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research article

The use of high-frequency short bipolar pulses in cisplatin electrochemotherapy in vitro

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Radiol Oncol 2019; 53(2): 194-205.

Received 4 February 2019
Accepted 23 April 2019

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Disclosure: No potential conflicts of interest were disclosed.

Background. In electrochemotherapy (ECT), chemotherapeutics are first administered, followed by short 100 µs monopolar pulses. However, these pulses cause pain and muscle contractions. It is thus necessary to administer muscle relaxants, general anesthesia and synchronize pulses with the heart rhythm of the patient, which makes the treatment more complex. It was suggested in ablation with irreversible electroporation, that bursts of short high-frequency bipolar pulses could alleviate these problems. Therefore, we designed our study to verify if it is possible to use high-frequency bipolar pulses (HF-EP pulses) in electrochemotherapy.

Materials and methods. We performed *in vitro* experiments on mouse skin melanoma (B16-F1) cells by adding 1–330 µM cisplatin and delivering either (a) eight 100 µs long monopolar pulses, 0.4–1.2 kV/cm, 1 Hz (ECT pulses) or (b) eight bursts at 1 Hz, consisting of 50 bipolar pulses. One bipolar pulse consisted of a series of 1 µs long positive and 1 µs long negative pulse (0.5–5 kV/cm) with a 1 µs delay in-between.

Results. With both types of pulses, the combination of electric pulses and cisplatin was more efficient in killing cells than cisplatin or electric pulses only. However, we needed to apply a higher electric field in HF-EP (3 kV/cm) than in ECT (1.2 kV/cm) to obtain comparable cytotoxicity.

Conclusions. It is possible to use HF-EP in electrochemotherapy; however, at the expense of applying higher electric fields than in classical ECT. The results obtained, nevertheless, offer an evidence that HF-EP could be used in electrochemotherapy with potentially alleviated muscle contractions and pain.

Key words: electroporation; electrochemotherapy; high-frequency bipolar pulses; cisplatin; cell survival; drug uptake

Introduction

When a cell is exposed to a sufficiently high electric field, the permeability of the cell membrane rapidly increases due to membrane electroporation. This transiently increased membrane permeability allows for the exchange of ions and molecules between inside and outside of the cells.[1-4] If cells recover and survive, electroporation is called reversible. If the damage is too extensive, resealing too slow, cells cannot restore the homeostasis, and they die, electroporation is called irreversible. Electroporation depends on the characteristics of the cells (shape, size, cytoskeleton structure, membrane composition) and the electrical parameters (amplitude, duration, number of electrical pulses and repetition frequency). Electroporation is used in medicine[5–10] (electrochemotherapy, gene therapy, irreversible electroporation as an ablation technique and transdermal drug delivery), in biotechnology[11,12], (inactivation of microorganisms, extraction of biomolecules from microorganisms and plants, genetic transformation of microorganisms) and food processing.[13,14]

Electrochemotherapy (ECT) is used in clinics to treat patients with various types of cancer (*e.g.*, melanoma, head-neck tumors, breast, liver, intestinal tract, brain cancer).[15] The standard operating procedures for electrochemotherapy include intratumoral or intravenous delivery of the chemother-

doi: 10.2478/raon-2019-0025

apeutic drug, followed by the application of high-voltage 100 μs long monopolar pulses to the tumor area.[16–19] Two chemotherapeutics are currently used in clinics - bleomycin[20,21] and cisplatin (cis-diaminodichloroplatin (II), CDDP).[22,23] The cytotoxicity of the chemotherapeutic drugs is increased as the delivered pulses increase cell membrane permeability, and facilitate the influx of drugs into the tumor cells.[24,25] Drawbacks of the application of 100 μs long monopolar, high-voltage electric pulses at repetition frequency 1 Hz are pain, muscle contractions[26-28], the need to use muscle relaxants and general anesthesia[29] and to synchronize pulses with the heart rhythm.[30,31] These problems can be alleviated for example by applying pulses at higher frequency[26], by using special designs of electrodes[32,33], or, as it was recently demonstrated, by delivering bursts of short high-frequency bipolar pulses, *i.e.*, the so-called high-frequency irreversible electroporation (H-FIRE) pulses.[33-37] Treatment with H-FIRE pulses, however, comes at the expense of delivering pulses of considerably higher amplitudes.[38]

Mostly, H-FIRE pulses have been used to achieve irreversible electroporation. However, they can also be used to increase the uptake of molecules into cells[38] which could be applied in achieving reversible electroporation to treat tumors with electrochemotherapy. Thus, this study aimed to determine whether H-FIRE pulses could also be used in electrochemotherapy which we call high-frequency electroporation (HF-EP).

We delivered 8 bursts of 50 bipolar pulses, each consisting of 1 μs long positive and negative pulse, with a 1 μs delay between them with electric field from 0.5–5 kV/cm. We compared HF-EP to classic eight monopolar 100 μs long pulses, delivered at frequency 1 Hz, with electric field from 0.4–1.2 kV/cm. Cisplatin concentration was from 1 μM to 330 μM. We showed that HF-EP pulses indeed cause higher cytotoxicity of cisplatin *in vitro*; however, in comparison to the standard 100 μs long monopolar pulses, higher voltage pulses must be delivered to obtain comparable effect.

Materials and methods

Cell preparation

Mouse skin melanoma cell line B16-F1, obtained from the European Collection of Authenticated Cell Cultures (ECACC, cat. no. 92101203, Sigma Aldrich, Germany, mycoplasma free), was grown 2–4 days in 75 cm^2 cell culture flasks (TPP, Austria) until 80% confluency in Dulbecco's Modified Eagle's Medium (DMEM, cat. no. D5671, Sigma Aldrich, Germany) in an incubator (Kambič, Slovenia) at 37°C and humidified 5% CO_2. DMEM, used in this composition for all *in vitro* experiments, was supplemented with 10% fetal bovine serum (cat. no. F7524, Sigma Aldrich, Germany), 2 mM L-glutamine (cat. no. G7513, Sigma Aldrich, Germany) and antibiotics, 50 μg/ml gentamycin (cat. no. G1397, Sigma Aldrich, Germany), 1 U/ml penicillin-streptomycin (cat. no. P11-010, PAA, Austria).

