Trujillo <i>et al</i> 2013)
Liver	Blood perfusion rate	ω_b	$6.4 \times 10^{-3} \text{ s}^{-1}$	(Trujillo <i>et al</i> 2013)
Liver	Blood density	ρ_b	1000 kg m^{-3}	(Trujillo <i>et al</i> 2013)

Results

Muscle contractions

Figure 4(a) shows an example of the recorded acceleration during the application of conventional IRE pulses (100 μ s dc pulses).

Figure 4 – (a) Recorded acceleration (anteroposterior axis) during the application of conventional IRE pulses (100 μ s dc pulses) with a magnitude of 115 V. (b) Measured acceleration peak (mean + SD) when a conventional IRE pulse (100 μ s dc pulse) is applied at different voltage magnitudes.

All the conventional IRE pulses delivered during the experiments caused strong muscular contractions which could be observed both visually and by means of the accelerometer recording. The measured peak values for the recorded acceleration signal (anteroposterior axis) show a certain degree of dependence on the applied magnitude of the pulses (Figure 4(b)).

Muscle contractions during delivery of sinusoidal bursts showed a strong dependence on the signal frequency. Such dependence is clearly illustrated in a sample recording obtained during a pre-treatment frequency sweep at constant voltage amplitude of 250 V (Figure 5(a)).

RF bursts

Figure 5 – a) Recorded acceleration (anteroposterior axis) during the application of RF bursts with an amplitude of 250 V at different frequencies. b) Measured acceleration peak (mean + SD) when a sinusoidal burst at 250 V is applied at different frequencies.

Figure 5(b) shows the measured acceleration peak during the application of a radiofrequency burst of 250 V at different frequencies. The acceleration peak magnitude shows a significant correlation with the frequency ($p < 0.0001$, Spearman rank test). Note that acceleration measurements at frequencies above 50 kHz do not show statistical significance in comparison with the control values when no pulses were delivered at all ($p < 0.0001$, one-way ANOVA).

Histological observation of the lesions

Both in the case of dc pulses and RF bursts, the resulting lesions around the electrode tract present a necrotic area where hepatocytes are swollen, with a pale or with an intense eosinophilic cytoplasm stain. Using the digitalized images and a post processing software (NDP.view2, Hamamatsu Photonics K.K., Japan) this area was delimited for each one of the lesions (Figure 6 black line). As the needles could present a slight misalignment with the normal the slice plane, instead of using the necrotic area for assessing the lesion, the minimum lesion diameter was determined to quantify the extent of the lesion (Figure 6, red double arrow line).

Figure 6 – Sample of a lesion created by RF bursts (H&E stain). The black line delimits the necrotic perimeter defining the lesion extension. The red double arrow line denotes the minimum diameter.

Table 3 summarizes the performed treatment groups and the treatment lesion sizes. Regrettably, the outcome of some of the performed treatments could not be evaluated because the liver lobe was not properly fixed.

Pulse or burst type	Pulses/ Bursts	Duration (μs)	Frequency	Magnitude (V)	Diameter (μm)			n
					mean	min	max	
CONTROL	100	100	dc	0 V	166	45	293	11
dc pulses	100	100	dc	75 V	324	294	353	2
dc pulses	100	100	dc	90 V	492	294	689	2
dc pulses	100	100	dc	100 V	494	351	611	11
dc pulses	100	100	dc	115 V	462	341	582	2
dc pulses	100	100	dc	150 V	873	773	953	8

dc pulses	100	100	dc	200 V	937	852	1095	4
dc pulses	100	100	dc	300 V	1050	1050	1050	1
dc pulses	100	100	dc	400 V	1540	1540	1540	1
RF bursts	100	100	50 kHz	250 V	735	666	803	2
RF bursts	100	100	100 kHz	250 V	694	694	694	1
RF bursts	100	100	250 kHz	250 V	472	388	539	7
RF bursts	100	100	500 kHz	250 V	364	239	433	6
RF bursts	100	100	1000 kHz	250 V	255	205	305	2
RF bursts	100	100	2000 kHz	250 V	162	145	178	2
RF bursts	100	100	500 kHz	500 V	451	413	500	8
RF bursts	100	100	500 kHz	1000 V	989	989	989	1

Figure 7 shows the lesion diameter produced by RF bursts with an amplitude of 250 V at different frequencies. It can be observed a strong dependence on the frequency.

