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Selective cytotoxicity of intense nanosecond-duration electric pulses in mammalian cells

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Abstract

Background—Nanosecond electric pulses (EP) disrupt cell membrane and organelles and cause cell death in a manner different from the conventional irreversible electroporation. We explored the cytotoxic effect of 10-ns EP (quantitation, mechanisms, efficiency, and specificity) in comparison with 300-ns, 1.8- and 9-μs EP.

Methods—Effects in Jurkat and U937 cells were characterized by survival assays, DNA electrophoresis and flow cytometry.

Results—10-ns EP caused apoptotic or necrotic death within 2–20 hrs. Survival (S , %) followed the absorbed dose (D , J/g) as: $S = \alpha D^{(-K)}$, where coefficients K and α determined the slope and the “shoulder” of the survival curve. K was similar in all groups, whereas α was cell type- and pulse duration-dependent. Long pulses caused immediate propidium uptake and phosphatidylserine (PS) externalization, whereas 10-ns pulses caused PS externalization only.

Conclusions—1.8- and 9-μs EP cause cell death efficiently and indiscriminately (LD_{50} 1–3 J/g in both cell lines); 10-ns EP are less efficient, but very selective (LD_{50} 50–80 J/g for Jurkat and 400–500 J/g for U937); 300-ns EP show intermediate effects. Shorter EP open propidium-impermeable, small membrane pores (“nanopores”), triggering different cell death mechanisms.

General significance—Nanosecond EP can selectively target certain cells in medical applications like tumor ablation.

Keywords

nanosecond pulses; electroporation; cell death; dose effect; pulsed electric field; nanoelectroporation; nanopores; membrane permeabilization

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1. Introduction

Extensive research has focused on the breakdown of phospholipid bilayer membranes by micro- and millisecond duration EP as a method to introduce otherwise impermeable molecules into cells [1–3]. This process, termed electroporation, has enabled the successful transfection of plasmid DNA, gene silencing by siRNA, and introduction of exogenous proteins into various cell types providing a critical tool for biological and clinical investigators [4–7]. Introduction of cytotoxic substances such as bleomycin and cisplatin into cells by electroporation, or electrochemotherapy, has been recognized as an efficient and safe way of tumor ablation [8–11]. Recent studies have also focused on the use of high intensity pulse exposures for killing cancerous tissue by irreversible electroporation (IRE) [12–16]. IRE is an advantageous technique for cancer treatment because collateral damage to neighboring cells is contained to areas within the maximum electric field. *In vivo* studies in animal models and humans have shown elimination of cancer in diverse tissues including skin, prostate, and liver.

Multiple research groups have proposed metrics such as electric charge and delivered energy or dose to relate the exposure parameters to plasma membrane breakdown and mammalian cell killing [17–18]. Delivered energy has also been shown to be a reliable exposure metric for decontamination of bacteria using intense electric pulses [19–21]. However, other studies found that neither charge nor energy adequately predicted membrane breakdown and death in DU 145 prostate cancer cells or in CHO cells [22–24]. To date, there is no consensus if any single exposure metric could adequately and universally predict cell death due to EP exposure.

In the last decade, electroporation studies have extended from the traditional milli- and microsecond durations into the nanosecond range. Early experimental studies showed that cells exposed to nanosecond EP (nsEP) externalized PS within minutes after exposure, but showed just minimal or no immediate uptake of propidium [25–28]. PS externalization is a known early marker of apoptosis, so these data suggested that nsEP triggered a fast apoptotic process, without immediate poration of the plasma membrane. Progression of these cells to apoptotic death eventually culminated in membrane destruction and delayed influx of propidium, but, in contrast to direct membrane poration by EP, the delayed propidium uptake was diffuse (did not start from the anodic pole of the cell).

Cell survival following nsEP exposure was investigated across multiple cell lines and using various pulse durations (10, 60, 300, and 600ns). Stacey et al. [29] reported that suspension-based cell lines were more sensitive than adherent lines to 60-ns EP. The cytotoxic effects were accompanied by DNA damage and delaying of cell progression through the mitotic cycle. Other reported signs of nsEP-induced apoptosis included cytochrome C release, Bax expression, caspase activation, and PARP cleavage [25–26,30–32].

However, later studies found that nsEP-induced PS externalization was essentially immediate (within seconds), which was too fast for an orderly apoptotic process [33–35]. Instead, the likely mechanism of the effect was lateral drift of PS to the outer face of the membrane alongside the lipid-water interface of *de novo* opened membrane nanopores. In view of these data, the use of PS externalization as an apoptotic marker for nsEP-treated cells has become questionable. While the fact of apoptotic death is not disputed (it has been demonstrated by multiple methods), such questions as the exact percentage of cells that undergo apoptosis and its dependence upon the treatment conditions may need to be re-visited.

