The feasibility of enhancing susceptibility of glioblastoma cells to IRE using a calcium adjuvant

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Abstract

Irreversible electroporation (IRE) is a cellular ablation method used to treat a variety of cancers. IRE works by exposing tissues to pulsed electric fields which cause cell membrane disruption. Cells exposed to lower energies become temporarily permeable while greater energy exposure results in cell death. For IRE to be used safely in the brain, methods are needed to extend the area of ablation without increasing applied voltage, and thus, thermal damage. We present evidence that IRE used with adjuvant calcium (5mM $CaCl_2$) results in a nearly two-fold increase in ablation area *in vitro* compared to IRE alone. Adjuvant 5mM $CaCl_2$ induces death in cells reversibly electroporated by IRE, thereby lowering the electric field thresholds required for cell death to nearly half that of IRE alone. The calcium-induced death response of reversibly electroporated cells is confirmed by electrochemotherapy pulses which also induced cell death with calcium but not without. These findings, combined with our numerical modeling, suggest the ability to ablate up to 3.2X larger volumes of tissue *in vivo* when combining IRE and calcium. The ability to ablate a larger volume with lowered energies would improve the efficacy and safety of IRE therapy.

Keywords: Electrochemotherapy, ablation volume, finite element modeling, irreversible electroporation, brain cancer, combined therapy

Introduction

Grade IV astrocytoma, also known as glioblastoma (GBM), is among the most aggressive cancers. Standard therapies such as surgical resection, radiation, and chemotherapy aim to eliminate the primary tumor in the hopes of alleviating neurological symptoms. Even with state-of-the-art treatment, the increase in median survival time is merely 14 months[39,40].

As an alternative to standard treatments, energy-based therapies that utilize electric fields with high magnitudes (400-3000V/cm), short durations (100μs) and low frequencies (~1Hz) are being used to induce cell death and enhance drug delivery. These therapies rely on a phenomenon known as electroporation, which occurs when an applied electric field causes the transmembrane potential of the cell membrane to rise above a threshold limit (~200-1000mV). The rise in transmembrane potential results in the formation of nanoscale defects, allowing otherwise impermeant ions and molecules to enter. At lower voltages, this permeabilization process may allow for membrane recovery with removal of the applied field (reversible electroporation), while cell death through loss of homeostasis occurs at a higher voltage threshold (irreversible electroporation)[27].

Irreversible electroporation (IRE) has been shown to treat spontaneous gliomas in canine patients[16,17] while sparing blood vessels and extracellular matrix[35]. An IRE treatment consists of delivering electrical pulses through two needle electrodes that are inserted into the bulk tumor[2]. Since electrical pulses are delivered at short duration and low frequencies, this causes minimal heating of the tissue, thereby ensuring ablation while mitigating thermal damage[9]. IRE treatment results in a sharp delineation between

treated and untreated tissue with submillimeter resolution both *in vivo*[11] and *in vitro*[3], making it possible to develop finite element simulations for predicting lesion volumes before treatment.

Reversible electroporation can be achieved using lower voltage pulse parameters that cause permeabilization of the membrane, allowing molecules to enter the cell, while permitting subsequent recovery. This technique has been used in gene transfection[30], blood-brain barrier disruption[7] and drug delivery[21]. Electrochemotherapy (ECT) utilizes reversible electroporation to enhance transport of cell impermeant chemotherapy drugs[21], which has shown to be effective in treating brain tumors[1,29,36].

Because ECT uses lower applied electric field magnitudes and fewer pulses than IRE[18], and the extent of electroporation largely depends on pulse duration and number[5], the zone of treatment is limited. IRE is more versatile, enabling control of the ablation zone in tissue. IRE is effective as a stand-alone therapy for ablating the primary tumor without the need for chemotherapy drugs, yet still induces reversible electroporation further away from the electrodes, making cells susceptible to adjuvant therapies. Neal et al., confirmed a 2-3X larger zone of cell death *in vitro* using IRE treatment in combination with chemotherapeutic drugs compared to IRE treatment alone[31].

Our hypothesis is that IRE efficacy may be improved when combined with adjuvant calcium, for a treatment that is safer than combined treatment with chemotherapeutics. This hypothesis is motivated by results which demonstrate that ECT pulses used in conjunction with calcium cause more cell death and a greater decrease in cellular ATP than electroporation alone[14]. Frandsen et al., hypothesized that this may be due to ATP depletion resulting from calcium ATPase pumps in the plasma membrane going into overdrive to pump calcium out of the cell, although further investigation is needed to confirm this mechanism and rule out others.