Cell suspension was prepared by detaching the cells in the exponential phase of growth with 10x trypsin-EDTA (cat. no T4174, Sigma Aldrich, Germany), diluted 1:9 in Hank's basal salt solution (cat. no. H4641, Sigma Aldrich, Germany). After no more than 3 minutes, trypsin was inactivated by adding DMEM, and cells were transferred to a 50 ml centrifuge tube. Then, the cells were centrifuged (5 min, 180 g, 21°C) and re-suspended in DMEM at concentration $5x10^6$ cells/ml (experiments to measure the optimal parameters of electroporation and resealing rate of cells), $5x10^4$ cells/ml (experiments to measure the cytotoxicity of cisplatin without electroporation) or $2.2x10^7$cells/ml (experiments to measure the cytotoxicity of cisplatin with electroporation). We performed experiments with different cell densities due to different requirements for cell number and sensitivities of the chosen assays. Even at the highest concentration ($2.2x10^7$ cells/ml) we were still well below the concentration where shielding of the electric field and decreased uptake were observed.[39]

Electroporation setup

Two types of pulses were applied – 100 μs long monopolar pulses (*i.e.* classical electrochemotherapy) and bursts of short bipolar pulses (HF-EP pulses). They were applied between plate stainless-steel electrodes with 2 mm distance.[40] Between pulses, electrodes were cleaned in potassium-phosphate buffer (KPB, 10 mM KH_2PO_4/K_2HPO_4 in ratio 40.5:9.5, 1 mM $MgCl_2$, 250 mM sucrose) and dried with sterile gauze. 100 μs long monopolar pulses (8 pulses, delivered at repetition frequency 1 Hz, Figure 1A) of different voltages (80, 120, 160, 200, 240 V) were delivered by the commercially available BetaTech pulse generator (Electro cell B10, BetaTech, France) or BTX Gemini X2 pulse generator (Harvard Apparatus, USA). Short bipolar pulses of different voltages (HF-EP protocol, 100 V to 1000 V with a step of 100 V, Figure 1B) were delivered by a laboratory prototype pulse generator (University of Ljubljana) based on H-bridge digital

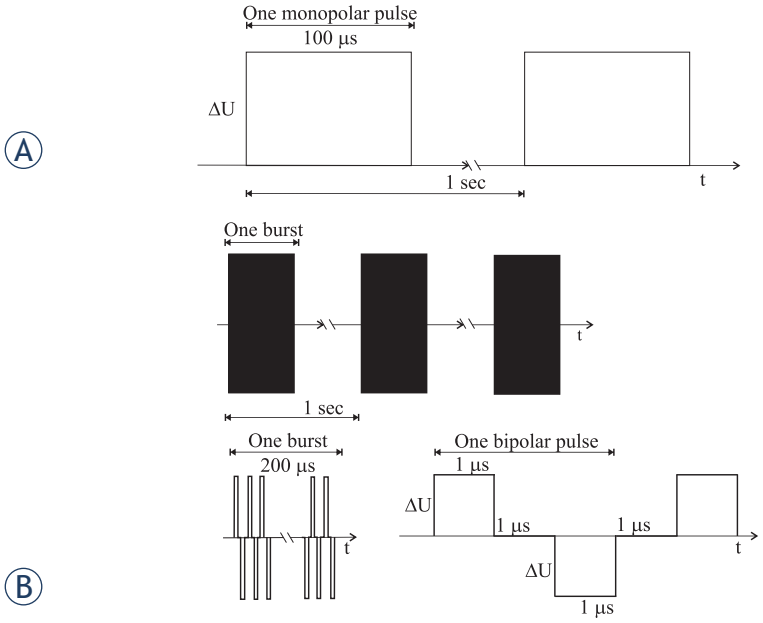

FIGURE 1. Scheme of the applied pulses. **(A)** 100 µs long monopolar pulses of amplitude ΔU (80 V – 240 V in a step of 40 V) were applied with a repetition frequency of 1 Hz. **(B)** Short bipolar pulses (HF-EP). Above: 8 bursts were applied with a repetition frequency of 1 Hz. Down left: One burst was 200 µs long and consisted of 50 bipolar pulses. Below right: One bipolar pulse of amplitude ΔU (100 V – 1000 V in a step of 100 V) consisted of 1 µs long positive pulse, 1 µs long negative pulse (both of voltage ΔU) with a 1 µs long delay between pulses.

amplifier with 1 kV MOSFETs (DE275-102N06A, IXYS, USA).[38,41] Short bipolar pulses were delivered in 8 bursts at repetition frequency 1 Hz, each containing 50 short bipolar pulses of 1 µs positive and 1 µs negative pulse. The delay between short bipolar pulses and between positive and negative pulse was 1 µs. The on-time (the time when the voltage was different from zero) of the HF-EP pulses was 800 µs, equivalent to the duration of the eight 100 µs long monopolar pulses. The duration of one short bipolar pulse was chosen as it successfully permeabilized cell membranes as previously demonstrated by an increased uptake of a fluorescent dye.[38] The voltage and the current were monitored in all experiments with an oscilloscope Wavesurfer 422, 200 MHz, a differential voltage probe ADP305 and a current probe CP030 or AP015, all from LeCroy, USA to ensure that delivered voltage and current were consistent at the same settings even if delivered with different generators.

