Bioelectrochemistry 110 (2016) 1–12

Contents lists available at ScienceDirect

Bioelectrochemistry

journal homepage: www.elsevier.com/locate/bioelechem

# Effects of high voltage nanosecond electric pulses on eukaryotic cells (*in vitro*): A systematic review

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ARTICLE INFO

*Article history:*
Received 13 August 2015
Received in revised form 23 February 2016
Accepted 23 February 2016
Available online 27 February 2016

*Keywords:*
Electroporation
Nanosecond electric pulses
Systematic review
Cells

ABSTRACT

For this systematic review, 203 published reports on effects of electroporation using nanosecond high-voltage electric pulses (nsEP) on eukaryotic cells (human, animal, plant) *in vitro* were analyzed. A field synopsis summarizes current published data in the field with respect to publication year, cell types, exposure configuration, and pulse duration. Published data were analyzed for effects observed in eight main target areas (plasma membrane, intracellular, apoptosis, calcium level and distribution, survival, nucleus, mitochondria, stress) and an additional 107 detailed outcomes. We statistically analyzed effects of nsEP with respect to three pulse duration groups: A: 1–10 ns, B: 11–100 ns and C: 101–999 ns. The analysis confirmed that the plasma membrane is more affected with longer pulses than with short pulses, seen best in uptake of dye molecules after applying single pulses. Additionally, we have reviewed measurements of nsEP and evaluations of the electric fields to which cells were exposed in these reports, and we provide recommendations for assessing nanosecond pulsed electric field effects in electroporation studies.



**Contents**



*Abbreviations:* nsEP, nanosecond electric pulses; FWHM, full width at half maximum; PI, propidium iodide; TB, trypan blue; EtH, ethidium homodimer; YOPRO, YO-PRO®-1 iodide; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; PEG, polyethylene glycol; PM, plasma membrane; SEM, scanning electron microscope; TDDS, time domain dielectric spectroscopy; TDR, time domain reflectometry; ER, endoplasmic reticulum; PARP, poly (ADP-ribose) polymerase; PS, phosphatidylserine; $Tl^{+}$, thallium ion; $Ca^{2+}$, calcium ion; BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid); EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; ROS, reactive oxygen species; MAPK, mitogen-activated protein kinase; AMPK, AMP-activated protein kinase; cyt c, cytochrome c.

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http://dx.doi.org/10.1016/j.bioelechem.2016.02.011

## 1. Introduction

Exposure of biological materials (cell suspensions, tissues) to high voltage electric pulses provokes a phenomenon that is nowadays termed electroporation: cell membranes become more permeable to molecules that otherwise cannot cross them [1–3]. Although not completely understood, electroporation has been widely studied and used as a basis for applications in medicine and biotechnology such as electrochemotherapy [4,5], gene electrotransfer [6], tissue ablation [7,8], extraction of various compounds [9,10], and microbial inactivation in food preservation [11].

Early theoretical predictions [12,13] and experiments [12,14,15] showed that shorter electric pulses of nanosecond duration (nsEP stands for nanosecond electric pulses; often called "nanosecond pulsed electric field" or nsPEF) have more profound effects on the cell interior than longer pulses of micro- and millisecond duration, and thus nsEP emerged as a promising tool for intracellular manipulation without any chemical intervention. With the development of new pulse generators that produced ultrashort pulses of very high electric fields of several tens of MV/m [16], researchers were able to show effects on cell organelles. In the last 15 years we have gained considerable knowledge about how nanosecond electric pulses affect cells: they affect cell organelles [15,17,18], increase intracellular calcium [19–21], and provoke apoptosis [22–24] and stress responses [25].

Researchers gradually discovered that the plasma membrane is also affected. The pores produced by nsEP are small, of nanometer scale, and are thus sometimes called "nanopores" [26–28]. Cells exposed to nsEP exhibit membrane permeability to both propidium (PI) and trypan blue (TB), classical indicators of membrane permeabilization, but detecting influx of these dyes and other small molecules after nanosecond pulse exposure requires methods with greater sensitivity than those used for longer pulses.

However, researchers using different cells, pulse parameters, exposure configuration and detection methods have described results that are often contradictory. Therefore, the first aim of this study was to review published results in a systematic and comprehensive way. Secondly, we used statistical analysis of published results to determine whether nsEP of different durations affect cells differently.

We classified nsEP into three distinct categories: A: 1–10 ns, B: 11–100 ns and C: 101–999 ns. With the use of nanosecond pulses, as pulses shorten, more intracellular effects and less effects on plasma membrane can be expected [15,19,29,30]. The first category (A) includes very short pulses, 1–10 ns, with rise times of a few ns (mostly shorter than the electrolyte relaxation time). In this regime the dielectric properties of the membrane and the intracellular and extracellular media dominate pulsed electric field effects on membranes, and the Maxwell–Wagner polarization of the membrane by migration of mobile charges is less important than it is for longer pulses [31]. Moreover, proportionally greater effects are expected on intracellular membranes with 1–10 ns pulses than with longer pulses [32,33]. In the second category (B), the pulse duration is less than the charging time of the plasma membrane [15,34,35]. The third category (C) includes nsEP with durations longer than the plasma membrane charging time.

The main focus of this review was the role of pulse duration in reported effects of nsEP on biological cells. With the use of statistical methods we analyzed all published data of experimental studies that examined effects of nsEP on eukaryotic human, animal, and plant cells *in vitro*. We tested several hypotheses. The main two were the following: 1. the occurrence of changes in the plasma membrane significantly depends on nsEP pulse duration, and 2. the occurrence of intracellular effects significantly depend on nsEP pulse duration. We hypothesized that PM effects are greater with longer pulses and intracellular effects are greater with shorter pulses. We also tested similar hypotheses for a number of effects of nsEP on cells *in vitro* that are reported in the literature.

Our second focus was the evaluation of the nanosecond pulsed electric fields used in experimental studies of electroporation (electropermeabilization). During the electroporation process biological cells are exposed to pulsed electric fields with specific electrical parameters, namely amplitude, duration, shape, number of pulses and pulse repetition rate. The duration of the pulse is usually specified as the full width at half maximum (FWHM) and a description of the pulse shape is usually enhanced with rise and fall times of the pulse [36]. In order to exactly specify the experimental method and thus to enable the reproduction of experiments under the same conditions, researchers should exactly determine and describe these electrical parameters. Some electrical parameters such as number of pulses and pulse repetition rate are relatively easy to state. Other electrical parameters are more difficult to determine, because it is currently not possible to measure the time course and distribution of the nanosecond electric field within the exposure configuration during the delivery. Our hypothesis was that in the existing literature on the electroporation of biological cells by nsEP there is not enough emphasis on the determination and description of the electric field to which biological cells are exposed or they are not described in adequate detail. By analyzing the data from the published reports, we compiled a list of recommendations for the evaluation of nanosecond electric field to which cells are exposed in electroporation studies.

## 2. Methods

### 2.1. Eligibility criteria

In our systematic review, we included reports of experimental studies in which eukaryotic human, animal, and plant cells were exposed to electric pulses of nanosecond duration *in vitro*.