The formation of nanopores in cell plasma membrane was further supported by electrophysiological and fluorescent detection methods [36–38]. These nanopores remained stable for minutes, and the resulting loss of electrolytes and cell volume changes could reasonably contribute to or even become the primary cause of cell death. Indeed, we recently found that blockage of nanopores effectively attenuated the cytotoxic effect of 60-ns pulses

[39]. At a fixed pulse duration, cell survival curves followed the absorbed dose [28,40], and a more general “scaling law” concept was recently proposed in an attempt to provide a more universal metric for different pulse durations [28,41]. Nonetheless, the exact mechanisms of cell death caused by nsEP, the balance of apoptotic and necrotic processes, and the dependence of the cytotoxic effect upon exposure parameters remain poorly understood.

Despite this incomplete knowledge, nsEP have already been employed to destroy tumors in animal experiments and even in a human trial [25,42–44]. The results were regarded as very successful, often enabling full elimination of cancerous cells without recurrence; the treatment caused little scarring and no significant side effects. Understanding the mechanisms and quantitation of nsEP effects with respect to the treatment parameters could greatly assist the progress of such *in vivo* trials.

At present, both the conventional IRE and nsEP look promising for clean removal of cancerous tissue with minimal collateral damage. However, there is no consensus as to whether nsEP offer a unique treatment or any advantages compared to IRE, or which nsEP parameters should be used. The choice of proper exposure parameters may be critical for the success of nsEP therapy. For instance, our findings with 10-ns pulses [32] were very different compared to what was reported by other authors for 60-, 300-, and 600-ns pulses: 10-ns pulses were highly inefficient in cell killing (it took hundreds of pulses even at a very high E-field) and a large fraction of cells died by necrosis rather than apoptosis. However, the exact cause of such differences could not be identified due to multiple methodological differences between these studies.

The present study was intended to further analyze the cytotoxic effect of 10-ns EP and quantitatively compare it to the effect of longer EP under the same experimental conditions. The focus of this study was to compare the mechanisms, efficiency, and selectivity of different EP.

2. Methods

2.1. Cell lines and propagation

Experiments were performed in two suspension cell lines, Jurkat clone E6-1 (human T-lymphocytes) and U-937 (human monocytes). The cells were obtained from ATCC (Manassas, VA) and propagated at 37 °C with 5% CO₂ in air, in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin. The media and its components were purchased from Mediatech Cellgro (Herdon, VA) except for serum (Atlanta Biologicals, Norcross, GA). For EP treatments, cells were harvested during the logarithmic growth phase, pelleted by centrifugation, and resuspended at a desired concentration in a fresh growth medium.

2.2. EP exposure procedures and cell survival assays

Cell suspension in complete growth medium was dispensed into conventional electroporation cuvettes with 1- or 2-mm gap between the electrodes (BioSmith Biotech, San Diego, CA). The cell density was adjusted for different assays as follows: Typically, it was 0.2×10^6 cells/ml for assessment of cell survival by hemocytometer counts, 1.2×10^6 cells/ml for metabolic cell survival assay using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent, and up to 40×10^6 cells/ml for detection of internucleosomal DNA cleavage by agarose gel electrophoresis. The electroporation cuvettes were exposed to EP at room temperature (21–23 °C). In each series of experiments, different EP treatments, including sham exposure, were alternated in a random sequence. Once filled with the cell suspension, cuvettes were subjected to EP treatment as soon as possible, usually within 10–20 min.

For MTT assay (BioAssay Systems, Hayward, CA), exposed cells were aseptically aliquoted into a 96-well plate, in triplicates at 50×10^3 cells/well, and diluted to 100 μ l with fresh growth medium. The plate was incubated at 37 °C, with 5% CO₂ in air. At 20 hr after EP treatment, 10 μ l of MTT reagent were added to each well, and incubation continued for additional 4 hr. Formed blue formazan crystals were dissolved by adding the solubilization buffer (100 μ l/well) and placing the plate on an orbital shaker overnight. Absorbance at 570 nm was read the next day using Synergy 2 or Synergy HT microplate reader (BioTEK, Winooski, VT), and the readings in EP-exposed samples were normalized to the parallel control.

For Trypan blue exclusion assay, 100- μ l aliquots of exposed and control samples were placed in the incubator until analysis, for a maximum of 72 hrs. A 0.4% solution of the dye (ATCC) was added to cell suspension at 25% v/v 1–2 min prior to cell counting. Live and dead cells were counted in eight fields of hemocytometer, following guidelines outlined in [45].

Most survival values shown on the graphs represent the MTT assay measurements, with the exception of Figs. 1 and 2, which are based on hemocytometer cell counts. Overall, these two cell survival assays complemented each other and produced closely matching data.

