

Application of Nisin as a Potential Agent for Nano-Electrochemotherapy: Effects on Mitochondria and Cell Death

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Abstract

Background

Electrochemotherapy (ECT) is a prominent cancer treatment method based on reversible electroporation of cell plasma membrane facilitating initially impermeable drug delivery and cell death. In this work, we have characterized the effects of nisin and its applicability for cancer treatment with pulsed electric fields. This study aims to test the hypothesis that application of nisin with nanosecond pulses (200 ns) can facilitate higher (than the conventional ECT pulses) mitochondrial stress (including depolarization and oxidation) followed by changes in cell death type. As model, 4T1 breast cancer cell line was used. We have employed bursts of 100 x 200 ns pulses (3–11 kV/cm) delivered at different repetition frequency (2–3.3 MHz) and compared the results with conventional ECT protocol (ESOP) of 1.3 kV/cm x 100 μ s x 8, 1 Hz. Cell plasma membrane permeability, mitochondrial oxidation and depolarization and viability including death type characterization were evaluated.

Results

This study shows that nisin can be successfully used as a cytotoxic agent with electroporation. Introduction of high frequency nanosecond pulses (3.3 MHz) triggers significantly higher oxidation of mitochondria and higher electroporation efficacy when compared to 2 or 2.5 MHz equivalent intensity bursts. Both nanosecond and ESOP protocols can trigger depolarization of mitochondria and complete loss of cell viability when combined with nisin, however, the fraction of apoptotic cells is significantly higher with nanosecond pulses.

Conclusions

It is concluded that nisin improves cell membrane permeability, is synergistic with electroporation resulting in increase of both the mitochondrial ROS and depolarization, while at the same time featuring excellent cytotoxicity as a possible ECT agent.

1 Background

Electroporation (EP) and electrochemotherapy (ECT) have been already established as widely recognized, minimally invasive biomedical technologies for treatment of cancer [1–3]. Supported by clinical guidelines (such as European Standard Operating Procedures for Electrochemotherapy (ESOP) [4, 5]) and expanding clinical trials, ECT has transitioned from a specialized palliative technique for superficial lesions to a versatile local control strategy for primary and metastatic tumors across various anatomical sites [6–8]. ECT combined reversible electroporation (transient permeabilization of cell membrane) with the administration of low-dose, highly cytotoxic chemotherapeutic agents with low membrane permeability (primarily bleomycin (BLM) and cisplatin (CDP)) [1, 9], which under normal

conditions poorly penetrate the hydrophobic cell membrane. As a result, the cytotoxicity of the drugs can be potentiated from 10-fold to thousand-fold depending on the employed cytotoxic agent. At the same time, the research of other drugs, which can be used in the context of ECT is constantly performed due to the challenges of multi-drug-resistant cancer types [10–12], varying susceptibility between cell lines and to maximize the immunomodulatory outcomes for metastases prevention [12–15]. Subsequently, experimental studies on ECT and doxorubicin, mitomycin C, paclitaxel and others have appeared, which depending on the cell line can induce comparable effect to BLM-based ECT increasing the flexibility of the methodology in case of BLM-resistant tumor cell lines. However, it should be noted that BLM and CDP are still the dominant drugs, which are used clinically [12, 16–18].

Application of non-standard potentially cytotoxic agents for ECT is gaining upmost interest and popularity, e.g., Pakhomova *et al.*, have shown the excellent potentiation of gelonin-induced cytotoxicity by pulsed electric fields [19]. Gelonin is a ribosome-inactivating protein with extreme intracellular toxicity but poor permeation into cells, thus with membrane permeabilization by EP the cytotoxicity and electrotransfer of gelonin is greatly improved. At the same time, our group has recently proposed the application of nisin for ECT and showed the first proof of concept for cancer treatment [20, 21]. Nisin is a Type A lantibiotic, which binds to Lipid II and constructs stable pore channels in cell membrane, which as a result, triggers rapid cytoplasmic leakage and accelerated cell death [22–24]. We have speculated that nisin can be used in combination with EP for treatment of cancer, resulting in stable long-living pores (irreversible), but triggered in the range of reversible electroporation pulses preventing thermal damage, minimizing inflammation and muscle contractions. The first *in vitro* proof of concept showed that the resealing of the cell membrane is hindered resulting in controlled cell death forming a base for nisin application as a main or adjuvant agent for ECT [21]. Available data covers only the cytotoxic effects of nisin, however, the effects of nisin on mitochondria and cell death type are not covered yet.

The capability to modulate apoptosis during ECT-based treatments, potentially introduces a highly synchronized mechanism driving clean tumor regression and immunogenic cell death. Previously, it was reported that shorter pulses (i.e., nanosecond) are superior in triggering an apoptotic pathway due to selective oxidation of mitochondria [25, 26]. Therefore, the effects of ECT on mitochondria are governed not only by the cytotoxic agent itself, but also by the pulsing protocols. Currently established ESOP protocols based on BLM or CDP drugs are very effective for drug-sensitive tumors, however, ECT-induced tumor cell death involves multiple mechanisms, however, predominantly necrosis [15, 18, 27, 28]. Derivation of alternative ECT strategies based on nanosecond pulses and other cytotoxic agents not only increases the treatment flexibility in terms of drug resistance, but also potentially can contribute to apoptosis-initiated immunomodulatory effects to generate Damage-Associated Molecular Patterns (DAMPs) and tumor-associated antigens [13, 15, 29, 30]. Finally, during apoptosis only the intracellular machinery is targeted, while the structural scaffolding of surrounding healthy tissues (blood vessels, collagen matrices) remains structurally unharmed. Potentially it may lead to better cosmetic healing and minimal scarring in cutaneous tumors.

Therefore, in this work we have characterized the effects of nanosecond pulses (200 ns, up to 11 kV/cm) delivered at ultra-high frequencies (up to 3.3 MHz) and evaluated the potential on nisin and high frequency electroporation for successful ECT *in vitro*. We have characterized the permeabilization of cells with and without nisin, the extent of mitochondrial depolarization and oxidation followed by cytotoxicity studies and cell death type determination. To ensure consolidation of knowledge and comparison, we have used the ESOPE protocol (1.3 kV/cm) as a reference. The experimental workflow and summary of methods involved in the paper are summarized in Fig. 1.

2 Methods

2.1 Cells

The murine mammary carcinoma cell line 4T1 (ATCC® CRL-2539) was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (all reagents obtained from Gibco, Thermo Fisher Scientific, New York, NY, USA). Cells were maintained as adherent monolayers in a humidified incubator at 37°C with 5% CO₂ and were routinely passaged before reaching complete confluency.

For experiments, cells were used during the logarithmic growth phase at approximately 80% confluency. Cells were examined for normal morphology and were confirmed to be free of mycoplasma contamination before experimental use. Mycoplasma testing was routinely performed using the MycoBlue® Mycoplasma Detection Kit (Vazyme, Nanjing, China), according to the manufacturer's instructions. Cells were enzymatically detached using trypsin-EDTA (Thermo Fisher Scientific, Grand Island, NY, USA). Following detachment, cells were collected by centrifugation and transferred to electroporation (EP) buffer (10 mM HEPES, 250 mM Sucrose, 1 mM MgCl₂, adjusted to pH 7.1) for further processing, depending on the experiment procedure.

2.2 Preparation of Nisin

Nisin Z (> 95% purity; CAS No. 137061-46-2; Cat. No. 0305; Handary, Brussels, Belgium) was dissolved in sterile distilled water to prepare a 5 mg/mL stock solution. Based on previous research [21] 50 µg/mL was selected as the highest non-cytotoxic concentration for evaluating the effect of nisin during electroporation. Prior to electroporation, cells were resuspended in EP buffer supplemented with nisin at a final concentration of 50 µg/mL, whereas control cells were resuspended in the same EP buffer without nisin. Following incubation under the specified experimental conditions, the cell suspensions were transferred to electroporation cuvettes and subjected to the appropriate electroporation protocols.