### 2.2. Search strategy and study selection

For the purpose of our review, a systematic search through eight online bibliographic databases (Science Direct, IEEE Xplore, SpringerLink, Web of Science, HighWire Press, Compendex, IngentaConnect, PubMed) was performed by TBN on August 9, 2013, by employing keywords with Boolean operators ("nanosecond electric pulses" OR "nanosecond pulsed electric field") AND "cells". In most cases the search was confined to the years 1990–2013. We included scientific articles and conference reports (also reports accepted for publication and published online) written in English, and excluded books, abstracts, and unpublished data. Briefly, records clearly not related to the theme (from other research fields such as physics and chemistry) were excluded on the

basis of abstract content. Some additional records were included on the basis of reviews' references and personal library. Further records were excluded based on reading the full text. Specifically, review, abstract only, non-English records, or records that pertained only to *in vivo* experiments, non-ns pulses, bacteria and yeast, pulse generators, or theory and modeling (without experimental data) were excluded from the study. From publications that reported the same results (*e.g.* conference reports and original research papers), the less comprehensive report was excluded from the review. An update of a systematic search was done on December 11, 2014, using the same search strategy. A database was established in open-source reference management software Zotero (Center for History and New Media, Fairfax, VA, USA).

### 2.3. Data collection and assessment of risk of bias

Each relevant report was assigned an identification number and was carefully read. The following data were extracted by TBN: publication year, leading research group, pulse parameters (pulse number, duration, amplitude, repetition rate), pulse duration classification (A: 1–10 ns, B: 11–100 ns, C: 101–999 ns), exposure configuration, research/detection methods, type of cells, main outcomes identification (plasma membrane effect, intracellular effect, apoptosis, calcium, survival, nucleus, mitochondria, stress) and a detailed description of outcomes.

Reports were classified into subgroups regarding the main outcomes studied (Table 1). For each main outcome, more data was extracted from subgroups for the detailed outcomes: each report has been annotated for detailed outcomes by "yes", "no" or blank (not studied), except for survival ("H"—higher, "S"—same and "L"—lower than control). In some cases, a "yes" was followed by an explanation that was further analyzed. When pulses of different durations were used, they were analyzed separately for each group of pulses (A, B, C). In the case of plasma membrane (PM) and intracellular effects, the outcomes were analyzed again for reports of studies regarding single or multiple pulses. Any pulses regardless of pulse number (single or multiple) were in the "all pulses" category. For details, see Table 1.

TBN and PTV collected the data independently. Disagreements over classification and categories were discussed until consensus was reached. In three cases, a third reviewer (DM) served as arbitrator.

The collected data was organized in Excel files (Microsoft Corp., Redmond, WA, USA).

When reading the reports and collecting data, a risk of bias was assessed, following recommendations of the Cochrane Collaboration [37]. TBN and PTV harmonized the criteria for risk of bias assessment. TBN assessed most possible sources of bias: sequence generation (not stating the exact method of randomization of experiments or the lack of it), not blinding (prone to subjective evaluation of researchers), incomplete outcome data (if some methods described in the Methods part were not presented in the Results section), selective outcome reporting (if any experimental series was omitted from statistics) and methodological sources of bias (if a method was clearly not suitable for presenting results), with the standard ratings (low, high, and unclear risk of bias). Reports without peer review ("not a journal") were also regarded as with a high risk of bias. PTV determined the methodological risk of bias and re-examined all the papers with a high-risk of bias and consensus about them was reached. Reviewers were not blinded to the authors of the reports. Overall risk of bias was determined by the guidelines of Cochrane Collaboration [37].

### 2.4. Data analysis and sensitivity analysis

For the synopsis, reports were analyzed for publication date, methods (types of cells, exposure configuration, pulse duration classification) and main research outcomes.

Numbers of reports were counted for each detailed outcome ("yes" and "no") and for each pulse duration group (A, B, C) and arranged into tables $2 \times 3$ (except for survival, where "yes" and "no" were replaced by "H"—higher, "S"—same, and "L"—lower than control, leading to $3 \times 3$ table). Pearson's Chi-square test was used for all the tables in order to test the dependence between pulse duration groups and the effects. When the expected frequencies of the events were too low — more than 20% of events had expected values below 5 (indicating a small underlying probability, and/or a small number of observations); the test was not regarded as valid. Pearson's Chi-square test was performed using SigmaPlot 11.0 (Systat Software Inc., Chicago, IL, USA).

In sensitivity analysis, reports with overall high risk of bias were excluded from statistical analysis. The results thus obtained were compared to those including reports with a high risk of bias.

### 2.5. Evaluation of nanosecond electric fields

We established a list of recommendations for evaluation of the electric field to which cells are exposed in electroporation studies. The

**Table 1**
Data extraction: main and detailed outcomes. Abbreviations: PI: propidium iodide, TB: trypan blue, EtH: ethidium homodimer, YOPRO — YO-PRO®-1 iodide, FITC: fluorescein isothiocyanate, GFP: green fluorescent protein, PEG: polyethylene glycol, PM: plasma membrane, SEM: scanning electron microscope, TDDS: time domain dielectric spectroscopy, TDR: time domain reflectometry, ER: endoplasmic reticulum, PS: phosphatidylserine, PARP: poly (ADP-ribose) polymerase, cyt c: cytochrome c.

| Main outcome | Pulses | Detailed outcomes |
|---|---|---|
| Plasma membrane effects | All pulses, single, multiple | PI, TB, EtH, YOPRO, other dyes and large molecules (calcein, FITC, bleomycin, GFP, fluorescein, PEG), ions uptake, PS externalization (first 30 min; detected by annexin-V or FM 1-43), other detecting systems for PM effects (SEM, TDDS, TDR, Annine-6, swelling, blebbing), pulse parameters' effects (amplitude, number, duration, repetition rate), delayed effect, recovery, up- and downregulating conditions of PM effects (conditions that lead to higher or lower PM effects than basic conditions, respectively) |
| Intracellular effect | All pulses, single, multiple | Organelles (not nucleus — ER, mitochondria, other), nucleus, apoptosis, $Ca^{2+}$ released from internal stores, other (cyt c, cytoskeleton), molecular level (caspases, DNA, other), PM also affected |
| Apoptosis | All pulses | Morphology, externalization of PS (more than 30 min after pulsing), caspases, $Ca^{2+}$-dependent, mitochondria, cyt c, mitochondrial membrane potential, DNA fragmentation, PARP cleavage, pro/anti-apoptotic factors, up- and downregulating conditions of apoptosis (conditions that lead to higher or lower apoptosis rate than basic conditions, respectively) |
| Calcium | All pulses | $Ca^{2+}$ elevation in cells, extracellular, intracellular, not defined source, ER release, store-operated (capacitive) $Ca^{2+}$ entry, $Ca^{2+}$-induced $Ca^{2+}$ release, conditions that block $Ca^{2+}$ elevation, only downstream effects of $Ca^{2+}$ observed (indirect methods through $Ca^{2+}$ blockers) |
| Survival | All pulses | Apoptosis, necrosis, up- and downregulating conditions of survival, the pulse parameters' effects (amplitude, number, duration, repetition rate) |
| Nucleus | All pulses | Nuclear morphology, nuclear envelope, DNA, chromosomes, nuclear proteins, transfection, cell cycle, other detection methods (TDDS, TDR, telomeres, centrosomes) |
| Mitochondria | All pulses | Mitochondrial membrane potential, cyt c, $Ca^{2+}$ release from mitochondria, pro/anti-apoptotic factors |
| Stress | All pulses | Stress |

recommendations address three categories: 1) the measurement protocol, 2) the time course of the pulse, and 3) the electric field determination. A thorough description of how this should be done properly is written in section 4.9.