The motivation for adjuvant calcium combined with IRE is based on the knowledge that electric field magnitude during an IRE treatment decreases as you travel away from the electrodes. A high electric field magnitude will develop close to the electrodes (irreversible electroporation) and a low electric field magnitude far from the electrodes (reversible electroporation). Cells in the irreversibly electroporated zone will die through loss of homeostasis resulting from electroporation, while the influx of calcium will exacerbate cell death in the reversibly electroporated zone. Calcium IRE may accentuate the treatment margin without applying additional energy. Furthermore, it may provide an advantage over microwave and radiofrequency ablation since the mechanism is non-thermal and spares vital structures. Though efforts have been made to extend the margin of energy based treatments, to our knowledge, we are the first to investigate IRE pulses in combination with calcium.

To test our hypothesis, we cultured glioblastoma cells in 3D collagen scaffolds and tested ECT and IRE pulses in combination with two concentrations of $CaCl_2$ solution. The electric field thresholds calculated from *in vitro* results were then used to inform a numerical model that simulates an *in vivo* treatment with the purpose of predicting

treatment volumes. These results suggest that using IRE with a calcium adjuvant enhances lesion size without increasing thermal damage.

Materials and Methods

Cell culture

U251 malignant glioma (MG) cells (Sigma Aldrich) were cultured in Dulbecco's Modified Eagle Medium (Life Technologies) containing 10% fetal bovine serum (Atlanta Biologicals), 1% penicillin/streptomycin (Life Technologies) and 0.1% non-essential amino acids (Life Technologies). Cells were routinely passaged at 70-90% confluence and kept in a humidified incubator at 37°C and 5% CO_2. Prior to fabricating the 3D collagen scaffolds, cells were removed from their flask using trypsin (Life Technologies) and centrifuged at 120g for five minutes. Cells were re-suspended in fresh medium and added to the collagen solution for a final concentration of $1x10^6$cells/mL.

Collagen scaffold fabrication

3D cell cultures are now recognized as more appropriate tumor models than 2D monolayer cultures[13]. This technique has been used previously by Arena et al.[3] and Ivey et al.[24] to study the effects of IRE on different tumor cell lines using similar matrix composition and stiffness. Concentrated collagen stock solutions (10mg/mL) were created using rat tail collagen type I as described previously[3]. While the brain consists of relatively low amounts of fibrous proteins, collagen provides a convenient scaffold material that produces relevant 3D geometry, integrin engagement with surrounding extracellular matrix, and appropriate cell-cell interactions. Collagen stock solution was mixed with 10X DMEM (10% of total solution volume) and 1N NaOH (2% of total collagen volume) until homogenous and adjusted to obtain a pH of 7.0–7.4. Cells in media were mixed into the collagen solutions to produce a final collagen concentration of 5mg/mL. Collagen was injected into Polydimethylsiloxane (PDMS) wells of controlled geometry (10mm diameter, 1mm height) to ensure uniformity of the electric field distribution across experiments. Injected collagen was molded flat in the PDMS wells and placed in the incubator to polymerize at 37ºC and 5% CO_2 for 20min. Fresh media was added to the wells and they were cultured in the incubator for 24hr before treatment. The electrical conductivities of the gel-cell mixtures were measured with a conductivity meter to ensure similar electrical properties. Collagen hydrogels without cells had a conductivity of 1.08 ± 0.06 S/m. Collagen hydrogels with cells seeded in the bulk had a conductivity of 1.17 ± 0.08 S/m.

Electroporation Protocol

Concentrations of 1mM and 5mM $CaCl_2$ were used in our study to determine the effect of a range of $CaCl_2$ concentrations on lesion size *in vitro*. These concentrations have shown effect on cell viability[14]. Media was aspirated from each well and a concentration

of 1mM or 5mM $CaCl_2$ in HEPES buffer was added for 30min at room temperature to ensure complete diffusion into the collagen scaffold. Calcium solutions were then aspirated from each well and scaffolds washed with new $CaCl_2$ solution to ensure all cell culture media had been replaced. Fresh calcium solutions were added immediately prior to pulsing. A control solution of NaCl in HEPES buffer was used, as it has similar conductivities and osmotic concentrations to the $CaCl_2$ solutions (Table1). All solutions were within the isotonic range of 260-320 mOsm/L and had pH values between 7.0-7.2 to better emulate the acidic tumor microenvironment[8,19,23]. Sham treatments were carried out by adding the NaCl and $CaCl_2$ solutions to each well and inserting the electrodes without pulsing.