2.3. Internucleosomal DNA cleavage

DNA fragmentation was visualized by agarose gel electrophoresis and staining with ethidium bromide. DNA isolation and processing were performed using a Quick Apoptotic DNA Ladder Detection Kit (BioVision, Mountain View, CA). For a positive control, we induced apoptosis in U937 cells by a moderate heat shock, 44 °C for 40 min [46–47].

To maximize the assay sensitivity, we used larger cell quantities. Cells were concentrated by a mild centrifugation (5 min at 300–400 g) and exposed at up to 40×10^6 cells/ml. Immediately afterwards, the samples were diluted to less than 10^6 cells/ml and moved to the incubator until lysis (from 1 to 24 hrs after exposure). Live and dead cells were counted in a hemocytometer prior to exposure and prior to lysis. Within the accuracy of the employed assays, concentration/dilution procedures *per se* had no effect on cells' growth rate or viability.

2.4. Flow cytometry

The fraction of cells that display propidium uptake and/or PS externalization following EP exposure was measured using a flow cytometer Accuri C6 (Accuri Cytometers, Inc., Ann Arbor, MI) or FACS Aria™ (Becton-Dickinson, Franklin Lakes, NJ). Prior to exposure, cell suspension in the growth medium (1.2×10^6 cells/ml) was supplemented with 5 μ l/ml of Annexin V-FITC (BD Pharmingen, San Diego, CA) and/or 1 μ l/ml of propidium iodide (Sigma-Aldrich, St. Louis, MO). Measurements were performed within 10 min after EP treatment. Sham-exposed samples and those treated with 0.04% digitonin were used as negative and positive controls, respectively.

2.5. EP exposure systems, dosimetry, and thermometry

The 10-ns exposure system was described previously [32,48], and principles of its operation were outlined in [49]. In brief, to produce 10-ns EP, a Blumlein line was charged from a high-voltage DC power supply until a breakdown voltage was reached across a spark gap in a pressurized switch chamber. The breakdown voltage (and, consequentially, the voltage of EP delivered to the sample) was varied from 10 to 30 kV by changing the pressure of SF₆ gas in the switch chamber. Pulser control system included a programmable gas regulator, pulse counter, and GPIB outputs for communication with the high voltage power supply and digital high speed oscilloscope (TDS3052B, Tektronix, Wilsonville, OR). The control system communicated with a PC using a custom program written in LabVIEW (National Instruments, Austin, TX).

Longer pulses (300 ns, 1.8 and 9.0 μ s) of up to 1-kV amplitude were produced by AVTECH AVOZ-D2-B-ODA generator (AVTECH Electrosystems, Ottawa, Ontario, Canada). To produce pulse trains of predetermined duration at selected pulse repetition rates, this generator was triggered externally from model S8800 stimulator (Grass Instruments Co., Quincy, MA). The pulse amplitude and shape (trapezoidal, with rise and fall times (20%–80%) of <100 ns) were monitored using a Tektronix TDS3052B oscilloscope. Pulses were delivered to a standard electroporation cuvette using a 50- to 10-Ohm transition module (AVOZ-D2-T, AVTECH Electrosystems) modified into a cuvette holder.

The E-field values reported in this paper were obtained by dividing the mean pulse voltage (as measured by the oscilloscope) by the width of the gap in the electroporation cuvette. To compare the cytotoxic effect of different pulse durations (section 3.2), we tested EP at a near maximum pulser output voltage (i.e., 29 kV for 10-ns pulses and 0.9 kV for all others), and at 2-fold reduced voltages. To evaluate EP cytotoxicity, the number of pulses at each voltage was varied widely, from 1 to 4,800 per exposure (see Table 1 for detail). In most experiments, pulses were delivered at 1–2 Hz; however, for the highest pulse numbers, the rate had to be increased to 5 Hz (see section 3.4.2). The absorbed dose was calculated as the energy delivered to the sample normalized to the mass of the sample [32].

In isolated experiments, sample temperature during and after EP exposure was monitored using a fiber optic REFLEX-4 thermometer (Nortech Fibronic, Quebec City, Quebec, Canada).

3. Results and Discussion

3.1. Timeline and pathways to cell death following exposure to 10-ns EP

The time dynamics of cell populations affected by 10-ns pulses was studied in two cell lines, U937 and Jurkat. The total cell density and the fraction of dead cells were measured using Trypan blue exclusion assay at time intervals from 0.5 hr to 72 hr after the treatment.

In both cell lines, 10-ns EP caused immediate (in 30 min or less) and delayed cell death (Fig. 1). The live cell population diminished gradually, reaching minimum at intervals from 2 hrs to as late as 24 hrs after the treatment. Exposure to a higher number of pulses caused a stronger and earlier effect, with Jurkat cells being much more vulnerable than U937 (this difference will be discussed in more detail below). At about 24 hrs, exposed cell populations started to grow at a pace close to that of the control groups.