We have analyzed how many evaluations of nanosecond electric field described in 203 existing reports on the electroporation of biological cells by nsEP comply with our recommendations (evaluation was done by MR). In some reports (45) more than one pulse delivery setup was used, which necessitated more than one evaluation of nanosecond electric field. We carefully analyzed all the descriptions separately. Each description was analyzed to determine whether it complied appropriately, poorly, or inappropriately with each of our recommendation categories: measurement protocol, time course of the pulse, and electric field determination. A description complies appropriately with our recommendation if the authors address the content of the recommendation adequately and comprehensively. A description complies poorly with our recommendation if the authors address the content of the recommendation but do it incompletely or with minor irregularities. A description complies inappropriately with our recommendation if the methods do not address the content of the recommendation or do it improperly. Overall compliance was given for each description by the worst rated category.

## 3. Results

### 3.1. Search results

Altogether, 203 reports were identified as relevant for the systematic review of the effects of nanosecond electric pulses (nsEP) on eukaryotic cells *in vitro* (153 original research papers and 50 conference reports) (the list of all 203 reports can be found in Supplement 1). A detailed description of the systematic search and report selection process is shown in Schematic 1.


**Schematic 1.** A systematic search of relevant reports and a report selection process.

### 3.2. A field synopsis

The first relevant report of the effects of nsEP on eukaryotic cells (human, animal, plant) *in vitro* [12] was published in 1997 (Fig. 1). The number of published reports increased steadily, reaching 33 in 2013. Three of the reports that were available online already in 2014 were later published in 2015. The trend clearly shows increased interest of researchers into effects of ns pulses; all reports were however treated equally — irrespective of their publication date.

In 203 reports of experimental studies, 84 different cell types were used. Most popular cells are Jurkat, a human T-cell leukemia cell line which was used in 79 reports (38.9%), followed by HeLa, a human cervical adenocarcinoma cell line (used in 21 reports, 10.3%), and HL-60, a human promyelocytic leukemia cell line (17–8.4%). Out of all 84 cell types used, 58 (69.0%) were of human, 23 (27.4%) of animal and three (3.6%) of plant origin. Only 13 cell types used were primary cultures, all the rest (71) were immortalized cell lines.

Researchers used two main configurations for exposure of cells to nanosecond electric pulses. Cuvettes with built-in electrodes used for bulk cell suspension treatment maintain sterile conditions. Cells can be further cultivated and propagated and used for analyses requiring larger numbers of cells. With cuvettes, however, the effects cannot be observed immediately after pulse application since the manipulation of suspended cells requires some time. For real-time, microscope observations of individual cells during and after pulse exposure, microelectrodes have been developed. Because microelectrodes can be made of more inert materials, electrochemical reactions on the electrodes as side effects [38,39] can be reduced.

Cuvettes were used in slightly more reports (126) than microelectrodes (89). In a few reports (2) other exposure configurations such as atomic force microscopy probes and loop antennas were used. In 14 of the reports more than one exposure configuration was used.

Most researchers (121 reports) used nanosecond electric pulses of moderate duration (B: 11–100 ns). The use of shorter (A: 1–10 ns) and longer (C: 101–999 ns) pulses was slightly less frequent (74 and 82 reports, respectively). In some of the reports different pulses were used, and therefore reports were associated with more than one pulse duration category.

We identified eight main outcomes of research in the field of nsEP effects on cells: plasma membrane (PM) effect, intracellular effect, apoptosis, calcium, survival, nucleus, mitochondria, and stress. Fig. 2 represents the number of reports with respect to the research outcomes. In the highest number of reports researchers addressed the effects of nsEP on PM (144).

### 3.3. Pearson's Chi-square tests

We identified eight main outcomes and classified the reports into subgroups with respect to these outcomes. Then we analyzed all the reports for additional detailed outcomes and counted how many reports noted positive or negative effects on detailed outcomes for each pulse length group (A, B, C). We sorted them into 107 tables that represented the results of 107 hypotheses.

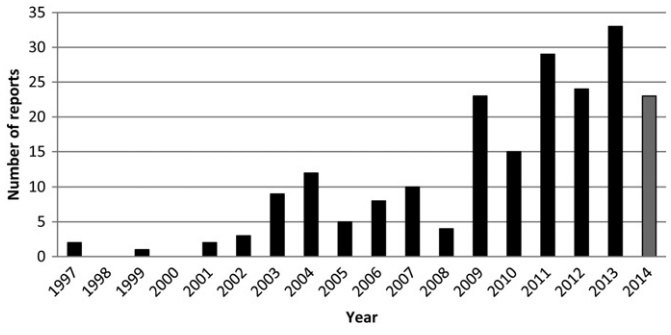


**Fig. 1.** The distribution of 203 relevant reports with respect to the year of publication. For the most recent year, 2014, only reports available before December 11 were included. Three of the reports that were available online already in 2014 were later published in 2015.

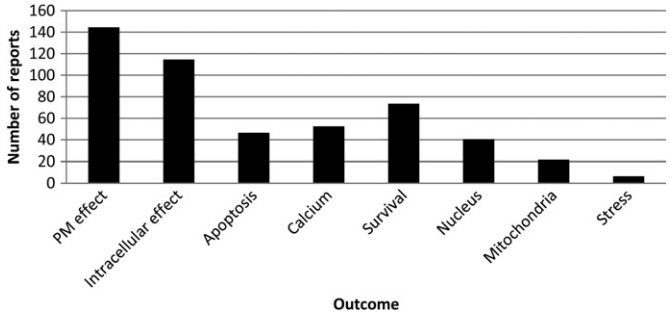


**Fig. 2.** The number of published reports regarding the research outcomes. PM — plasma membrane.

We tested 107 hypotheses regarding the effects of single or multiple nanosecond pulses of three different pulse durations (A: 1–10 ns, B: 11–100 ns and C: 101–999 ns) on eukaryotic (human, animal, plant) cells. Six of the hypotheses were completely overlapping and three were partially overlapping. We used Pearson's Chi-square test for all 107 tables to test the independence between the effects and pulse duration groups.

In only 13 cases were the tests valid (Table 2). In all other cases the expected frequencies of the events were too low (more than 20% of events had expected values below 5) so the Chi-square tests were not regarded as valid. Two of the 13 hypotheses were completely overlapping (intracellular + intracellular $Ca^{2+}$, calcium + intracellular source), therefore only twelve hypotheses were valid at the end. Out of 13 tested hypotheses, only six were showing a significant relation (dependence) between pulse duration and distinct effects on cells: PI uptake caused by any pulse (regardless of pulse number), the occurrence of changes in PM caused by any pulse (regardless of pulse number), the uptake of dye molecules (PI, TB, EtH — ethidium homodimer, YOPRO — YO-PRO®-1 iodide, other) by a single pulse, the occurrence of changes in PM caused by a single pulse, the occurrence of intracellular changes caused by any pulse (regardless of pulse number), and the occurrence of changes in PM caused by a single pulse that provoked intracellular effects.

### 3.4. Risk of bias and sensitivity analysis

Fifty reports with overall high risk of bias (Fig. 3) were identified during the analysis (for having a high risk of bias for incomplete outcome data, methods used, and/or for not being published in a peer-reviewed journal).