Determination of permeability and resealing

In permeability experiments, just before pulse application, 60 µl of cell suspension was mixed with 6 µl of 1.5 mM propidium iodide (PI) (136 µM final concentration). In resealing experiments, PI was not added before pulse application but after electroporation. 60 µl of the cell suspension was electroporated, and 50 µl of the treated sample was transferred to a 1.5 ml centrifuge tube. In resealing experiments, 5 µl of PI (136 µM final concentration) was added to 50 µl of the treated sample 2 min, 5 min, 10 min or 20 min after pulse delivery. Two minutes after electroporation (permeability experiments) or PI addition (resealing experiments), the samples were diluted in 100 µl of KPB, and vortexed. The uptake of propidium was measured on the flow cytometer (Attune NxT; Life Technologies, Carlsbad, CA, USA). Cells were excited with a blue laser at 488 nm, and the emitted fluorescence was detected through a 574/26 nm band-pass filter. The measurement was finished when 10,000 events were acquired. Single cells were separated from all events by gating. Obtained data were analyzed using the Attune NxT software. The percentage of permeabilized cells was determined from the histogram of PI fluorescence.

Cell survival following electroporation only

60 µl of the cell suspension was electroporated, 50 µl was transferred to a 15 ml centrifuge tube, and two minutes after pulses delivery, the samples were diluted in 450 µl of DMEM and mixed with a pipette. When all the samples were finished, $5x10^4$ cells were transferred in each well on a 96-well plate in three technical repetitions. After 24 h of incubation at 37°C and humidified 5% CO_2, the survival assay was performed. 20 µl of MTS (CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS), Promega, USA) was added per well according to manufacturer's instructions and left in an incubator for 2h. MTS assay was used to quantify the number of viable cells evaluating their metabolic activity by measuring the formazan absorbance at 490 nm. After 2 h, the absorbance was measured on a spectrofluorometer (Tecan Infinite 200; Tecan, Grödig, Austria). Cell survival was calculated by first subtracting the background (only DMEM and MTS) from all measurements and then normalizing the absorbance of the treated samples to the absorbance of the control samples.

Cytotoxicity of cisplatin without electroporation

On the first day, $5x10^3$ B16-F1 cells were seeded per well on a 96-well plate and left for one day in an in-

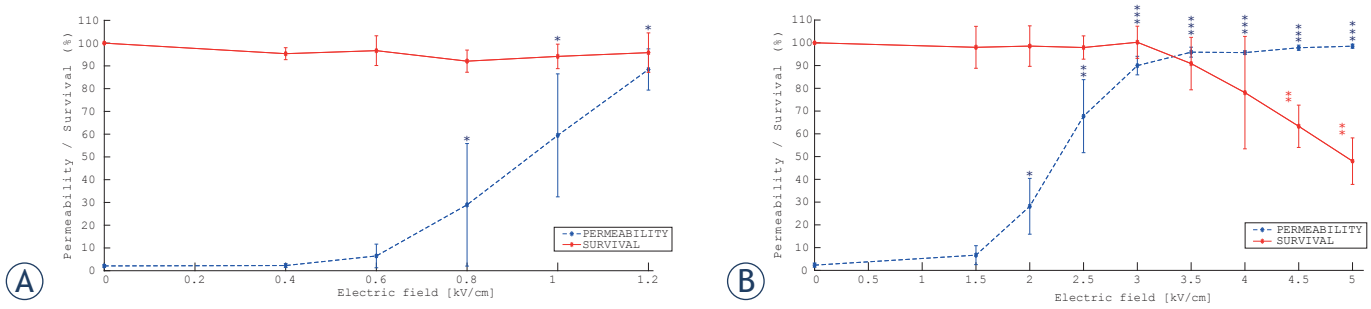

FIGURE 2. Cell membrane permeability and cell survival as a function of electric field for **(A)** 8 x 100 µs long monopolar pulses, delivered at repetition frequency 1 Hz; **(B)** 8 bursts of short bipolar pulses (HF-EP) of 1-1-1-1 µs, delivered at repetition frequency 1 Hz. Each data point was repeated 3–4 times (mean ± standard deviation). In the control sample, no pulses were applied. Note different scales on the x-axes. On **(A)**, the threshold of electroporation was at 0.8 kV/cm (P = 0.029, t-test) and survival did not decrease in comparison with control (one-sample t-test). On **(B)** the threshold of electroporation was at 2 kV/cm (P = 0.022, t-test), while the survival decreased at 4.5 kV/cm (P = 0.004, one-sample t-test). In Figure **2B**, blue asterisks refer to permeability curve and red asterisks to the survival curve.

cubator (Kambič, Slovenia) at 37°C and humidified 5% CO_2. On the second day (24 h after cell seeding), the 3.3 mM stock cisplatin (Accord HealthCare, Poland) was diluted in 0.9% NaCl (physiological solution) to obtain the 10x higher concentration of cisplatin than desired with the cells (1, 10, 100, 330 µM). Diluted cisplatin was then mixed with the DMEM in ratio 1:9 and cells were incubated in DMEM with cisplatin for 10 min, 1 h, 24 h or 48 h. After the indicated time, DMEM with cisplatin was substituted with DMEM only. On the fourth day (72 h after cell seeding), the MTS survival assay was performed as described in the subsection *Cell survival following electroporation only*.

Electroporation with cisplatin

We performed two types of experiments. We applied: 1) different electric fields at fixed cisplatin concentration (100 µM) to evaluate the effect of electric field on cell death; 2) fixed electric field (optimal value – long monopolar pulses E = 1.2 kV/cm and short bipolar (HF-EP) pulses E = 3 kV/cm) with different cisplatin concentrations to evaluate the effect of cisplatin concentration on cell survival. Optimal parameters of electroporation were determined with experiments described in the subsections *Determination of permeability and resealing*, and *Cell survival following electroporation only* and were chosen as those where the highest uptake of propidium iodide (*i.e.*, highest cell membrane permeability) and the highest cell survival were obtained.