TABLE1

Hollow stainless steel, blunt tip needles (Howard Electronic Instruments) with diameters 0.914mm (OD) and 0.635mm (ID) were used as electrodes. A custom-made part housed the electrodes to ensure uniform spacing (4mm center-to-center) and placement in each collagen scaffold (Figure1). IRE pulses were delivered using an ECM 830 pulse generator (Harvard apparatus) and consisted of eighty 450V pulses, frequency of 1Hz and pulse duration of 100μs. ECT pulses consisted of the same parameters except eight pulses were delivered. After treatment, $CaCl_2$ and NaCl solutions were removed from each well, replaced with cell culture media and the well plate was returned to the incubator.

Determining area and cell death electric field thresholds in collagen scaffolds

Scaffolds were kept in the incubator for 24hr after treatment. It has been reported that this is sufficient time to allow transient pores formed in the cell membrane to recover[6] and evaluate the effects of calcium electroporation[14,22]. Scaffolds were incubated with 2μM Calcein AM (Invitrogen) and 15μM propidium iodide (Life Technologies) in PBS for 30min at room temperature. Calcein AM labels living cells green while propidium iodide labels cells lacking membrane integrity red. Images were taken of each well using an inverted DMI 6000B microscope (Leica Microsystems) with a 5x objective. A custom algorithm developed in MATLAB was used to measure lesion area (see Supplementary Material for details). Details describing the numerical model simulating collagen scaffold experiments and calculation of electric field thresholds have been previously reported[3,24] and are described in Supplementary Material.

Numerical model to simulate lesion volumes in the brain

A simplified finite element model of the brain was created to simulate the increased lesion size that would occur *in vivo* using IRE and calcium. The brain was modeled as a 3D domain with dimensions sufficiently large to mitigate any boundary effects that may influence lesion size and shape (12cmX12cmX12cm). This assumption provided us with an ideal case to separate the effectiveness of treatment from any influences geometry and boundary conditions may have on lesion size. Two stainless steel, cylinder electrodes (1mm diameter), spaced 2.0cm apart (center-to-center) with a length of

1.5cm were inserted into the center of the 3D domain. The governing equation that determines potential distribution in a material is defined below:

$$\frac{\partial \varphi}{\partial t} = \nabla \cdot (\sigma \nabla \varphi) \quad (1)$$

Here, φ is electric potential and σ is electrical conductivity of the brain. We were unable to assume constant conductivity since it has been shown that conductivity of tissue increases more dramatically after electroporation than cells in suspension due to the large ratio of cell volume to extracellular fluid volume[25]. Ions leak out of the cells when they are electroporated[38] therefore increasing conductivity. Joule heating effects also increase conductivity and act as a volumetric heat source. To model this change in conductivity, we employed a smoothed Heaviside function that is dependent on electric field[16]. We can indirectly relate the change in conductivity to the applied electric field since the extent to which the tissue is electroporated depends on this magnitude. Conductivity also depends on the change in temperature that occurs in the tissue, so an additional linear heating term is used. Here α, is the coefficient that describes how conductivity changes with temperature (3.2%/℃)[10] and T_0 is the initial temperature of the tissue (37℃). To ensure the solution will converge, we smoothed the function using a continuous second derivative. The function used to define conductivity is shown below as it was written in COMSOL.

$$\sigma(E, T(t)) = \sigma_0\left[1 + 2 \cdot flc2hs\left(E_{norm} - E_{delta}, E_{range}\right) + \alpha(T(t) - T_0)\right] \quad (2)$$

Here E_{norm} is the magnitude of the electric field, E_{range} is the range over which the function transitions (±120V/cm), E_{delta} is the electric field value at which this transition occurs (580V/cm) and σ_0 is a baseline conductivity value for grey matter (0.285S/m)[10,16]. In using this function, we assumed that conductivity increases by a factor of three since this was reported for other organs during electroporation[38]. We must also account for metabolic heat generation, conduction and convection due to blood perfusion. We included the Penne's Bioheat equation with a modified Joule heating term to account for resistive losses[26].

$$\rho c_p \frac{\partial T}{\partial t} = \nabla \cdot (k \nabla T) - \omega_b c_b \rho_b (T - T_a) + q''' + \sigma |\nabla \varphi|^2 \cdot \frac{d}{\tau} \quad (3)$$

