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Effect of interphase and interpulse delay in high-frequency irreversible electroporation pulses on cell survival, membrane permeabilization and electrode material release

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ABSTRACT

To achieve high efficiency of electroporation and to minimize unwanted side effects, the electric field parameters must be optimized. Recently, it was suggested that biphasic high-frequency irreversible electroporation (H-FIRE) pulses reduce muscle contractions. However, it was also shown for sub-microsecond biphasic pulses that the opposite polarity phase of the pulse cancels the effect of the first phase if the interphase delay is short enough. We investigated the effect of interphase and interpulse delay (ranging from 0.5 to 10,000 μs) of 1 μs biphasic H-FIRE pulses on cell membrane permeabilization, on survival of four mammalian cell lines and determined metal release from aluminum, platinum and stainless steel electrodes. Biphasic H-FIRE pulses were compared to 8 × 100 μs monophasic pulses. We show that a longer interphase and interpulse delay results in lower cell survival, while the effects on cell membrane permeabilization are ambiguous. The cancellation effect was observed only for the survival of one cell line. Application of biphasic H-FIRE pulses results in lower metal release from electrodes but the interphase and interpulse delay does not have a large effect. The electrode material, however, importantly influences metal release – the lowest release was measured from platinum and the highest from aluminum electrodes.

1. Introduction

Electroporation (also termed electropermeabilization or pulsed electric field treatment) is the phenomenon of increased cell membrane permeabilization due to exposure of cells/tissue to short electric pulses [1]. It is used in numerous applications including cell transfection/transformation, electrochemotherapy (ECT), tissue ablation, extraction of biomolecules from cells, inactivation of microorganisms in water and liquid foods [2–5]. Efficacy of electroporation depends on several physical and biological parameters. In electroporation-based applications, the electric field parameters like electric field strength, pulse shape, pulse duration, pulse polarity, delay between pulses and number of pulses must be adjusted to specific biomedical or biotechnological applications, i.e. to achieve specific electroporation objectives [6]. For example, in the case of cell transfection/transformation or ECT the aim is to achieve high cell permeabilization and high cell survival to allow the entry of the desired molecule (a plasmid or chemotherapeutic agent) into the cells [7]. However, for tissue ablation or microbial inactivation, an efficient electroporation protocol results in low survival of the target cells (tumor or arrhythmogenic substrate) or microorganisms in food or water treatment [3,8,9].

In the past decade, irreversible electroporation (IRE) emerged as a new non-thermal ablation modality [10]. IRE is showing promising results in early clinical research of ablation of intra-abdominal tumors [11–13] and cardiac ablation [9,14–16]. During IRE treatment, electric pulses temporarily increase the semi-selective permeability of the cell membrane, thus allowing non-selective transport of molecules in and out of the targeted cells (through the compromised cell membrane). In IRE, different pulse parameters and delivery protocols are used in different studies. Most frequently, 70–100 pulses of 50–100 μs duration and higher amplitude are used [8,17,18] compared to the standard eight 100 μs pulses used in ECT [19]. In contrast to reversible electroporation, in IRE the membrane may reseal after the treatment, but the cell dies nevertheless. General anesthesia and the administration of neuromuscular blocking drugs are required in IRE to prevent pulse-induced muscle contractions [20] and pulse delivery must be synchronized with the electrocardiogram (ECG) to prevent the

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induction of cardiac arrhythmias [21]. Recently, short biphasic pulses used for high-frequency irreversible electroporation (H-FIRE) have attracted considerable attention since they have shown reduced muscle contractions compared to treatments using monophasic pulses [22–26]. It also seems that H-FIRE limits the likelihood of cardiac interference [27]. In several studies, authors have shown that H-FIRE with short biphasic pulses necessitate higher amplitudes of pulses to be used, i.e. requiring higher electric field strengths compared to monophasic pulses to achieve the same biological effect—being it cell membrane permeabilization or cell kill [22,28–30]. At the same time, it was reported for nanosecond biphasic pulses that the opposite polarity phase of the pulse cancels the effect of the first phase if the interphase delay is short enough—a phenomenon called "cancellation effect"—which may explain why higher amplitudes are needed when using biphasic pulses. This cancellation effect was also observed in microsecond range of pulses, yet it is still not fully understood [31–35].

Another "side-effect" of electroporation are also electrochemical processes taking place at the electrode-electrolyte interface, such as electrolysis, generation of radicals and release of metal ions from the electrodes which results in electrode wear and fouling, sample contamination or/and chemical modification of the medium. Electrochemical processes occurring during the delivery of high-voltage electric pulses with an emphasis on food processing were described by Pataro et al. [36] and Saulis et al. [37]. These effects are often neglected although they change the composition of the electroporation medium, can affect cells or food that has been treated and even cause experimental errors [38–49]. On the other hand, the chemical interaction between the products of electrolysis and cells are exploited to cause cell death in electrolytic tissue ablation [50] and in the combination of electroporation and electrolysis (E2) [51].

Electrodes for electroporation procedures are most often made of aluminum, stainless steel or platinum [37]. Metal ions released from electrodes during electroporation can change the solution pH [43,49], precipitate proteins and nucleic acids [44,52], impact flavor and mouth feeling of treated food [47] and can be cytotoxic and/or affect the biochemistry of the exposed cells [42,45]. Released metal ions can also affect the methods we use to monitor electroporation, e.g. membrane permeabilization after electroporation with calcein since metal ions can form complexes with fluorescent dyes and quench their fluorescence [53]. Proposed strategies for reducing the intensity of electrochemical reactions include reduction of the voltage, shortening of the pulse duration, lowering of medium conductivity or the use of biphasic pulses [37]. It was confirmed experimentally that contamination with released metal ions can be largely reduced by using 100 μs biphasic pulses instead of monophasic pulses [45] and that the shortening of the pulse limits electrochemical reactions and electrode corrosion [54]. However, when using shorter pulses, a stronger electric field (or higher number of pulses) must be applied to achieve the same electroporation efficiency [37,55]. It thus remains unclear whether shortening the pulse duration with concomitantly increased voltage reduces electrochemical reactions.

In this study, we investigated the effect of interphase delay and interpulse delay between biphasic pulses (i.e pulse repetition rate) of 1 μs symmetric rectangular biphasic H-FIRE pulses on cell membrane permeabilization and survival of CHO-K1 (Chinese hamster ovary), H9c2 (rat cardiomyoblast), C2C12 (mouse myoblast) and HT22 (mouse neuronal) cells. The interphase and interpulse delay ranged from 0.5 μs to 10,000 μs. We compared biphasic H-FIRE pulses to 8 × 100 μs monophasic pulses widely used in ECT. For all pulses, the total energized time was 800 μs. We show that not only longer interphase delay but also longer interpulse delay between biphasic pulses results in lower cell survival (i.e. in more efficient cell kill) while the effects on cell membrane permeabilization are more ambiguous. The previously reported cancellation effect of the first phase of the pulse by the second was observed only for the survival of CHO cells. We also measured metal release from aluminum, platinum and stainless steel 304 wire electrodes. The electrode material has a big influence on the amount of released metal ions – we measured the lowest concentration of released ions from platinum electrodes and highest from aluminum electrodes. Our results suggest that contrary to cell survival and membrane permeabilization, the interphase and interpulse delay in the investigated range does not largely affect the concentration of released metal ions. We showed, however, that application of short biphasic H-FIRE pulses results in lower metal release from aluminum, platinum and stainless steel 304 electrodes compared to standard 100 μs monophasic ECT and IRE pulses.