The 3.3 mM stock cisplatin was diluted in 0.9% NaCl to obtain the desired concentrations of cisplatin with the cells (1, 10, 100, 330 µM) in both experiments. The drug was prepared fresh for each experiment. Right before experiments, 120 µl of cell suspension was mixed with 13.3 µl of cisplatin. 60 µl of the cell suspension with added cisplatin was transferred between the electrodes, and long monopolar or short bipolar (HF-EP) pulses were delivered (electroporation+cisplatin). The remaining 60 µl was used as a control and was transferred between the electrodes, but no pulses were delivered (only cisplatin). 50 µl of the treated and control sample were transferred in a 15 ml centrifuge tube. 10 minutes after pulse delivery, the samples were diluted 40x in full DMEM and vortexed. $5.5x10^3$ cells were transferred in each well on a 96-well plate in triplicates. The survival assay was performed as described in the subsection *Cell Survival* after 72 hours as previously suggested.[42]

Statistical analysis

Statistical analysis was performed using the software SigmaPlot v11 (Systat Software, San Jose, CA). We performed the t-test or one sample t-test when comparing two groups or one group towards normalized control. We performed the 1-way or 2-way ANOVA if the normality test was passed or the ANOVA on ranks if the normality test failed with the post-hoc Tukey test. The details on the performed test and the obtained P-value are written in respective figure captions in the Results section. On figures, one asterisk (*) signifies $P < 0.05$, two (**) $P < 0.01$ and three (***) $P < 0.001$.

Results

Electroporation with propidium iodide

First, we performed experiments to determine the optimal parameters of electroporation to be later

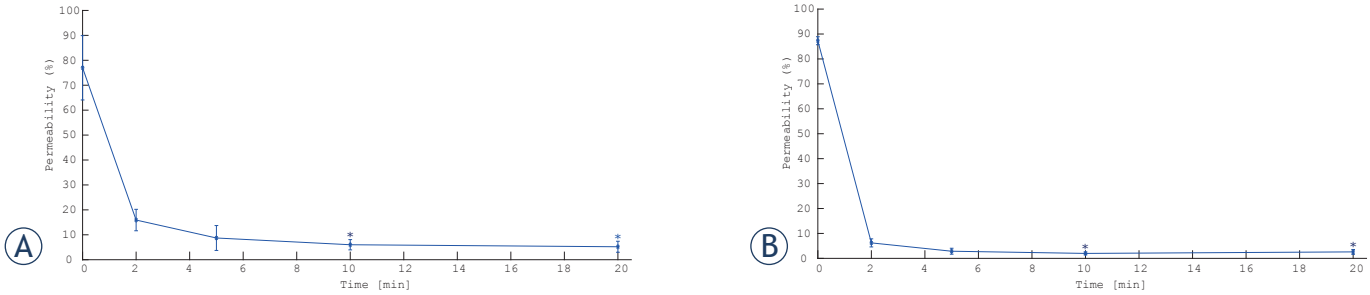

FIGURE 3. Cell membrane permeability as a function of different time of propidium iodide administration after electroporation for **(A)** 8 x 100 µs long monopolar pulses, delivered at a repetition frequency 1 Hz; **(B)** 8 bursts of short bipolar pulses (HF-EP) of 1-1-1-1 µs, delivered at repetition frequency 1 Hz. Each data point was repeated 4 times (mean ± standard deviation). We performed a 1-way ANOVA on ranks. For both types of pulses, there was a significant difference between 0 min vs 10 min and 20 min ($P < 0.05$), other pairwise comparisons were not significant.

used in the experiments with cisplatin. As optimal parameters of electroporation were considered those where the highest cell membrane permeability and the highest cell survival were achieved. In Figure 2 we can observe the permeability curves (blue dashed line) and the survival curves (red solid line) as a function of electric field amplitude for (A) 100 µs long monopolar pulses and (B) bursts of short bipolar (HF-EP) pulses. In Figure 2A we can see that the threshold of electroporation was at 0.8 kV/cm and highest uptake and survival were achieved at 1.2 kV/cm which was considered as the optimal point of electroporation. In Figure 2B we can see that the threshold of electroporation was at 2 kV/cm, the threshold for irreversible electroporation at 4.5 kV/cm and the highest uptake and survival for HF-EP pulses were obtained at 3 kV/cm which was chosen as the optimal point of electroporation with short bipolar pulses. Electric pulses of 1.2 kV/cm with 100 µs monopolar pulses and 3 kV/cm in HF-EP protocol were thus considered to be equivalent and were used in further experiments.

With the optimal parameters of electroporation, we measured the resealing of cell membranes after electroporation. Figure 3 shows the permeability curves obtained as a function of different time of exposure to propidium iodide after electroporation delivering (A) long monopolar pulses at E = 1.2 kV/cm and (B) HF-EP pulses at E = 3 kV/cm. Figure 3A and Figure 3B show a peak of permeability at 0 min, *i.e.*, right after the pulses are applied. Then, we can see a decrease in permeability that reaches a plateau after 10 min. We chose 10 min as the time after which cell membranes resealed. Accordingly, in the subsequent experiments, electroporated samples with cisplatin were diluted after 10 minutes.

