Understanding the role of calcium-mediated cell death in high-frequency irreversible electroporation

Elisa M. Wasson\textsuperscript{a,e}, Nastaran Alinezhadalalamib,e, Rebecca M. Brock\textsuperscript{c,d}, Irving C. Allen\textsuperscript{c,d,e}, Scott S. Verbridge\textsuperscript{b,e}, Rafael V. Davalos\textsuperscript{a,b,e}

\textsuperscript{a}\textsuperscript{b}Department of Mechanical Engineering, Virginia Tech, Goodwin Hall, 635 Prices Fork Road, Blacksburg, VA 24061, USA
\textsuperscript{b}\textsuperscript{b}Department of Biomedical Engineering and Mechanics, Virginia Tech- Wake Forest University, 325 Stanger Street, Blacksburg, VA 24061, USA
\textsuperscript{c}\textsuperscript{c}Graduate Program in Translational Biology, Medicine, and Health, Virginia Tech, 1 Riverside Circle, Roanoke, VA 24016, USA
\textsuperscript{d}\textsuperscript{d}Department of Biomedical Sciences and Pathobiology, Virginia Tech, 205 Duck Pond Drive, Blacksburg, VA 24061, USA
\textsuperscript{e}\textsuperscript{e}Institute for Critical Technology and Applied Sciences Center for Engineered Health, Virginia Tech, Kelly Hall, Blacksburg, VA 24061, USA

Abstract

High-frequency irreversible electroporation (H-FIRE) is an emerging electroporation-based therapy used to ablate cancerous tissue. Treatment consists of delivering short, bipolar pulses (1–10 μs) in a series of 80–100 bursts (1 burst/s, 100 μs on-time). Reducing pulse duration leads to reduced treatment volumes compared to traditional IRE, therefore larger voltages must be applied to generate ablations comparable in size. We show that adjuvant calcium enhances ablation area in vitro for H-FIRE treatments of several pulse durations (1, 2, 5, 10 μs). Furthermore, H-FIRE treatment using 10 μs pulses delivered with 1 mM CaCl\textsubscript{2} results in cell death thresholds (771 ± 129 V/cm) comparable to IRE thresholds without calcium (698 ± 103 V/cm). Quantifying the reversible electroporation threshold revealed that CaCl\textsubscript{2} enhances the permeabilization of cells compared to a NaCl control. Gene expression analysis determined that CaCl\textsubscript{2} upregulates expression of EIFB5 and 60S ribosomal subunit genes while downregulating NOX1/4, leading to increased signaling in pathways that may cause necroptosis. The opposite was found for control treatment without CaCl\textsubscript{2} suggesting cells experience an increase in pro survival signaling. Our study is the first to identify key genes and signaling pathways responsible for differences in cell response to H-FIRE treatment with and without calcium.

1. Introduction

Irreversible electroporation (IRE) treatment is an alternative to standard cancer therapies and has been shown to successfully treat the most aggressive, and sometimes unresectable tumors such as pancreatic adenocarcinoma in human patients [1–4] and glioblastoma in canine patients [5–8]. In a typical electroporation treatment, needle electrodes are inserted into the tumor and a series of electrical pulses are delivered to the tissue. The applied electric field induces a rise in the cells’ transmembrane potential (TMP). This rise in TMP lowers the energy needed for hydrophilic pores to form in the membrane [9,10] in a phenomenon known as electroporation. These pores allow otherwise impermeable molecules and ions to enter the cell. Additionally, recent studies have shown that voltage gated channels may affect transport during electroporation, also allowing exchange of molecules [11] that may lead to a loss of cellular homeostasis. Pulse parameters that are applied (field strength, pulse duration, and number of pulses) can be varied to elicit different cellular responses. Below a critical energy threshold, cells can recover from the loss of homeostasis and the pores may reseal over time. The cells are then said to experience reversible electroporation. Taking advantage of the enhanced cell membrane permeability and transport into the cell, reversible electroporation has commonly been used in cancer therapy [12,13] as well as for the treatment of tumors in a technique known as electrochemotherapy (ECT) [14–17]. However, if the applied energy surpasses the critical threshold, cells cannot recover from the loss of homeostasis induced by the electrical field. The permanent loss of homeostasis results in cell death and cells are said to experience irreversible electroporation. IRE has been shown to cause minimal thermal damage to tissue [18], unlike techniques such as radiofrequency and microwave ablation, therefore sparing critical structures such as vasculature and nerves [19,20].
Despite the promising outlook of IRE for treatment of unresectable tumors [21], clinical application of IRE is known to cause muscle contractions in human and animal patients, requiring the use of neuromuscular blocking agents [22,23]. We have developed a second generation modality of IRE treatment known as high-frequency irreversible electroporation (H-FIRE) to address these limitations [24]. While traditional IRE treatment uses square, unipolar pulses of 100 μs, H-FIRE treatment uses bipolar pulses that are much shorter in duration (1–10 μs) and are delivered in a series of 80–100 bursts (1 burst/s), as in the case of IRE (1 pulse/s). It has been hypothesized that delivering pulses at a higher frequency diminishes the dependence of IRE on cell size and tissue geometry [25–27]. Nonlinear changes in tissue conductivity occur to a lesser degree than in IRE treatment [28,29] and therefore ablations using H-FIRE have been shown to be more predictable and uniform [30,31]. H-FIRE treatment also mitigates muscle contractions seen with IRE treatment [24]. However, reducing pulse duration in H-FIRE treatment leads to a reduction in treatment volume compared to traditional IRE. This limitation necessitates the use of much larger applied voltages to generate ablations comparable in size. Higher voltages may increase thermal damage and the likelihood of inducing muscle contractions [31–33], therefore negating some of the benefits that H-FIRE treatment has over traditional IRE.