2. Materials and methods

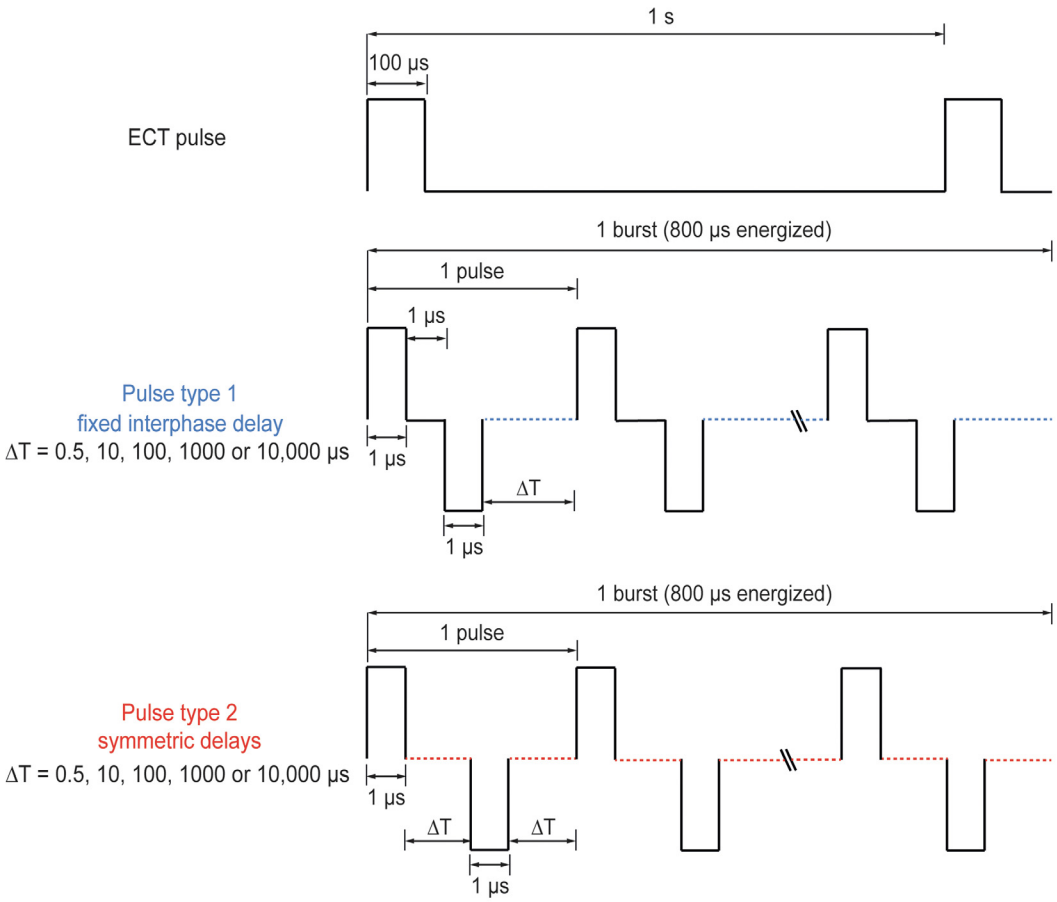
2.1. Electroporation set-up

We used a laboratory prototype pulse generator (University of Ljubljana), based on H-bridge digital amplifier with 1 kV MOSFETs (DE275-102N06A, IXYS, USA) [29], in the experiments. In cell membrane permeabilization, cell survival and metal release experiments, we applied 8 standard ECT rectangular pulses of 100 μs duration with 1 Hz repetition rate or 1 burst of 400 biphasic H-FIRE rectangular pulses with the same amplitude (for all the total energized time was 800 μs). 1 pulse in the case of H-FIRE pulses consists of the positive phase, negative phase and the interphase delay (see Schematic 1). The duration of the positive phase is 1 μs and the duration of the negative phase is 1 μs for all the H-FIRE pulses. For H-FIRE pulses, we varied the duration of the interphase delay and interpulse delay between pairs of biphasic pulses, and based on that named them as pulses of type 1 (fixed interphase delay) and type 2 (symmetric delays). For pulses of type 1 (fixed interphase delay), the interphase delay is fixed to 1 μs, while the interpulse delay was set to 0.5, 10, 100, 1000 or 10,000 μs. For pulses of type 2 (symmetric delays), the interphase and interpulse delay are of same duration: 0.5, 10, 100, 1000 or 10,000 μs. The voltage and the electrical current were monitored in all experiments with the oscilloscope Wavesurfer 422 or Wavepro 7300A, differential voltage probe ADP305 and current probe CP030 or CP031A (all from Teledyne LeCroy, New York, USA). The voltage and current waveforms of some of the pulses are shown in Fig. 1. The measured voltage pulse shape looks very similar in the cell (1A, E) and metal release (cell-free) experiments (1B, F). However, the measured current pulse shape clearly looks different in the cell (1C, G) and the metal release experiments (1D, H).

Two different electrode configurations were used. For cell experiments, we used two parallel plate stainless steel 304 electrodes with distance between the inner edges of the electrodes set at 2 mm (Fig. 2A). In metal release experiments, we used two parallel rod-shaped wire electrodes with 1 mm diameter and distance between the inner edges of the electrodes set at 4 mm (Fig. 2B). The electrode materials were 99.999% aluminum (cat. no. AL005182, Goodfellow Cambridge, England, UK), 99.99% platinum (cat. no. PT005155, Goodfellow Cambridge) and stainless steel 304 (cat. no. FE225150, Goodfellow Cambridge) composed of 17–20% Cr, <2% Mn, 8–11% Ni, <800 ppm C and Fe balance.

2.2. Cell lines and cell culture

Chinese hamster ovary CHO-K1 cell line, obtained directly from the European Collection of Authenticated Cell Cultures (ECACC, cat. no. 85051005, mycoplasma free), was grown in 25 cm^2 culture



Schematic 1. Pulses used in the study. We applied 8 standard ECT pulses of 100 µs duration with 1 Hz repetition rate or 1 burst of 400 H-FIRE pulses (for all the total energized time was 800 µs). 1 pulse in the case of H-FIRE pulses consists of the positive phase, negative phase and the interphase delay. The duration of the positive phase is 1 µs and the duration of the negative phase is 1 µs for all H-FIRE pulses. For pulses of type 1 (fixed interphase delay) the interphase delay is 1 µs, while the interpulse delay between pairs of biphasic pulses is 0.5, 10, 100, 1000 or 10,000 µs. For pulses of type 2 (symmetric delays) the interphase delay and interpulse delay between pairs of biphasic pulses are of same duration: 0.5, 10, 100, 1000 or 10,000 µs.

flasks (TPP, Switzerland) in Nutrient Mixture F-12 Ham (cat. no. N6658, Sigma-Aldrich, Missouri, United States) for 2–4 days in an incubator (Kambič, Slovenia) at 37 °C and humidified atmosphere with 5% CO_2. The growth medium (used in this composition through all experiments) was supplemented with 10% fetal bovine serum (FBS, cat. no. F9665, Sigma-Aldrich), 1.0 mM L-glutamine (cat. no. G7513, Sigma-Aldrich) and antibiotics: 1 U/ml penicillin/streptomycin (cat. no. P0781, Sigma-Aldrich) and 50 µg/ml gentamycin (cat. no. G1397, Sigma-Aldrich). Rat cardiac myoblast cell line H9c2, obtained directly from ECACC (cat. no. 88092904, mycoplasma free), was grown in 75 cm^2 culture flasks (TPP) in Dulbecco's Modified Eagle Medium (DMEM, cat. no. D6546, Sigma-Aldrich) for 2–4 days in an incubator (Kambič) at 37 °C and humidified atmosphere with 10% CO_2. The growth medium (used in this composition through all experiments) was supplemented with 10% FBS (cat. no. F2442, Sigma-Aldrich), 4.0 mM L-glutamine and antibiotics: 1 U/ml penicillin/streptomycin and 50 µg/ml gentamycin. Mouse myoblast cell line C2C12, obtained directly from ECACC (cat. no. 91031101, mycoplasma free), was grown in 75 cm^2 culture flasks in Dulbecco's Modified Eagle Medium (DMEM, cat. no. D6546, Sigma-Aldrich) for 2–4 days in an incubator at 37 °C and humidified atmosphere with 10% CO_2. The growth medium (used in this composition through all experiments) was supplemented with 10% FBS (cat. no. F9665, Sigma-Aldrich), 2.0 mM L-glutamine and antibiotics: 1 U/ml penicillin/streptomycin and 50 µg/ml gentamycin. Mouse neuronal cell line HT22, obtained directly from The Salk Institute for Biological Studies in California, USA, was grown in 25 cm^2 culture flasks in Dulbecco's Modified Eagle Medium (DMEM, cat. no. D5671, Sigma-Aldrich) for 2–3 days in an incubator at 37 °C and humidified atmosphere with 5% CO_2. The growth medium (used in this composition through all experiments) was supplemented with 10% FBS (cat. no. F9665, Sigma-Aldrich), 2.0 mM L-glutamine and antibiotics: 1 U/ml penicillin/streptomycin and 50 µg/ml gentamycin.

On the day of the experiment, cell suspension was prepared by detaching the cells with 1 × trypsin-EDTA (cat. no T4174, Sigma-Aldrich) diluted in 1 × Hank's basal salt solution (cat. no. H4641, Sigma-Aldrich). Trypsin was inactivated by F-12 Ham (CHO) or DMEM (H9c2, C2C12 and HT22) complete growth medium. Cells were transferred to a 50 ml centrifuge tube (TPP) and centrifuged 5 min at 180 g and 23 °C. The supernatant was aspirated, and cells were re-suspended in the complete growth medium F-12 Ham (CHO) or DMEM (H9c2, C2C12 and HT22) which was used as electroporation buffer.

2.3. Cell survival

For cell survival experiments, cells were re-suspended at a cell density of 2 × 10^6 (CHO), 7.5 × 10^5 (H9c2), 1 × 10^6 (C2C12) or 9 × 10^5 (HT22) cells/ml. 50 µl of the cell suspension was transferred between plate stainless steel 304 electrodes, followed by pulse treatment (for the sham control no pulses were applied).