Delayed occurrence of cell death appears consistent with previous reports that ns-range EP trigger the apoptotic process, in contrast to micro- and millisecond range EP that cause immediate membrane rupture and necrosis. On the other hand, a significant fraction of cells died at intervals considerably shorter than it typically takes to complete the apoptotic process. Here, we employed internucleosomal DNA fragmentation as a more reliable and definitive sign of apoptosis than PS externalization. DNA fragmentation occurs relatively late in the apoptotic process, but (in *in vitro* assays) precedes plasma membrane degradation and the uptake of Trypan blue. Therefore, a comparison of the time course of cell death (as detected by dye exclusion) with the time course of appearance of the “DNA ladder” can give an estimate of the fraction of apoptotic cells after nsEP treatment.

Fig. 2 compares apoptotic DNA cleavage in U937 cells exposed to 10-ns EP and subjected to a heat shock. At 2 hrs after the nsEP treatment, the number of “live” cells (impermeable to Trypan blue) decreased almost twofold, but no apoptotic ladder could be detected. In contrast, heat-treated cells displayed DNA cleavage, but no reduction in live cells density. A distinct DNA ladder developed in both treated groups by 3 hrs. At 5 hrs post exposure, the Trypan blue

exclusion dropped to 21% in nsEP-exposed cells, but still was at 90% in the heat-shocked group, even though the DNA ladder for this group developed earlier.

These data demonstrated that 10-ns EP induce apoptosis which develops at the same pace or even slower than a “classic” apoptosis caused by the heat shock. However, consistently with our earlier observations [32], the data also showed that apoptosis was not the only or even the main cell death pathway, as a large number of cells already became Trypan blue-permeable before the DNA cleavage could be detected. In fact, the prevalence of necrotic cell death is not surprising, since the most commonly seen cytophysiological effect of nsEP is necrotic cell swelling [27,37,50], as opposed to apoptotic shrinking. Cell swelling occurs even in isosmotic media by the so-called colloid osmotic mechanism [50–53] when the plasma membrane is made permeable to small electrolytes, e.g., as a result of nanoelectroporation [35,37,39,50]. If pores are not sealed, colloid osmotic swelling inevitably leads to eventual membrane rupture and cell death, although the process may take long time. That said, one can reasonably expect that tuning the exposure conditions (pulse amplitude, number, duration), as well as treating cells within tissue (when cell swelling is limited) may change the balance of necrotic and apoptotic processes.

3.2. Cytotoxic efficiency of 10-ns EP in comparison with 300-ns, 1.8- and 9- μ s EP

In our previous studies, analysis of the cytotoxic efficiency of varied 10-ns EP treatments (different E-field values, numbers of pulses, and pulse repetition rates) established that, in most cases, the same values of the absorbed dose caused the same decrease in cell survival [28,40]. For both Jurkat and U937 cells, the survival curve started with a plateau (“shoulder”) at lower doses, and then decreased exponentially to about 10%. However, for values under 10–15%, the survival was higher than predicted by the exponential fit, either due to the presence of a resistant cell fraction in the population, or because of an unidentified bias in the method that was used (dye exclusion).

In the present study, we employed a different method (MTT assay) and observed the same deviation from the exponential fit at the highest doses of EP treatment. Furthermore, this effect was also characteristic for treatments using longer EP. Instead of the exponential fit, we were able to fit the survival data within a wider range with a power function $S = \alpha D^{(-K)}$, where D is the absorbed dose (J/g), and coefficients K and α determine the slope and the “shoulder” of the survival curve on a double-logarithmic scale (Fig. 3). Interestingly, the slope (K) displayed only minor fluctuations across the range of tested conditions. In contrast, the “shoulder” width (α) was the same only for long pulses (1.8 and 9 μ s) for both Jurkat and U937 cells, but increased profoundly and in a cell type-specific manner when shorter EP were used (Table 2).

Combined survival data for different exposure conditions are presented in Fig. 4. In Jurkat cells (top graph), the survival data are clearly split into two major groups, corresponding to a difference in the efficiency of the tested EP treatments. One group, reflecting the higher EP efficiency, included all datapoints for 1.8 and 9- μ s pulses, at either 2.25 or 4.5 kV/cm, as well as for 300 ns at 4.5 kV/cm. The other group, for which the EP efficiency was much lower, included all datapoints for 300 ns at 2.25 kV/cm and for 10 ns at both 150 and 290 kV/cm. Notably, within each group, the cytotoxic efficiency of EP was determined largely (if not entirely) by the dose, no matter what combination of the E-field, pulse duration, and pulse number was employed to reach a particular dose.