Figure 7 – Diameter of the lesions produced by sequences of 100 sinusoidal bursts with duration of 100 μs and amplitude of 250 V at different frequencies. Dashed line shows the mean lesion diameter in control cases attributable to mechanical damage.

By increasing the frequency of the bursts, the produced lesion size rapidly decreases. This indicates that the minimum electric field magnitude required to damage the tissue increases with the frequency. According to the electric field distribution obtained by the numerical simulations each of the lesions diameter measured in the histological preparations corresponds to a specific electric field threshold to produce irreversible electroporation (Figure 8). That is, in Figure 8 it is indicated the simulated electric field magnitude that appears at distance $D/2$ from the needle where D is the diameter of the lesion (necrotic area) according to the corresponding histological observation.

Figure 8 – Estimated electric field threshold to achieve hepatic IRE using sequences of 100 sinusoidal bursts with a duration of 100 μ s at different frequencies.

For different amplitudes, Figure 9 displays the lesion sizes for the dc pulses and for the RF bursts at 500 kHz. It is clearly observable that the RF bursts at 500 kHz are less effective than the dc pulses. The performed treatments using conventional IRE dc pulses produced lesions with a size corresponding to a threshold value of 500 ± 49 V/cm. On the other hand, the estimated threshold value for radiofrequency bursts at 500 kHz is 2984 ± 791 V/cm.

Figure 9 – Lesion diameter according to the pulse magnitude by using conventional IRE dc pulses and radiofrequency bursts at 500 kHz.

Simulated thermal effects

The numerical simulations were carried out aiming to assess the possible thermal damage produced by the radiofrequency bursts. Figure 10 shows the temporal evolution of the maximum temperature detected at any point of the whole tissue when radiofrequency bursts are applied with three different magnitudes. The location of these temperature maxima evolved during bursts delivery but always appeared at locations very close to the needle (from 0 μ m to 50 μ m). It can be observed that with the

maximum applied voltage (1000 V), the maximum temperature reaches values (61°C) which cannot be considered negligible.

Figure 10 – Temporal evolution of the maximum temperature detected at any point of the whole tissue during the 100 sinusoidal bursts with duration of 100 μ s delivered at 1 Hz using different amplitudes of voltage.

However, it must be noted that this temperature is present in a very small spatial region and is only sustained for a very short period of time (< 10 ms). That prevents thermal damage. The computed CEM43 thermal dose is of 122 minutes which is lower than the 340 minutes defined threshold for liver damage (Figure 11).

Figure 11 – Computed CEM43 thermal dose for the assayed treatments consisting of radiofrequency bursts with an amplitude of 1000 V, 500 V and 250 V.

Discussion

As hypothesized, the present study confirms that sinusoidal bursts can produce non-thermal lesions in liver equivalent to those produced by conventional IRE pulses whilst avoiding neuromuscular stimulation. This is a noteworthy conclusion because it indicates that it may be possible to eliminate the need for neuromuscular blockade in IRE treatments of internal organs thus avoiding one of the major current limitations of those treatments (Lyu *et al* 2017). In addition, since this conclusion indicates that motor nerves are not stimulated, it can be inferred that sensory nerves will also not be

stimulated – or will be minimally stimulated – and the need for anesthetics to suppress pain will be alleviated. However, it must be noted that this deduction related to pain is not explicitly tested in the present study as the animal was anesthetized.

It is worth noting that the above result was obtained using a monopolar electrode configuration. This configuration, which consists of an active electrode that applies the treatment and a skin return electrode of large area that disperses the current density thereby preventing injury to the underlying tissue, represents a phenomenal challenge for avoiding stimulation in IRE treatments. In monopolar configurations, since currents are forced to flow through large portions of the body, unintended severe stimulation is very likely to occur (Srimathveeravalli *et al* 2013). Until now, in contrast to radiofrequency and microwave ablation modalities, and despite the use of anesthesia and neuromuscular blockade, IRE clinical treatments have been limited to bipolar configurations mainly because this issue.