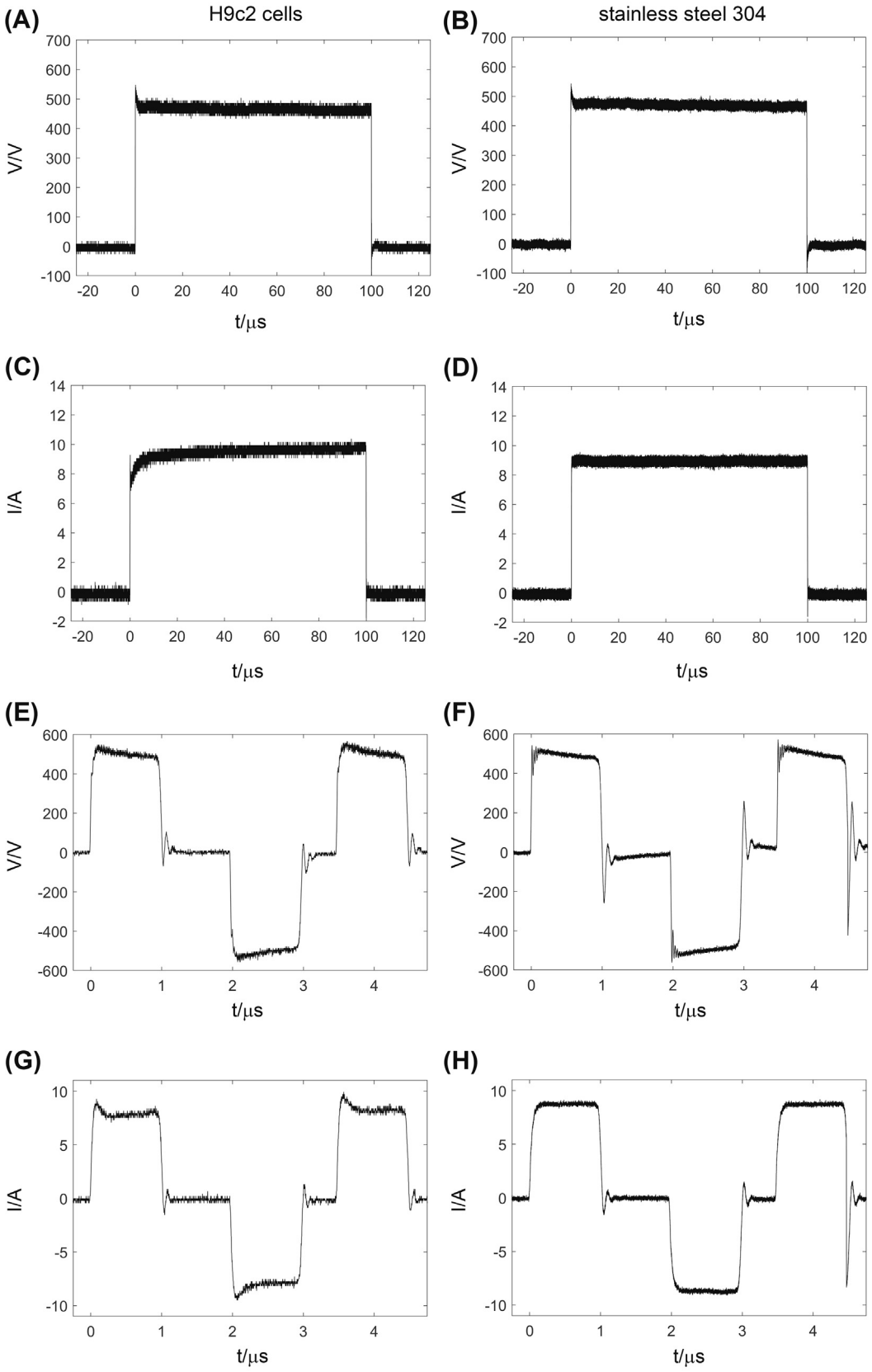
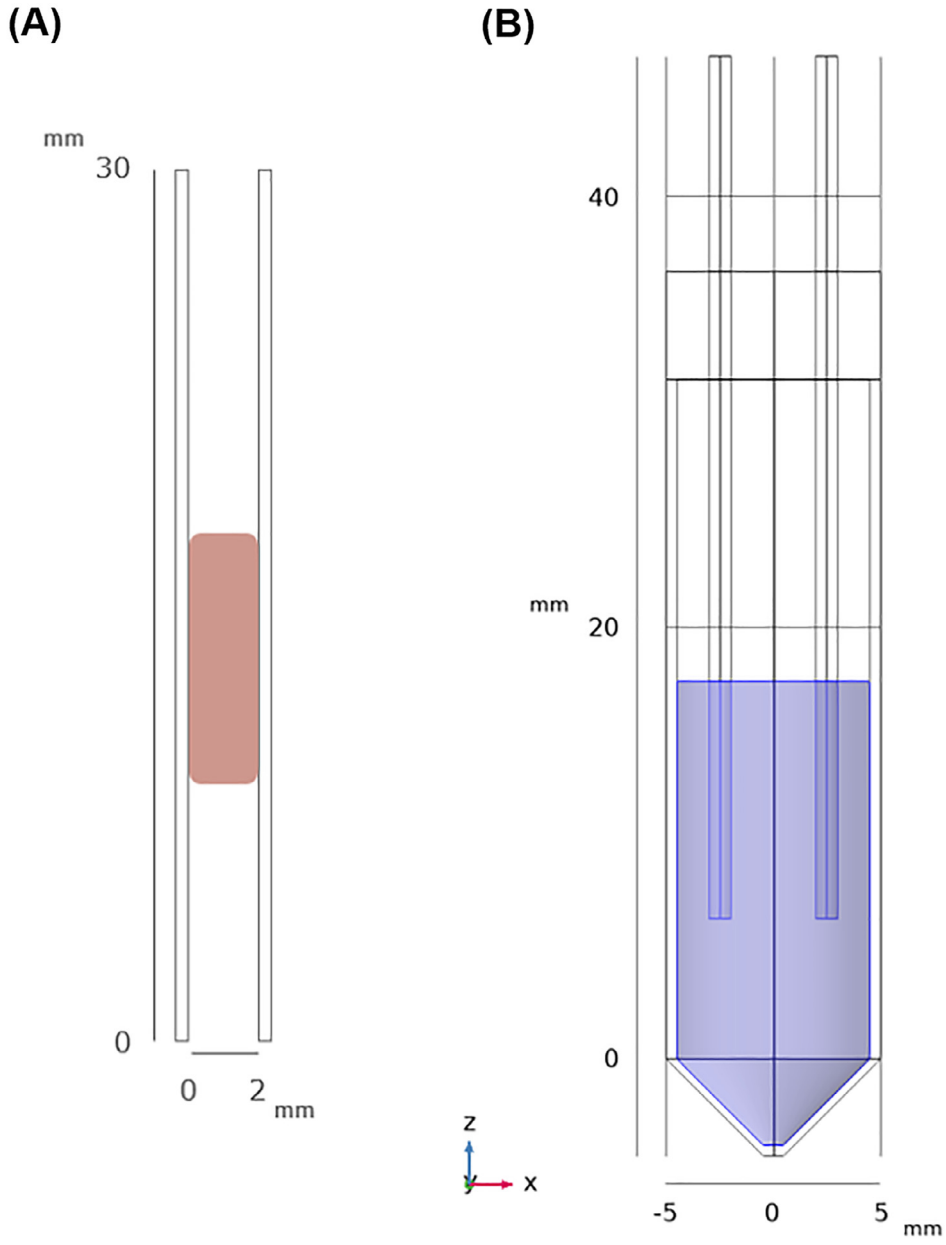


Fig. 1. Measured voltage and electrical current in (A,C, E, G) cell membrane permeabilization experiments with H9c2 cells and (B, D, F, H) metal release experiments with stainless steel 304 electrodes. (A, B, C, D) The first 100 μs pulse in the burst of 8 × 100 μs monophasic pulses at 1 Hz repetition rate (ECT pulse, see Schematic 1) and (E, F, G, H) the first 4.75 μs of the burst of 1 μs biphasic pulses of type 1 (interphase delay = 1 μs) and interpulse delay of 0.5 μs. Note the different scales.



Schematic 2. Scheme of (A) plate electrodes used in cell experiments and (B) wire electrodes used in metal release experiments. (A) The electrodes are presented as white rectangles, the distance between the inner edges of the plate electrodes is 2 mm, the cell suspension between the electrodes is colored red. (B) The wire electrodes are shown as they were used in metal release experiments: immersed in a 2 ml microcentrifuge tube filled with 1.1 ml of 0.9% NaCl. The electrodes are presented as two rectangles and they are 4 mm (inner edge-inner edge) apart, the boundaries of the microcentrifuge tube are presented by a double grey line, the 0.9% NaCl solution is colored blue.