Cytotoxicity of cisplatin without electroporation

We measured the cytotoxicity of cisplatin without electroporation at different cisplatin concentrations and incubation times on attached confluent cell monolayers (Figure 4). Cells were more affected if they were exposed to cisplatin for a longer time (24 h and 48 h incubation caused significantly higher cell death than 10 min and 1 h incubation). There was no difference if cells were incubated for 10 min vs 1 h and 24 h vs 48 h. There was no difference between 1 µM and 10 µM, but in general, cytotoxicity increased with higher cisplatin concentrations. After 10 min and 1 h of incubation (red solid and green dashed curve, respectively) there was a decrease in cell survival with increasing cisplatin concentration and at the highest tested concentration

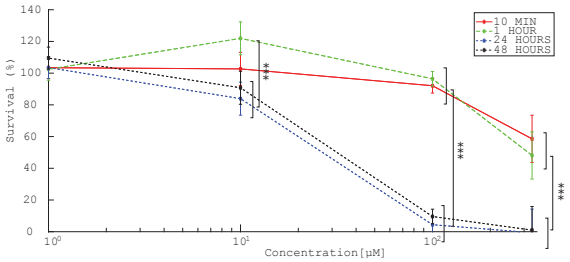

FIGURE 4. Cytotoxicity of cisplatin without electroporation at different concentrations and time of incubation. Each data point was repeated 4 times (mean ± standard deviation) and is normalized to the control sample in which cisplatin was substituted by 0.9% NaCl. A 2-way ANOVA was performed. 10 min or 1 h of incubation was different from 24 h or 48 h ($P < 0.001$) while there was no difference between 10 min vs 1 h and 24 h vs 48 h. 330 µM cisplatin was more cytotoxic than other tested concentrations ($P < 0.001$). There was no significant difference between 1 µM and 10 µM cisplatin; all other comparisons were significantly different ($P < 0.001$).

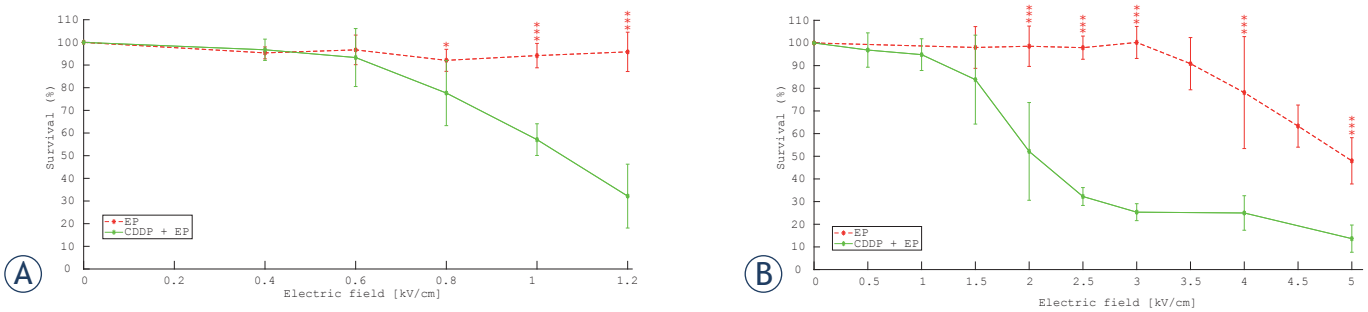

FIGURE 5. Cytotoxicity of cisplatin in combination with electroporation (EP) at fixed value of cisplatin (CDDP) 100 μM as a function of electric field: **(A)** 8 x 100 μs long monopolar pulses (ECT) were delivered at repetition frequency 1 Hz; **(B)** 8 bursts of short bipolar pulses (HF-EP) of 1-1-1-1 μs were delivered at repetition frequency 1 Hz. Each data point was repeated 3–6 times (mean ± standard deviation). Results are normalized to the control sample without an electric field and with 100 μM cisplatin. We performed a **(A)** 2-way ANOVA or **(B)** 2-way ANOVA on ranks. (A) At 0.8 kV/cm ($P = 0.036$) and 1 kV/cm and 1.2 kV/cm ($P < 0.001$) EP samples were significantly different from CDDP+EP samples. **(B)** At electric fields equal to or higher than 2 kV/cm EP samples were significantly different from CDDP+EP samples ($P < 0.001$).

(330 μM) we obtained 58.55% ± 14.90% and 48.12% ± 14.01% survival for 10 min and 1 h, respectively. After 24 h and 48 h (blue dotted and black dash-dot curve, respectively) of incubation, cell survival decreased rapidly to less than 10% already with 100 μM of cisplatin.

Cytotoxicity of cisplatin with electroporation - electrochemotherapy

First, we measured the cytotoxicity of cisplatin with electroporation at different electric fields and selected cisplatin (CDDP) concentration of 100 μM. In Figure 5, we can observe cell survival as a function of applied electric field, on Figure 5A for long monopolar pulses and Figure 5B for HF-EP pulses. The solid green line shows cell survival after electroporation with cisplatin and red dashed line survival after only electroporation without cisplatin. The red dashed curves of Figure 5A and B are already shown in Figure 2A and B. We can see in both Figure 5A and B that the combination of electric pulses and cisplatin is more efficient in achieving cell death than applying only electric pulses or only cisplatin (100% survival at 100 μM cisplatin and 10 min incubation time, Figure 4) and that cytotoxicity of cisplatin increases with increasing electric field, starting at 0.8 kV/cm for 100 μs long monopolar pulses and 2 kV/cm for short bipolar pulses, which coincides with the thresholds for reversible electroporation (Figure 2). In Figure 5A we can see that at $E = 1.2$ kV/cm with cisplatin 32.16% ± 14.08% of cells survive while when we apply only electric pulses, all cell survive. Similarly, in Figure 5B at E = 3 kV/cm 25.33% ± 3.73% of cells survive electroporation with cisplatin opposed to 100% when only electric pulses are applied.

