Decreases in Cell Viability Resulting from Metal Ions Present in Stainless Steel in Electroporated and Nonelectroporated Cells

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Abstract

Unintentional metal ion release has been observed with the application of electroporation pulses using metal electrodes, but little research has been conducted evaluating impacts on cell viability. This study focused on the four principal metals found in stainless steel (Fe, Ni, Cr, and Mn), a common electrode material, and analyzed differences in cell viability of rat cardio myoblast cell line following reversible electroporation. The results demonstrate that increases in metal ion concentration decreases cell viability and found significant differences between electroporated and nonelectroporated cells. The results of our study emphasize on the importance of optimizing pulse parameters to reduce ion metal release to avoid undesired cell death in applications utilizing reversible electroporation.

1 Introduction

Electroporation (also referred to as electropermeabilization or pulsed electric field treatment) is an emerging technology that uses a series of short, high-voltage electric pulses to transiently disrupt the integrity of the lipid bilayer of the cell membrane, allowing for an increased non-selective permeabilization of the cell [1]. Based on the cell's ability to reseal the pores in the lipid structure resulting from the high-voltage pulses, electroporation is differentiated into reversible and irreversible. Several physical and biological parameters can be adjusted to achieve the specific objectives of electroporation; primarily, electric field strength (largely influenced by voltage applied, distance between electrodes, tissue dielectric properties, and electrode geometry) and characteristics of the applied pulses such as shape, duration, number, delay, and polarity. Dependent on the modification of these variables, multiple applications for electroporation have been investigated and devised in recent years. Certain situations desire specific molecules to enter the cell and survive, such as in electrochemotherapy (ECT) or gene electrotransfer, and utilize the techniques of reversible electroporation. Other applications implement irreversible electroporation when success is defined by low cell survival, such as cardiac tissue ablation or the inactivation of microorganisms in food processing [2]–[4].

It is known that the cell membrane is selectively impermeable to ions and molecules, employing specific ion pumps, transmembrane protein channels and transport mechanisms to facilitate physiologically necessary ion and molecule exchange across the cell membrane. With the application of reversible electroporation, the selectivity of the membrane is temporarily compromised allowing for previously impermeant molecules and ions, to enter the cell and become enclosed inside with the resealing of the membrane. While this outcome is favorable in the implementation of reversible electroporation-based applications, the lack of selectivity also results in the opportunity for undesired molecules to enter the cell alongside the desired. It has previously been observed through experimentation that when high-voltage pulses are applied to a metal electrode, electrochemical processes occur at the electrode-electrolyte interface releasing ions from the electrodes into the solution [5]–[8]. The release of metal ions can result in chemical modification of the medium (including changes in pH), sample contamination, electrode wear, precipitation of proteins and nucleic acids, and/or increased cytotoxicity for reversibly electroporated cells [5]–[7], [9] .

While the field of metal cytotoxicity has many medical implications [10], little research has been conducted exploring how the unintentional addition of the metal ions released from electrodes during electroporation impacts cell viability. Kotnik et al. [5] briefly addressed a potential decrease in cell viability of electroporated cells due to metal ions. It was determined by the study that Al ions, at the examined concentrations, were inconsequential for cell viability. However, difference in cell viability of electroporated and nonelectroporated cells as a function of extracellular concentration of the Fe ions was observed. Fe was studied because it represents a large percentage of the composition of stainless steel, a common electrode material, but Ni, Cr, and Mn ions have all been observed to be released from stainless steel electrodes with the application of electroporation pulses [7], [8] .

The objective of our study was thus to analyze how the metal ions present in stainless steel (Fe, Ni, Cr, and Mn) affect cell viability of H9c2 (rat cardio myoblast) cells following reversible electroporation.

2 Methods and Materials

2.1 Pulse Delivery

The ELECTROcell B10 HV-LV (LEROY Biotech, France) pulse generator was used to deliver 40 biphasic symmetric rectangular pulses of 10 μs duration and interphase delay at 1.03 Hz repetition rate. The nominal voltage of the applied pulses ranged from 100 to 1000 V and increased in increments of 100 V. The voltage and electric current were monitored on a WaveSurfer 422 200 MHz oscilloscope using the high-voltage differential probe ADP305 and current probe CP030 (all from LeCroy, New York, USA) respectively.