2.3 Electroporation Parameters and Setup

Pulsed electric field (PEF) was delivered to cells with a custom-made square-wave high-voltage pulse generator (VilniusTECH, Vilnius, Lithuania) [31]. Pulses were applied to the cells in commercially

available electroporation cuvettes with a 1 mm electrode gap (Biorad, Hercules, CA, USA), using a sample volume of 60 μ L per cuvette.

Voltages ranging from 0.13 to 1.1 kV were applied to the cuvette, generating electric fields between 1.3 and 11 kV/cm, respectively. Nanosecond pulsed electric fields (nsPEF) consisted of 100 unipolar pulses (3–11 kV/cm, 200 ns pulse duration). The duration of delay was adjusted to achieve repetition frequencies of 2 MHz, 2.5 MHz or 3.3 MHz, corresponding to delays of 100, 200 and 300 ns, respectively. Based on the permeabilization results, the most appropriate protocols were selected for subsequent experiments. The microsecond pulses (0.5–2 kV/cm $\times$ 100 μ s $\times$ 8 sequences, 1 Hz) were used as reference protocols for incorporation and comparison of new knowledge and data on nsPEF. Microsecond pulses (μ sPEF) (1.3 kV/cm, 100 μ s, 8 pulses delivered at 1 Hz) were used as reference protocols to facilitate comparison with and contextualization of existing and newly generated data on nsPEFs.

2.4 Cell Membrane Permeabilization

Cell permeabilization induced by electroporation in 4T1 cells (4×10^6 cells/mL) was assessed using the green, fluorescent dye YOPRO-1 (YP, Sigma–Aldrich, St. Louis, MO, USA). Following incubation with nisin (50 μ L/ml), cells were mixed with YP dye to achieve a final concentration of 1 μ M and transferred into EP cuvettes. After PEF treatment, the cell suspensions were transferred to FACS FlowTubes (Corning, New York, USA). Following a 10-minute incubation at room temperature, 300 μ L of a 0.9% NaCl solution was added to each tube. Untreated control samples were used to establish gating parameters. Samples were analyzed using a BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA). YP fluorescence was detected in Channel FL1, with excitation at 491 nm and emission collected at 533/30 nm bandpass filter (BPF). Results analysis performed with BD Accuri C6 Software (BD, Franklin Lakes, NJ, USA) and later post-processed in OriginPro software (OriginLab, Northampton, MA, USA).

2.5 Mitochondrial Membrane Potential Assay

Mitochondria membrane potential (MMP) was detected with the MitoProbe_DiIC1(5) Assay Kit (M34151, Invitrogen, Thermo Fisher, USA). DiIC1(5) penetrates the cytosol of eukaryotic cells and accumulates primarily in mitochondria with active MMP, producing bright, far-red fluorescence. A decrease in DiIC1(5) fluorescence intensity therefore indicates a reduction in MMP. On the day of the experiment, cells were collected by centrifugation, resuspended in EP buffer at a concentration of 3×10^6 cells/mL, with and without nisin and incubated on ice for 20 min. After the incubation the cells were stained with 50 nM of DiIC1 (5) and immediately 60 μ L of the cell suspension was placed between the electrodes and subjected to the respective PEF protocols. After the treatment the cells were incubated at 37°C for 30 min. Changes in MMP using DiIC(5) (Ex. 640/657) were detected and analyzed with flow cytometer FlowSight (Amnis, Seattle, WA, USA) using 637 nm red laser and 670/14 nm band-pass filter. Data on median fluorescence of individual cells (1×10^4) were normalized to untreated control and used for further analysis.

2.6 Determination of Mitochondrial ROS

Mitochondrial ROS (mtROS) levels were assessed using MitoSOX™ Red (Thermo Fisher Scientific, Waltham, MA, USA), a fluorogenic probe that selectively detects mitochondrial superoxide and is relatively insensitive to ROS generated by non-mitochondrial sources. Cells were centrifuged, resuspended in EP buffer (6×10^6 /ml) and incubated on ice for 20 min with and without nisin. The 60 μ l samples were placed between the electrodes and treated with different PEF protocols. After PEF application the cells were stained for 20 min containing 5 μ M of MitoSOX at 37°C. MtROS was detected and analyzed with flow cytometer FlowSight (Amnis, Seattle, WA, USA) using 488 nm laser and a 577/35 band-pass filter. Data on median fluorescence of individual cells (1×10^4) were normalized to untreated control and used for further analysis.

2.7 Cell Viability Assay

The 4T1 cells viability 24 h post-treatment was characterized based on PrestoBlue metabolic activity assay (Thermo Fisher Scientific, Waltham, MA, USA). Prepared cell suspension (900×10^5 /ml) was 20 min incubated on ice with and without nisin and later treated by different PEF protocols. The samples were transferred to a 96-well flat bottom plate (TPP, Trasadingen, Switzerland) and incubated in room temperature for 10 min. After the incubation growth medium was added to each well and incubated at 37°C and 5% CO₂ for 24 h. Each sample was imaged using a CellCyte X live-cell imaging system (Cytex, Breisgau, Germany), which is housed directly within a laboratory incubator, enabling time-lapse imaging.

The following day, the wells underwent two washes with PBS. Afterwards, 150 μ l of PBS and 2.5 μ l of cell viability reagent PrestoBlue, were added to each well. The cells were incubated for the next 2 h at 37°C, 5% CO₂ and the fluorescence was measured with a spectrofluorometer (Tecan Infinite M200, Tecan, Austria). The excitation wavelength was 540/20 nm and the emission was evaluated at 620/40 nm. A percentage of viable cells was obtained by the normalization of sample absorbance to the absorbance of the control. Each sample was performed in four technical repetitions and measurements for each sample were repeated four times.

2.8 Death Type Characterization

Apoptosis of 4T1 cells induced by PEF was analyzed using an Annexin V Alexa Fluor 488 /propidium iodide (PI) apoptosis detection kit (Thermo Fisher Scientific, Waltham, MA, USA). Annexin V Alexa Fluor 488 detects the externalization of phosphatidylserine, an early event during apoptosis, whereas PI identifies cells with compromised plasma membrane integrity. Annexin V conjugates are made from a highly purified cys-annexin, resulting in higher binding efficiency and extremely accurate characterization of the apoptotic process. Based on Annexin V Alexa Fluor 488 and PI staining, cells were classified as viable (Annexin V-/PI-), early apoptotic (Annexin V+/PI-), necrotic (Annexin V+/PI+), and naked nuclei cells (Annexin V-/PI+).

The cells (2.5×10^6 cells) were incubated on ice for 20 min with and without nisin and later treated by different PEF protocols. The samples were transferred to 24-well and incubated in room temperature for 10 min. After the incubation growth medium was added to each well and incubated at 37°C and 5% CO₂ for 24 h. After the incubation, following treatment with nisin, nsPEF and their combination, cells were collected, including both floating and adherent cells. The cells were collected by centrifugation and resuspended using 1× Annexin V Binding Buffer at a concentration of $1-5 \times 10^6$ cells/mL. Alexa Fluor 488 and PI were added to the cell suspension, followed by 15 min incubation under light-protected conditions according to the manufacturer's protocol. Finally, 100 µL of 0.9% NaCl was added after the incubation. Fluorescence intensity was analyzed by the flow cytometer (Attune NxT; Life Technologies, Carlsbad, CA, USA).

2.9 Statistical Analysis

One-way analysis of variance (ANOVA; $p < 0.05$) was used to compare different treatments. Tukey HSD multiple comparison test for the evaluation of the difference was used when ANOVA indicated a statistically significant result ($p < 0.05$ was considered statistically significant). The data was post-processed in OriginPro software (OriginLab, Northampton, MA, USA). All experiments were performed at least in three repetitions, and the treatment efficiency was expressed as mean $\pm$ standard deviation.