Then, we measured cytotoxicity of cisplatin with electroporation at a fixed electric field (optimal point of electroporation with the highest cell membrane permeability and lowest survival - long monopolar pulses at E = 1.2 kV/cm and HF-EP pulses at E = 3 kV/cm) and different cisplatin concentrations. In Figure 6 we can see two cell survival curves obtained by applying 1) only cisplatin (red dashed curve) and 2) cisplatin in combination with electroporation (solid green curve). From the red dashed curve in Figure 6A and B we can see that cell survival does not decrease with increasing cisplatin concentration due to short incubation time (see also Figure 4). From the solid green curve in Figure 6 A and B we can see that the cytotoxicity of cisplatin increases when electric pulses are applied with increasing cisplatin concentration. A similar trend in survival is observed for both types of pulses.

Discussion

We aimed to determine whether it is possible to use bursts of short bipolar pulses (HF-EP) in *in vitro* electrochemotherapy (ECT) treatments instead of standard long monopolar pulses (classical ECT). We thus performed *in vitro* experiments on mouse skin melanoma cells, as melanoma is one of the cancers successfully treated with electrochemotherapy.[43]

Optimal treatment parameters

First, we determined the cytotoxic effects of cisplatin on a confluent monolayer of cells, because survival after longer exposure time was not possi-

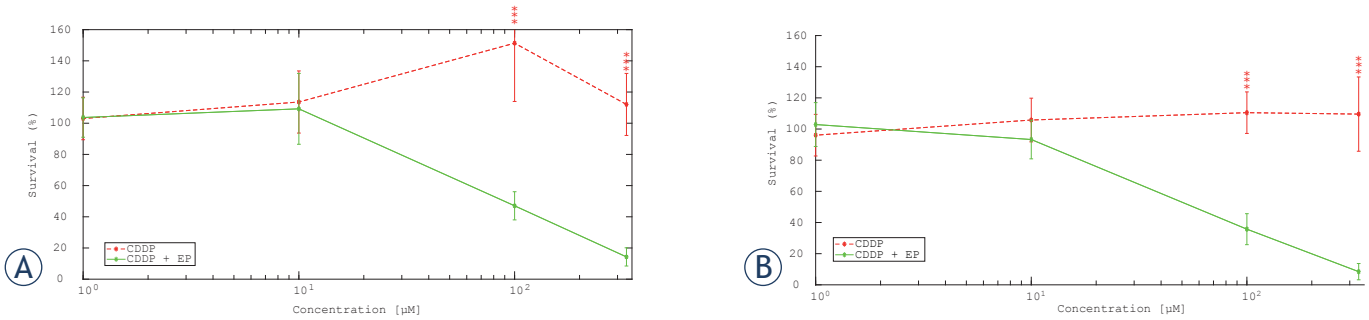

FIGURE 6. Cytotoxicity of cisplatin at different concentration of cisplatin (CDDP) and electroporation (EP) at a fixed value of electric field **(A)** 1.2 kV/cm, 8x100 μs long monopolar pulses, delivered at repetition frequency 1 Hz; **(B)** 3 kV/cm, 8 bursts of short bipolar pulses (HF-EP) of 1-1-1-1 μs, delivered at repetition frequency 1 Hz. Each data point was repeated 3-7 times (mean ± standard deviation). Each data was normalized to the control sample electroporated and with 0.9% NaCl instead of cisplatin. We performed a 2-way ANOVA. For both types of pulses, at 100 μM and 330 μM the CDDP samples were significantly different from the CDDP+EP samples ($P < 0.001$).

ble to evaluate on cell suspension (Figure 4). At 100 μM, short exposure (1 hour or less) did not affect survival. We decided to perform experiments with electroporation at 100 μM cisplatin in order to see possible potentiation of the cytotoxic effect of cisplatin after electroporation. Namely, using higher concentration could already decrease survival without applying electric pulses and we could not asses, if electroporation increases cytotoxicity. In the experiments assessing survival after incubation with cisplatin as determined by the MTS assay, 24 h and 48 h time points were not different one from another and we assumed that also 72 h exposure (which was used in the electroporation experiments) would yield similar results. However, we did not make experiments also at 72 h exposure time.

We determined the optimal parameters for experiments with cisplatin and electric pulses, *i.e.*, the optimal voltage of electric pulses, incubation time with cisplatin after pulse application and cisplatin concentration with a) 100 μs long monopolar pulses (ECT) and b) short bipolar pulses (HF-EP). In experiments with 8x100 μs monopolar pulses, the optimal electric field (highest uptake of propidium and the highest cell survival) was 1.2 kV/cm (Figure 2A) which is in agreement with other studies [44] and corroborates our existing data where cell permeabilization was detected via intracellular platinum measurements.[45] Unfortunately, we could not apply voltages higher than 240 V (1.2 kV/cm) due to the current limitations of the pulse generator. We determined that the optimal electric field with HF-EP pulses was 3 kV/cm (Figure 2B). With bipolar pulses, we had to apply 2.5-times higher electric field than with monopolar pulses to obtain comparable effect, which is in agreement with the results reported by Sweeney *et al*. for propidium uptake[38] and with the *in vitro* data on irreversible electroporation, where irreversible electroporation threshold increased 2.1-times, when 1 μs long pulses were applied in bursts instead as 100 μs long pulses.[46]