For the sensitivity analysis, reports with high risk of bias were excluded from statistical analysis (Table 3). Compared to all publication analysis (Table 2), in sensitivity analysis only five hypotheses were valid but the results for all five were similar. Only two of them showed that the effect is related to nsEP pulse duration: the uptake of dye molecules (PI, TB, EtH, YOPRO, other) by a single pulse, and the occurrence of changes in PM caused by a single pulse is more likely at longer pulses.

### 3.5. Evaluation of nanosecond electric field

We analyzed each description of pulse delivery setup in all 203 reports for compliance with our recommendations for the evaluation of the nanosecond electric field to which cells are exposed in electroporation studies (regarding measurement protocol, time course of the pulse, and electric field determination). Each description was determined by three categories: if it complies appropriately, poorly, or inappropriately with our recommendations. Overall compliance was given by the worst

**Table 2**
Hypotheses tested by Chi-square test (only valid) — sensitivity analysis. Reports with high risk of bias were excluded from statistical analysis. *p* — Chi-square test probabilities are listed for each detailed outcome (nsEP effect). If *p* is 0.05 or less, the distinct nsEP effect statistically depends on pulse duration (detailed outcome is marked with asterisk *). The last six columns represent percentages of reports that noted positive (yes) or negative (no) results for each detailed outcome using nsEP with three pulse durations (A, B, C) with the number of reports in brackets. Abbreviations: PM: plasma membrane, PI: propidium iodide, TB: trypan blue, EtH: ethidium homodimer, YOPRO — YO-PRO®-1 iodide.

| Main outcome | Detailed outcome | *p* | A | | B | | C | |
|---|---|---|---|---|---|---|---|---|
| | | | yes | no | yes | no | yes | no |
| PM all pulses | PI* | 0.044 | 56.3 (9) | 43.8 (7) | 79.3 (23) | 20.7 (6) | 88.9 (24) | 11.1 (3) |
| PM all pulses | Molecules together (PI, TB, EtH, YOPRO, other) | 0.055 | 70.7 (29) | 29.3 (12) | 79.2 (42) | 20.8 (11) | 91.1 (41) | 8.9 (4) |
| PM all pulses | PM affected* | 0.014 | 83.9 (47) | 16.1 (9) | 87.2 (68) | 12.8 (10) | 98.5 (67) | 1.5 (1) |
| PM single pulse | Molecules together (PI, TB, EtH, YOPRO, other)* | <0.001 | 15.8 (3) | 84.2 (16) | 65.5 (19) | 34.5 (10) | 75.0 (18) | 25.0 (6) |
| PM single pulse | PM affected* | <0.001 | 41.4 (12) | 58.6 (17) | 76.6 (36) | 23.4 (11) | 97.6 (40) | 2.4 (1) |
| PM multiple pulses | Molecules together (PI, TB, EtH, YOPRO, other) | 0.108 | 75.0 (27) | 25.0 (9) | 78.8 (26) | 21.2 (7) | 93.8 (30) | 6.3 (2) |
| Intracellular all pulses | $Ca^{2+}$ intracellular | 0.146 | 30.0 (3) | 70.0 (7) | 66.7 (12) | 33.3 (6) | 62.5 (10) | 37.5 (6) |
| Intracellular all pulses | PM also | 0.093 | 75.9 (22) | 24.1 (7) | 85.4 (35) | 14.6 (6) | 94.6 (35) | 5.4 (2) |
| Intracellular all pulses | Intracellular all together* | 0.025 | 73.2 (30) | 26.8 (11) | 92.0 (69) | 8.0 (6) | 83.7 (41) | 16.3 (8) |
| Intracellular single pulse | $Ca^{2+}$ intracellular | 0.140 | 33.3 (3) | 66.7 (6) | 70.6 (12) | 29.4 (5) | 69.2 (9) | 30.8 (4) |
| Intracellular single pulse | PM also* | 0.020 | 33.3 (5) | 66.7 (10) | 70.0 (14) | 30.0 (6) | 80.0 (12) | 20.0 (3) |
| Intracellular single pulse | Intracellular all together | 0.233 | 52.2 (12) | 47.8 (11) | 65.5 (19) | 34.5 (10) | 77.8 (14) | 22.2 (4) |
| Calcium all pulses | Intracellular | 0.146 | 30.0 (3) | 70.0 (7) | 66.7 (12) | 33.3 (6) | 62.5 (10) | 37.5 (6) |

rated category. We found out that only 15.8% of the descriptions complied appropriately with all our recommendations (Fig. 4).

To determine progress/improvement in nanosecond electric field evaluation, the analysis was done separately for the last five years of our review. 5% of 15 descriptions in 2010, 21.9% of 29 descriptions in 2011, 29.2% of 24 descriptions in 2012, 26.5% of 33 descriptions in 2013 and 34.8% of 23 descriptions in 2014 complied appropriately with our recommendations.

## 4. Discussion

A systematic analysis of published reports was used as a tool to gain insight into the wide variety of effects that are provoked in cells by nsEP.

### *4.1. Plasma membrane effects*

Plasma membrane permeability increases after exposure of cells to a pulsed electric field of adequate duration, pulse number, and amplitude (reviewed in [3]). However, initial studies of the effects of electric pulses in the nanosecond range indicated that nsEP had a significant effect on internal membranes with less or almost no effect on the plasma membrane [12,15,40]. In subsequent reports [26–28,41] it was shown that this is not the case and that even single nsEP can provoke the formation of pores smaller than the diameter of dyes used for detection in earlier studies (propidium iodide — PI, trypan blue — TB, ethidium homodimer — EtH). With the use of different detecting methods, such as patch-clamp [27,28], $Tl^+$ uptake [28,42], cell swelling [43,44] and phosphatidylserine (PS) externalization [26], it was suggested that plasma membrane pores of diameter 1 nm or less [43–46] can be detected, even after exposing cells to a single nanosecond electric pulse.

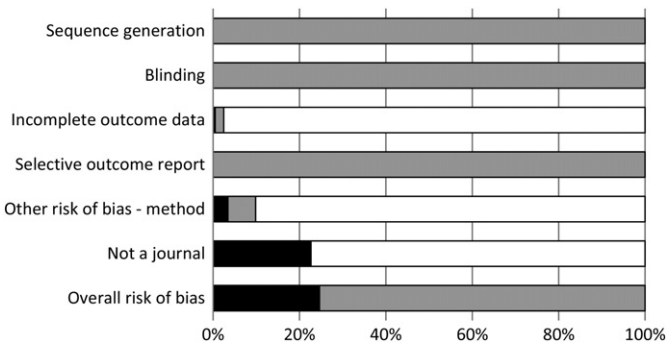


**Fig. 3.** Risk of bias assessment for reports included in the systematic review. The rates of reports with high risk of bias are shown in black, unclear in gray and low risk of bias in white.

In our systematic review, we identified 144 reports in which researchers studied nsEP effects on plasma membrane. In eight of the reports, authors did not detect any PM changes at any of the conditions (pulse parameters) tested. The other 136 reported that nsEP affected PM in at least one of the pulsing conditions used. 99 reports showed that pulse parameters (amplitude, duration, number and/or repetition rate) affect the impact of nsEP on PM.