A limitation of the present study is the small size of the induced IRE lesions. In part, this was intentional: we wanted to maximize the number of assays per animal to comply with the second R (reduce the number of animals used) in the Three Rs guiding principles for ethical animal testing (Russell and Burch 1959). However, this limitation was also caused by two other restrictive factors: the maximum voltage amplitude provided by the generators (1000 V for 500 kHz and 250 V for other frequencies) and the use of the monopolar configuration through very thin needles. The use of the monopolar configuration implies that the electric field magnitude drops rapidly moving away from the needle and that causes the radius of the IRE lesion – where the field magnitude is above the IRE threshold – to be small. Presumably, with the same needles, larger lesions would be possible by delivering higher voltage amplitudes or longer bursts but then thermal damage around the needles would occur making it more complex to interpret the results.

The numerical analysis contained in the present study indicates that thermal damage could not occur in any of the performed assays. In the worst scenario (1000 V), the simulations indicate that no area of the liver is exposed to a thermal insult equivalent to that caused by an exposure to a temperature of 43°C for 122 minutes (i.e. $CEM_{43} = 122$ minutes) and this insufficient to cause thermal damage. It has been estimated that the minimum CEM_{43} for liver damage is 320 minutes (Chang 2010). It must be noted that we attempted to record the assays with a thermographic camera (E60, FLIR Systems, Inc., USA) but, despite its good thermal sensitivity (0.05 K), we were not able to distinguish any temperature increase due to its limited spatial resolution.

It is interesting to note that the IRE field magnitude thresholds increase with frequency but that such dependency is not as strong as direct proportionality (Figure 8). This is aligned with previous observations from in vitro experimental studies (Marszalek *et al* 1990) and with an equivalent observation reported in our previous study (Mercadal *et al* 2017): cellular IRE field magnitude thresholds for bipolar square pulses increase as the length of the pulses (t_p) is shortened (i.e. frequency increases) but such dependency is not as strong as $1/t_p$. This is relevant because the field magnitude thresholds for stimulation are directly proportional to the frequency, as evidenced in our previous numerical study (Mercadal *et al* 2017), and by experimental observations (Reilly 1988). Therefore, as frequency increases, the stimulation and IRE thresholds will depart and it will be easier to induce IRE lesions without causing stimulation. This hypothetical

behavior is observed in the present study: at frequencies lower than 50 kHz muscle contractions are observable while at higher frequencies those contractions disappear and similar IRE lesions are induced. As reported in our previous study, we propose that the different dependence of IRE and stimulation thresholds on the frequency appears because transmembrane charging for nerve fibers is much slower than that of cells treated by electroporation because of their geometrical differences (Mercadal *et al* 2017).

It can be stated that tissue irreversible electroporation by radiofrequency fields is a novel ablation modality. Irreversible electroporation by radiofrequency fields applied in short bursts is essentially different from conventional radiofrequency ablation (RFA) because it does not involve thermal damage and it is also significantly distant from conventional irreversible electroporation with dc pulses (IRE) or irreversible electroporation with high-frequency bipolar pulses (H-FIRE) because different waveforms are employed and the required generators are substantially different. We propose the term Non-Thermal Radiofrequency Ablation (NT-RFA) to designate this novel ablation modality.

Conclusions

It was found that sinusoidal bursts with a frequency equal or above 100 kHz induce IRE lesions in liver rabbit which are equivalent to those obtained with conventional dc pulses but, in contrast to these dc pulses, the sinusoidal bursts do not cause perceptible muscle contractions. This indicates that it may be possible to eliminate the need for neuromuscular blockade in IRE treatments of internal organs by using sinusoidal bursts instead of dc pulses. This possibility reinforced by the fact that the present study was conducted using a monopolar electrode configuration.

It was also found that IRE electric field magnitude thresholds increase with the frequency of the sinusoidal bursts but that such dependency is not as strong as direct proportionality.

Irreversible electroporation by radiofrequency fields applied in short bursts can be considered to be a novel tissue ablation modality. We propose the term Non-Thermal Radiofrequency Ablation (NT-RFA) to designate this novel ablation modality.

According to numerical simulations (not reported) based on the results obtained here, a sequence of 100 bursts with a duration of 100 μ s, a frequency of 500 kHz and an amplitude of 3500 V would produce a lesion of 14.4 mm $\times$ 6.4 mm in liver if applied across two parallel needle electrodes with a diameter of 1 mm and a separation of 10 mm.