2.2 Cell Culture

H9c2 rat cardio myoblast cell line was cultured according to the methods described previously [9]. For experiments, cells were detached with trypsin and were re-suspended at a cell density of 1×10^6 cells/ml (for permeabilization and survival experiments after electroporation) or 2×10^6 cells/ml (for survival experiments in the presence of metal ions) in KPB buffer of pH 7.4 composed of 10 mM KH_2PO_4 (cat. no. 104873, Merck, New Jersey, USA), 10 mM K_2HPO_4 (cat. no. 04248, Riedel de haen Honeywell, Germany), 1 mM $MgCl_2$, (cat. no. M-8266, Sigma-Aldrich) and 250 mM sucrose (cat. no. 16104, Sigma-Aldrich).

2.3 Cell Membrane Permeabilization

Immediately before application of electric pulses, 3.2 μl of diluted YO-PRO1 iodide (cat. no. Y3603, Thermo Fischer Scientific, Massachusetts, USA) was added to 80 μl of cell suspension to a final concentration of 0.025 mM YO-PRO1 and mixed by pipetting. 50 μl of the mixture was then transferred to a 2 mm gap aluminum electroporation cuvette (cat. no. 732-1136, VWR, Belgium) and the pulses were applied. 40 μl of the electroporated cells were transferred to a new 1.5 ml microcentrifuge tube. Three minutes after electroporation, 150 μl of complete medium DMEM was added to the microcentrifuge tube, gently vortexed, and analyzed on the flow cytometer Attune NxT (Thermo Fischer Scientific) using 488 nm laser and 530/30 nm band-pass filter until 10,000 events were recorded. The percentage of permeabilized cells was determined from the histogram of YO-PRO1 fluorescence. 5-6 iterations were performed for each electric field strength value. The sham controls were treated in the same way with the exception of no pulses being applied.

2.4 Cell Survival After Electroporation

Just before pulse delivery, 50 μl of cell suspension was pipetted to an electroporation cuvette with a 2 mm gap. Ten minutes after pulse delivery (no pulses were applied in the case of the sham controls), 10 μl of the treated cells suspension was transferred to a 1.5 ml microcentrifuge tube with 440 ml of complete medium DMEM, gently vortexed, and plated (100 μl) in a well of a flat bottom 96-well plate as a triplicate. The plate was then incubated for 72 hours at 37 °C in a humidified, 10 % CO_2 atmosphere. After 72 hours, 11.1 μl of PrestoBlue (Thermo Fisher Scientific, cat. no. A13261) assay was added to each well and the plate was then incubated for another hour. After the hour, fluorescence (560/590 nm excitation/emission) was measured with the spectrofluorometer Infinite 200 (Tecan, Austria). The survival was calculated by first subtracting the measured fluorescence of the blank and then normalizing the average fluorescence of the three technical replicates of the sample to the fluorescence of the sham controls. The experiments were repeated 5-6 times per voltage value.

2.5 Cell Survival in the Presence of Metal Ions

10 mM stock solutions of $FeCl_3$ (cat. no. 31237, Sigma-Aldrich), $NiCl_2$ (cat. no. 654507, Sigma-Aldrich), $CrCl_3$ (cat. no. 27096, Sigma-Aldrich), and $MnCl_2$ (cat. no. M1787, Sigma-Aldrich) were diluted in KPB buffer to reach a desired working solution which upon addition to the cells would result in final concentrations of 0.1, 0.5, 1.0, 1.5, or 2.0 mM Fe^{3+}, 0.05, 0.1, 0.15, 0.2, or 0.25 mM Ni^{2+}, 0.2, 0.4, 0.6, 0.8, or 1.0 mM for Cr^{3+}, and 0.1, 0.15, 0.2, 0.25, or 0.3 mM for Mn^{2+} respectively. Concentration ranges were determined following preliminary testing performed following the same protocol.