It has been shown that utilizing ECT pulses in combination with adjuvant calcium results in ATP depletion of cancer cells for as long as 8 h after treatment, compared to only 1 h after electroporation treatment alone, leading to enhanced cell death [34–38]. Motivated by these studies, we have previously demonstrated that using adjuvant calcium in combination with IRE treatment induces a larger zone of cell death without the need to increase the energy applied to the tissue [39]. We hypothesize that using adjuvant calcium will enhance the lethality of H-FIRE treatment. Utilizing adjuvant calcium with H-FIRE treatment will allow us to retain its clinical advantages while eliminating the need to use higher voltages to produce ablations similar in size to traditional IRE treatment.

We investigated our hypothesis using a simplified collagen hydrogel model of a tumor that allowed visualization of treatment ablations. Cancer cells were seeded in collagen scaffolds and treated using H-FIRE waveforms of varying pulse durations (1, 2, 5, and 10 μs) with and without calcium. Reversible and irreversible electroporation thresholds were quantified and compared to our previously tested calcium IRE treatments [39] to elucidate differences in cell death response to extracellular calcium. Finally, we began work to uncover the biological effects on cells in response to H-FIRE treatment with and without calcium, which to our knowledge, has not been previously investigated. Our results show that adjuvant calcium enhances ablation size for all H-FIRE treatments. Furthermore, 10 μs pulses delivered with calcium result in ablations comparable in size to IRE treatment without calcium. Additional mechanistic studies identified signaling pathways associated with NAPDH oxidase activity, ROS production, and the translation of stress related mRNA that were differentially dysregulated under CaCl₂ and no CaCl₂ conditions, impacting cell death.

2. Materials and methods

2.1. Cell culture

U251 malignant glioma cells (Sigma Aldrich, 09063001) were maintained at 5% CO₂ and 37 °C in Eagle’s Minimum Essential Medium (Sigma Aldrich) supplemented with 1% penicillin/streptomycin (Life Technologies), 10% fetal bovine serum (Atlanta Biologicals), 1% non-essential amino acids (Sigma Aldrich) and 1 mM sodium pyruvate (Sigma Aldrich). Cells were routinely passaged at 80–90% confluence.

2.2. Collagen scaffold preparation

Sterile polydimethylsiloxane (PDMS, SYLGARD™ 184, Dow Corning) wells (10 mm diameter, 1 mm height) were placed in a 24 well plate to ensure uniform collagen scaffold geometry and electric field distribution between each replicate. PDMS wells were treated with 1% PEI (Acros Organics) for 10 min, 0.1% glutaraldehyde (Fisher Scientific) for 20 min, and then washed twice with deionized water prior to collagen seeding to ensure collagen adhesion during treatment. Commercial rat tail collagen type I (BD Biosciences) was neutralized using a solution of 10× Dulbecco’s Modified Eagle Medium (10% total volume, Sigma Aldrich), 1 N NaOH (2% collagen volume, Sigma Aldrich), and 1× Dulbecco’s Modified Eagle Medium (Sigma Aldrich) to a final concentration of 5 mg/mL. U251 cells were detached from flasks using 0.25% trypsin/EDTA (Thermo Fisher Scientific) solution and added to the neutralized collagen solution at a concentration of 1 × 10⁶ cells/mL. The collagen/cell solution was dispensed into PDMS wells and PDMS tops were used to mold the collagen flat while they polymerized in a cell culture incubator for 20 min. PDMS tops were then removed and cell culture media was added. Collagen scaffolds were maintained in the incubator for 24 h prior to treatment.