After pulse application, 40 μl of the cell suspension was immediately transferred to a 1.5 ml microcentrifuge tube with 360 μl of complete growth medium F-12 Ham (CHO) or DMEM (H9c2, C2C12 and HT22). The cell suspension was gently vortexed. Then, 100 μl of the cell suspension was plated in a well of a flat bottom 96-well plate (TPP) in three technical repetitions. The plate was transferred to the incubator heated to 37 °C with 5% (CHO, HT22) or 10% (H9c2, C2C12) CO_2 for 24 h. Cell survival was assessed via the CellTiter 96® AQueous One Solution Cell Proliferation Assay (cat. no. G3580, Promega, Wisconsin, USA) which is a colorimetric method for determining the number of viable cells. The CellTiter 96® AQueous One Solution Cell Proliferation Assay contains the tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and the electron coupling reagent phenazine ethosulfate (PES). The MTS is bioreduced by cells into a colored formazan product that is soluble in growth medium. The quantity of formazan product as measured by absorbance at 490 nm is directly proportional to the number of living cells in culture. 20 μl of the CellTiter 96® AQueous One Solution Cell Proliferation Assay was added per well and after 2 h and 15 min incubation at 37 °C in incubator with 5% (CHO, HT22) or 10% (H9c2, C2C12) CO_2, the absorbance at 490 nm was measured with the spectrofluorometer Infinite® 200 (Tecan, Austria). The survival was calculated by first subtracting the absorbance of the blank (complete growth medium without cells) and then normalizing the average absorbance of the three technical repetitions of the sample to the absorbance of the sham controls. The experiments were repeated 3–5 times per each pulse treatment with different order of the pulse treatments.

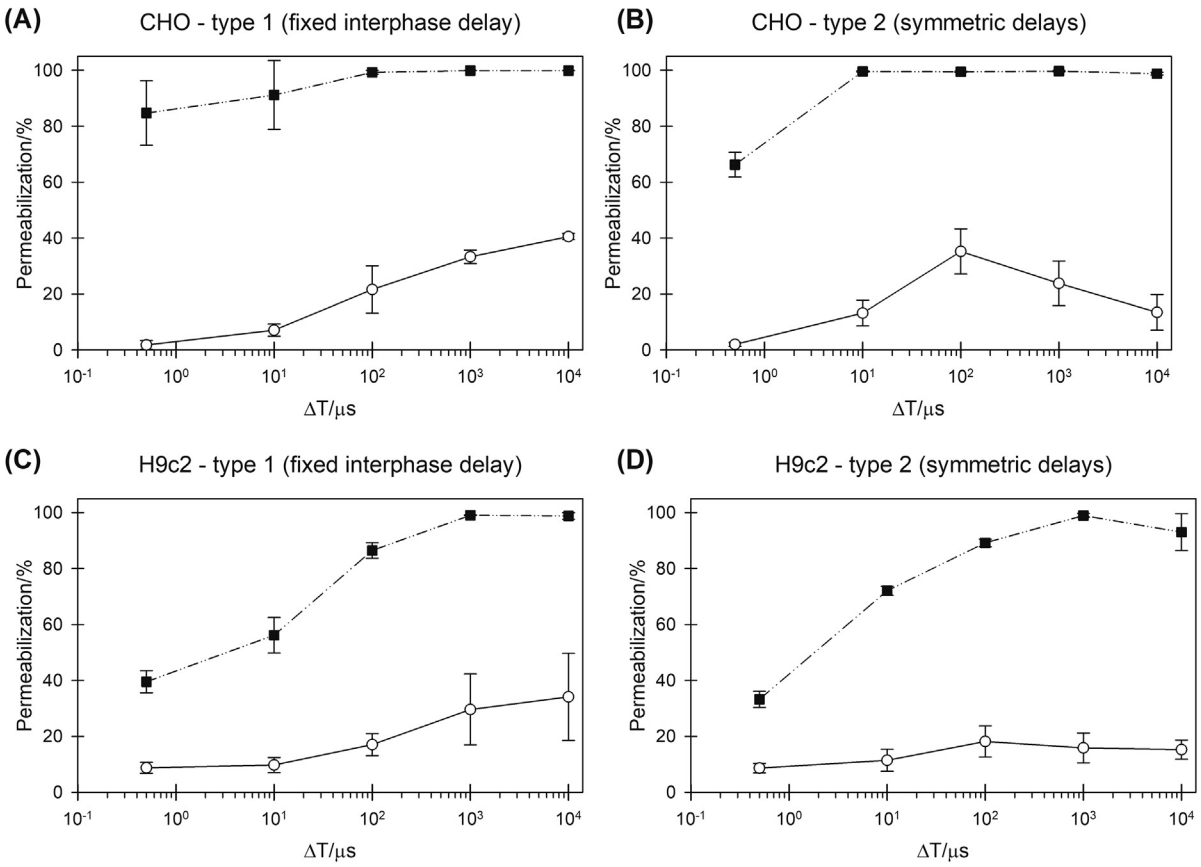


Fig. 2. Cell membrane permeabilization of CHO and H9c2 cells at different electric field strengths as a function of delay (ΔT) of biphasic H-FIRE pulses of (A, C) type 1 (fixed interphase delay) and (B, D) type 2 (symmetric interphase delay) (see Schematic 1). White circles and solid line represent the percentage of permeabilized cells at 1.5 kV/cm, black squares and dashed line represent the percentage of permeabilized cells at 2.5 kV/cm. Results are presented as an average of 3–5 repetitions. Bars represent standard deviation. Note the logarithmic scale on the horizontal axis.

2.4. Cell membrane permeabilization

For cell membrane permeabilization experiments, we used cells in suspension at a cell density of 2×10^6 (CHO) or 1×10^6 (H9c2, C2C12 and HT22) cells/ml. We wanted to use the same cell concentration as in cell survival experiments, however, we could not record 10,000 events on the flow cytometer if we used a concentration lower than 1×10^6 cells/ml. We thus decided to use 1×10^6 cells/ml for H9c2, C2C12 and HT22 cells. At this concentration, the cells should be sufficiently far apart from one another that they do not locally alter the electric field experienced by neighboring cells [56,57]. Right before application of electric pulses, the cell suspension was mixed with propidium iodide (PI, cat. no. P1304MP, Thermo Fisher Scientific, Massachusetts, USA) to final concentration of 136 μM. PI is a non-permeant fluorescent dye, which emits strong fluorescence after entering the cell and thus allows easy determination of cell electroporation and discrimination between electroporated and non-electroporated cells. 50 μl of the cells-PI mixture was transferred between plate stainless steel 304 electrodes, followed by pulse treatment. 40 μl of the treated cell suspension was transferred to a new 1.5 ml microcentrifuge tube. Three minutes after the last pulse, 150 μl of complete growth medium F-12 Ham (CHO) or DMEM (H9c2, C2C12 and HT22) was added to the cell suspension and the sample was gently vortexed and analyzed on the flow cytometer Attune NxT (Thermo Fisher Scientific). Cells were excited with blue-light laser at 488 nm, and the emitted fluorescence was detected through a 574/26 nm band-pass filter. The measurement was stopped when 10,000 events were acquired. The obtained data was analyzed using the Attune NxT software (Thermo Fisher Scientific). Single cells were separated from all events by gating. The percentage of cells with permeabilized cell membrane was determined from the histogram of PI fluorescence. The experiments were repeated 3–5 times per each pulse treatment with different order of the pulse treatments. The sham control was handled in the same way as the samples with the exception that no pulses were delivered to the cell suspension.

2.5. Metal release

0.9% (w/v) NaCl in water solution was prepared from water for ultratrace analysis (cat. no. 14211, Sigma-Aldrich) and 99.999% pure NaCl (cat. no. 204439, Sigma-Aldrich). Before the application of each pulse treatment, aluminum, platinum or stainless steel 304 wire electrodes were cleaned with sonication in the ultrasonic bath Elmasonic P (Elma Schmidbauer, Germany) filled with 1% solution of the detergent Kemex A (Kemika, Croatia) in deionized water for 2 min at room temperature. After sonication, electrodes were first rinsed with deionized water and then with acetone (cat. no. 32201, Sigma-Aldrich) and let to dry in air. Electrodes were placed in a 2 ml microcentrifuge tube (ISOLAB, Germany) filled with 1.1 ml of 0.9% NaCl solution so that 11.5 mm of the electrodes was immersed in the 0.9% NaCl solution (see Schematic 2). After application of different H-FIRE biphasic or ECT monophasic pulses (see Schematic 1) with amplitude 500 V, 1 ml of the treated 0.9% NaCl solution was transferred to a new 15 ml centrifuge and 2.5 μl of 65% HNO_3 (Merck, Germany) was added. For the sham control, the electrodes were immersed in 0.9% NaCl solution for the

Table 1
ICP-MS operating parameters for determination of elements.