3 Results

3.1 Permeabilization of Cell Membrane Using Nanosecond Pulses

Firstly, we have characterized the cell membrane permeabilization using ultra-high frequency electroporation and 200 ns x 100 pulses. The results are summarized in Fig. 2.

There is a dose-dependent response based on applied electric field amplitude, i.e., higher PEF amplitude results in higher permeabilization of cell plasma membrane, which is an expected result. For example, both at 2 and 2.5 MHz pulse repetition frequencies (Fig. 2C and 2B) the increase in cell membrane permeability to YP is almost linear in the 3–11 kV/cm electric field amplitude range. The higher is the pulse repetition frequency, the higher is the permeabilization if other parameters of PEF are not altered. For example, 5 kV/cm x 200 ns x 100 pulses protocol delivered at 3.3 MHz results in > 75% cell membrane permeabilization, while the same protocol delivered at 2 MHz frequency triggers sub-40% electroporation efficacy. The result is influenced by the residual transmembrane potential (TMP) accumulation phenomenon occurring when the pulses are compressed (closely spaced). Basically, the cell membrane depolarization is longer than the delay between the consequent pulses resulting in the cells being polarized throughout the whole burst affecting the stability and size of the pores. The phenomenon allows triggering saturated electroporation using pulse amplitude/duration parameters, which would be sub-threshold otherwise enabling the use of nanosecond pulses with significantly lower amplitudes. Subsequently, 7–10 kV/cm electric pulses delivered at 3.3 MHz trigger saturated permeabilization (i.e., with increase of amplitude the efficiency of electroporation does not improve),

which is not the case in other instances (Fig. 3B and 3C). The ESOPE protocol (1.3 kV/cm x 100 μ s x 8, 1 Hz), which was used as a reference also triggers saturated permeabilization of $92 \pm 3\%$ (data not shown).

The same protocols were tested with nisin, and the results are presented in Fig. 3. Nisin significantly improves permeabilization efficacy in the whole range of pulses, where statistically significant electroporation without nisin is triggered. There is still a clear dependence on frequency similar to Fig. 2, however, the differences between 2 and 2.5 MHz treatments are not as profound.

When the 3.3 MHz is used, the high cell membrane permeabilization ($> 90\%$) is triggered already at 5 kV/cm, which is an excellent result – nisin affects the efficacy of electroporation, allows reduction of permeabilization thresholds, however, requires above-threshold electric field for the synergistic effect to be triggered. Further, characterization of PEF on mitochondria depolarization and oxidation with and without nisin were studied. The protocols were limited to marginal cases (*i.e.*, 3.3 MHz and 2 MHz) where the highest differences between treatments are expected. Also, the amplitude range was limited to 5–11 kV/cm where high permeabilization of the cell plasma membrane is expected (refer to Figs. 2 and 3).

3.2 Effects of Electroporation and Nisin on Mitochondria Potential

The depolarization of mitochondria was evaluated based on DiIC1 fluorescence, which enters active mitochondria in live cells based on the negative electrical potential across the mitochondrial membrane. The results are summarized in Fig. 4.

The depolarization of mitochondria scales with the applied PEF amplitude, *i.e.*, the higher is the amplitude the higher is the depolarization. The 11 kV/cm using both 3.3 and 2 MHz protocols resulted in saturated depolarization of mitochondria (equivalent to positive control level). As predicted by the YP data (Fig. 1), there is a tendency that 2 MHz pulse bursts trigger inferior electroporation resulting in significantly (*i.e.*, 7 kV/cm and 9 kV/cm protocols) lower depolarization when compared to the 3.3 MHz equivalent energy pulses.

The differences due to pulse repetition frequency became less profound when nisin was added, which is also in agreement with the permeabilization data (Fig. 3). The 9 and 11 kV/cm protocols reached saturated level of depolarization equivalent to positive control. In all cases addition of nisin triggered higher depolarization of mitochondria.

As a reference, ESOPE protocol was tested (data not shown). Without and with nisin the normalized DiIC1 fluorescence was $78 \pm 6\%$ and $51 \pm 3\%$, respectively. High permeabilization of the cell plasma membrane is a prerequisite for significant depolarization of mitochondria independently on the employed pulse range.

3.3 Effects of Electroporation and Nisin on Mitochondria Oxidation

The same protocols were characterized in the context of mitochondrial oxidation. The results are summarized in Fig. 5.

Mitochondrial ROS scales with the PEF amplitude almost linearly in the 5–11 kV/cm PEF range independently on the used pulse repetition frequency. As predicted by both the membrane permeabilization and mitochondria depolarization data, the 2 MHz pulse bursts are inferior to 3.3 MHz protocols. In all cases there is tendency that 3.3 MHz burst induce higher ROS. The 9 and 11 kV/cm pulse protocols induce higher ROS than the positive control (2 kV/cm x 100 μ s x 8, 1 Hz).

Addition of nisin increased mitochondria ROS profoundly when 2 MHz protocols were used, independently on the PEF amplitude. A similar change in ROS is observed for 3.3 MHz protocols; however, the increase was less profound.

The 1.3 kV/cm x 100 μ s x 8, 1 Hz ESOPE protocol did not trigger any detectable changes in mitochondrial ROS (data not shown).

3.4 Effects of Electroporation and Nisin on Cell Viability and Death Type

Changes in cell morphology over time were assessed using a CellCyte X live-cell imaging system, with images acquired at 4, 10, 16, and 22 h following treatment (Fig. 6).

It can be seen (Fig. 6A, B and C) that with the passage of time the absolute majority of cells successfully attached and started dividing indicating the non-toxicity of involved treatments, i.e. both the nisin by itself or ESOPE PEF protocol are predominantly non-toxic. However, when the PEF treatment was combined with nisin not a single cell was able to attach, indicating toxicity of the procedure.

The results were confirmed by cell viability data 24 h post treatment and are summarized in Fig. 7. All nanosecond protocols without nisin trigger reversible electroporation (similar to ESOPE) independently on the amplitude, while the cytotoxicity of the treatment is increased when nisin is added.

The 5 kV/cm protocols (both the 3.3 and 2 MHz) induce similar cell death resulting 30–35% of cells remaining viable. The result is in good agreement with the permeabilization data presented in Fig. 2. Further increase of the PEF amplitude (7–11 kV/cm) induces a complete response for most of the protocols, which is in excellent agreement with the permeabilization data. The ESOPE protocol also triggers a complete response.

Finally, we have evaluated the cell death type following PEF + nisin 24 h post-treatment. The ESOPE protocol triggered $95 \pm 1\%$ necrosis and $2 \pm 1\%$ early apoptosis (data not shown), which is an expected result. The results for nsPEF are summarized in Fig. 8.

Data is good agreement with metabolic activity assay (Fig. 7). The number of necrotic cells increases with the increase of PEF amplitude, while for all the protocols the number of viable cells is below 30%. There is a clear tendency that 3.3 MHz protocols induce higher fraction of necrotic cells when compared

to the 2 MHz bursts of equivalent intensity, which agrees with experimental data above. The 5 kV/cm protocols result in the highest fraction of early apoptotic cells (up to 30%), which is reduced to sub-10% with increase of amplitude to 11 kV/cm. The number of early apoptotic cells for all the nsPEF protocols is significantly ($P < 0.05$) higher when compared to ESOP (2 ± 1%).

4 Discussion

Nisin is vastly used in food processing, however it has gained attention as a repurposing candidate for oncology in recent years. Long utilized as an FDA- and WHO-approved food preservative (E234), its therapeutic scope has expanded due to its preferential cytotoxicity toward malignant cells relative to non-cancerous lines [32, 33]. The main mechanism driving nisin's anticancer effect is membrane disruption. Unlike normal human cells, the outer plasma membrane of cancer cells features a net negative charge due to elevated levels of phosphatidylserine, O-glycosylated mucins, and sialic acid [34–36]. Nisin's cationic (positively charged) structure binds electrostatically to these anionic surfaces, forming transient, influx-permeable pores [37, 38]. Therefore, previously, we have speculated that combination of electroporation with nisin should have a synergistic effect due to similar mechanism of effect (pore formation) resulting in long lasting non-reversible pores but in the reversible electroporation range. The first proof of concept has confirmed the hypothesis, indicating excellent cytotoxicity of nisin as an alternative to ECT drugs [21] or as an adjuvant agent [20].