2.3. Electroporation treatment

After 24 h, the media was aspirated from scaffolds and replaced with either CaCl₂ (Fisher Scientific) or NaCl (Fisher Scientific) solutions (1 mM or 5 mM). Each solution contained the same base components; 250 mM sucrose (Fisher Scientific), 1 mM MgCl₂ (Sigma Aldrich) and 10 mM HEPES buffer (Sigma Aldrich) in deionized water with pH of 7.2–7.4. Supplementary Table 1 lists the osmolality and conductivity of buffers used in this study. Solutions incubated in the wells at room temperature for 30 min, were aspirated, and then replaced with fresh CaCl₂ or NaCl solutions for another 10 min to ensure that all media was removed. Fresh solutions were added immediately prior to pulsing. A custom-designed electrode housing was used to ensure precise placement (4 mm center-to-center spacing) of two hollow, stainless-steel blunt tip needles with 0.914 mm outer diameter and 0.635 mm inner diameter (Howard Electronic Instruments) into the collagen scaffold. To investigate whether enhanced cell death was unique to calcium, other ions that are involved in removing calcium from the cell were tested, namely KCl (1 mM, Alfa Aesar) and a combination of CaCl₂ and NaCl (1 mM each). These treatments followed the same protocol described above.

Schematic 1. Schematic of bipolar H-FIRE waveforms. The same inter-pulse delay (1 μs) and varying pulse durations (1, 2, 5, 10 μs) were investigated in this study. A series of 80 bursts were delivered at the frequency of one burst/s and amplitude of 800 V for a total on time of 100 μs. H-FIRE waveforms are described in this study by the following name scheme (positive pulse duration – inter-pulse delay – negative pulse duration).
Several H-FIRE pulses were investigated in this study and delivered using a custom-built pulse generator (EPULSUS®-FBM1-5, EnergyPulse Systems). Waveforms were captured using a voltage probe (BTX, Harvard Apparatus) and oscilloscope (DPO 2012, Tektronix). An H-FIRE waveform can be described by pulse duration, inter-pulse delay, number of pulses, number of bursts, and frequency of burst delivery (Schematic 1). Here, we describe the treatment delivered by the parameters of one bipolar pulse (positive pulse duration, inter-pulse delay, and negative pulse duration). Inter-pulse delay was kept constant at 1 μs while pulse duration was varied (1, 2, 5, and 10 μs). Eighty bursts with a total on-time of 100 μs (100, 50, 20, and 10 pulses respectively) were delivered at a frequency of 1 burst/s and a voltage of 800 V. For irreversible threshold experiments, CaCl₂ and NaCl solutions were aspirated based on z-score. Gene expression was profiled for 89 genes using a Qiagen Calcium Signaling RT² Profiler PCR Array and data was evaluated utilizing the ΔΔCt. Method. The resulting fold changes in gene expression were used to determine the effects of H-FIRE treatment parameters on biological functions and complex signaling networks using Ingenuity Pathway Analysis (IPA), as previously described [44–46]. Results were normalized respectively to untreated cells. The gene list for each array and functional categories for each gene are available through the manufacturer. Ingenuity Pathways Analysis (IPA) and the manufacturer’s array software (Qiagen) was used to analyze gene expression data. IPA data were ranked and evaluated based on z-score.

2.6. Statistical analysis

Irreversible experiments were repeated 6–12 times for each condition. Reversible experiments were repeated at least six times. Discrepancies in the number of replicates between conditions were due to bubbles or other defects that may have changed the electric field distribution or prohibited a reliable measurement of ablation area. These scaffolds were excluded from analysis. Two-way ANOVA was used to test for differences in cell death area due to the different applied solutions and pulse waveforms. Tukey post-hoc comparisons were used to examine differences among treatment groups. Statistical analyses were performed with a confidence level of α = 0.05 (JMP Pro 14). Results are shown as means ± standard deviation.

3. Results

3.1. Collagen scaffold allows testing a range of electric field magnitudes and determination of electroporation thresholds using finite element analysis

Our numerical model allows simulation and visualization of the electric field and temperature distribution in the collagen scaffold (Fig. 1). Using surface integration, we can determine the area encompassed by different electric field magnitudes, enabling us to determine the electric field threshold that results in different ablation areas for each treatment. This platform provides measurements that are more relevant to in vivo treatments on tissue since the cells experience a 3D environment and are exposed to a range of electric field magnitudes. Simulating treatment resulted in a maximal temperature increase of 4.4 °C.

3.2. Adjuvant calcium increases ablation size for all conditions and is necessary to produce ablations for short pulse durations (<10 μs)

We tested a range of H-FIRE pulse durations (1, 2, 5, 10 μs) and calcium concentrations (1 mM and 5 mM) on cells seeded in collagen scaffolds to quantify cell death with and without calcium. Fig. 2 shows that the ablations produced in the collagen scaffolds by calcium H-FIRE treatment are larger than NaCl controls for all pulse parameters tested. For short pulse durations (<10 μs), it seems that the cells treated with NaCl can recover from the treatment and no ablations are produced. Only in the presence of CaCl₂ or when longer pulse durations (>10 μs) are used, do continuous ablations form. Results suggest that the cell death response to CaCl₂ is dose dependent since treatments with 5 mM CaCl₂ result in larger ablations than those using 1 mM CaCl₂. As IRE and H-FIRE were delivered at different voltages, we directly compared the electric field thresholds evaluated by first quantifying ablation areas using ImageJ and a custom algorithm previously developed in MATLAB [39].