Parameter	Agilent 7700 ICP-MS Type/Value		Agilent 8800 ICP-MS
Sample introduction			
Nebulizer		Micromist	
Spray chamber		Scott	
Skimmer and sampler cone		Ni	
Plasma condition			
Forward power		1550 W	
Plasma gas flow		15.0 l min^{-1}	
Carrier gas flow	0.95 l min^{-1}	0.85 l min^{-1}	0.95 l min^{-1}
Dilution gas flow	0.15 l min^{-1}	0.20 lL min^{-1}	0.10 l min^{-1}
Sample depth		8.0 mm	
Cell gas flow	10 ml He min^{-1}	/	10 ml He min^{-1}
Energy discrimination	4.5 V	3.5 V	7.0 V
Data acquisition parameters			
Isotopes monitored	^{27}Al	^{195}Pt	^{52}Cr, ^{55}Mn, ^{56}Fe, ^{60}Ni
Isotopes of internal standards	^{115}In	^{193}Ir	^{103}Rh

same duration as for other samples, but no pulses were applied. Experiments were performed in triplicates. For the 0.9% NaCl solution only, 5 μl of 65% HNO_3 was added to 2 ml of 0.9% NaCl solution in a 15 ml centrifuge. Samples were kept at 4 °C until analysis.

Total concentrations of Al, Pt, Fe, Ni, Cr and Mn in the analyzed samples were determined by inductively coupled plasma mass spectrometry (ICP-MS) against an external calibration curve. Concentrations of Al and Pt were determined on Agilent 7700 and those of Fe, Ni, Cr and Mn on Agilent 8800 ICP-MS instruments (Agilent Technologies, Tokyo, Japan). Optimized measurement parameters for the ICP-MS instruments are presented in Table 1. Calibration standard solutions of Al and Pt were prepared from Al stock solution (1000 μg Al ml^{-1} in 2–3% HNO_3) and Pt stock solution (1000 μg Pt ml^{-1} in 8% HCl), respectively, while calibration standard solutions of Fe, Ni, Cr and Mn were prepared from multi-element stock solution (containing 1000 μg/ml of each element in 6% HNO_3). All stock solutions were obtained from Merck (Germany). Calibration standards were prepared in 0.1% HNO_3 in the concentration range of 0.1–100 μg/l. The samples were, prior ICP-MS measurements, diluted 4-times with 0.1% HNO_3 for the determination of Fe, Ni, Cr and Mn and measured directly (without any dilution) for the determination of Al and Pt. All dilutions of the samples were made with ultrapure water (18.2 MΩ cm) obtained from a Direct-Q 5 Ultrapure water system (Millipore, Massachusetts, USA). To evaluate the accuracy of the ICP-MS analysis, the solution of 0.9% NaCl was spiked with standard solution containing all elements of interest to reach the final concentration of 10 μg/l in the spiked sample. Recoveries (the ratio between the measured and expected concentrations) were between 95% and 128% (N = 4) for all the elements – accuracy and precision of ICP-MS measurement for each element are listed in Table S1 in Supplementary Material.

2.6. Statistical analysis

Levene's median test was used to assess equal variance and the Shapiro-Wilk test to test normality of data ($\alpha = 0.05$).

Analysis of cell survival and membrane permeabilization data was performed separately for all the cell lines. Cell membrane permeabilization data for C2C12 were, for statistical purposes, transformed to a logarithmic scale to approximately conform to normality. Cell survival data for CHO and H9c2 and cell membrane permeabilization data for CHO and C2C12 were analyzed with analysis of variance (ANOVA). One factor was "pulse type" with two levels: type 1 (fixed interphase delay) and type 2 (symmetric delay), and the second factor was "delay" with five levels: 0.5, 10, 100, 1000 or 10,000 μs. Where statistically significant interaction or influence of one factor exists, Tukey's multiple comparison test was performed to test pairs of averages among treatments ($\alpha = 0.05$). Cell survival data for C2C12 and HT22 cells and cell membrane permeabilization data for H9c2 and HT22 cells were analyzed using the nonparametric Kruskal–Wallis test and p-values were adjusted with the post-hoc Holm method test ($\alpha = 0.05$) because the assumptions of the ANOVA were not met.

Metal release data were compared separately for Al, Pt, Fe, Mn, Cr and Ni. The concentration of released Al, Fe and Ni was, for statistical purposes, transformed to a logarithmic scale to approximately conform to normality and analyzed with one-way ANOVA. Tukey's multiple comparison test was performed to test pairs of averages among treatments ($\alpha = 0.05$). The concentration of released Pt, Cr and Mn was analyzed with the nonparametric Kruskal–Wallis test and p-values were adjusted with the post-hoc Holm method test ($\alpha = 0.05$) because the assumptions of the ANOVA were not met.

Data were processed and visualized using Microsoft Excel 2016, SigmaPlot 11.0 and R 3.5.2 [58].

3. Results

3.1. Membrane permeabilization and cell survival

First, we measured cell membrane permeabilization and cell survival of CHO and H9c2 cells after exposure to different pulses at two different electric field strengths (Fig. 2). In order to compare the effects of the delay, we opted for an electric field strength - where the differences between pulse treatments were most pronounced. In the case of membrane permeabilization, that value was determined to be 1.5 kV/cm—with increasing the electric field strength we achieved >90% membrane permeabilization with the majority of pulse treatments and thus the differences between pulses became less evident (or even undetectable). For cell survival, we chose to set the electric field strength at 2.5 kV/cm because at lower strengths we did not achieve a decrease in survival (data not shown).

Cell membrane permeabilization increased with increasing the delay of type 1 (fixed interphase delay) pulses, while for pulses of type 2 (symmetric delays) no increase or even a decrease was observed when pulses with delay of 1000 or 10,000 μs were used for all tested cell lines (Fig. 3). Because of the previously reported cancellation effect of the first phase by the second, we would expect that pulses of type 1 (which have a fixed interphase delay of 1 μs) are eqiuvalent (i.e. permeabilize the same portion of the cells) as pulses of type 2 (symmetric delays) with short interphase delay. Prolonging the interphase delay in pulses of type 2, however, should abolish the "cancellation effect" making pulses of type 2 (symmetric delays) more efficient than pulses of type 1 (fixed interphase delay) [59]. For cell membrane permeabilization, we thus did not observe "cancellation effect" irrespective of the tested cell line. For all four cell lines, we measured lower permeabilization when cells were treated with pulses of type 2 (symmetric delays) of longer delays compared to type 1 (fixed interphase delay). Exposure to monophasic 8 × 100 μs pulses of the same electric field strength (1.5 kV/cm) resulted in > 99% permeabilized cells (data not shown), which indicates that biphasic H-FIRE pulses are less effective for membrane permeabilization (consistent with previous report by Sweeney et al. [29]).

The survival of all four cell lines decreased when increasing the interphase and/or interpulse delay (Fig. 4). When increasing the delay, the total duration of the burst is increased, while the pulse repetition rate is lowered. In other words, cell survival decreased at lower pulse repetition rates. Only for CHO cells, survival was significantly lower for pulses of type 2 (symmetric delays) with 1000 μs interphase delay or longer compared to type 1 (fixed interphase delay). This is in agreement with the "cancellation effect" according to which pulses with longer interphase delay are expected to be more effective (i.e. result in lower cell survival). The lowest survival (6.0% for CHO, −2.0% for H9c2, −5.0% for C2C12 and 1.3% for HT22) was achieved with monophasic 8 × 100 μs pulses of the same electric field strength (2.5 kV/cm) (data not shown) thus suggesting that 1 μs biphasic H-FIRE of the same total duration (i.e. 800 μs) are less effective also in terms of reducing cell survival, i.e. cell kill. In other words, higher electric field strengths are needed to achieve the same biological effect when using biphasic H-FIRE pulses compared to standard ECT/IRE monophasic pulses of the same cumulative duration.

The biphasic H-FIRE pulses that most effectively permeabilized the cell membrane at 1.5 kV/cm, however, were not the most effective ones in terms of decreasing the cell survival at 2.5 kV/cm. For example, for CHO cells statistically significant higher membrane permeabilization was achieved after treatment with type 1 (fixed interphase delay) pulse with 10,000 μs delay (40.5%) than type 2 pulse (symmetric delays) with 10,000 μs delay which permeabilized 13.5% of cells. Treatment with the respective type 2 (symmetric delays) pulse, however, resulted in significantly lower cell survival (19.2%) compared to the type 1 (fixed interphase delay) pulse (48.7%). Membrane permeabilization of CHO cells after application of the type 2 (symmetric delays) pulse with 10,000 μs delay was significantly lower even than with the type 2 (symmetric delays) pulse with 100 μs delay (35.2%). However, the type 2 (symmetric delays) pulse with 100 μs delay did not decrease the cell survival at all, while the application of type 2 (symmetric delays) pulse with 10,000 μs delay resulted in 19.2% cell survival.