At the same time, unlike necrosis, which causes the cell membrane to rupture, triggering inflammatory responses and surrounding tissue damage, apoptosis generally preserves membrane integrity and facilitates rapid phagocytic clearance, thereby limiting excessive inflammation and collateral tissue damage [39, 40]. Therefore, introducing a cytotoxic agent, which stimulates apoptosis is beneficial in the context of ECT. Nisin is a suitable candidate, since it elevates intracellular reactive oxygen species (ROS) levels and collapses the mitochondrial membrane potential, causing cytochrome C release into the cytosol. This is accompanied by an up-regulation of the pro-apoptotic protein Bax and a down-regulation of anti-apoptotic Bcl-2 [41].

Therefore, in this work we have characterized the effects of nisin-based ECT in the context of mitochondria oxidation, depolarization and apoptosis induction. Additionally, we have employed short nanosecond pulses delivered with extreme repetition frequency (up to 3.3 MHz) to have a higher frequency component of the burst and trigger reduction of permeabilization threshold due to residual TMP formation [42]. It is known that nanosecond pulses are more likely to induce apoptosis, affect intracellular structure and selective oxidation [43–45], however, real life applications of nsPEF are limited (when compared to microsecond pulses) due to the requirement of high PEF amplitudes, which are challenging to achieve in clinical context either due to engineering or safety challenges. Additionally, nsPEF delivered at few Hz or kHz frequency are inferior for molecular delivery due to lack of significant electrophoretic component, smaller induced pore size and their stability, which limits their applicability for ECT [46]. However, reduction of permeabilization thresholds due to the residual TMP phenomenon [25, 47], allows to overcome all the challenges described above introducing sub-10 kV/cm nanosecond

pulse protocols, which was not possible before. These protocols are effective for drug delivery [46], while still featuring all the benefits of nanosecond pulse range (reduced muscle contractions, more uniform treatment due to impedance mitigation, capability to affect intracellular structures).

According to acquired data 3.3 MHz 200 ns pulses were significantly superior for induction of electroporation when compared to 2 MHz procedures (at the same parameters). Effective permeabilization was achieved at significantly lower electric fields, which motivates for further development of pulse compression techniques. When combined with nisin, the membrane permeabilization rate increased significantly. The selected concentration of nisin does not cause permeabilization of cell membrane by itself, however, when combined with above-electroporation threshold electric field the synergy is induced. Based on the results, we are speculating that nisin at low concentrations stabilizes electroporation-induced pores and prevents them from closing, which was previously confirmed by cell membrane resealing experiments [21]. Also, it is known that nisin causes mitochondrial membrane potential collapse and a spike in reactive oxygen species [48], however, our data indicate that nisin is not causing mitochondrial depolarization and oxidation when used at low non-toxic concentrations. However, when combined with electroporation, the synergistic effects of nisin to induce depolarization of mitochondria are evident (i.e., at 5 kV/cm). In the context of oxidative stress, nisin also increases the oxidation of mitochondria when combined with electroporation. As predicted, nanosecond pulses induced high mitochondrial oxidative stress, which scales both with amplitude and pulse repetition frequency. It should be noted that all the nsPEF protocols involved in the study are causing reversible electroporation (as confirmed by viability data), however, the mitochondrial oxidation is higher (9 and 11 kV/cm at 3.3 MHz) than the one induced by irreversible electroporation protocol (2 kV/cm, ESOPE). The 1.3 kV/cm ESOPE protocol did not induce any significant oxidation of mitochondria. It could be summarized, that all the three target mechanisms of electroporation, which were focused in the study (i.e., permeabilization of cell plasma membrane, mitochondria oxidation and depolarization) were improved/boosted by the presence of low concentrations of nisin.

Finally, we have characterized the cytotoxicity of the treatments involved including characterization of the cell death type. It was shown that 200 ns PEF when combined with nisin can be successfully used for killing of cancer cells, featuring amplitudes as low as 7 kV/cm to be effective, which is an excellent result. The 5 kV/cm protocols returned ~ 60–70% cytotoxicity due to non-saturated permeabilization of cell plasma membrane (even with nisin). The number of apoptotic cells with protocols featuring nsPEF was significantly higher when compared to ESOPE, which could be also the consequence of the induced mitochondrial oxidative stress (not the case for ESOPE pulses). Mitochondrial oxidation in apoptosis acts both as a trigger signal that initiates the cell-death cascade and as a biochemical requirement for execution [49–51]. Further optimization of nisin concentration and nsPEF protocols is required in the future to derive optimal protocols; however, our data already supports the synergistic combination potential of nisin with electric fields. An unsolved uncertainty is the stability of nisin when used *in vivo*. The stability and biological activity of nisin are strongly dependent on pH, temperature and can also be affected by factors such as storage conditions, proteolytic enzymes, and interactions with food or other

biological components [52–54], which could affect its efficacy, therefore, *in vivo* validation of the effects is required including derivation of the effective concentration, which expected to be a lot higher.

5 Conclusion

Nisin, a polycyclic cationic bacteriocin produced by *Lactococcus lactis*, is widely known as an FDA-approved food biopreservative. In this work we have shown that it can be successfully used for treatment of cancer cells when combined with electroporation due to synergy of the mechanism of effect (pore formation). The methodology is effective both in the nanosecond and microsecond range of pulses, however, shorter pulses introduce an additional degree of freedom (i.e., significantly higher oxidation of mitochondria), which is known to be one of the main stresses to induce apoptosis. Our data show that the fraction of apoptotic cells is increased when nanosecond pulses are involved, therefore, further optimization of pulse protocols and nisin concentrations is required in the future. Also, we have shown, that higher repetition frequency nanosecond pulses enable reduction of the permeabilization thresholds to achieve saturated permeabilization and cytotoxic effect. Further *in vivo* validation is required in the future.

Declarations

Ethical approval and consent to participate

Not applicable.

Consent for publication

All authors reviewed and approved the manuscript for Publication.

Competing interests

The authors have no disclosures or potential conflicts of interest.

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Author Contribution

Conceptualization, V.N.; data curation P.M. and V.N.; formal analysis, P.M.; funding acquisition, V.N.; investigation P.M. and V.N.; methodology P.M. and V.N.; project administration, V.N. resources, V.N; supervision, V.N.; validation, P.M. and V.N.; visualization P.M; writing original draft preparation – P.M. and

V.N.; writing—review and editing - P.M. and V.N. All authors have read and agreed to the published version of the manuscript.

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Data availability

The data supporting the findings of this study are available from the corresponding authors (P. M.) upon request.