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Fig. 1. Simulating H-FIRE treatments using the finite element method allows us to determine the electric field and temperature distribution in the collagen scaffold. (A) The mesh was refined until there was <1% change in electric field and temperature values along a cutline between the electrodes. The mesh consisted of 102,615 total elements. (B) Solving for the electric field distribution and quantifying the area of electroporation allows us to determine the electric field threshold for each treatment. 800 V was applied with an electrode spacing of 4 mm (center-to-center). (C) Using the highest conductivity buffer (\(\sigma = 0.131 \text{ S/m}\)) in the simulation led to a predicted increase in temperature of 4.4 °C in the collagen scaffold.

Fig. 2. Ablation areas for CaCl\(_2\) are significantly larger than ablations for NaCl for all pulse durations. Pulse durations below 10 \(\mu\)s, need CaCl\(_2\) to produce an ablation larger than 5 mm\(^2\) (***\(p < .001\), **\(p < .05\), scale bar 1 mm). (A) In vitro collagen scaffolds consisted of 5 mg/mL rat tail collagen type I and U251 malignant glioma cells \((1 \times 10^6 \text{cells/mL})\). Electrodes were spaced 4 mm apart (center-to-center). For IRE treatments, 80, 100 \(\mu\)s pulses were delivered at 450 V and 1 Hz frequency. For H-FIRE treatments, 80 bursts of various pulse durations were delivered at 800 V at a frequency of 1 burst/s for a total on time of 100 \(\mu\)s. Live cells are stained with calcein AM (green) while dead and/or membrane compromised cells are stained with propidium iodide (magenta). Propidium iodide stains cells red, but the images are shown in magenta for visualization purposes \((n \geq 6 \text{ for all conditions})\). (B) Ablations were measured using a custom algorithm developed in MATLAB and ImageJ. The total area of the scaffold was 78.54 mm\(^2\) (10 mm diameter) with a thickness of 1 mm. Results are shown as mean ± std. IRE* data was taken from a previous study (Wasson et al., 2017) [39]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2 clearly demonstrates enhanced ablation area in response to calcium H-FIRE treatment. The increase of ablation area for 1 mM CaCl\(_2\) compared to its NaCl control is nearly 2.13x for the 1-1-1 waveform, 2.79x for the 2-1-2 waveform, 5.69x for the 5-
1-5 waveform, and 3.3× for the 10-1-10 waveform. For 5 mM CaCl₂, the 1-1-1 waveform results in a 3.47× increase in ablation area, for the 2-1-2 waveform there is a 4.37× increase, for the 5-1-5 waveform there is a 5.23× increase, and for the 10-1-10 waveform there is a 2.69× increase. As pulse duration increases, the area of ablation for CaCl₂ increases dramatically while for NaCl, ablation areas increase moderately. The 5-1-5 waveform results in the largest increase of ablation area between CaCl₂ and NaCl treatments. For 1 mM CaCl₂, the 5-1-5 resulted in a 5.69× increase in ablation area compared to NaCl and for 5 mM CaCl₂, the 5-1-5 resulted in a 5.23× increase in ablation area. Differences seen between calcium concentrations is more pronounced for shorter pulse durations (1 and 2 μs).

3.3. Reversible electroporation zones using CaCl₂ are larger than NaCl controls

To investigate how CaCl₂ and NaCl treatments affect permeabilization of the cells, we characterized areas for reversible electroporation and their corresponding reversible electric field thresholds. Reversible electroporation thresholds for H-FIRE treatment have not been extensively characterized, making our study one of the first to quantify reversible thresholds for a range of unexplored pulse durations [47]. Fig. 3 shows that both NaCl solutions had smaller reversible areas than their CaCl₂ counterparts, therefore the buffers used seemed to affect the extent that the cells were being permeabilized. In addition, it appeared that the difference between NaCl and CaCl₂ reversibly electroporated zones was similar for all waveforms. To compare the reversible zones more quantitatively, area was measured using our custom MATLAB algorithm.

Fig. 3B shows that for 1 mM CaCl₂, the reversible zone was 1.56× larger than the 1 mM NaCl zone for the 2-1-2 waveform, 1.7× larger for the 5-1-5 waveform, and 1.42× larger for the 10-1-10 waveform. For 5 mM CaCl₂, the reversible zone was 1.66× larger than the 1 mM NaCl zone for the 2-1-2 waveform, 1.73× for the 5-1-5 waveform, and 1.46× larger for the 10-1-10 waveform. There was no statistically significant difference between 1 mM and 5 mM treatments for the reversible case, although, after 24 h, the 5 mM CaCl₂ areas are larger than the 1 mM CaCl₂ for shorter pulse durations suggesting that cells exposed to lower levels of calcium recover to some extent.