When we analyzed PM effects after applying nsEP regardless of the number applied (single and multiple pulsing) we confirmed our hypothesis that the occurrence of PM effects significantly depends on the duration of the applied pulses. In 83.9%, 87.2%, and 98.5% of reports, authors noted that they detected PM effects after applying pulses of short (A: 1–10 ns), moderate (B: 11–100 ns), and long (C: 101–999 ns) duration, respectively. This is in agreement with our hypothesis that longer pulses are more likely to cause PM effects than shorter pulses. The PM effects were all effects taken together: from the uptake of larger molecules such as dyes (PI, TB, EtH, YOPRO) to inorganic ion uptake and immediate phosphatidylserine (PS) externalization. PS externalization is usually used for detecting apoptosis, however, in the case of PM permeabilization it is more likely that PS passes from the inner to the outer leaflet through the pores [26]. Therefore, we treated the immediate PS externalization (within the first 20 min after exposure to electric pulses) as the sign of PM permeabilization rather than the onset of apoptosis [47–49], even though it is hard to distinguish between the two processes. The uptake of PI was also dependent on pulse duration: 56.3%, 79.3% and 88.9% of reports noted that authors detected PI uptake after applying pulses of short (A), moderate (B) and long (C) duration, respectively. The uptake of larger molecules such as dyes (PI, TB, EtH, YOPRO), and some others — calcein, bleomycin, fluorescein, fluorescein isothiocyanate — FITC, polyethylene glycol — PEG, green fluorescent protein — GFP also showed the same trend (70.2%, 79.2% and 91.1% of reports showed dye uptake after A, B, C pulses, respectively), but was not statistically significant.

We also analyzed the reports of studies where single nsEP were used. The occurrence of PM effects significantly depended on the duration of the applied single pulse. The occurrence of PM effects was higher after applying longer single pulses: 41.4%, 76.6% and 97.6% of reports on effects of single pulses confirmed PM effects after applying single pulses of short (A), moderate (B) and long (C) duration, respectively. When we analyzed reports on the uptake of molecules after single nsEP application we confirmed that the uptake of these dyes significantly depends on the duration of the applied pulse. In shorter pulses (A), only 15.8% of reports showed uptake of the dyes, whether for moderate (B) and long (C) pulses the uptake is noted in 65.5% and 75.0% of reports.

**Table 3**
Hypotheses tested by Chi-square test (only valid) — sensitivity analysis. Reports with high risk of bias were excluded from statistical analysis. *p* — Chi-square test probabilities are listed for each detailed outcome (nsEP effect). If *p* is 0.05 or less, the distinct nsEP effect statistically depends on pulse duration (detailed outcome is marked with asterisk *). The last six columns represent percentages of reports that noted positive (yes) or negative (no) results for each detailed outcome using nsEP with three pulse durations (A, B, C) with the number of reports in brackets. Abbreviations: PM: plasma membrane, PI: propidium iodide, TB: trypan blue, EtH: ethidium homodimer, YOPRO: YO-PRO-1.

| Main outcome | Detailed outcome | *p* | A | | B | | C | |
|---|---|---|---|---|---|---|---|---|
| | | | yes | no | yes | no | yes | no |
| PM all pulses | Molecules together (PI, TB, EtH, YOPRO, other) | 0.147 | 72.7 (24) | 27.3 (9) | 81.4 (35) | 18.6 (8) | 91.2 (31) | 8.8 (3) |
| PM single pulse | Molecules together (PI, TB, EtH, YOPRO, other)* | 0.001 | 17.6 (3) | 82.4 (14) | 66.7 (14) | 33.3 (7) | 75.0 (12) | 25.0 (4) |
| PM single pulse | PM affected* | <0.001 | 45.8 (11) | 54.2 (13) | 78.1 (25) | 21.9 (7) | 100.0 (30) | 0.0 (0) |
| PM multiple pulses | Molecules together (PI, TB, EtH, YOPRO, other) | 0.205 | 75.9 (22) | 24.1 (7) | 77.4 (24) | 22.6 (7) | 92.6 (25) | 7.4 (2) |
| Intracellular single pulse | Intracellular all together | 0.171 | 52.6 (10) | 47.4 (9) | 66.7 (14) | 33.3 (7) | 84.6 (11) | 15.4 (2) |

Unfortunately, Chi-square tests for the uptake of most used dyes separately (PI, EtH, TB) as well as ions (Ca, Na, K, Co, Tl) failed to prove the dependency on pulse duration due to too low expected frequencies of reports. However, ion uptake was detected in 58.3%, 78.9% and 95.7% of reports on applying single pulses of short (A), moderate (B) and long (C) duration, respectively. This confirms that longer nsEP provoke the appearance of larger pores that are permeable to the dyes such as PI and TB but all nsEP pulses were able to cause smaller pores permeable for ions. This is in accordance with theoretical predictions [13,50]. It was suggested that smaller pores can convert into larger pores [28] as is the case expected for longer pulses [51,52].

We also analyzed the effects on PM using multiple nsEP (*i.e.* more than one pulse is applied). The occurrence of the uptake of molecules (dyes and other, see above) did not depend on pulse duration. This means that the uptake of dyes can be achieved also with shorter pulses (A) if we apply a train of pulses. The uptake of molecules was detected in 75.0%, 78.8% and 93.8% of reports using short (A), moderate (B) and long (C) pulses, respectively. For all other effects, Chi-square tests failed due to too low expected frequencies of reports. However, pore size and the size of the molecule/ion are not the only things that are important in detecting plasma membrane permeabilization, they are also the interactions of permeants with the plasma membrane [53] and the sensitivity of the method itself.

Out of all pulse parameters, repetition rate is the most intriguing. Repetition rate in the range of 0.001–10,000 Hz was considered in five reports regarding PM effects and four reports regarding survival. In four reports, higher repetition rate produced more effects [41,44,54,55]. According to three reports, higher repetition rate produced less effects [26,56,57] and in two reports, the authors noted that the effect of repetition rate is bell-shaped: at first, observed effects are increasing with increasing repetition rate and after reaching the peak they are decreasing again [58,59]. We believe that with further investigations of pulse repetition rate impact on nsEP effects on cells we will gain important information on pore dynamics and transport as in classical electroporation [60].

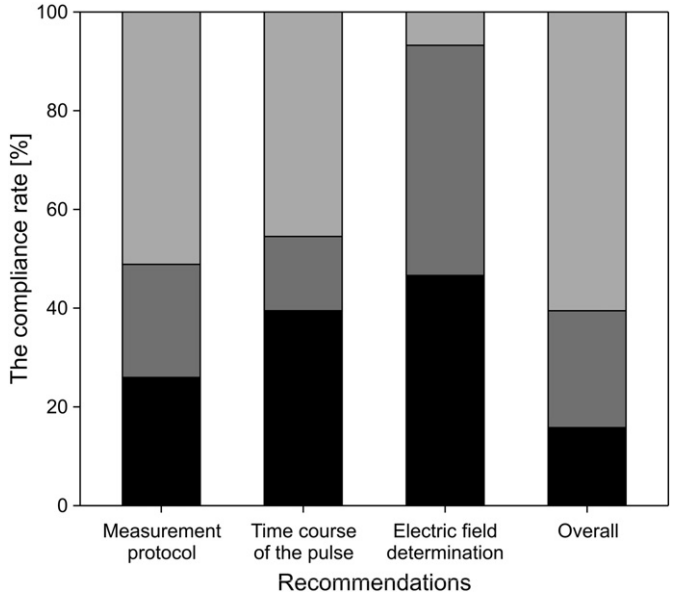


**Fig. 4.** The compliance rate of all evaluations of nanosecond electric field described in reports included in a systematic review on the recommendations for the evaluation of nanosecond electric field to which cells are exposed in electroporation studies. The rates of descriptions that were evaluated as appropriate are shown in black, poor in dark gray and inappropriate in light gray.