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Figures

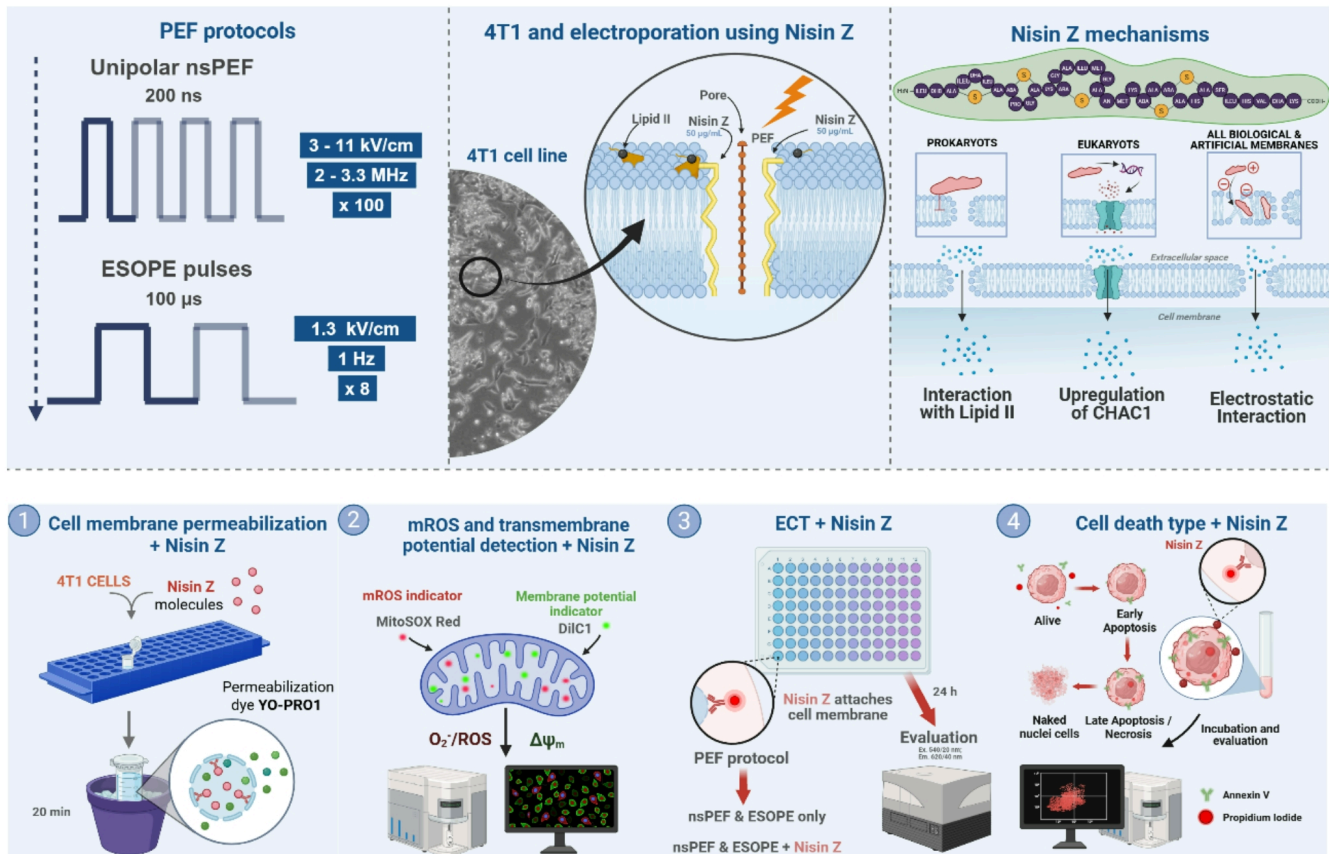


Figure 1

Schematic overview of the experimental workflow. 4T1 cells were incubated in the presence or absence of nisin Z, followed by pulsed electric field (PEF) treatment. The effects of the treatments were subsequently evaluated by assessing membrane permeabilization, mtROS, transmembrane potential detection, ECT *in vitro* and cell death type determination at defined time points using flow cytometry and metabolic assays

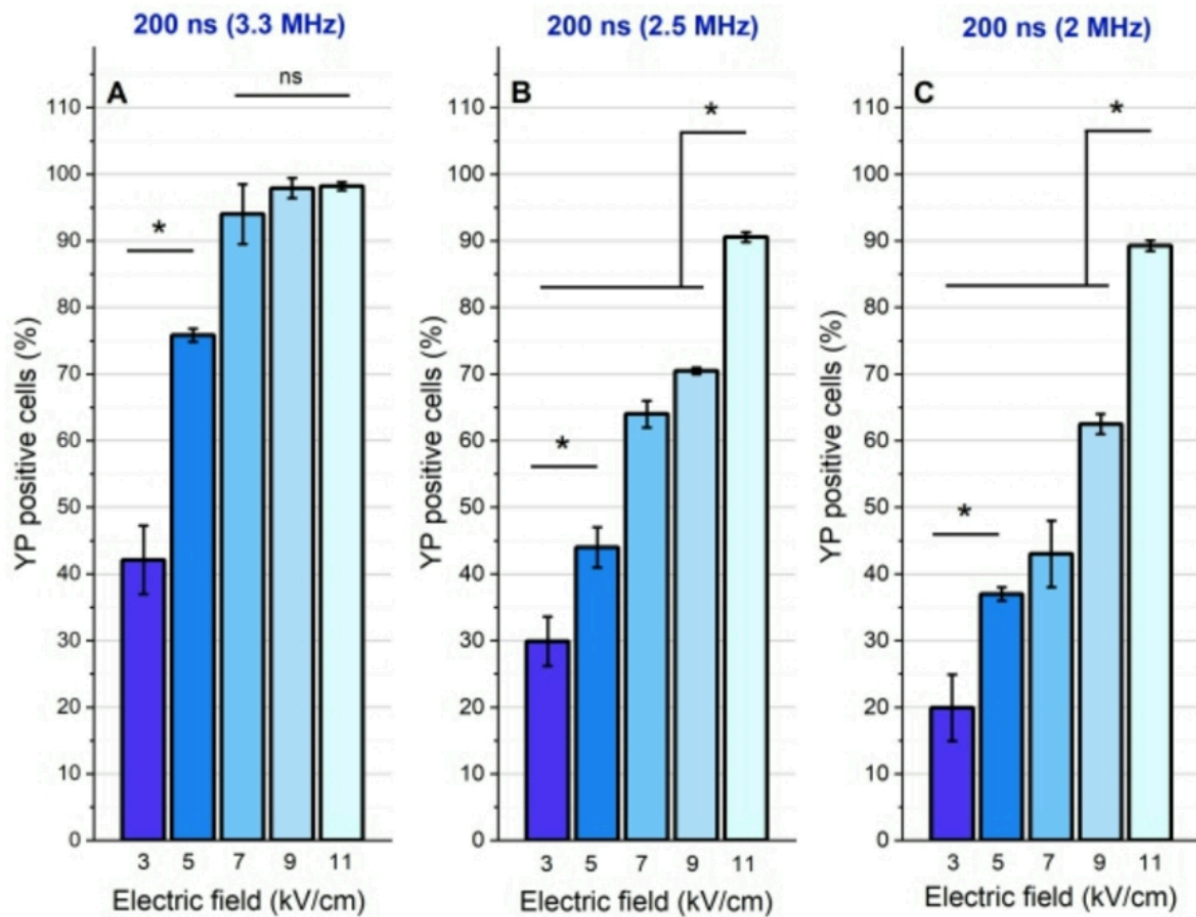


Figure 2

Cell membrane permeabilization dependence on applied electric field parameters, where A) 3.3 MHz pulses; B) 2.5 MHz pulses and C) 2 MHz pulses. In each instance 200 ns x 100 pulses have been used. Asterisk (*) corresponds to statistically significant changes between treatments ($P < 0.05$), ns – non-significant ($P > 0.05$). Untreated control is $9 \pm 4\%$ (data not shown)