3.2. Metal release

We also measured the concentration of released Al ions from wire electrodes made from pure aluminum, concentration of released Pt from platinum wire electrodes and concentration of released Fe, Cr, Mn and Ni ions from stainless steel 304 wire electrodes in 0.9% (w/v) NaCl solution after delivery of different pulses (biphasic H-FIRE or monophasic ECT). As reported in Table S2 in the Supplementary Material, the metal ions of interest were detected also in the sham control sample in which the electrodes were immersed in the 0.9% NaCl solution only for a few seconds and no pulses were applied. For all the different pulse treatments, the lowest concentration of all measured metal ions was measured from platinum electrodes followed by stainless steel 304 electrodes and aluminum electrodes (Table S2 in Supplementary Material and Fig. 5). Significantly higher concentration of released Al from aluminum electrodes and Fe and Ni from stainless steel 304 electrodes was detected after treatment with 8 × 100 μs monophasic pulses than any of the biphasic H-FIRE pulses (Table S3, S5 and S8 in Supplementary Material). Although the measured Pt from platinum electrodes after treatment with ECT monophasic pulses was approximately 10 to 100 times higher than after the application of biphasic H-FIRE pulses, the differences are statistically sig-

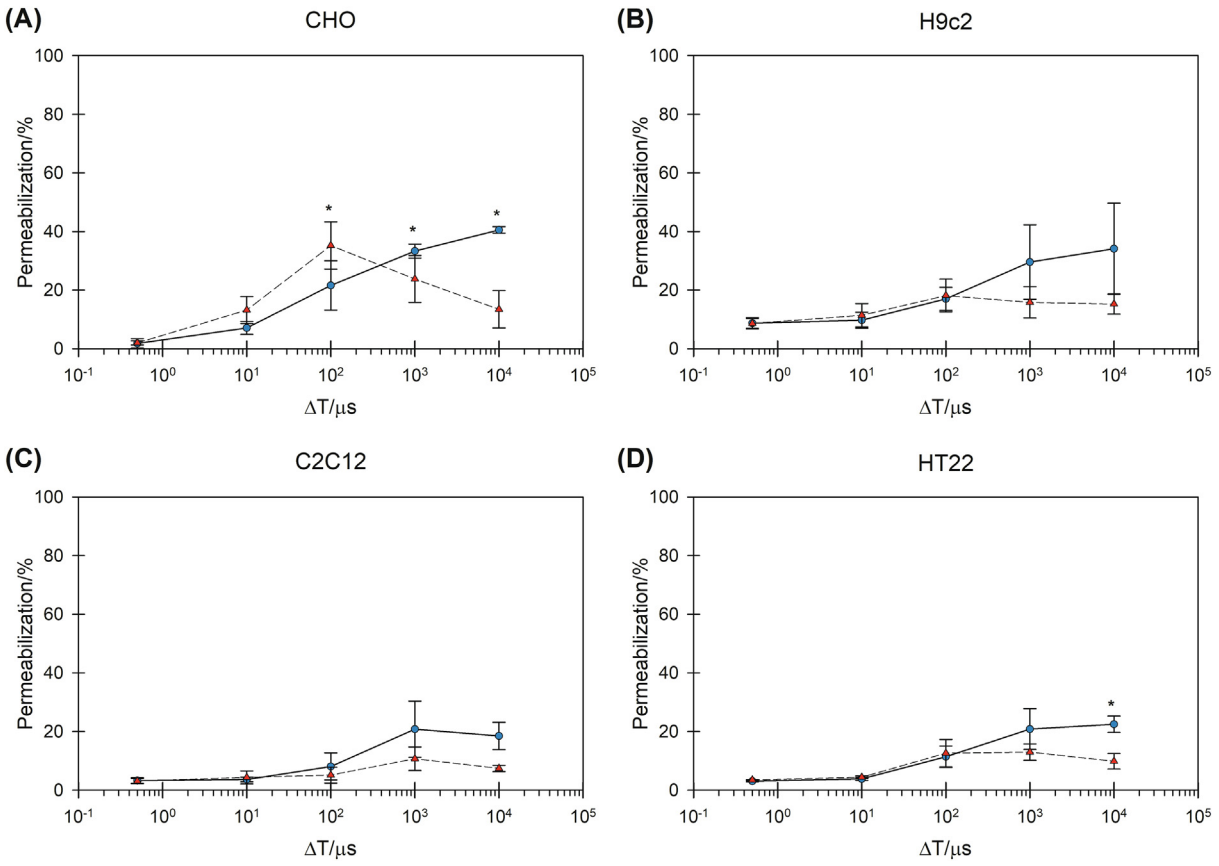


Fig. 3. Cell membrane permeabilization of (A) CHO, (B) H9c2, (C) C2C12 and (D) HT22 cells at 1.5 kV/cm as a function of delay (ΔT) of H-FIRE pulses. Blue circles and solid line represent pulses of type 1 (fixed interphase delay), red triangles and dashed line represent pulses of type 2 (symmetric delays) (see Schematic 1). Results are presented as an average of 3–5 repetitions. Bars represent standard deviation, asterisks (*) represent statistically significant ($p < 0.05$) difference between type 1 (fixed interphase delay) and type 2 (symmetric delays) pulses with the same delay. Note the logarithmic scale on the horizontal axis.

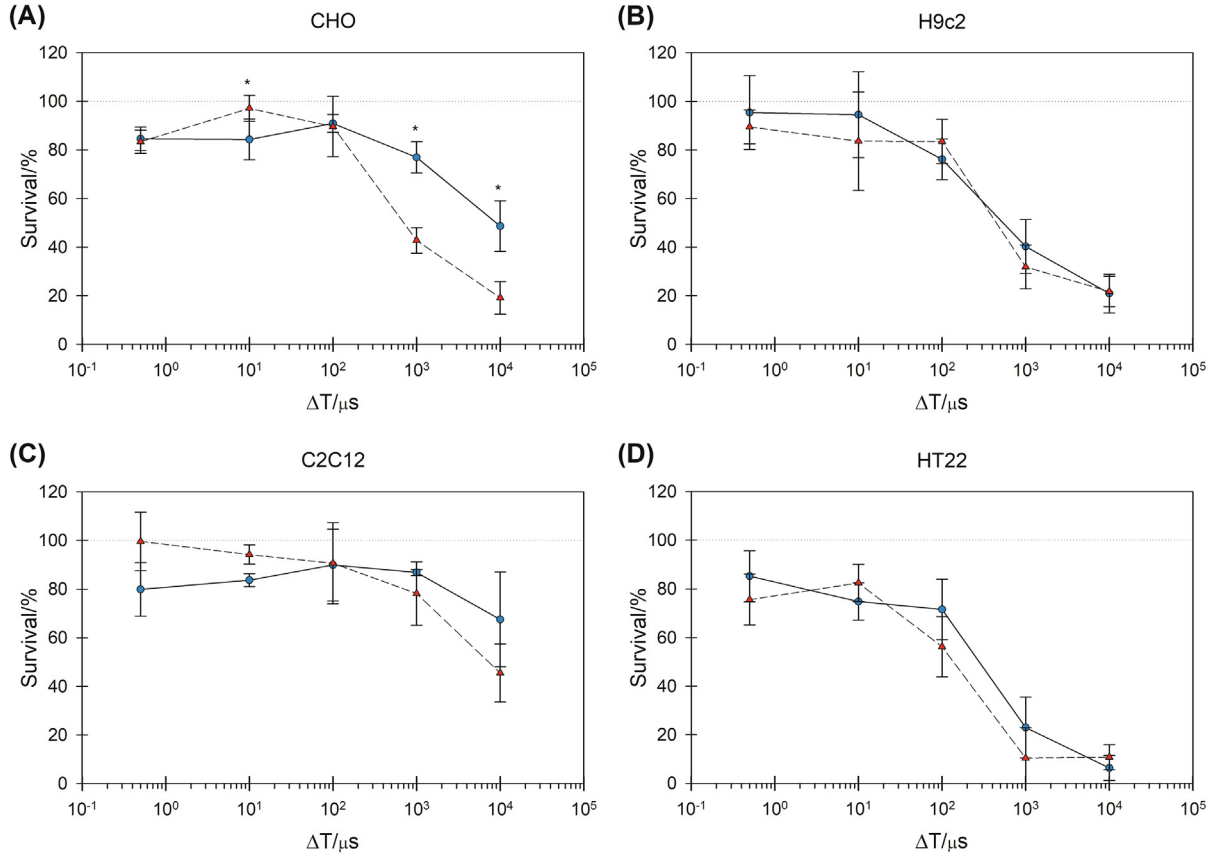


Fig. 4. Cell survival of (A) CHO, (B) H9c2, (C) C2C12 and (D) HT22 cells at 2.5 kV/cm as a function of delay (ΔT) of H-FIRE pulses. Blue circles and solid line represent pulses of type 1 (fixed interphase delay), red triangles and dashed line represent pulses of type 2 (symmetric delays) (see Schematic 1). Results are presented as an average of 3–5 repetitions. Bars represent standard deviation, asterisks (*) represent statistically significant ($p < 0.05$) difference between type 1 (fixed interphase delay) and type 2 (symmetric delays) pulses with the same delay. Note the logarithmic scale on the horizontal axis.