The data for U937 cells (bottom graph) also fell into distinctly different groups. However, the 300-ns data did not merge into the other groups, and instead formed a smoother “transition” between them. Same as with Jurkat, 300-ns pulse duration was the only exception from the “dose rule,” i.e., the exposures at 2.25 and 4.5 kV/cm resulted in different survival even though the dose was the same.

Very different efficiency of long and short pulses in causing cell death can be quantified by the dose that decreases cell survival to 50% (LD_{50}). Fig. 5 shows LD_{50} values and respective confidence intervals as calculated from the power fits of the survival curves separately for each of the studied groups (Table 2). Long pulses were the most efficient, with LD_{50} at 1–3 J/g, whereas shortening pulses to 10 ns increased LD_{50} by more than two orders of magnitude.

Importantly, this change of efficiency was not the same for two tested cell lines. Long pulses (1.8 and 9 μ s) killed both Jurkat and U937 equally well, whereas 10-ns pulses were 5- to 8-fold more efficient for killing Jurkat than U937. As a result, 10-ns EP at certain doses (e.g., 200 J/g, see Fig. 6) can selectively eliminate most of the Jurkat population while leaving U937 cells practically unaffected. Such selectivity cannot be accomplished when using microsecond pulses. (Note that data in Fig. 6 are not direct measurements, but values predicted for 200 J/g using the best fit equations in Table 2)

To summarize, long EP killed cells efficiently, but indiscriminately; short pulses were far less efficient, but showed profound selectivity. One can conclude that long pulses would be best suited for applications that require complete elimination of all cell types at the lowest energy expense, such as disinfection and sterilization. Instead, short pulses may find the best application in tissue and tumor ablation, when selectivity against a certain type of cells is the major factor and the energy expenditure is of little concern. At this point, however, we do not know what makes some cells more sensitive than others to nsEP; future studies will be needed to reveal the sensitivity mechanism and select what kinds of cancer might become potential candidates for ns EP treatment.

3.3. Cell membrane permeabilization by long and short EP

Clustering of the survival data into separate groups (Fig. 4), as well as very different efficiency and selectivity of long and short EP suggested that their cytotoxic effect could be caused by different mechanisms.

Based on observations of nanopore formation by nsEP exposure [33,35,50], we hypothesized that the difference in the cytotoxic effect of long and short pulses might result from qualitatively different types of plasma membrane impairment. Using flow cytometry, we quantified PS externalization (as a marker for nanopore formation) and propidium uptake (as a marker for conventional electroporation); all measurements were performed within 10 min after exposure to either 10-ns or 1.8- μ s pulses.

As anticipated, longer pulses efficiently caused both PS externalization and propidium uptake (Fig. 7), indicating that membrane pores opened by these pulses are large enough for propidium passage. In contrast, 10-ns pulses triggered PS externalization with little or no propidium uptake, reflecting the formation of nanopores. Although we did not extend these experiments to 300-ns pulses, the survival data for this intermediate pulse duration suggested a mixture of the nanoporation and conventional electroporation mechanisms, with the former one prevailing at the lower E-field intensity, and the latter one prevailing at the higher intensity.

It is not known exactly how the formation of nanopores leads to cell death, but the pathway will likely be different from the “classic” IRE. One can reasonably expect that nanopores are less injurious to the cell than larger electropores and can be more readily resealed, which would explain much higher tolerance of cells to nsEP. In contrast to the IRE, the formation of nanopores will not cause immediate loss of cell constituents other than the smallest solutes, i.e. small inorganic ions. The loss of transmembrane gradient of these ions will not only eliminate the membrane potential, but will also trigger water uptake by the colloid osmotic mechanism. The cell will likely attempt to repair the membrane by endo- and exocytosis of damaged sites [54–55], and activate ionic pumps to restore transmembrane ion balance and

membrane potential. The success or failure of the repair will depend on multiple factors such as the extent of damage; composition of the extracellular medium; the strength of the cytoskeleton, which will help to withstand swelling; the availability of membrane components necessary for repair; and the supply of ATP and/or ATP loss due to leakage via damaged plasma membrane. If the attempts to repair cell membrane were not successful, the cell will most likely undergo necrotic death due to continued swelling and eventual membrane rupture. However, even in case of successful repair, the ATP depletion may be excessive, triggering either apoptosis or necrosis [56–58]. Intracellular damage by nsEP, but not by longer pulses, may also be a reason for apoptosis [26,28]. Regardless of the exact pathway to cell death, the processes triggered by nsEP appear more complex and dependent on cell physiology than in the case of IRE. Understanding of exact nature of these processes will help to explain and predict selective cytotoxic effects of nsEP in different cell lines and facilitate medical applications of this modality.