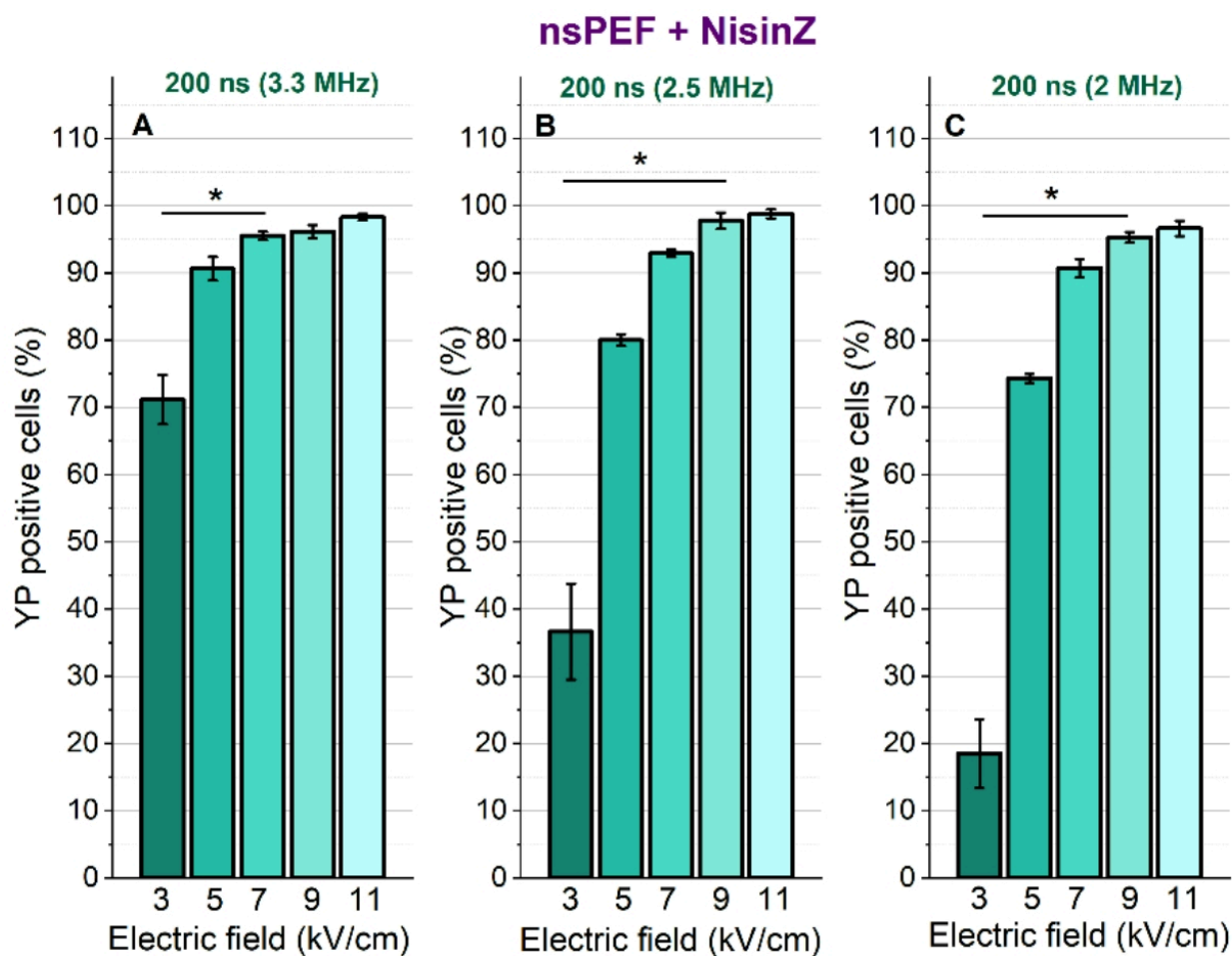


Figure 3

Cell membrane permeabilization dependence on applied electric field parameters with added nisin, where A) 3.3 MHz pulses; B) 2.5 MHz pulses and C) 2 MHz pulses. In each instance 200 ns x 100 pulses have been used. Asterisk (*) corresponds to statistically significant changes ($P < 0.05$). Untreated control is $11 \pm 5\%$ (data not shown)

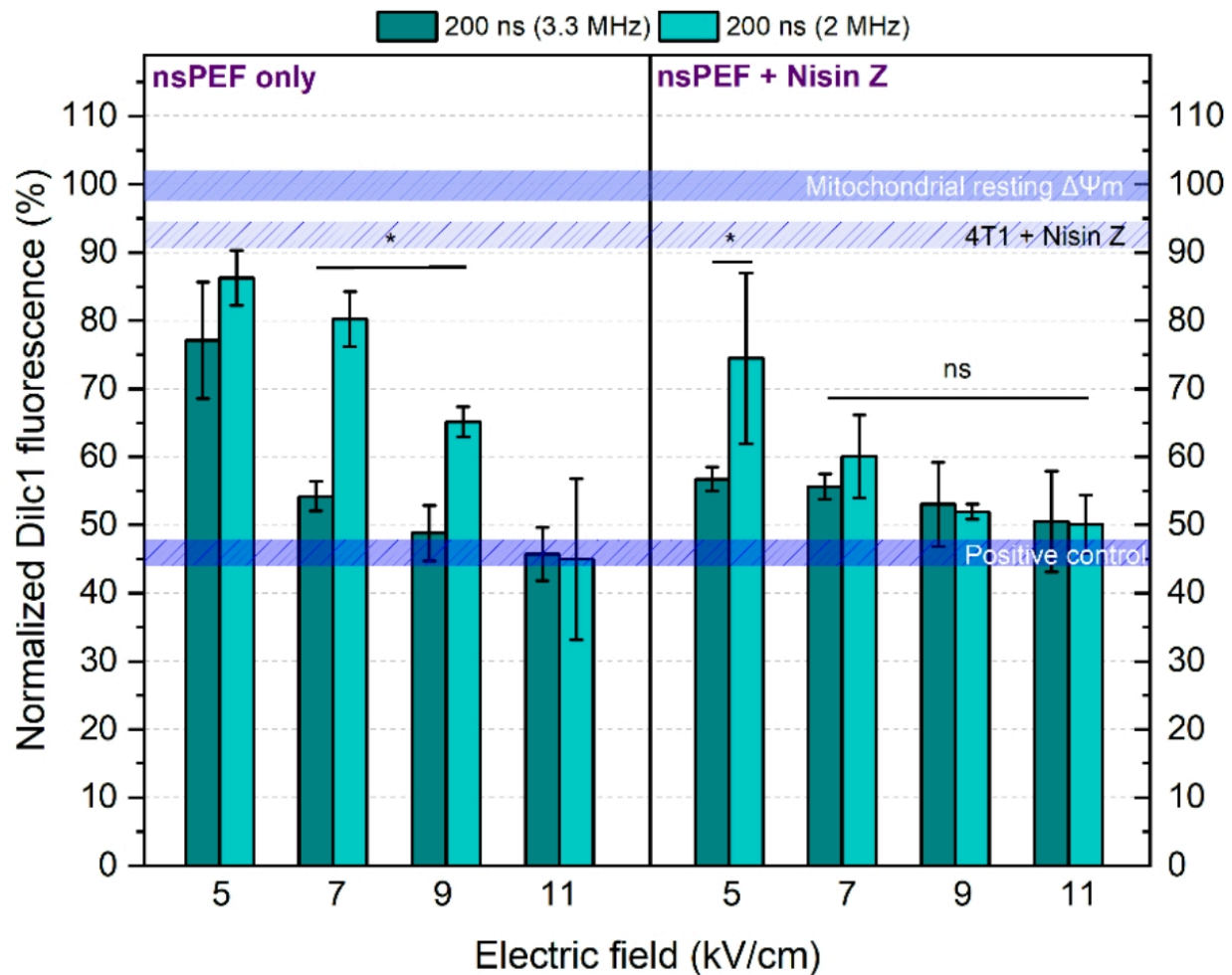


Figure 4

The effects of nanosecond electric fields with and without nisin on depolarization of mitochondria. Asterisk (*) corresponds to statistically significant changes between treatments ($P < 0.05$), ns – non-significant ($P > 0.05$). Shaded area represent untreated control (i.e., mitochondrial resting potential) and positive PEF control (2 kV/cm x 100 μ s x 8, 1 Hz) serving as a saturated depolarization reference. All data are normalized versus untreated control