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References

- Arena C B, Sano M B, Rossmeisl J H, Caldwell J L, Garcia P a, Rylander M N and Davalos R V 2011 High-frequency irreversible electroporation (H-FIRE) for non-thermal ablation without muscle contraction. *Biomed. Eng. Online* **10** 102
- Bai-Lin Qin, Qinghua Zhang, Barbosa-Canovas G V, Swanson B G and Pedrow P D 1994 Inactivation of microorganisms by pulsed electric fields of different voltage waveforms *IEEE Trans. Dielectr. Electr. Insul.* **1** 1047–57
- Ball C, Thomson K R and Kavnoudias H 2010 Irreversible electroporation: a new challenge in “out of operating theater” anesthesia. *Anesth. Analg.* **110** 1305–9
- Berjano E J 2006 Theoretical modeling for radiofrequency ablation: state-of-the-art and challenges for the future. *Biomed. Eng. Online* **5** 24
- Berjano E J, Burdío F, Navarro A C, Burdío J M, Güemes A, Aldana O, Ros P, Sousa R, Lozano R, Tejero E, de Gregorio M A and Gregorio M a De 2006 Improved perfusion system for bipolar radiofrequency ablation of liver: preliminary findings from a computer modeling study *Physiol. Meas.* **27** N55–66
- Castellví Q, Sánchez-Velázquez P, Moll X, Berjano E, Andaluz A, Burdío F, Bijnsens B and Ivorra A 2017 Modeling Liver Electrical Conductivity during Hypertonic Injection *Int. j. numer. method. biomed. eng.* 1–23
- Chang I A 2010 Considerations for Thermal Injury Analysis for RF Ablation Devices *Open Biomed. Eng. J.* **4** 3–12
- Dieckerhoff S, Ruan M J and De Doncker R W 1999 Design of an IGBT-based LCL-resonant inverter for high-frequency induction heating *Conf. Rec. 1999 IEEE Ind. Appl. Conf. Thirty-Forth IAS Annu. Meet. (Cat. No.99CH36370)* **3** 2039–45
- Garcia P A, Rossmeisl J H, Neal R E, Ellis T L and Davalos R V 2011 A Parametric Study Delineating Irreversible Electroporation from Thermal Damage Based on a Minimally Invasive Intracranial Procedure *Biomed. Eng. Online* **10** 34
- Geveke D J and Brunkhorst C 2004 Inactivation of Escherichia coli in Apple Juice by Radio Frequency Electric Fields *J. Food Sci.* **69**FEP134-FEP138
- González-Sosa J, Ruiz-Vargas A, Arias G and Ivorra A 2014 Fast flow-through non-thermal pasteurization using constant radiofrequency electric fields *Innov. Food Sci. Emerg. Technol.* **22** 116–23
- Graham S J, Chen L, Leitch M, Peters R D, Bronskill M J, Foster F S, Henkelman R M and Plewes D B 1999 Quantifying tissue damage due to focused ultrasound heating observed by MRI *Magn. Reson. Med.* **41** 321–8
- He X, Bhowmick S and Bischof J C 2009 Thermal therapy in urologic systems: a comparison of arrhenius and thermal isoeffective dose models in predicting hyperthermic injury. *J. Biomech. Eng.* **131** 74507
- Heller R and Heller L C 2015 Gene Electroporation Clinical Trials *Advances in Genetics* vol 89(Elsevier Ltd)pp 235–62
- Irla M, Saade M, Kissenpfennig A, Poulin L F, Leserman L, Marche P N, Jouvin-Marche E, Berger F and Nguyen C 2008 ZAP-70 Restoration in Mice by In Vivo Thymic Electroporation ed J A Gold *PLoS One* **3** e2059
- Ivorra A 2010 Tissue Electroporation as a Bioelectric Phenomenon: Basic Concepts *Irreversible Electroporation* Series in Biomedical Engineering ed B Rubinsky (Berlin, Heidelberg: Springer Berlin Heidelberg) pp 23–61
- Ivorra A and Rubinsky B 2007 In vivo electrical impedance measurements during and after electroporation of rat liver. *Bioelectrochemistry* **70** 287–95