3.4. Possible confounding factors: temperature rise, pulse rate, exposure duration, and electrochemical reactions

The data presented above clearly show higher cytotoxic efficiency of longer pulses, higher selectivity of shorter EP, and suggest two different mechanisms of initial cell damage. However, one cannot limit the variability between the diverse exposures to strictly the desired variables (the pulse duration, E-field amplitude, and pulse number). For example, longer trains and higher doses employed for 10-ns pulses inevitably caused more sample heating than in case of μ s-duration pulses. Therefore, the conclusions presented above would not be fully valid without detailed analysis of all confounding factors.

3.4.1. Sample heating by EP exposure—It is immediately obvious from Fig. 4 that the cytotoxic effect of various EP was not thermal in nature: If the effect were just thermal, equivalent doses delivered by pulses of differing width would have caused the same sample heating and decreased cell survival to the same extent. Although this was not the case, possible contribution of the EP-induced temperature rise to cell killing needs to be analyzed.

In principle, the doses that were delivered to cell samples could be sufficient to heat them from the room temperature to potentially lethal levels (the “worst case scenario” heating, i.e., without any heat dissipation, equals approximately 0.24 °C per 1 J/g.) However, we determined earlier that heat dissipation from both 1- and 2-mm electroporation cuvettes is very efficient [32]. Nonetheless, sample heating was measured again for all exposure regimens.

We found that the temperature in exposed samples never exceeded 30 °C and therefore posed no concern, with the exception of 10-ns exposures at 290 kV/cm. At this field intensity, delivering 300 and 1,000 pulses at the maximum rate sustained by the pulser (1.8 Hz) could increase sample temperature to 32 and 42 °C, respectively. Considering that the entire duration of the EP treatment was less than 10 min, reaching even 42 °C was probably innocuous (compare to “moderate heat shock” induced by holding cells at 44 °C for 40 min, Fig. 2). Although heating was not the cause of cell death, it could modify the cytotoxic efficiency of EP treatments, making cell killing at the highest doses a combined effect of EP and heating.

3.4.2. Effects of pulse repetition rate—Most experiments in this study were performed at 1 Hz pulse repetition rate. We have previously demonstrated that 10-ns pulses at 0.1, 0.25, 1, and 2 Hz caused similar effects [32]. In the present study, the only EP treatment that required the use of still higher repetition rate (5 Hz) was exposure to 300-ns pulses: With relatively low efficiency of 300-ns EP at voltages sustained by the pulser, we had to deliver up to as many as 4,800 pulses to drop the survival under 50%. For such exposures, the pulse rate was increased to 5 Hz, to keep the overall treatment duration within reasonable limits (see 3.4.3 below).

Therefore, in a separate series of experiments we tested if modest changes in the pulse repetition rate could affect the cytotoxicity of 300-ns pulses (Fig. 8). We observed a trend to a lower efficiency of EP exposures at higher repetition rates, visible mostly in Jurkat cells. We conclude that increasing the pulsing rate to 5 Hz in the experiments presented in Figs. 4, 5, and 6 could not significantly bias measured cell survival levels.

3.4.3. Effects of exposure duration—One reason why we had to use the higher repetition rates for long pulse trains was that the time duration of EP exposure could be a confounding factor by itself. We found that delaying the onset of EP exposures after a cell sample has been transferred into the electroporation cuvette could increase the survival, particularly in U937 cells (Fig. 9). The protective effect became significant for delays of over 20 min, and could be countered by mixing of the cell sample immediately prior to EP exposure. The nature of this protective effect is not completely clear; it could be caused either by gradual cell precipitation, or, more likely, by depletion of oxygen in the sample, which would inhibit the cytotoxic effect [48]. Since such protective effects could also develop during long EP exposures, we had to increase pulse repetition rate to avoid them. In addition, in each series of experiments, the sequence of different EP treatments was carefully randomized, to prevent data bias due to different time delays between sample preparation and exposure onset.

3.4.4. Electrochemical alteration of the culture medium—Electrochemical reactions are unavoidable in experiments that involve passing of electrical current through a cell suspension. At least under some conditions, these reactions could lead to significant pH changes and solubilization of the electrode material [59]. However, without special experiments, it is difficult to estimate the extent to which such electrochemical reactions affect cell survival.

To address this question, in a separate set of experiments, we split the exposed cell samples into two identical aliquots immediately following EP exposure. In both the aliquots, cells were separated from the medium by mild centrifugation, and the supernatant was removed. Next, one aliquot was resuspended in a fresh culture medium, whereas the other one was resuspended in the same supernatant that had just been removed (“EP-exposed medium”, which supposedly contained all potentially harmful electrochemically-created species). These experiments established that cell survival in both aliquots was identical (data not shown); thus we conclude that, for the studied conditions, electrochemical reactions did not affect the EP treatment results.