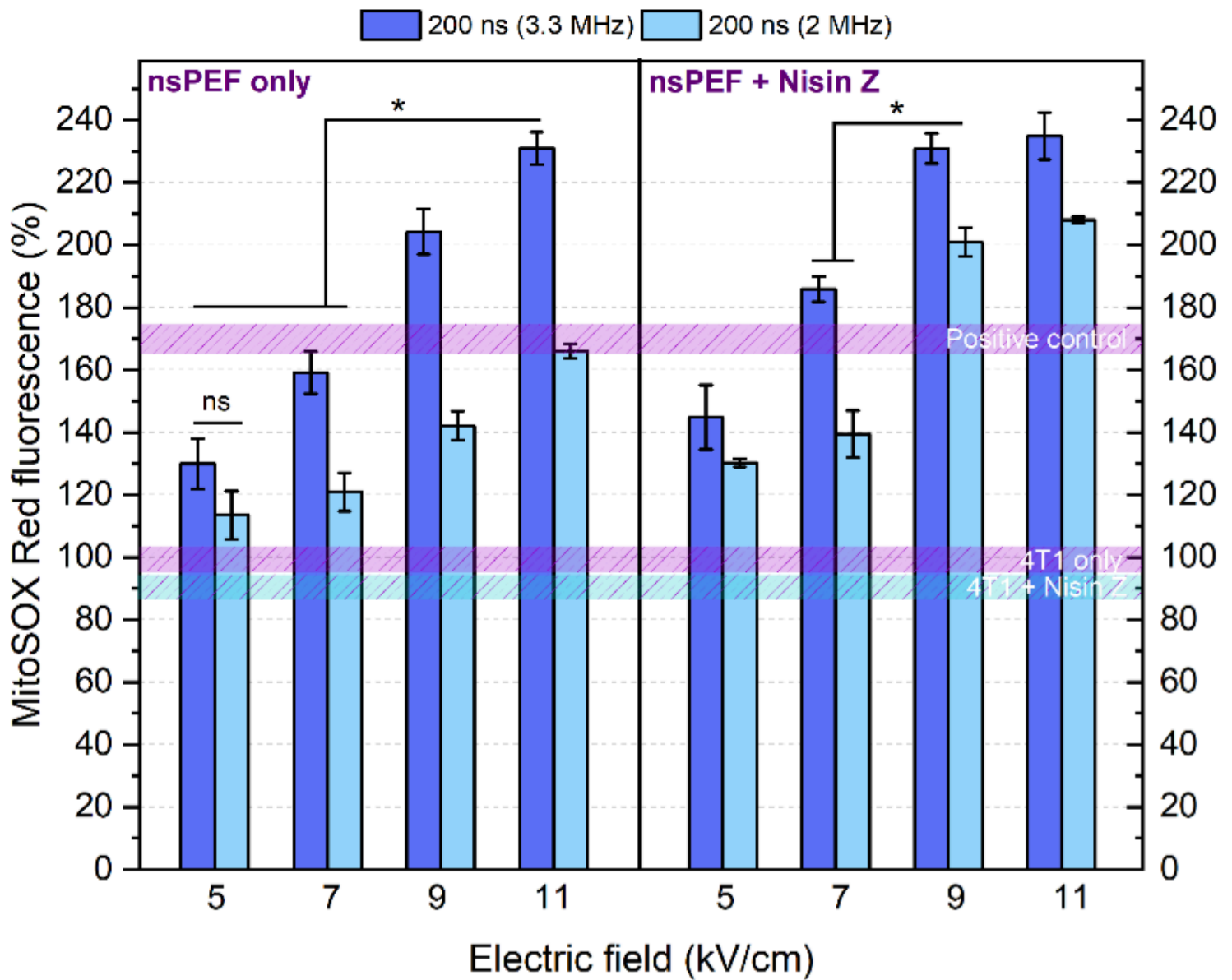


Figure 5

The effects of nanosecond electric fields with and without nisin on oxidation of mitochondria. Asterisk (*) corresponds to statistically significant changes between treatments ($P < 0.05$), ns – non-significant ($P > 0.05$). Shaded area represent untreated control (i.e., 4T1 only) and positive PEF control (2 kV/cm x 100 μ s x 8, 1 Hz). All data are normalized versus untreated control

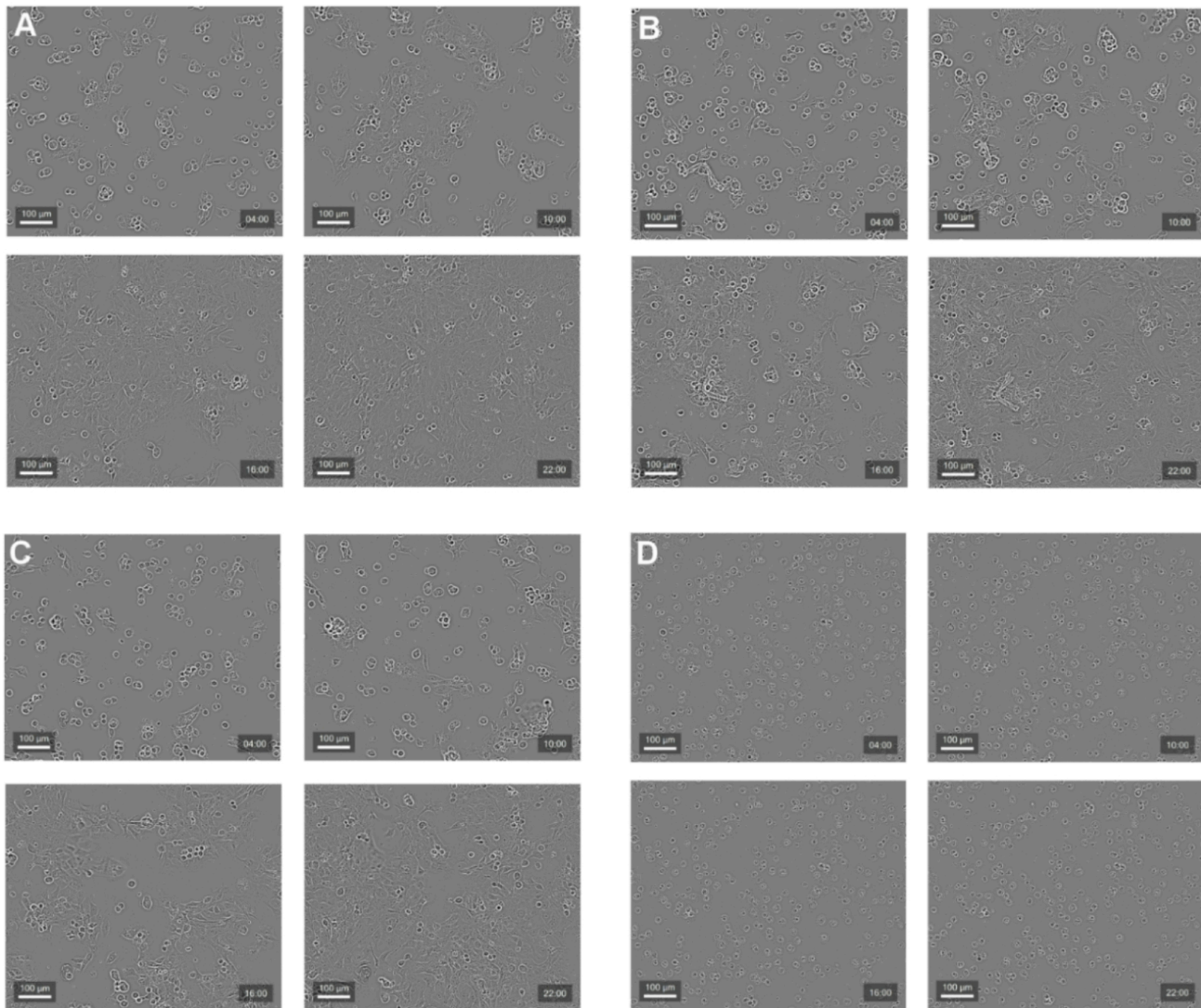


Figure 6

Cell morphology was assessed by time-lapse imaging using a CellCyte X live-cell imaging system for all the protocols involved in the study, with images acquired at 4, 10, 16, and 22 h following the treatment. Examples of typical microscopic images are shown, where **A** – untreated cells, **B** – cells treated only with Nisin Z, **C** – 1.3 kV/cm ESOP, **D** – 1.3 kV/cm ESOP + Nisin Z