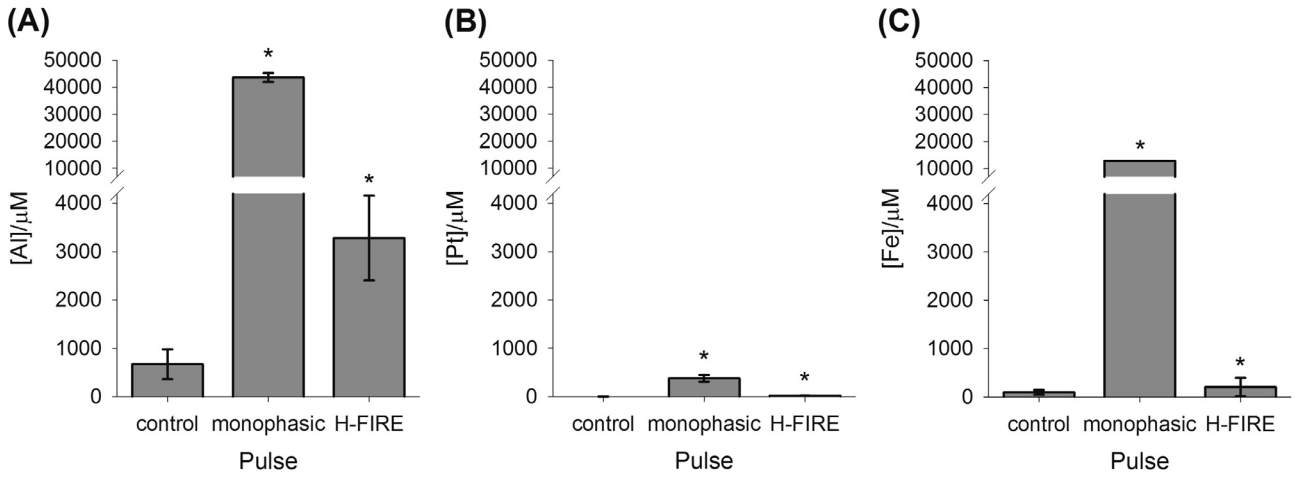


Fig. 5. Concentration of released (A) Al ions from aluminum wire electrodes, (B) Pt ions released from platinum wire electrodes and (C) Fe ions from stainless steel 304 wire electrodes in 0.9% NaCl solution determined by ICP-MS. The concentration of metal ions was measured after the electrodes were only immersed in the 0.9% NaCl solution (control), after delivery of 8 × 100 μs monophasic pulses (monophasic) with 500 V amplitude and after the delivery of a burst of 400 type 1 (interphase delay fixed at 1 μs) biphasic H-FIRE pulses with 10,000 μs interpulse delay with amplitude 500 V (H-FIRE). Results are presented as an average of 3 repetitions. Bars represent standard deviation, asterisks (*) represent statistically significant difference ($p < 0.05$) to control. Note the scale break.

nificant only between certain biphasic H-FIRE pulses and the ECT monophasic pulses (Table S2 and S4 in Supplementary Material). The interphase and interpulse delay did not have a significant effect on metal release from aluminum or from stainless steel 304 electrodes (Table S3, S5, S6, S7 and S8 in Supplementary Material). For platinum electrodes, however, significantly higher metal release was measured after the application of biphasic H-FIRE pulses with longer interphase and interpulse delay compared to biphasic H-FIRE pulses with shorter delays. For pulses of type 1 (fixed interphase delay) with 1000 and 10,000 μs interpulse delay and type 2 (symmetric delays) pulse with 1000 μs interphase and interpulse delay, we measured more Pt than for other H-FIRE

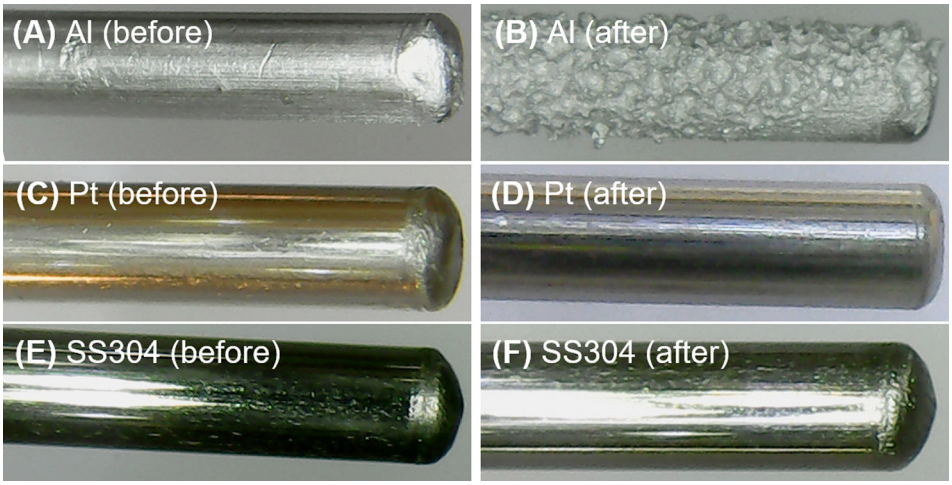


Fig. 6. Pictures of aluminum, platinum and stainless steel 304 wire electrodes (A, C, E) before and (B, D, F) after delivery of biphasic H-FIRE and monophasic ECT pulses in metal release experiments.

pulses (Table S2 and Table S4 in Supplementary Material). Electrode corrosion after pulse delivery was apparent for the aluminum electrodes (Fig. 6).

4. Discussion

The aim of this study was to investigate the effect of the interphase delay and interpulse delay between pairs of biphasic pulses (i.e pulse repetition rate) of symmetric 1 μs rectangular H-FIRE pulses on cell membrane permeabilization, cell survival/cell kill of four different cell lines—CHO (Chinese hamster ovary), H9c2 (rat cardiomyoblast), C2C12 (mouse myoblast) and HT22 (mouse neuronal)—and release of metal ions from aluminum, platinum, and stainless steel 304 electrodes.

4.1. Cell survival and membrane permeabilization

We showed on four cell lines that it is possible to increase the effectiveness (i.e. achieve lower cell survival) of short biphasic H-FIRE pulses by increasing the interphase and interpulse delay, i.e. reducing pulse repetition rate. This is in agreement with previous reports that lower pulse repetition rates are more effective [60–62] and also with the findings of Arena et al. [22] that the addition of a delay between the positive and negative phase in H-FIRE pulses results in more efficient cell kill. However, even biphasic H-FIRE pulses with longer delays were less effective than 8 × 100 μs monophasic pulses, requiring the use of higher electric field strengths to achieve the same biological effect. For CHO cells, we showed that the previously reported cancellation effect of the positive phase by the negative phase [29,31,35,59] exists for cell survival for interphase delay of up to 100–1000 μs. Pulses of type 2 with interphase delays of 0.5, 10 or 100 μs were no more effective (i.e. they did not decrease the cell survival) than pulses of type 1 (with 1 μs of interphase delay). However, when prolonging the interphase delay of pulses of type 2 (symmetric delays) to 1000 and 10,000 μs and keeping the interphase delay of type 1 pulses at 1 μs, the "cancellation effect" was abolished and pulses of type 2 became more effective than pulses of type 1. We did not observe the cancellation effect for cell survival with the three other tested cell lines (H9c2, C2C12 and HT22). However, knowing the composition of the electroporation medium can influence the response of the cells to the electric pulses [63–65], it is important to note that the cells were electroporated in different media (CHO in F-12 Ham and the others in variations of the DMEM medium).

The effect of the interphase and interpulse delay on membrane permeabilization on the other hand seems to be more complex. The shape of the permeabilization curve (Fig. 3) is surprisingly different from the previously reported cancellation effect for biphasic nanosecond and microsecond pulses [31,34,35,59] since we observed lower membrane permeabilization with pulses of type 2 (symmetric delays) of longer interphase delays (1000 or 10,000 μs) than pulses of type 1 with 1 μs interphase delay. Our results also suggest that higher membrane permeabilization does not always result in lower cell survival and vice versa that low cell survival is not necessarily a consequence of high membrane permeabilization. This indicates a more complex interplay between membrane permeabilization and cell survival and suggests that cell survival is affected also by other factors besides membrane permeabilization.