3.5. Search for the universal metric of EP treatment efficiency

With the introduction of intense EP treatments in early 1960s, many authors attempted to develop a universal metric to quantify their efficiency. The proposed metrics included charge, dose, and so-called “scaling parameter” [17–24,28,41], but the experimental findings that would (or would not) support the applicability of these metrics remain highly contradictory.

Here, we chose dose as a metric for the following reasons: (1) it was dose that determined primary membrane damage when applying single EP [36], (2) dose adequately defined cytotoxic effects of various E-field amplitudes and pulse numbers for a fixed pulse duration [28,40], and (3) dose effects could be well approximated by power fit and can readily be interpreted. Although application of dose proved very useful for data analysis, we concur with previous reports that dose alone cannot serve as a universal metric that works equally well for all pulse durations.

In fact, the data presented in Fig. 4 show that such a universal metric probably does not exist at all, because of different mechanisms that underlie the cytotoxic effect, and because of the significant “biological” component that defines whether a certain degree of primary damage will or will not cause cell death. This component markedly varies from one cell line to another,

hampering any attempts to develop a universal exposure metric. Indeed, assuming that some model would accurately describe the survival of Jurkat cells, as shown in Fig. 4, this model would inevitably fail to match the data for U937 cells; and vice versa. We conclude that understanding of the intrinsic mechanisms that define EP cytotoxicity would be more productive for predicting EP efficiency than relying solely on the physical parameters of exposure.

4. Summary

The principal finding of this study is that reducing the duration of EP from micro- to nanoseconds decreases the cytotoxic efficiency of the treatment, but may improve its selectivity against certain cell types. At the primary mechanisms level, this difference can be related to the transition from the conventional electroporation to nanoelectroporation (which is defined as formation of stable plasma membrane pores not exceeding 1–2 nm in diameter). Cell death triggered by opening of nanopores and, potentially, also by damage to organelles, is mostly delayed and can be either necrotic or apoptotic. In either case, the ability to repair the primary damage and the pathways to full recovery or death are, to a great extent, determined by intrinsic biological properties and physiology of the cell. This large “biological component” explains why physical parameters of exposure have limited predictive power for nsEP treatments, and why different cell types can vary so much in nsEP sensitivity. Further research will be focused on identifying the critical processes that determine cell fate after nsEP treatments, so that their efficiency could be reliably predicted for various cell types.

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Abbreviations

EP	electric pulses
IRE	irreversible electroporation
nsEP	nanosecond-duration electric pulse
PS	phosphatidylserine
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

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Figure 1.

Time dynamics of U937 and Jurkat cell populations following exposure to trains of 10-ns electric pulses (90 kV/cm, 2 Hz; the number of pulses delivered to the sample is indicated next to the curves). “Sham exposure” is the same treatment, but using zero pulses (control). The density of potentially viable cells (impermeable to Trypan blue) was measured by hemocytometer counts (mean $\pm$ s.e., for 10 to 25 independent experiments). For clarity, some error bars are shown in one direction only.

Figure 2.

Internucleosomal DNA fragmentation in nsEP-exposed and heat-shocked U937 cells. Cell samples were lysed at indicated time intervals after sham exposure (control), nsEP exposure (600 pulses, 10 ns, 100 kV/cm), or a heat shock (HS; 40 min at 44 °C). The numbers at the bottom show the percentage of cells that exclude Trypan blue, as determined by hemocytometer counts immediately prior to lysis at 2 and 5 hrs.

Figure 3.

Power function fit provides a more accurate prediction of dose-dependent survival changes in EP-treated cells than exponential fit. Survival was measured by MTT assay 24 hr after exposure of U937 cells to trains of 10-ns EP (top) or 9-μs EP. The dashed lines show the best fit using exponential function (left panels; semi-logarithmic scale) or power function (right panels; double-logarithmic scale). Note that the datapoints at higher doses (“resistive tail”) were excluded from the exponential fit calculations, and the data for the lowest doses (“initial shoulder”) were excluded from the power fit approximations.

Figure 4.

Dose-dependent changes in survival of Jurkat (top graph) and U937 cells exposed to trains of electric pulses. The exact numbers of pulses per train for each tested dose, pulse duration, and E-field amplitude are given in Table 1. Survival was measured at 24 hr after the treatment by MTT assay and expressed in % to sham-exposed parallel controls. Each datapoint is the mean $\pm$ s.e. for a minimum of 3 independent experiments (mostly 6 to 12 experiments); some error bars are shown in one direction only. Dashed lines represent the best fit curves using power function with 95% confidence intervals, shown by shaded areas. For clarity only, closely positioned datapoints (e.g., for 1.8- and 9- μ s EP) were pooled together for best fit approximation; see Table 2 for exact best fit parameters for each exposure regimen separately.