Here ρ and c_p are the density and specific heat of brain tissue respectively, k is thermal conductivity of brain tissue, ω_b is blood perfusion rate, c_b and ρ_b are the specific heat and density of blood, T_a is the arterial temperature (37°C), q''' is the metabolic heat generation, σ is electrical conductivity of the brain, and φ is electrical potential. We approximated the total resistive heating that occurs with a treatment using a duty cycle approach, as opposed to iterating over each pulse, to reduce computation time while still accounting for the heat generated during the total "on time" of the treatment. Here d is the pulse duration (50μs) and τ is the pulse period (1s). For treatments in the brain, it is preferred to use 50 μs pulses as opposed to 100 μs to mitigate thermal damage[16]. The surface of one electrode was treated as energized, one grounded and remaining boundaries as insulated. 3000V was simulated giving an applied electric field magnitude of 1500V/cm which is typical of IRE treatments delivered in the brain[16,17]. 1000 and 2000V were also tested to establish a relationship between applied voltage and lesion volume. All boundaries were treated as thermally $(\partial T/\partial n = 0)$ insulative to account for the case of maximum heating in the tissue. Material properties were defined as

stainless steel for the electrodes and grey matter for brain tissue (Table2)[2,4,10,41,42]. Standard IRE and ECT treatment were simulated in the numerical model of the brain and the electric field contour corresponding to its *in vitro* treatment threshold was located. The volumes within these boundaries were numerically integrated. It was assumed that homogenous concentrations of $CaCl_2$ and NaCl solutions would be achieved *in vivo*. The mesh was refined until there was less than a 2.0% change in calculated volumes.

TABLE2

Statistical Analysis

All IRE, ECT and sham conditions were tested ten or eleven times (n=10, n=11) as determined by a power analysis (Figure2). The discrepancy between these repetitions resulted from collagen detachment during treatment. Statistical analyses were performed with a confidence level of $\alpha=0.05$ (JMP Pro 13). Two-way ANOVA was used to test for differences in cell death area and cell death threshold. Tukey post-hoc comparisons were used to examine differences among treatment groups. Results are shown as arithmetic means±standard deviation.

Results

Experimental setup and numerical model simulating IRE pulses in collagen scaffolds

FIGURE1

Figure 1 shows our experimental platform. The electrode housing enabled precise positioning of electrodes into the collagen scaffold, eliminating variability that may occur in the electric field distribution due to varying exposure lengths or boundary effects if the electrodes were not centered.

Our numerical model was used to predict the field distribution in the collagen scaffolds during a typical treatment. The experimental platform enabled us to visualize a range of electric field magnitudes as opposed to a single value that is applied when testing cells in suspension using plate electrodes (Figure1C). This represents what occurs *in vivo* during a typical IRE or ECT treatment since cells in the tumor will experience a gradient of field magnitudes depending on their distance from the electrodes. For ECT treatments, reversible electroporation occurs at an electric field magnitude around 300V/cm whereas IRE occurs above 500-600V/cm[30]. In our numerical model of the collagen scaffolds, these zones can be distinctly visualized. By bounding regions of different electric field magnitudes using a contour plot, the model allowed us to determine the field threshold that causes an equivalent area of cell death for each of our treatments. This highlights our ability to precisely predict ablation sizes.

Experimental Results

Our experimental results confirmed our hypothesis that calcium IRE results in larger lesions than IRE alone. Sham treatments resulted in no cell death therefore confirming

that the $CaCl_2$ and NaCl solutions themselves do not affect cell viability (Figure2). Similarly, treating with ECT pulses and NaCl solutions did not result in cell death. Due to the small number of pulses used in an ECT treatment, the cells become permeabilized, but subsequently recover.

On the other hand, when we applied IRE pulses in the presence of NaCl solutions, we see a small region of dead cells formed in the collagen scaffold. This area experiences a higher electric field magnitude for a longer duration of time, therefore electroporating the cells to a greater extent and rendering them incapable of recovering from a loss of homeostasis.

FIGURE2

Since NaCl and $CaCl_2$ solutions have comparable conductivities, it can be concluded that the larger lesions observed for $CaCl_2$ treatments are due to the action of calcium and not additional heating effects that may occur due to the conductivities of our buffer solutions.

It should be noted that in some of our experimental images, the propidium iodide staining is difficult to see. This effect has also been seen in previous experiments using the same platform[24] and although the exact mechanism is not known, it seems as if the cells disappear from the scaffold 24 hours after treatment. Since Calcein AM only fluoresces when in the presence of intracellular esterases, we can conclude that cells in the dark region are dead and those stained green with Calcein AM are alive.