With the selected parameters of electroporation, we measured the resealing rate of cells after electroporation. We determined that after 10 min cell membrane is mostly resealed (Figure 3) and did all subsequent cisplatin experiments with 10 min incubation. Dilution of cells with permeable membranes would namely reduce or stop the influx too early or even cause efflux of cisplatin due to dilution and potential reversal of the direction of the concentration gradient.[47] This time range is in agreement with the existing *in vitro* studies, where the incubation time ranges from 5 minutes[23] to 60 minutes[48] as well as with the *in vivo* standard operating procedures where the pulses are applied between 8 and 28 minutes after intravenous drug injection.[19] With propidium iodide (PI) we could use shorter incubation times (2 minutes) as PI binds soon after entering the cell[49], but with cisplatin, we do not know how fast it binds, and we have to wait until cell membranes are completely resealed before the dilution is made. PI was used as a model for cisplatin as its molecular weight is in the same range as of cisplatin (668 g/mol and 300 g/mol for PI and cisplatin, respectively). The similarity in the shape of the permeabilization curve (Figure 2) and cell death due to cisplatin uptake (Figure 5) is another indicator that PI is an appropriate molecule to assess the uptake of cisplatin. Also, experiments with PI and flow cytometry are fast and easy to perform, enable screening of a wide range of parameters quicker than assessing cell survival or plati-

num uptake via mass spectrometry and are thus usually used to determine optimal parameters of electric pulses for electrochemotherapy *in vitro*.[50-52]

100 μM cisplatin concentration was chosen as we could (1) test several pulse parameters without reaching the limitations of the survival assay, (2) it is in a similar range as used in other *in vitro* studies.[23,45,48,53,54] Other tested concentrations (1, 10, 100, 330 μM) were chosen as they were already used in previous *in vitro* experiments.[23,45] (3) The IC50 value of cisplatin pooled together from several studies in[53] was determined to be between 0.83 μM and 1000 μM without electroporation and 0.083 μM and 106 μM with electroporation. As we determined graphically from Figure 6, the IC50 value was in our study 85 μM for monopolar, and 45 μM for bipolar pulses, which is in agreement with the literature and close to the 100 μM.

In our study, different cell densities were used due to different requirements for cell number and sensitivities of the chosen assays. However, even at the highest concentration ($2.2x10^7$ cells/ml) we were still well below the concentration where shielding of the electric field and decreased uptake were observed.[39] 72 h growth time after electrochemotherapy was chosen as it was shown that results of metabolic assays are highly dependent on evaluation time point and they correspond to the results of clonogenic assay better at later time points.[42]

Cytotoxicity of cisplatin with electroporation

We measured the cytotoxicity of cisplatin with electroporation at fixed cisplatin concentration of 100 μM and different electric fields (Figure 5). We were interested in the effect of electric field intensity on cisplatin cytotoxicity, as usually when treating tumors *in vivo*, the electric field distribution is inhomogeneous due to different dielectric properties of different tissues and various electrode configurations.[55,56] A similar tendency of cell survival as a function of the electric field was observed with monopolar as well as HF-EP pulses - we achieved greater cell death by applying cisplatin in combination with electric pulses than by only applying electric pulses. Survival decreased with increasing electric field. In Figure 5A, comparing the red curve with the green one, we can see that at $E = 1.2$ kV/cm cells die because of the cisplatin uptake and not due to irreversible electroporation. The survival after applying 1.2 kV/cm was still 100%, the survival with electric pulses and cisplatin dropped to 32.16% ± 14.08%. Similarly as with monopolar pulses, when applying bipolar pulses of E = 3 kV/cm, cells die due to the cisplatin uptake and not due to irreversible electroporation (Figure 5B). At E > 3 kV/cm cell death is due to the cytotoxic effect of cisplatin as well as irreversible electroporation. As expected and in accordance with previously published results for propidium iodide, we needed to deliver 2.5-times higher electric field with the HF-EP pulses to achieve a comparable effect.[38]

Interestingly, the shape of the permeabilization curve to propidium (Figure 2) corresponds perfectly to the shape of the survival curve after electrochemotherapy (Figure 5). The onset of membrane permeabilization is at 0.8 kV/cm for long monopolar pulses (Figure 2A) and at 2 kV/cm for HF-EP pulses (Figure 2B), which corresponds to the onset of the decrease in survival after electrochemotherapy (Figure 5). The plateau of membrane permeabilization for HF-EP pulses is reached at 3–3.5 kV/cm (Figure 2B) which corresponds to the reached plateau of survival (Figure 5B). Thus at our specific conditions, membrane permeability to propidium is a good indicator of cytotoxicity of cisplatin.

In Figure 6, we measured cytotoxicity of cisplatin with electroporation at a fixed electric field (monopolar pulses E = 1.2 kV/cm and short bipolar pulses E = 3 kV/cm) and different cisplatin concentrations. Namely, in tissues, inhomogeneous cisplatin concentration is expected, also initial cisplatin concentration is usually inhomogeneous after intratumoral injection.[45] Both (A) monopolar pulses at E = 1.2 kV/cm and (B) HF-EP pulses at E = 3 kV/cm show a similar behavior. In both Figures 6 A and B, the cytotoxicity of cisplatin increases more with cisplatin in combination with electric pulses than using only cisplatin.[23,25] Indeed, without electric pulses application, a high dose of cisplatin and/or longer incubation times need to be used to achieve a decrease in cell survival (Figure 4). However, applying 330 μM cisplatin with long monopolar pulses only 14.28% ± 5.84% of cell survived and with short bipolar pulses (HF-EP) only 8.45% ± 5.22% of cell survived. We must keep in mind, that with short bipolar pulses, 2.5-times higher electric field was applied to achieve a similar effect. From the red dashed curve in Figure 6A and B we can see that cell survival did not decrease with increasing cisplatin concentration. This result should be the same as in Figure 4 considering only the 10 min curve, but in Figure 4 cell survival slightly decreases with increasing cisplatin concentration. The reasons for this discrepancy could be the differences in the protocols: attached cell monolayers to measure the cytotoxicity of cisplatin without

electroporation and cells in suspension to measure the cytotoxicity of cisplatin in combination with electroporation. Also, the attached cells were diluted much less with fresh DMEM after exposure to cisplatin than cells in suspension. Besides, cell survival was measured after 48h for the attached cell and after 72 h for the cell in suspension.