Interestingly, when bipolar nsEP pulses were used instead of monopolar, the membrane damage and uptake of ions were significantly reduced [61,62]. The cancelation effect by reversing the electric field direction was not observed in experiments with longer pulses [63,64] suggesting different mechanisms of action specific to nsEP. A few possible mechanisms were suggested for this phenomenon [61,62] although the exact mechanism remains unknown.

We also identified the time frames in which the recovery of the functions studied (resealing of the membrane/gaining initial impermeability for dyes and ions, phospholipid asymmetry, membrane fluidity) was reported. 21 reports showed PM recovery from several minutes up to 5 h after delivery of nsEP. Resealing of the membrane (loss of increased permeability) was reported to be in the range of several minutes (up to 15), the time being strongly dependent on the sensitivity of a testing method.

Eleven reports noted a delayed PM effect (10 min–2 h) which points to secondary PM permeabilization due to apoptotic processes. However, the time of resealing of PM (up to 15 min) reported in the literature is also overlapping with the delayed effect so it depends on the conditions and methods used in each experiment. In any case, we did not take the delayed uptake of dyes into statistical analysis for apoptosis detection, we simply pointed out to the possibility of secondary effect.

### 4.2. Intracellular effects

In our review, intracellular effects of nsEP were considered in 114 reports. In these, researchers studied impact on cell organelles (nucleus, ER, mitochondria, endocytotic vesicles, vacuoles, granules, cytoskeleton), apoptosis, release of $Ca^{2+}$ from internal stores, and at molecular level (DNA, caspases, pro-/anti-apoptotic factors, ROS, cytochrome c). In only ten reports did researchers fail to detect any intracellular effects after any pulse parameters used, other 104 reports confirm that nsEP caused intracellular effects. This can be a result of not publishing negative results (for PM effects, publishing negative results was more often). This points to the fact that publishing negative results can be of great importance [65].

When we took into account all the reports regardless of the pulse number, we confirmed our hypothesis that the occurrence of any intracellular effect depends on pulse duration: intracellular effects were confirmed in 73.2%, 92.0% and 83.7% of reports using short (A), moderate (B) and long (C) pulses, respectively. However, we did not confirm our hypothesis that shorter pulses are more likely to cause intracellular effects: with moderate (B) nsEP we get more intracellular effects than

with the shortest pulses (A). On the one hand, with increasing duration of nsEP, the intracellular effects are expected to be similar to those observed with electroporation with longer, micro- and millisecond electric pulses. The theoretical evaluations point to that with pulses shorter than 100 ns the voltage induced on the organelle membranes can exceed those on plasma membrane [30]. On the other hand, the reasons for failure to observe intracellular effects with shorter pulses are: 1. increasing challenges of pulse delivery and metrology at 10 ns and below (not actually delivering the pulses to the target cells); 2. increasing difficulty of detecting the lower magnitude responses to pulses 10 ns and below.

Then we analyzed the impact of a single nsEP on intracellular effects all together. Statistical analysis revealed that with single pulses, the occurrence of any intracellular effect is not dependent on pulse duration: intracellular effects were confirmed in 52.2%, 65.5% and 77.8% of reports using short (A), moderate (B) and long (C) single pulses, respectively. This means that all single nsEP pulses, regardless of their duration, can cause intracellular effects, at least in the reports published (note that many of negative results may remain unpublished which can affect the conclusions of our study). For multiple pulses, Chi-square test failed to prove the dependency of intracellular effects on pulse duration due to too low expected frequencies of reports.

Seventy-three reports considered effects of nsEP on organelles (40 nucleus, 21 mitochondria, three granules, one lysosomes, ten endoplasmic reticulum — ER, one giant vacuoles, two endocytotic vesicles, one chloroplasts). Also, there were nine and 55 reports on the effects of nsEP on cytoskeleton and molecular level effects (DNA, caspases, pro-/anti-apoptotic factors, ROS, cytochrome c), respectively. Almost all of them reported that nsEP indeed have an effect with these endpoints (using all pulses, single or multiple pulses). For all these intracellular effects of single, multiple or all pulses (regardless of pulse number) nsEP, Chi-square tests failed to prove the dependency on pulse duration due to too low expected frequencies of reports.

There were also 34 reports that considered the effects of nsEP on intracellular $Ca^{2+}$ release. For all pulses (regardless of pulse number) as well as for single pulses, the Chi-square test was valid but showed that the release of $Ca^{2+}$ from internal stores is not related to pulse duration (see below, in section 4.4). For multiple pulses, Chi-square test was not valid.

In the 114 reports in which intracellular effects were reported, we also analyzed whether the pulses that affect the cell interior also affect the PM. We confirmed that with single pulses, the occurrence of changes in PM caused by single nsEP that caused intracellular effects significantly depends on the duration of the ns pulses. 33.3%, 70.0% and 80.0% of reports noted that PM was affected using single short (A), moderate (B) and long (C) pulses that caused intracellular effects, respectively. This means that longer single pulses have a more profound effect that can be detected by various methods, including those that are less sensitive ones (PI or other dye uptake). On the other hand, for the pulses of any given number (regardless of pulse number) the changes in PM were not dependent on pulse duration: 75.9%, 85.4% and 94.6% of reports confirmed PM changes after using short (A), moderate (B) and long (C) pulses that caused intracellular effects, respectively. For multiple pulses, Chi-square tests failed due to too low expected frequencies of reports.

### 4.3. Apoptosis

In 2002, Beebe first reported that nsEP induced apoptosis in cells. Apoptosis can be provoked by nsEP also *in vivo*, a promising new tool for cancer treatment [66–69]. Since the first report, 46 others addressed this issue for cells *in vitro*. Out of these studies, only five reports noted failure to detect apoptosis in any conditions tested. All others confirmed nsEP-induced apoptosis with different methods detecting hallmarks typical of apoptosis. Apoptosis was confirmed in 64.3%, 87.9% and 82.6% of reports after applying short (A), moderate (B) or long (C) nsEP, respectively, using at least one method. However, Chi-square test was not valid so our analysis does not support the hypothesis that the occurrence of apoptosis is dependent of pulse duration. Moreover, Chi-square tests of all separate hallmarks of apoptosis were also not valid.

Nine reports noted that nsEP cause morphological changes that point to apoptosis: shrinkage, micronuclei formation, fragmented and condensed chromatin, blebbing, and forming of apoptotic bodies. Various physiological and pathophysiological stimuli cause a self-destruction of cells through apoptosis (reviewed in [70]). This is a very complex process that involves two main apoptotic pathways, intrinsic and extrinsic, that are executed and regulated through an interconnected cascade of events where numerous apoptotic factors are involved [71]. Therefore, apoptosis provoked by nsEP was also studied biochemically to elucidate possible targets and downstream events. In 22 reports authors confirmed the activation of caspases, proteases that play an important role in apoptosis. However, six reports showed that caspases were not activated in cells or in certain conditions (presence of NaCl). Mostly, apoptosis was calcium-dependent (seven reports) but in one report authors showed that with shorter pulses (A and B duration) it was not.