60 μl of cell suspension and 60 μl of the working metal solutions were pipetted into a sterile 1.5 ml microcentrifuge tube. 50 μl of the mixed solution was then transferred to an electroporation cuvette of 2 mm gap and electroporated by delivering 600 V pulses (see Results for explanation). 10 minutes after electroporation, 14 μl of the treated solution was pipetted into a new microcentrifuge tube containing 386 μl of complete medium DMEM, gently vortexed, and plated (100 μl) as a triplicate in a 96-well plate. The plate was then incubated for 72 hours at 37 °C and 10 % CO_2. Cell survival was determined using the same protocol as described in "Cell Survival After Electroporation."

2.6 Statistical Methods

Differences in cell viability were evaluated at the α-level 0.05. After outliers, defined using the IQR method, were removed from the data, normal distribution (Q-Q) plot was used to evaluate if data are normally distributed and Levene test was used to assess equivalence of variances. If the data met both assumptions, normal distribution and equal variances, a two-way ANOVA and Tukey post-hoc were run. If the assumptions were not met, a Kruskal-Wallis and post-hoc Holm test was applied.

3 Results and Discussion

3.1 Permeabilization and Survival Post-Electroporation

Based on the survival-permeabilization results (Figure 1), we selected an amplitude of 600 V (3000 V/cm electric field strength - calculated by nominal voltage divided by the distance between the electrodes) for subsequent experiments in which the effect of metal ions on cell survival was investigated. This value, nominally 600 V, resulted in high percent (95 %) permeabilization of the cell membrane, before a decrease in cell viability resulting from the electroporation was noted, i.e. resulted in reversible electroporation.

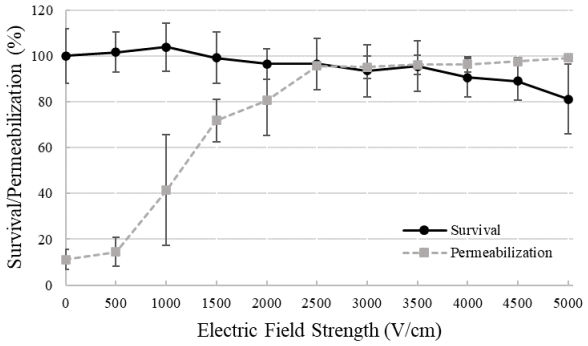

Figure 1: Survival-Permeabilization curve of H9c2 cells. Bars represent standard deviation.

3.2 Cell Survival in the Presence of Metal Ions

Cell viability of in the presence the four metals present in stainless steel (Fe, Ni, Cr, and Mn) was evaluated to consider potential consequences of ion release during electroporation pulses from metal electrodes. The results indicate that the release of metal ions could result in the most detrimental effect, loss of cell viability, when releasing a high enough concentration of ions from stainless steel electrodes. Analysis of the data for Fe^{3+}, Cr^{3+}, and Mn^{2+} (Figure 2A, B, and D respectively) revealed statistically significant differences in cell viability as ion concentrations increased. Fe^{3+} and Mn^{2+} also showed significant differences in cell viability between electroporated cells and their nonelectroporated counterparts but not at specific concentrations. Kotnik et al. [5] found significant loss in cell viability at concentrations of Fe^{2+}/Fe^{3+} above 1.5 mM while in our study we observed significant differences as low as 0.5 mM Fe^{3+}. This difference highlights potential variations among cell survival for different cells lines. Kotnik et. al used DC-3F Chinese hamster fibroblasts while in our study we used H9c2 rat cardio myoblasts, in the presence of metal ions. Evaluation of higher concentrations may continue to develop larger differences in the data and could be a focus in future studies.