Fig. 4 shows that reversible electroporation zones were larger than irreversible zones for all conditions tested. For the 2-1-2 waveform, reversible zones were 4.68× larger than irreversible zones for 1 mM NaCl, 3.88× for 5 mM NaCl, 2.62× for 1 mM CaCl₂, 1 mM CaCl₂ 2-1-2 10-1-10

Fig. 3. Reversible electroporation areas show that the NaCl buffer produces a smaller affected area than the CaCl₂ buffer indicating the cells are electroporated to a lesser extent. The NaCl buffer may provide protection against cell death whereas the CaCl₂ solution does not (*** p < .001, scale bar 1 mm). (A) The image on the left of each panel shows both the unpermeabilized (green) and permeabilized (violet) regions of cells while the image on the right (violet alone) shows permeabilized cells alone. In vitro collagen scaffolds consisted of 5 mg/mL rat tail collagen type I and U251 malignant gliomas (1 × 10⁶ cells/mL). H-FIRE treatments consisted of delivering 80 bursts of pulses with various durations at 800 V and a frequency of 1 burst/s for a total on time of 100 μs. The total area of the scaffold was 78.54 mm² (10 mm diameter) with a thickness of 1 mm. (B) Reversible electroporation areas were measured using Image J and a custom algorithm developed in MATLAB. Electrodes were spaced 4 mm apart (center-to-center). Results are shown as mean ± std. (n ≥ 6 for all conditions). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
and 1.47× for 5 mM CaCl₂. For the 5-1-5 waveform reversible zones were 5.58× larger than irreversible zones for 1 mM NaCl, 3.94× for 5 mM NaCl, 1.67× for 1 mM CaCl₂, and 1.3× for 5 mM CaCl₂. For the 10-1-10 waveform, reversible zones were 3.17× larger for 1 mM NaCl, 2.94× larger for 5 mM NaCl, 1.32× larger for 1 mM CaCl₂, and 1.59× larger for 5 mM CaCl₂ when compared to irreversible zones.

In Fig. 4B, the difference between reversible and irreversible electroporation areas was largest for NaCl solutions. When CaCl₂ was used, the difference between reversible and irreversible electroporation areas decreased. As pulse duration increased, the difference between reversible and irreversible electroporation areas also decreased for all conditions.

3.4. Using adjuvant calcium in combination with a 10-1-10 H-FIRE treatment reduces the irreversible electroporation threshold to that of an IRE treatment

Using our finite element model of H-FIRE treatment in the scaffold, we were able to find the corresponding electric field thresholds for each ablation area. Table 1 shows that using adjuvant calcium reduces the electric field threshold in all experimental conditions. When compared to the NaCl control, 1 mM CaCl₂ reduces the electric field threshold 1.24× for the 1-1-1 waveform, 1.48× for the 2-1-2 waveform, 2.05× for the 5-1-5 waveform, and 2.19× for the 10-1-10 waveform. When comparing 5 mM NaCl to 5 mM CaCl₂, the electric field threshold is reduced 1.46× for the

### Table 1

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Boxes highlight that 10-1-10 H-FIRE treatment with calcium results in comparable electric field thresholds to standard IRE treatment.
We profiled gene expression patterns while calcium upregulated expression of EIF5B and the enhanced ablation areas were unique to calcium. Testing a 1 mM KCl solution did not result in statistically significant ablation sizes (Fig. 6 A). Both 1 mM NaCl and 1 mM KCl results in ablations similar in size to CaCl2 alone (n = 9), therefore NaCl does not rescue the cells from the effect of excess calcium. Replacing CaCl2 with KCl (n = 8), results in an ablation that is similar to NaCl alone. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 5. Enhanced cell death using H-FIRE treatment with pulse durations below 10 μs is unique to CaCl2. Experiments were completed using a 5-1-5 waveform (**p < .001). (A) In vitro collagen scaffolds consisted of 5 mg/mL rat tail collagen type I and U251 malignant glioma cells (1 x 106 cells/mL). Electrodes were spaced 4 mm apart (center-to-center). Eighty bursts consisting of 5 μs pulses were delivered at 800 V, frequency of 1 burst/s for a total on-time of 100 μs. Live cells are stained with calcein AM (green) while dead and/or membrane compromised cells are stained with propidium iodide (magenta). Propidium iodide stains cells red, but the images are shown in magenta for visualization purposes (scale bar 1 mm). (B) Using a combined solution of 1 mM CaCl2 and 1 mM NaCl (n = 5) results in ablations similar in size to CaCl2 alone (n = 9), therefore NaCl is unable to rescue the cells from the effect of CaCl2. Experiments were completed using a 5-1-5 waveform (Fig. 6 B). These data demonstrate the global changes in gene expression across treatment groups, with multiple differences observed between CaCl2 and NaCl (Fig. 6B).