Mitochondria play an important role in apoptosis execution (reviewed in [72,73]). In eleven reports on apoptosis, authors noted mitochondria were affected (in one not). In six reports authors reported that cytochrome c was released from mitochondria but three reports showed that no cytochrome c was released. In eight reports researchers noted that mitochondrial membrane potential collapsed during apoptosis. Ten reports addressed various pro- and anti-apoptotic factors such as PARP, Bcl-2 family, IAP and SMAC/DIABLO. DNA fragmentation is also one of the significant hallmarks of apoptosis. DNA fragmentation was confirmed in nine reports, in two it was not.

Phosphatidylserine (PS) externalization is also one of the hallmarks of apoptosis [47]. However, PS can also migrate from inner to outer leaflet of PM through pores that emerge during electroporation [26, 41]. Since resealing of membrane takes several minutes (more than 15 min, see section 4.1), only reports in which authors detected PS externalization at least 30 min after nsEP application were considered as apoptosis detection (reported positive in 15 reports and in two not).

All these results point to the fact that nsEP of distinct pulse parameters act differently on apoptotic pathways in different cells. It is still not clear what are the primary targets of nsEP that activate the cascade of events leading to apoptosis. Moreover, in 16 reports out of 46 (34.8%), apoptosis was detected with one method only. For our analysis, this was a subject of debate in methodology in classifying reports as positive for apoptosis: PTV suggested that only reports that confirmed apoptosis with two or more different methods should be positive for apoptosis. Especially in cases where apoptosis is detected by PS externalization, we thus recommend using at least one additional method of apoptosis detection.

### 4.4. Calcium

Numerous researchers reported that nsEP increase the intracellular concentration of calcium ions ($Ca^{2+}$). We identified 52 reports that presented results on cell $Ca^{2+}$. In eleven of them authors observed indirect effect of $Ca^{2+}$ on other nsEP induced effects such as apoptosis, stress or reactive oxygen species (ROS) production. In other reports, intracellular $Ca^{2+}$ concentration was measured using different fluorescent dyes (Fura-2, Fluo-3) and all except one of them reported the elevation of intracellular $Ca^{2+}$ concentration.

$Ca^{2+}$ in the cytoplasm is maintained in low concentration and confined to internal stores [74]. Therefore, after applying nsEP, $Ca^{2+}$ can be either released from internal stores such as ER and mitochondria or taken up from outside the cell through PM permeabilization. In many

studies authors identified the source of elevated $Ca^{2+}$ concentration with the use of intra- and extracellular $Ca^{2+}$ chelators (BAPTA, EGTA, EDTA). Our statistical analysis showed that calcium release from intracellular stores does not significantly depend on the duration of the applied ns pulses: $Ca^{2+}$ release from intracellular stores was reported positive in 30.0%, 66.7% and 62.5% of reports in which they described intracellular release from internal stores after applying short (A), moderate (B) or long (C) nsEP, respectively. On the other hand, the source of $Ca^{2+}$ was confirmed to be extracellular in 81.8%, 73.3% and 89.5% of reports that considered extracellular $Ca^{2+}$ uptake for short (A), moderate (B) or long (C) nsEP, respectively, but Chi-square test was not valid. $Ca^{2+}$ can pass PM through lipid nanopores [75–77] or through protein channels [78]. Nevertheless, especially in the cases where $Ca^{2+}$ is released from the internal stores, we still do not know whether $Ca^{2+}$ release is a direct or indirect effect of nsEP.

In some cases, the nsEP-induced increase of $Ca^{2+}$ level was more complex: six of the reports noted store-operated (capacitive) $Ca^{2+}$ entry [19,21,79–82] and three reported Ca-induced $Ca^{2+}$ release [62,83,84].

### 4.5. Survival

The impact of nsEP on cell survival was described in 73 reports. In most cases, survival after nsEP exposure was lower than in controls: 72.2%, 87.5% and 100.0% of reports showed lower cell survival than control for short (A), moderate (B) or long (C) nsEP, respectively. However, Chi-square test for dependency between survival and pulse duration was not valid because the expected frequencies of reports were too low.

Some of the authors of these reports explored the mode of cell death: 23 reports noted that the reason for lower cell survival was apoptosis and 14 that it is necrosis (ten of them reported both). Several conditions have an effect on nsEP survival: either increasing (*e.g.* in adherent cells, hypoxic conditions and pulsing media) or decreasing survival (*e.g.* oxidation, pulse fractionation and surfactant Tween 80). In 57 reports the authors showed that at least one of the pulse parameters (amplitude, duration, number, repetition rate) has an impact on cell survival after nsEP exposure.

### 4.6. Nucleus

We identified 40 reports in which nsEP effects on the cell nucleus were described. In 16 of them authors reported that these pulses affect the morphology of the nuclei: irregular shape or fragmentation, enlarged, condensed intranuclear structures. Four reports noted that nsEP have an effect on the nuclear envelope (disintegration) [85–88]. NsEP have also an effect on DNA (fragmentation and DNA content) which was shown in 21 reports. Other reports revealed nsEP effects on chromosomes (two reports), nuclear proteins (one report) and cell cycle (six reports). However, for the effects of nsEP on nucleus, nuclear envelope, DNA or other observed effects our analysis of these reports does not support the hypothesis that there is a dependence on pulse duration (Chi-square tests were not valid).

NsEP were also used in combination with longer, milli-/microsecond pulses for gene electrotransfer. The hypothesis was that nsEP would permeabilize nuclear envelope and hence increase access of gene to the nucleus. In two reports [19,79] it was shown that nsEP increase gene transfection if used after EP pulses and in one report showed no effects [89]. On the contrary, nsEP alone were also successful for transfection in combination with peptide vehicle for DNA [90]. However, when nsEP pulses were used before EP pulses, the gene transfection increased, but the exact mechanism remains unknown [91].

### 4.7. Mitochondria

In our systematic analysis, we identified 21 reports in which the effects of nsEP on mitochondria were addressed. All except four [20, 92–94] reported that nsEP affect mitochondria. Twelve reports noted that nsEP affect mitochondrial membrane potential. In six reports authors showed that nsEP cause the release of cytochrome c from mitochondria, but in three reports [23,94,95] nsEP did not. In two reports [20,92] the researchers also reported studying the release of $Ca^{2+}$ ions from mitochondria but failed to detect it. The release of apoptotic factors from mitochondria is already described in chapter 4.3 Apoptosis. However, our analysis of these reports does not support the hypothesis that there is a dependence between the effects of nsEP on mitochondria and pulse duration because Chi-square tests were not valid.

### 4.8. Stress

Cells are exposed to different environmental and intracellular stimuli that can cause stress. In such circumstances different pathways are activated as stress responses: they protect the cell in order to survive and re-establish homeostasis. If the damage is too severe, they direct the cell to its death [96]. In six reports authors addressed the effect of nsEP on cellular stress responses and all of them showed that nsEP indeed cause stress in cells and activate stress responses [25,97–101]. However, our analysis of reports does not support the hypothesis that there is a dependence between the effects of nsEP on stress and pulse duration (Chi-square tests were not valid). During stress, cells responded through different stress response pathways: mitogen-activated protein kinases (MAPK) or AMP-activated protein kinase (AMPK) pathways or other stress-response kinases (PERK, GCN2) or led to stress response in growing plants.