The majority of previous studies has focused on the use of biphasic H-FIRE pulses for tissue ablation [22,23,25]. Tissue ablation is based on irreversible electroporation and thus an effective protocol must result in low cell survival. Recently, short biphasic H-FIRE pulses have also been explored for use in ECT [30]. ECT is based on reversible electroporation and the authors have shown *in vitro* that is possible to use also biphasic H-FIRE pulses (which they named high frequency electroporation (HF-EP) pulses) for cisplatin ECT—but again with higher electric field strengths than the commonly used 8 × 100 μs monophasic pulses. Our results suggest that for ECT and other applications based on reversible electroporation also 1 μs biphasic H-FIRE pulses with interphase delay of up to 100–1000 μs can be used since we achieved high membrane permeabilization without decrease in cell survival with their application.

H-FIRE pulses have attracted attention because it has been shown that their application results in reduced pain and muscle contractions compared to monophasic pulses of the same amplitude. The results of a numerical model study [66] indicate that it is possible to avoid nerve stimulation with the use of bursts of short biphasic pulses which achieve the same IRE efficacy as conventional 100 μs monophasic pulses because the stimulation thresholds raise faster than the irreversible electroporation thresholds. However, higher electric field strength is required to achieve the same effect as with monophasic pulses. It thus remains to be tested also experimentally if pain and muscle contractions remain reduced when using biphasic H-FIRE pulses at amplitudes that pro-

duce the same biological effect as monophasic pulses. Reduced muscle contraction was so far shown after the application of biphasic pulses of 1, 2, 5 or 10 μs duration of each phase and symmetric interphase delay and interpulse delay between biphasic pulses of 2 or 5 μs [22,23,25,26]. It would be thus necessary to test pulse-induced muscle contractions with the application of biphasic H-FIRE pulses of longer interphase and interpulse delays to see if such pulses do not cause more intense contractions.

4.2. Metal release

This is the first report of metal release from aluminum, platinum and stainless steel 304 wire electrodes after treatment with short biphasic H-FIRE pulses. We opted for electrodes made from pure aluminum in the absence of specification of material from which commercial aluminum cuvettes are made. The amount of metal release depends largely on the particular electrode material—the measured concentration of Al ions from aluminum electrodes was higher than the concentration of released Fe from stainless steel 304 and both were higher than the measured concentration of Pt from platinum electrodes. However, the application of some pulses resulted in higher concentration of released Pt from platinum electrodes than Cr and Mn from stainless steel 304 electrodes. The highest measured concentration of Pt ions from platinum electrodes (after the application of monophasic 8 × 100 μs pulses) was lower than the lowest measured concentration of Al ions released from aluminum electrodes (in sham control samples in which the electrodes were only immersed in the 0.9% NaCl solution and no pulses were delivered). In agreement with previous reports [45], the application of biphasic H-FIRE pulses resulted in significantly lower metal dissolution compared to monophasic 8 × 100 μs pulses for aluminum and stainless steel 304 electrodes, however, for platinum electrodes the metal release after application of biphasic H-FIRE pulses was not always statistically significant lower than for monophasic 8 × 100 μs pulses. Different delays of the 1 μs biphasic H-FIRE pulses did not result in significant differences in concentrations of released metals from aluminum and stainless steel 304 electrodes in the range of pulse parameters tested. However, more Pt ions were detected after biphasic H-FIRE pulses of type 1 (fixed interphase delay) and type 2 (symmetric delays) pulses with longer delays were applied compared to biphasic H-FIRE pulses with shorter delays. Additional work is needed to explain how the delays affect the release of Pt.

We measured an increase (although not statistically significant for some of the tested metals) in concentration of metal ions also in sham control sample where electrodes were only immersed in 0.9% NaCl solution and no pulses were delivered. This metal release could be explained by the fact that when an electrode is placed into an electrolyte, a so-called double layer is formed immediately, even if no external voltage is applied. The double layer consists of a layer of charged particles and/or orientated dipoles that exist at the electrode-electrolyte interface. Chemical reactions occur immediately and electrons are transferred between the electrode and the electrolyte which results in formation of an electric field between the electrode and the layer of ions that influences further chemical reactions and promotes oxidation reactions [54].

The differences in concentrations of Fe, Cr, Mn and Ni determined after the delivery of the same pulse with the stainless steel 304 electrodes are probably related to different concentrations of elements in stainless steel 304 and differences in standard potentials of reduction half reactions. The stainless steel 304 wire from which our electrodes were made is, according to manufacturer's specification, composed of 18% Cr, 10% Ni, <2% Mn, <800 ppm C and the rest is Fe. We measured the concentration of Fe, Cr, Ni and Mn. After the delivery of 8 × 100 μs monophasic pulses, the highest concentration of Fe ions was measured followed by Cr, Ni and Mn (proportional to the stainless steel 304 composition). However, after the application of biphasic H-FIRE pulses, we detected a similar concentration of released Cr and Mn, slightly higher concentration of released Ni and the highest concentration of Fe, which is not proportional neither to the stainless steel 304 composition or to the standard potentials of the oxidation reactions. Further work would be needed in order to understand the effect of different pulses on the concentration of released metals.

The medium in which metal release experiments were performed was a pure 0.9% NaCl solution in water. We are aware that such solution does not mimic real-life electroporation media or tissue, however, it allowed us to detect very small amounts of metal ions. In preliminary metal release experiments, we used growth medium F-12 Ham (data not shown), however, this medium already contains some metals, especially Fe and Mn, in concentrations of several orders of magnitude higher than the concentrations of released metal ions from electrodes measured in our experiments.

A limitation in our study was that we used electrodes of different geometry for the cell experiments (plate electrodes) and metal release experiments (wire electrodes) resulting also in different contact surface and current densities. The contact surface for the plate electrodes is approximately 1.5 times smaller than for wire electrodes, resulting in an approximately 1.5 times larger current density for plate electrodes. While the plate electrodes provide a relatively homogeneous field in the suspension, the field is nonhomogeneous when wire electrodes are used. We still believe that the following conclusion based on our results is valid: for biphasic H-FIRE pulses of 1 μs duration, it is possible to increase the delay up to 10,000 μs and to improve the effectiveness by reducing the pulse repetition rate without drastically increasing metal release from electrodes. It is important to note also that the delivery of pulses, especially 8 × 100 μs monophasic, caused visible corrosion of the aluminum electrodes that also changed the electrode geometry. No corrosion was observed for platinum and stainless steel 304 electrodes.

Aluminum, platinum and stainless steel are commonly used materials for electrode fabrication. It was shown previously that the use of aluminum, platinum and stainless steel electrodes results in release of the electrode material [41–43,45,47,48,54,67–69]. Pt metal is biologically inert [70], however, Al and Fe ions showed to be cytotoxic and to affect the biochemistry of electroporated cells [42,45,71]. The effects of other metals from which the stainless steel 304 electrodes are composed (Mn, Cr, Ni) on electroporated cells has not been studied yet to the best of our knowledge. However, these metals have been shown to be toxic and cancerogenic or to have reproductive and developmental toxicity [71–74]. In some *in vitro* cell studies, Mn in the concentration from 2 μM to a few hundred μM already affected cells [75–77]. The concentration of released Mn from stainless steel 304 electrodes in our experiments was also in this concentration range. Cr(VI) in submicromolar concentration has been shown to decrease the survival of cells *in vitro* [77,78], while in our experiments the concentration of released Cr was in the micro- and milimolar range (although we do not know the oxidation state of Cr). The concentration of different Ni compounds that reduced the cell survival/cloning efficiency and caused transformations in *in vitro* cell studies, was reported to be in the micro- and milimolar range [79–81], which is in the same range as the Ni released from stainless steel 304 electrodes in our experiments.

5. Conclusions

Short biphasic H-FIRE pulses with longer delays (i.e. lower pulse repetition rates) are more effective in terms of decreased survival

(achieving cell kill) and do not significantly increase electrolytic contamination with metal ions from the electrodes. Lower pulse repetition rates also reduce temperature increase [82], but prolong the treatment time. To achieve the same biological effect as with 8 × 100 μs monophasic pulses, however, a higher electric field strength is needed. Higher cell membrane permeabilization does not always result in lower cell survival which indicates a more complex interplay between cell membrane permeabilization and cell survival. It still has to be determined if application of short biphasic H-FIRE pulses with higher voltage results in reduced muscle contractions and lower metal release from electrodes compared to commonly used 8 × 100 μs monophasic pulses with equivalent biological effect.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioelechem.2020.107523.

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