- Jiang C, Davalos R V. and Bischof J C 2015 A Review of Basic to Clinical Studies of Irreversible Electroporation Therapy *IEEE Trans. Biomed. Eng.* **62** 4–20
- Kotnik T, Pucihar G and Miklavčič D 2010 Induced Transmembrane Voltage and Its Correlation with Electroporation-Mediated Molecular Transport *J. Membr. Biol.* **236** 3–13
- Lyu T, Wang X, Su Z, Shangguan J, Sun C, Figini M, Wang J, Yaghmai V, Larson A C and Zhang Z 2017 Irreversible electroporation in primary and metastatic hepatic malignancies *Medicine (Baltimore)*. **96** e6386
- Marszalek P, Liu D S and Tsong T Y 1990 Schwan equation and transmembrane potential induced by alternating electric field *Biophys. J.* **58** 1053–8
- Mercadal B, Arena C B, Davalos R V and Ivorra A 2017 Avoiding nerve stimulation in irreversible electroporation: a numerical modeling study *Phys. Med. Biol.* **62** 8060–79
- Paperman W D, David Y and Hibbetts J 2009 Electromagnetic interference in the hospital *Biomedical Engineering Desk Reference* (Elsevier Science) pp 493–504
- Pennes H H 1998 Analysis of tissue and arterial blood temperatures in the resting human forearm. 1948. *J. Appl. Physiol.* **85** 5–34
- Reilly J P 1988 No Title *Electrical Models for Neural Excitation Studies* (Johns Hopkins APL Technical Digest) pp 44–59
- Rogers W R, Merritt J H, Comeaux J A, Kuhnel C T, Moreland D F, Teltschik D G, Lucas J H and Murphy M R 2004 Strength-duration curve an electrically excitable tissue extended down to near 1 nanosecond *IEEE Trans. Plasma Sci.* **32** 1587–99
- Russell W M S and Burch R L 1959 *The Principles of Humane Experimental Technique* (London: Methuen & Co.)
- Sano M B, Arena C B, DeWitt M R, Saur D and Davalos R V. 2014 In-vitro bipolar nano- and microsecond electro-pulse bursts for irreversible electroporation therapies *Bioelectrochemistry* **100** 69–79
- Sel D, Cukjati D, Batiuskaite D, Slivnik T, Mir L M and Miklavcic D 2005 Sequential Finite Element Model of Tissue Electroporabilization *IEEE Trans. Biomed. Eng.* **52** 816–27
- Siddiqui I A, Latouche E L, DeWitt M R, Swet J H, Kirks R C, Baker E H, Iannitti D A, Vrochides D, Davalos R V. and McKillop I H 2016 Induction of rapid, reproducible hepatic ablations using next-generation, high frequency irreversible electroporation (H-FIRE) in vivo *Hpb* **18** 726–34
- Silve A and Mir L M 2011 Cell Electroporabilization and Cellular Uptake of Small Molecules: The Electrochemotherapy Concept *Clinical Aspects of Electroporation* vol 300(New York, NY: Springer New York)pp 69–82
- Srimathveeravalli G, Wimmer T, Monette S, Gutta N B, Ezell P C, Maybody M, Weiser M R and Solomon S B 2013 Evaluation of an Endorectal Electrode for Performing Focused Irreversible Electroporation Ablations in the Swine Rectum *J. Vasc. Interv. Radiol.* **24** 1249–56
- Sweeney D C, Reberšek M, Dermol J, Rems L, Miklavčič D and Davalos R V. 2016 Quantification of cell membrane permeability induced by monopolar and high-frequency bipolar bursts of electrical pulses *Biochim. Biophys. Acta - Biomembr.* **1858** 2689–98
- Trujillo M, Castellví Q, Burdío F, Sánchez Velazquez P, Ivorra A, Andaluz A and Berjano E 2013 Can electroporation previous to radiofrequency hepatic ablation enlarge thermal lesion size? A feasibility study based on theoretical modelling and in vivo experiments. *Int. J. Hyperthermia* **29** 211–8
- Yong-Nong C and Chih-Ming K 2013 Design of Plasma Generator Driven by High-

- frequency High-voltage Power Supply *J. Appl. Res. Technol.* **11** 225–34
- Zimmermann U, Pilwat G and Riemann F 1974 Dielectric Breakdown of Cell Membranes *Biophys. J.* **14** 881–99
- Zupanic A, Kos B and Miklavcic D 2012 Treatment planning of electroporation-based medical interventions: electrochemotherapy, gene electrotransfer and irreversible electroporation *Phys. Med. Biol.* **57** 5425–40