Based on the significant correlation between changes in NOX1, NOX4, EIF5B, and the 60S ribosomal subunit signaling with NaCl and CaCl2 and the diverse changes in global gene expression, IPA analysis identified a specific group of related biological functions impacted by H-FIRE treatment under the two cation conditions tested (Fig. 6C). Under NaCl conditions, H-FIRE induces as significant up-regulation in NOX1 and NOX4 expression. This upregulation results in an increase in genes associated with NADPH Oxidase activity (Fig. 6C). However, consistent with the limited Ca2+ in the media, we observed gene expression changes consistent with a defect in luminal calcium signaling. Because NOX proteins require Ca2+ for ROS generation, these data are also consistent with the attenuation of ROS production observed in the NaCl conditions (Fig. 6C). Together, these data suggest that the increase in NOX1 and NOX4 expression may be compensatory changes associated with the Ca2+ limited conditions in the NaCl buffer and likely signal attempts by the cell to generate ROS following H-FIRE treatment. Concurrently with the defect in ROS signaling, we also observed a significant decrease in EIF5B and the 60S Ribosomal Subunit signaling (Fig. 6C). Consistent with these observations, IPA identified a significant decrease in pathways associated with the translation of stress related mRNA and a significant increase in signaling pathways associated with pro-survival (Fig. 6C). These data suggest that low Ca2+ conditions in the NaCl buffer provides a protective effect to minimize cell death following H-FIRE treatment.

Compared to our findings for the NaCl buffer, we observed opposite effects under the CaCl2 conditions. Our results show a significant decrease in NOX1 and NOX4 associated signaling pathways, reduced unfolded protein response signaling, and attenuated apoptosis signaling (Fig. 6C). These data would suggest less cell death under these conditions. However, we also observed a significant increase in luminal calcium signaling pathways that were significantly correlated with increased cell death and a concurrent increase in signaling pathways associated with the translation of stress related mRNA (Fig. 6C). These data are consistent with the increase in EIF5B and 60S Ribosomal subunit signaling that was also observed (Fig. 6C). The increases in these pathways resulted

Translation Factor 5B that functions in translation initiation and interacts with the 60S Ribosomal Subunit. A heatmap was generated using the Average Linkage Euclidean Distance Measurement Method to better visualize the global changes in gene expression (Fig. 6B). This heatmap shows the global changes in gene expression across treatment groups, with multiple differences observed between CaCl2 and NaCl (Fig. 6B).
Fig. 6. Signaling pathways associated with NOX1/4 and EIF5B are differentially regulated following H-FIRE depending on CaCl₂ or NaCl environment. Gene expression profiling analysis identified biological functions specifically associated with NOX1, NOX4, EIF5B and the 60S ribosomal subunit that were significantly dysregulated following H-FIRE treatment and correlated with CaCl₂ or NaCl containing buffer. (A) Individual expression of each of these genes was significantly and differentially regulated based on CaCl₂ or NaCl. (B) Heatmap analysis reflects global changes in gene expression and revealed significant dysregulation among genes associated with NADPH oxidase activity, proliferation, cell death, calcium signaling, and cellular stress. Red is downregulated genes and green signifies upregulated genes. (C) Ingenuity pathway analysis of gene expression profiling data revealed differentially activated pathways, quantified by associated z-score, associated with increased proliferation and survival under NaCl conditions and increased necroptosis under CaCl₂ conditions following H-FIRE. Here, red represents upregulated genes and green signifies downregulated genes. **p < .01. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
in an increase in necroptosis signaling, rather than apoptosis, in the CaCl₂ conditions following H-FIRE (Fig. 6C).

4. Discussion

We have demonstrated that adjuvant calcium used in combination with H-FIRE treatment enhances ablation areas and reduces both reversible and irreversible electroporation thresholds. Pulse durations shorter than 10 μs require the use of adjuvant calcium to produce ablations larger than 5 mm² in vitro (Fig. 2). It has been shown that shorter, bipolar pulses are less efficient at permeabilizing cells compared to longer, monopolar pulses [33,48]. Longer pulse durations cause an increase in the number and/or size of pores that are created in the membrane [32,33,49], enhancing permeabilization and potentially cell death. Interestingly, the 5-1-5 waveform resulted in the largest increase of ablation area between CaCl₂ and NaCl treatments. The 5-1-5 waveform may be maximizing permeabilization, while minimizing cell death from irreversible electroporation in the absence of calcium. To confirm this, it was necessary to characterize the reversible ablation areas and thresholds.

When CaCl₂ was used, the difference between reversible and irreversible electroporation areas decreased for all waveforms (Fig. 4). This result further supports our hypothesis that cells which are reversibly electroporated are driven to undergo cell death in the presence of adjuvant calcium, therefore enhancing the area of irreversible electroporation. As pulse duration increased, the difference between reversible and irreversible electroporation areas also decreased for all conditions, suggesting that there is a transition to a higher number of cells dying. The difference between irreversible and reversible electroporation was highest for 1 mM NaCl and the 5-1-5 waveform confirming that permeabilization was maximized while cell death was minimized.