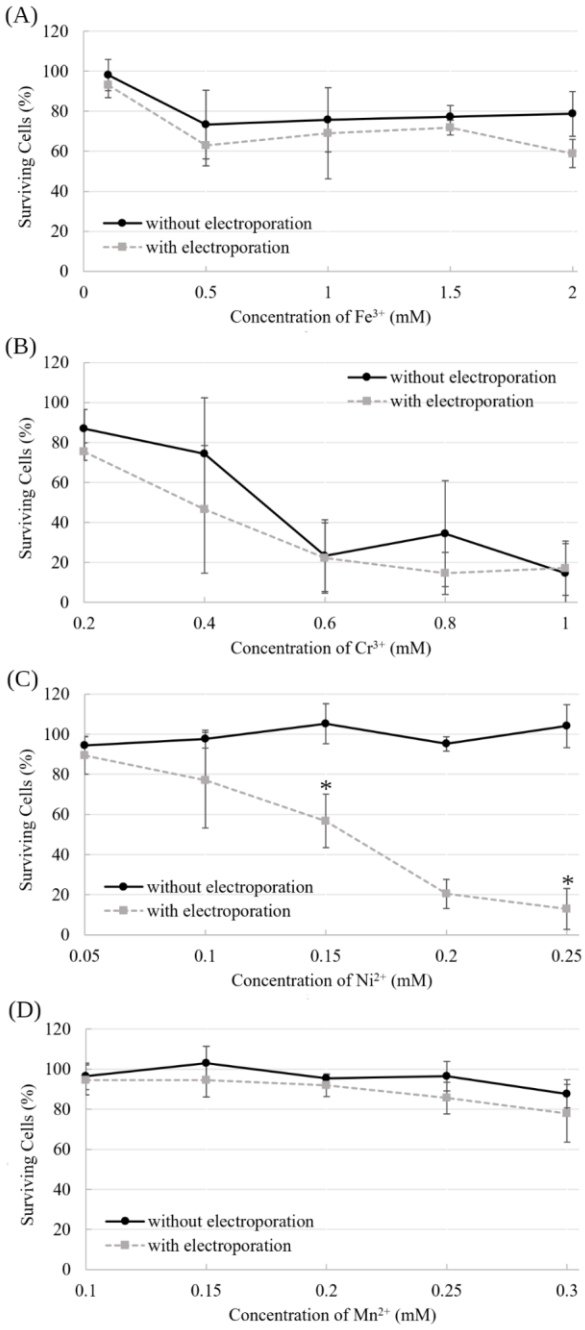

Figure 2: Comparison of cell viability between electroporated and nonelectroporated H9c2 cells for varying concentrations of (A) Fe^{3+}, (B) Cr^{3+}, (C) Ni^{2+}, and (D) Mn^{2+}. Bars represent standard deviation, asterisk (*) denotes statistical significance ($p < 0.05$) between electroporated and nonelectroporated cells.

Ni^{2+} (Figure 2C) revealed significant differences between cell viability in the electroporated and nonelectroporated cells at ion concentrations of 0.15 and 0.25 mM. Ni^{2+}, clearly demonstrating how metal cytotoxicity increases with the application of electroporation to cells. Biphasic pulses were chosen for this study because their application results in lower metal release from electrodes compared to monophasic pulses [5]. In preliminary metal release experiments, the measured concentrations of Fe, Ni, Cr, and Mn ions in KPB after delivery of pulses used in this study were

much lower than the concentrations used in cell experiments, indicating that pulse delivery negligibly increased the concentration of the metal of interest in cell experiments (data not shown). This distinction between the data sets emphasizes the importance of minimizing ion release when utilizing electroporation technology.

All four investigated metal ions provide insight into the effect of metal ions released from electrodes on cell viability. It needs to be stressed however, each of the ions exist in multiple oxidation states and the exact oxidation state of the ions released from the metal electrodes following electroporation was not determined. This study selected one oxidation state for each element and expanding on this limitation could be a focus of future studies.

The measured electric current of the final pulse was compared for cell suspensions electroporated in KPB only and in KPB with 2 mM Fe^{3+} to look into the potential changes in electric current as a result of the added metal ions. 2 mM Fe^{3+} was chosen because it represented the highest concentration of added metal ions. Graphing the values against one another verified that there was only a slight increase in current through the cell suspension resulting from the metal ions (less than 4 % increase). This indicates that cell death measured by the experiments can most probably be attributed to the presence of the cytotoxic effects of the metal ions and not increases in conductivity and consequently increased temperature.

Conclusion

The objective of this study was to analyze how metal ions present in stainless steel (Fe, Ni, Cr, and Mn) impact cell viability of H9c2 (rat cardio myoblast) cells following reversible electroporation. The outcomes of this research show how increase in metal ion concentrations decreases cell viability for all four tested metals and found significant differences between electroporated and nonelectroporated cells at specific concentrations for Ni^{2+}. Results of this study emphasize the importance of optimizing pulse parameters and physical characteristics of the electrodes to reduce ion metal release during pulse application in order to minimize undesired cell death in reversible electroporation applications.

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