Outlooks for using high-frequency electroporation in the clinics

HF-IRE pulses were reported to reduce muscle contractions in comparison with classic 100 μs pulses which was observed in several studies *in vivo*. For example, muscle contractions with HF-IRE pulses were much less noticeable than with 100 μs long monopolar pulses in experiments on rabbit liver.[33,57,58] Even in the absence of cardiac synchronization and paralytics, only minor muscle twitch was recorded in one out of 24 cases[59,60] when treating porcine liver. Sano *et al.* observed that HF-IRE waveforms reduced the intensity of muscle contractions in comparison with traditional IRE pulses on *ex-vivo* porcine model[34] and in *in vivo* murine tumor.[46] Arena *et al.* observed that HF-IRE pulses eliminated muscle contractions when electric pulses were applied to the brain of rats[37] and achieved blood-brain-barrier disruption without inducing local or distal muscle contractions.[61] Latouche *et al*. observed no evidence of muscle or nerve excitation or cardiac arrhythmia during any pulse delivery when treating intracranial meningioma in dogs.[35] In a first human study on high-frequency irreversible electroporation of prostate cancer, only a small amount of muscle relaxant was needed, and there were no visible muscle contractions during the pulse delivery process.[36] Additionally, the histological analysis in *in vivo* porcine experiments indicates that with HF-IRE rapid and reproducible ablation in the liver can be achieved, while preserving gross vascular/biliary architecture.[60] The mechanism for decreased muscle contractions is still unknown. However, different possible explanations were offered. It was suggested that (1) stimulation threshold raises faster than the threshold for irreversible electroporation with decreasing pulse length[62] which is a consequence of geometrical differences between nerve fibers and tumor cells.[63] (2) At around 1 μs there is an overlap of the depolarization threshold and electroporation threshold on the strength-intensity curve.[41] (3) The short negative pulse delivered after a positive pulse accelerated the passive repolarization and swamped the regenerative response, thus abolishing the action potential.[64] The pain was not yet evaluated, but promising results regarding muscle contractions indicate that we can expect less pain with HF-EP than with classical 100 μs pulses.

Before transfer to the clinical setting, more experiments *in vitro* as well *in vivo* need to be performed. In the scope of the current study, experiments with bleomycin are not feasible due to organizational reasons. However, we are planning to perform, in the future, experiments using bleomycin with HF-EP, as bleomycin is frequently used for ECT in the clinics. So, cytotoxicity of bleomycin and HF-EP needs to be assessed, and experiments determining intratumoral cisplatin/bleomycin concentration should be performed. The electric field needed to achieve cell death is with HF-EP higher than in classical EP, and thus the effect of high voltage on important structures in the vicinity of the tumors should be investigated, similarly as in[60] for hepatic veins. Also, temperature increase due to Joule heating has to be minimized for example by introducing a delay between bursts[36,59], limiting electric current or number of bursts[36,46,61] and avoiding increased temperature by optimizing treatment parameters.[35,37,58,61] The influence of HF-EP on muscle contractions, pain and heart rhythm should also be studied, as is being done for high-frequency irreversible electroporation. Currently, pulses in the published studies are being applied with laboratory prototypes - a clinical generator of bipolar pulses needs to be designed and certified before clinical use. However, electrode geometry could be the same as those used with the longer monopolar pulses, but electrical isolation of the wiring and stray capacitance should be re-evaluated.

Applying HF-EP pulses comes at the expense of delivering considerably higher pulse amplitude. However, we need to take into account that in our study, we focused on eight bursts in total on-time of 800 μs to enable comparison with the standard ECT protocol and be consistent with previous studies.[38] To obtain a good effect while keeping the applied voltage low, we could apply more bursts, longer pulses than 1 μs or asymmetrical bipolar pulses[34,65], although it was indicated that muscle contractions are increased with the asymmetrical waveforms. Also of importance is that with pulses in the range of a few microseconds, we are already in the range of the so-called cancellation effect which could be partially responsible for decreased effect of shorter pulses in comparison to longer pulses.[38,66] We can nevertheless conclude that HF-EP pulses can be successfully used in electrochemotherapy treat-

ments *in vitro*, however, at the expense of delivering electric pulses of higher amplitudes.[38]

Although still at the *in vitro* testing stage, we believe that the use of HF-EP pulses for electrochemotherapy in the clinics could potentially decrease the discomfort connected with muscle contractions and pain, simplifying the treatment procedure by lowering dose of muscle relaxants and anesthesia, and avoid synchronization with the electrocardiogram, while potentially achieving more homogeneous electric field distribution[67] and reducing the electrolytic contamination.[68]

Conclusions

In conclusion, with long monopolar and short bipolar pulses (HF-EP), we achieved similar efficiency of electrochemotherapy with cisplatin *in vitro*, however, with short bipolar pulses, we had to apply a much higher electric field for the same effect. Nevertheless, we believe that HF-EP pulses could eventually be translated into the clinical setting to be used in electrochemotherapy treatments to alleviate pain, reduce muscle contractions, decrease the needed dose of anesthetics and muscle relaxants while maintaining high treatment efficacy. Further studies of the HF-EP pulses for electrochemotherapy with bleomycin *in vitro* and *in vivo* are needed.

Acknowledgments

This work was supported by the Slovenian Research Agency (ARRS) [research core funding No. P2-0249 and IP-0510]. The research was conducted within the scope of the electroporation in Biology and Medicine (EBAM) European Associated Laboratory (LEA). Authors would like to thank L. Vukanović and D. Hodžić for their help in the cell culture laboratory and dr. T. Jarm for his help with the statistical analysis and M. Bernik for the linguistic revision of Slovenian abstract. M.S. would like to thank dr. E. Sieni for her help and acknowledge the Erasmus+ grant.

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