Results suggest that the cell death response to CaCl₂ is dose dependent for shorter pulse durations (1–5 μs). Fig. 4 shows that for 5 mM CaCl₂, irreversible ablation areas are almost the same as reversible electroporation areas whereas for 1 mM CaCl₂, the irreversible areas are much smaller. Cells may also be able to pump lower concentrations of calcium out through the plasma membrane ATPase pump [34,50]. In addition, Ciofani et al., demonstrated that cells electroporated in the presence of calcium (< 0.5 mM) had a lower relative fluorescence emission when exposed to PI 5 min post pulse [51] than cells that were electroporated without calcium, suggesting that low levels of calcium may aid in the resealing process [52]. The dose dependence seen in our results seems to diminish as pulse duration increases. When a 10-1-10 waveform is used, the cells may be electroporated to an extent that they cannot recover and the difference in ablation area for the two calcium concentrations decreases, suggesting that there is a transition to a higher number of cells being irreversibly electroporated.

Using a 10-1-10 waveform with CaCl₂ decreases the cell death threshold (771 ± 129 V/cm) to a value comparable to an IRE treatment without CaCl₂ (698 ± 103 V/cm). This finding may allow researchers to ablate areas similar in size to traditional IRE treatments, while eliminating some of the side effects associated with IRE such as conductivity changes and dependence on tissue geometry [29]. It has been shown that decreasing pulse duration as well as the use of bipolar pulses leads to a decrease in nerve excitation [53], however elimination of muscle contractions using a 10-1-10 waveform is yet to be confirmed. In addition, using a 10 μs waveform may eliminate the need for custom built electronics to deliver the pulses, therefore reducing the complexity of pulse generator design and cost of H-FIRE treatment.

We found that CaCl₂ buffer lowers the electroporation threshold, causing larger reversible electroporation zones compared to NaCl buffer (Fig. 3). These results match the findings of Pakhomova et al., where the authors found that cells treated in a sucrose and NaCl buffer were rescued from cell swelling and early cell death while showing increased caspase activation [54,55]. The authors hypothesized that sucrose is unable to enter pores formed during nsPEF treatment, therefore preventing cell uptake of water and subsequent rupture of the membrane. When buffer contained both CaCl₂ and sucrose, blebbing of the membrane was suppressed initially, followed by sudden bleb formation and massive uptake of propidium iodide leading to necrosis. Sucrose prevented cell swelling initially, but over time as more CaCl₂ entered the cell, existing pores either expanded or new large pores formed, allowing sucrose and PI to enter the cell. Therefore, the authors concluded that CaCl₂ causes cell death through a non-osmotic mechanism, although they did not specify the biological mechanism responsible. It is important to note that Pakhomova et al., used trapezoidal pulses with a duration of 300 ns whereas our pulses are square wave with longer duration (1–10 μs). It is known that pulse shape as well as duration affects pore formation during electroporation, therefore it is possible that the dynamics of CaCl₂ and sucrose/NaCl uptake are different during H-FIRE treatment. However, we do see similar results in terms of CaCl₂ causing more permeabilization than the NaCl/sucrose solution. This similarity may suggest that pore formation during H-FIRE treatment may be similar to nsPEF treatment, however, to directly compare our results we would need to monitor cell swelling and uptake in response to H-FIRE treatment.

To investigate the differences in cellular response to CaCl₂ and NaCl conditions, we analyzed the regulation of calcium signaling genes. The genes chosen were pre-selected and validated by the vendor (Qiagen) to provide the broadest overview of calcium signaling pathways using a commercially available array. When the data are processed and evaluated using the vendors software and Ingenuity Pathway Analysis (also from Qiagen), the 89 genes selected are optimized to identify biological pathways and functions impacted by treatment. Our gene expression profiling identified several distinct biological functions that were significantly altered and correlated with NaCl or CaCl₂ environments. Under the NaCl conditions, the U251 cells were more resistant to cell death than cells treated in CaCl₂ conditions due in part to the downregulation of EIF5B and the eventual reduction in stress associated mRNA translation following H-FIRE in the NaCl conditions. The increase in NOX1 and NOX4 signaling should have increased cell death through increased ROS in these cells under these conditions. However, we paradoxically observed minimal ROS signaling, indeed even an attenuation (Fig. 6). This is likely due to the requirement for Ca²⁺ for NOX1 signaling. In the absence of calcium, it is likely that the cell death effects associated with NOX1 and ROS production are minimized [56]. Thus, we are likely observing increased gene transcription of NOX1 as a compensatory mechanism following H-FIRE in cells attempting to initiate ROS signaling. This is also likely associated with the increase in NOX4 transcription. Unlike NOX1, NOX4 is constitutively active and likely upregulated at the expression level as compensation for the low calcium conditions where it can ultimately compensate for the defect in NOX1 signaling [56]. However, this delay in NOX4 signaling would be predicted to stall apoptosis in our models, as reflected in the reduced levels of cell death following H-FIRE treatment. In addition to its roles in cell death, increased expression of NOX1 also plays a role in cell proliferation where it has been demonstrated to mediate cell growth and transformation when overexpressed [57]. NOX1 regulation also targets cyclin D1 and ERK1/2 activity [58]. Thus, in addition to reduced cell death in the NaCl conditions, we are also likely observing some differences associated with increased cell...
proliferation and pro-survival signaling. Interestingly, Pakhomova et al. did not observe a cell sparing 24 h after treatment with NaCl and sucrose buffer, which does not appear consistent with our findings. Perhaps the pulse parameters or cell type that we have chosen to study are responsible for this contradiction. Previous research has shown that the mechanism of cell death may differ between cell types as well as pulse strength and duration [47,59].