Figure 5.

Calculated doses that cause 50% death (LD_{50}) in Jurkat and U937 cells for EP treatments using different pulse durations (μs) and E-field amplitudes (kV/cm). The error bars represent 95% confidence intervals for mean LD_{50} values, as determined by a power fit separately for each type of treatment (see Table 2 for exact fit coefficients). Note the logarithmic scale for LD_{50} and its pronounced increase as the pulse duration decreases. Also note that LD_{50} for Jurkat and U937 cells was essentially the same for long pulses, but 5–8-fold different for short pulses.

Figure 6. Predicted survival rates in Jurkat and U937 cells at a dose of 200 J/g for different pulse durations (μs) and E-field intensities (kV/cm). These values and confidence intervals were calculated from the best fit data (Table 2). See text for further detail. Note that exposure using 10-ns pulses can selectively eliminate most of the Jurkat cells, while leaving the U937 cell population minimally affected.

Figure 7. Effect of 10-ns and 1.8- μ s EP at different doses on propidium uptake and PS externalization in U937 cells. The data were collected by flow cytometry measurements within 10 min after exposure (mean $\pm$ s.e., from 3 to 6 experiments per datapoint).

Figure 8.

Effect of pulse repetition rate on the cytotoxic efficiency of 300-ns, 4.5 kV/cm EP in U937 and Jurkat cells. Shown are mean values $\pm$ s.e. for 4 to 12 experiments per datapoint. The dashed lines are best fit approximations using a logarithmic function. Within the studied range (1–10 Hz), increasing the pulse rate slightly decreased the nsEP efficiency or had no effect.

Figure 9.

Prolonged holding of U937 cells in an electroporation cuvette may decrease the cytotoxic efficiency of the subsequent nsEP exposure. The graph shows cell survival at 24 hr (mean $\pm$ s.e., n=4) as a function of the time delay (min) between placing cells into the cuvette and the onset of nsEP exposure. Mixing of the cell sample immediately prior to exposure eliminated the protective effect.

Table 1

The number of pulses (n) and corresponding absorbed dose values (J/g) that were tested in Jurkat and/or U937 cell lines to produce cell survival data presented in Figs. 3–6.

0.01 μs				0.3 μs				1.8 μs				9 μs			
150 kV/cm		290 kV/cm		2.25 kV/cm		4.5 kV/cm		2.25 kV/cm		4.5 kV/cm		2.25 kV/cm		4.5 kV/cm	
n	J/g	n	J/g	n	J/g	n	J/g	n	J/g	n	J/g	n	J/g	n	J/g
10	28	10	104	100	1.8	25	1.8	5	0.54	1	0.42	1	0.54	1	2.1
30	84	30	313	200	3.6	50	3.6	10	1.1	2	0.86	2	1.1	2	4.3
100	279	100	1043	400	7.1	100	7.1	20	2.1	3	1.3	4	2.1	4	8.6
300	837	300	3129	800	14.3	200	14.3	40	4.3	4	1.7	5	2.7	5	10.7
1000	2790	1000	10428	1200	21.4	300	21.4	50	5.4	5	2.1	7	3.8	7	15
				1600	28.6	400	28.6	60	6.4	7	3	10	5.4	10	21.4
				2400	42.9	600	42.9	70	7.5	10	4.3	15	8	12	25.7
				3200	57.2	800	57.2	80	8.6	15	6.4	20	10.7	15	32.2
				4800	85.8	1000	71.5	120	12.9	20	8.6	25	13.4	17	36.5
						1200	85.8	160	17.2	30	12.9	30	16.1	20	42.9
						1600	114.4	200	21.4	40	17.2	40	21.4	25	53.6
								240	25.7	50	21.4	50	26.8	30	64.3
									60	25.7		60	32.2		
												80	42.9		

Table 2
Power fit parameters for Jurkat and U937 cells survival values at 24 hours after exposure to electric pulses of different duration.

Pulse duration, μ s	E-field, kV/cm	Power fit parameters*			
		α		K	
		Jurkat	U937	Jurkat	U937
0.01	150	608	3157	0.65	0.69
	290	550	4326	0.55	0.71
	2.25	236	678	0.48	0.62
0.3	4.5	163	270	0.74	0.62
	2.25	116	81	0.68	0.54
	4.5	86	60	0.73	0.58
9.0	2.25	103	99	0.65	0.63
	4.5	62	73	0.60	0.59

*The power fit equation was $S=\alpha D^{\alpha}(-K)$, where S is the cell survival (% to sham-exposed control) and D is the absorbed dose (J/g). Coefficients K and α , respectively, determine the slope and the “shoulder” of the survival curve. See text and Fig. 4 for more detail.