### 4.9. Recommendations for the evaluation of nanosecond electric field to which cells are exposed in electroporation studies

The determination and description of the nanosecond electric fields to which cells are exposed during experimental work were not adequately addressed, or at least were not reported in sufficient detail in most reports. The nanosecond electroporation report should: 1) describe exactly how the electric pulses were measured; 2) provide time-domain waveforms of the electric pulse at the electrodes (irrespective of electrodes used); and 3) calculate or otherwise determine the distribution of the electric field within the exposure chamber/biological sample, *i.e.* describe what electric field the cells were exposed to.

We recommend the following: a) measurement of the pulse should be done with calibrated equipment; b) measurement of the pulse should be conducted with oscilloscope and voltage/current/electric-field probes with higher bandwidth than the bandwidth of the pulse. Also, maximal non-destructive amplitude of the voltage/current/electric-field probes should be higher than the amplitude of the pulse; c) measurements of the pulse should be conducted on the chamber electrodes or, if that is not possible, the voltage/current on the electrodes should be determined by evaluating losses and reflections of the pulse between the measuring point and the electrodes; d) measurement of the pulse amplitude should be made and checked in each experiment where pulses were delivered; e) time course of the pulse should be displayed or described in such a manner that the amplitude and polarity of the reflected waves are visible/described, even if they are very small. Therefore at least double pulse traveling time through the system should be displayed/described; and f) electric field within the biological sample induced by the delivered electric pulse should be determined analytically or by numerical methods. All the necessary data to determine the electric field within the chamber/biological sample should be written or cited in the report, *e.g.*, the electrical properties or the material and the dimensions of the chamber/electrodes and the biological sample.

Because it is currently not possible to measure the time course and distribution of the nanosecond pulsed electric field within the exposure chamber during the delivery, the determination of the time course and distribution of the electric field within the exposure chamber must be

divided into two steps. In the first step investigators should measure/determine the time course of the electric pulse delivered to the exposure system, and in the second step the distribution of the electric field within the chamber/sample should be calculated/determined.

A nanosecond electroporation system usually consists of: nanosecond pulse generator, transmission line or delivery system, and electroporation chamber/electrodes. During the evaluation of the induced electric field in the electroporation chamber researchers should take into account that nanosecond pulses travel approximately 20 cm/ns in coaxial cable; that nanosecond pulses reflect on impedance change and that especially the high frequency components of the nanosecond pulse lose power in the transmission lines. Due to reflections the parameters of pulse exposure change. If the load impedance is too high, reflections will be positive, and the amplitude on the load will be higher than with a matched load. If the impedance of the load is too low, amplitude on the load will be lower, and the pulse will become bipolar [34]. Therefore, the electric pulse delivered to the exposure system should preferably be measured directly on the electrodes of the exposure system. If not, pulse reflections and pulse loss should be evaluated from the measurement point to the electroporation chamber [102].

Researchers in the field of nanosecond electroporation should also take into consideration results reported in the recent literature that bipolar nanosecond pulses (which can easily be generated by incorrect matching of the generator and the load) may have much lower effect on biological cells than unipolar pulses [62]. Therefore, the researchers should also evaluate the reflections of the pulse. Rise and fall times of a nanosecond pulse are rarely specified or discussed in the nanosecond literature, mainly because these two electrical parameters are difficult to adjust or vary. However, they are likely to be important in the field of nanosecond electroporation and should therefore be measured and reported.

We have analyzed the evaluations of nanosecond electric field described in reports included in our systematic review with respect to our recommendations. In some reports more than one pulse delivery setup was used, therefore more than one evaluation of the nanosecond electric field was described. Only 15.8% of the descriptions complied with all our recommendations. The descriptions that did not comply with our recommendations typically had the following inadequacies regarding: 1) the measurement protocol: a) not describing measurement setup, b) not describing voltage/current probes or place of measurement, c) not using validated probes, and d) measuring the output of the generator and not the delivered pulse; 2) the time course of the pulse: a) time course of the pulse not displayed, and b) displayed only single pulse traveling time through the system; and 3) the electric field determination: a) used microelectrodes, which induce inhomogeneous electric field, and estimated electric field by dividing U/d, and b) did not specify how they determined the electric field.

Even in the last year of analysis, *i.e.* 2014, only 34.8% of 23 descriptions complied with our recommendations.

## 5. Summary and conclusions

In our systematic review, we analyzed 203 reports (obtained with a systematic search) in which authors noted results of studies of the effects of nsEP on cells (human, animal, plant) *in vitro*. We have presented a field synopsis and reviewed eight main research outcomes (effects of nsEP on plasma membrane, intracellular, apoptosis, calcium, survival, nucleus, mitochondria, and stress) and also their detailed outcomes.

Moreover, we analyzed all these outcomes for three different pulse durations: short (A: 1–10 ns), moderate (B: 11–100 ns), and long (C: 101–999 ns) nsEP. We confirmed our hypothesis that the occurrence of PM effects is dependent on pulse duration. Longer pulses are more likely to cause PM effects (overall PM effects, dye uptake) than shorter pulses. The choice of electroporation detection method significantly affects experimental results: when using shorter nsEP, the smaller pores that are formed are more difficult for dye molecules to pass through, reducing the likelihood that influx will be detected.

We also confirmed our hypothesis that the occurrence of intracellular effects is dependent on pulse duration: however, not the shortest pulses (A) but moderate (B) pulses were most likely to cause intracellular effects. With shorter pulses (A), the pulse delivery, metrology and detection are more challenging than with moderate pulses. With longer pulses (C) the intracellular effects are expected to be similar to those from electroporation with longer, micro- and millisecond electric pulses that are usually used for electroporation (electrochemotherapy, gene electrotransfer). Moreover, the results on the intracellular effects of nsEP can be very much affected by the fact that negative results on this issue are not published, contrary to PM.

We are aware that we tried to analyze different methods that yield to similar endpoints for the biological effects of nsEP, but even with that in mind we were able to draw solid conclusions. Another limitation of our study is the fact that we evaluated the effect of nsEP with a simple "yes" and "no". If we instead had used quantitative data (trends) the results could turn out differently. But considering the different methods used in the studies, our conclusion would be even more prone to errors of the studies.

Apart from that, we analyzed thoroughly the measurements of nsEP and the evaluations of nanosecond electric field in nanosecond electroporation reports. We have identified main features: the measurement protocol, time course of the pulse, and electric field determination that should be described in every paper on nanosecond electroporation. Moreover, we outlined recommendations for appropriate measurement of nsEP and evaluation of nanosecond electric field to which cells are exposed. We believe that with these in mind researchers will conduct and present their results in a more comprehensive way which will allow faster progress in this interesting field of research.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bioelechem.2016.02.011.

## Acknowledgments

The study was supported by the Slovenian Research Agency (ARRS), grant number P2-0249. It was conducted in the scope of the LEA EBAM: International Laboratory of Pulsed Electric Fields Applications in Biology and Medicine (2011–2018) and COST TD1104: European network for development of electroporation-based technologies and treatments (www.electroporation.net, 2011–2016).

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