Conversely, the U251 cells were more sensitive to H-FIRE treatment in the CaCl₂ conditions than they were in the NaCl conditions. This is due, in part, to the upregulation of EIF5B and the eventual induction of necroptosis. EIF5B represents a regulatory node in cancer cells, whereby under some conditions increased gene expression is associated with cell evasion of apoptosis by promoting the translation of pro-survival proteins, while under other conditions overexpression promotes cell cycle defects resulting in cell death in response to stress [60,61]. Our data is consistent with the later findings, whereby in conditions of calcium enrichment followed by cellular stress and damage associated with H-FIRE, we observe significantly increased cell death. Likewise, the anti-apoptotic functions of EIF5B, associated with the upregulation of XIAP and BCL-XL, may function as a significant driver of the increased necroptosis signaling as the predominate form of early cell death in the U251 cells following H-FIRE in the CaCl₂ conditions (Fig. 6). Finally, it should be noted that dysregulated increases in intracellular calcium can promote cell death through necrosis following cellular damage and/or stress [62]. One limitation of our study is that we analyzed gene expression of cells that were exposed to a range of electric field magnitudes which may be experiencing differential gene expression. To accommodate for this limitation, we normalized our results to sham controls to observe general trends. However, in future work it may be useful to investigate gene expression in response to a uniform electric field magnitude. Such investigations would allow for precise characterization of cell response for different electric field strengths. Our results can be used to inform future studies as to which electric field magnitudes may be of interest for future investigations into H-FIRE treatment with and without calcium.

While the exact mechanism distinguishing cell death for NaCl and CaCl₂ still needs further investigation, several molecular dynamics studies have shown that calcium interacts with and can even bind to the lipid head groups of the membrane [63]. These interactions may influence pore formation, pore lifetime, and pore resealing [63,64]. It has also recently been demonstrated that lipid composition varies between cell type and can further influence pore formation and interaction of ions with the membrane [65]. Furthermore, previous studies have shown that an excess of intracellular calcium may activate proteases and phospholipases which further contribute to the lifetime of pores and exacerbate membrane damage, subsequently preventing resealing of the membrane [66].

5. Conclusion

We have demonstrated successful enhancement of H-FIRE ablations using adjuvant calcium. Utilizing a 10–1–10 H-FIRE waveform with 1 mM CaCl₂ reduces the irreversible electroporation threshold to a value comparable to standard IRE treatments without calcium, therefore enhancing the efficacy of H-FIRE without increasing the applied voltage. This finding may potentially allow clinicians to use commercially available generators instead of custom-made generators for treatment, simplifying translation to the clinic. We have also uncovered several distinct biological functions that were significantly altered and correlated with NaCl or CaCl₂ environments.

Calcium electroporation has recently been investigated in several clinical trials for cutaneous metastases, colon as well as head and neck cancer [36,67,68]. We envision using calcium H-FIRE treatment in vivo in a similar fashion to enhance ablation size by injecting calcium chloride directly into the tumor and delivering H-FIRE pulses through needle electrodes inserted into the tumor. However, translating to the clinic still presents challenges such as optimizing CaCl₂ concentration as well as injection volume as to avoid necrosis of surrounding tissue and hypercalcemia.

Following our observation that sucrose and NaCl may prevent cell death, we also envision injecting a buffer that contains sucrose and lacks calcium to control electroporation treatments in vivo, therefore preserving tissue that is outside of the desired ablation zone. This sucrose buffer could be injected into blood vessels near tumors prior to treatment to prevent electroporation of the endothelial cells in the vessel. Spatial control over ablations and sparing of blood vessels may be beneficial for treatments such as blood-brain barrier disruption. Electrodes may be designed to inject CaCl₂ into the tumor during treatment while also delivering NaCl and sucrose buffer on the tumor borders to protect surrounding tissue. Venofer is an Iron Sucrose solution (300 mg/ml sucrose w/v) that is administered intravenously and utilized to treat anemia. Side effects are relatively minor and include muscle cramps, nausea, vomiting, and dizziness. Most side effects are associated with the speed of administration. However, it would be reasonable to utilize something like Venofer to increase sucrose concentrations as supported by the data provided here.

In addition, these results suggest that adding calcium to electroporation buffers prior to gene transfection procedures may enhance permeabilization and therefore cell uptake [35]. Our study is the first to demonstrate enhancement of H-FIRE ablations using adjuvant calcium, in addition to identifying key genes and signaling pathways responsible for differences in cell death with and without calcium.

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Appendix A. Supplementary data

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References