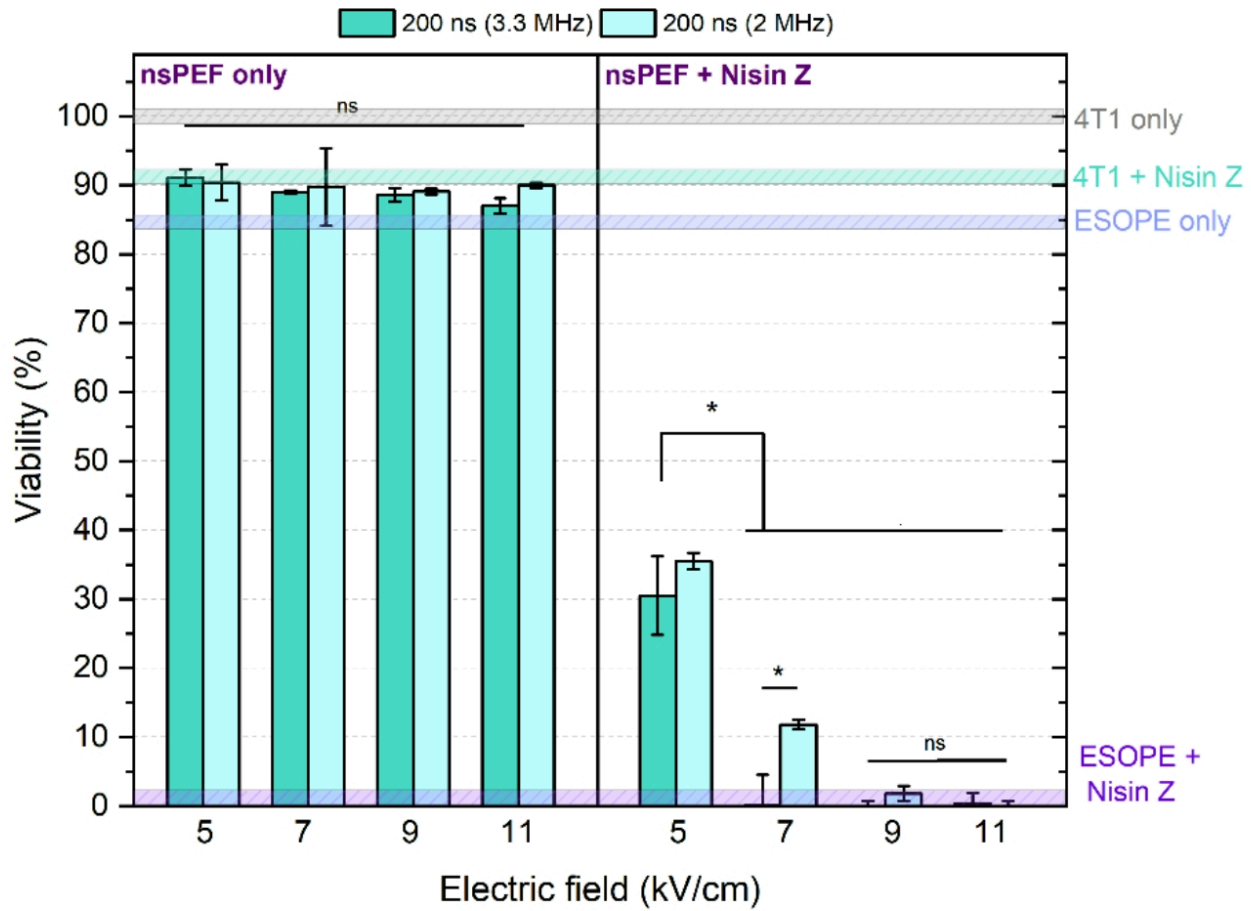


Figure 7

The effects of nanosecond electric fields with and without nisin on cell viability. Asterisk (*) corresponds to statistically significant changes between treatments ($P < 0.05$), ns – non-significant ($P > 0.05$). All data are normalized versus untreated control

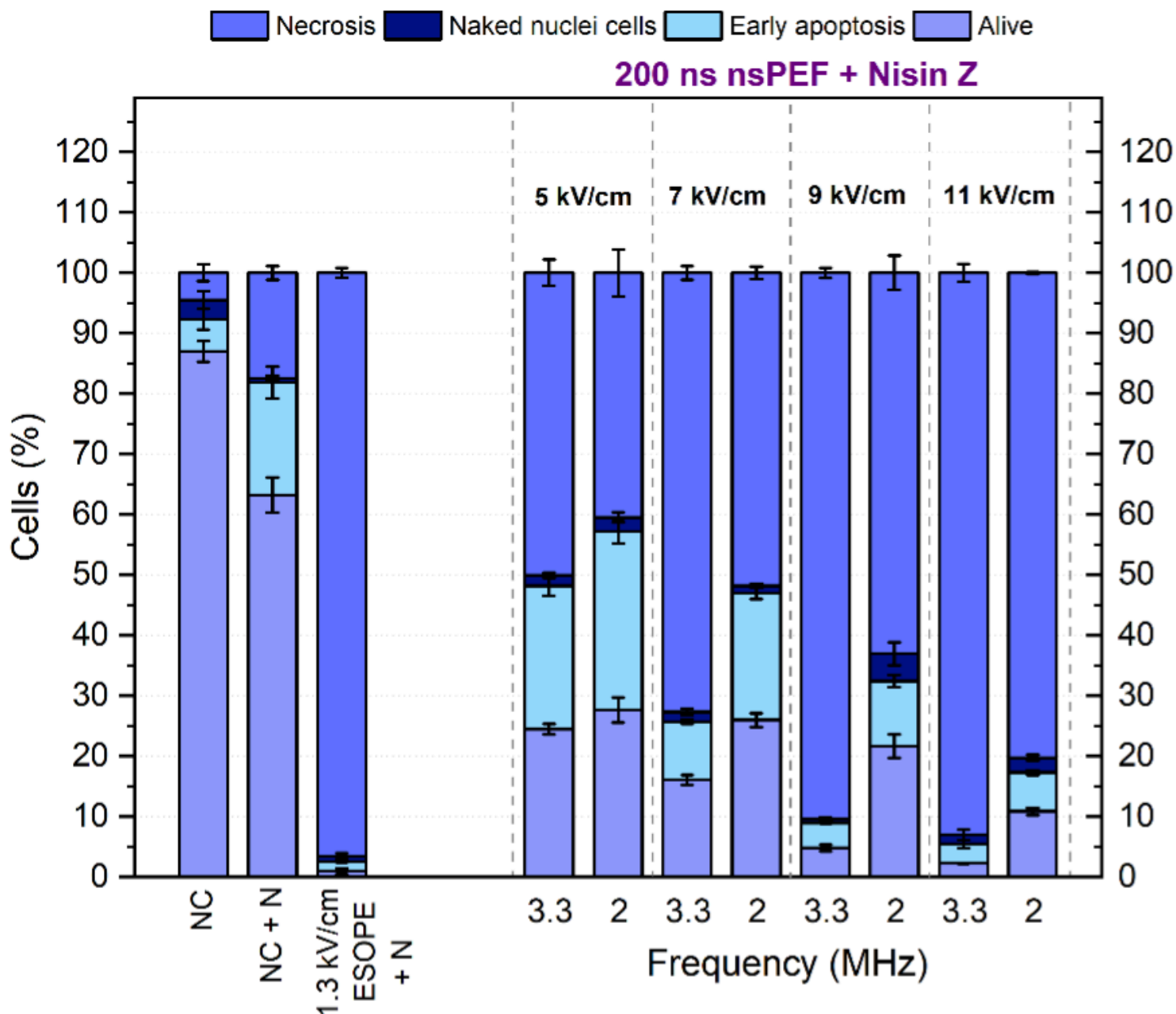


Figure 8

The effects of nanosecond electric fields with nisin on cell death type switching