FIGURE3

Combining all area measurements from the image processing algorithm shows that 1mM and 5mM $CaCl_2$ treatments lead to an increase in lesion size of nearly double that of their respective NaCl controls when using IRE pulses (Figure3). The 1mM $CaCl_2$ treatment combined with IRE, resulted in an average lesion area of $25.1\pm3.9mm^2$ whereas its NaCl control resulted in an average lesion area of $14.4\pm2.9mm^2$. Combinatorial treatment using 5mM $CaCl_2$ solution with IRE resulted in an average lesion area of $32.5\pm2.0mm^2$ whereas 5mM NaCl combined with IRE resulted in an average lesion area of $13.2\pm3.6mm^2$. Comparing 1mM $CaCl_2$ and 5mM $CaCl_2$ concentrations in combination with IRE resulted in a significant difference in lesion area. There was no significant difference between 1mM NaCl and 5mM NaCl treatments ($p=0.9553$).

For ECT pulses, it is evident that calcium treatments also had a significantly larger lesion area than their NaCl controls for both 1mM $CaCl_2$ and 5mM $CaCl_2$. Lesion areas were not significantly different for 5mM $CaCl_2$ and 1mM $CaCl_2$ treatments ($p=0.1926$). Data for both NaCl concentrations are not shown in Figure 3 for ECT treatments since these pulses did not result in a lesion and were treated as having zero area.

When comparing ECT pulses to IRE pulses, for the 1mM $CaCl_2$ solution, there was no significant difference between lesion areas ($p=0.9451$) whereas for the 5mM $CaCl_2$ solution, there was a significant difference. As mentioned previously, an IRE treatment

of 80 pulses leads to an average lesion area of 32.5±2.0mm^2, whereas an ECT treatment of eight pulses lead to an average lesion area of 26.7±2.1mm^2 for the 5mM $CaCl_2$ solution.

Calculating electric field threshold required for cell death

FIGURE4

From our numerical model of the collagen scaffold, we determined a relationship between lesion area and electric field magnitude by performing numerical integration on the surface of the scaffold for a range of electric field magnitudes (100-1500V/cm). We constructed a curve and fit this data using least squares fitting in MATLAB. This resulted in a sixth order polynomial equation shown in Figure 4A. Despite this equation not having relevance to the physics in our model, we were able to use it to accurately back out the electric field thresholds for each treatment condition without needing to manually determine them using COMSOL. Least squares fitting resulted in a maximum relative error of 4.7%.

Increasing the applied voltage from 450V to 800V demonstrates that it is possible to increase the lesion area by applying a higher voltage during treatment, however, the applied voltage in our experiment was limited by the size of the scaffold. If we were to apply 800V in combination with 5mM $CaCl_2$, the lesion size and shape may be influenced by the electrically insulated boundaries of the collagen scaffold and an accurate area may be difficult to measure. An applied voltage of 450V was chosen because it provides a wide range of electric field magnitudes over a broad range of areas. Figure 4B shows electric field contours taken from the numerical model of the collagen scaffold, overlaid onto a treatment using IRE in combination with 5mM $CaCl_2$ solution. Electric field lines of high magnitude are plotted to locate the electrodes in the image and place the overlaying solution accurately. The average field threshold using least squares fitting was calculated to be 377±18.6V/cm. An electric field contour of 400V/cm demonstrates the accuracy of the numerical solution in predicting cell death thresholds.

FIGURE5

Figure 5 shows the electric field thresholds that were calculated using the polynomial equation. IRE treatments used in combination with $CaCl_2$ solutions had significantly lower field thresholds than their NaCl controls. A treatment using IRE and 1mM $CaCl_2$ resulted in a field threshold of 467±67V/cm whereas IRE used in combination with 1mM NaCl resulted in a threshold of 698±103V/cm. Increasing the amount of calcium in our solution to a 5mM concentration resulted in a further reduced threshold of 377±19 V/cm. This threshold was significantly lower than its control using 5mM NaCl (745±139 V/cm). IRE treatments using 5mM $CaCl_2$ resulted in a lower threshold than ECT treatments using 5mM $CaCl_2$ (440±26V/cm), but the difference was not statistically significant ($p=0.3853$). The same is true for a $CaCl_2$ concentration of 1mM, which resulted in an average threshold value for an ECT treatment of 481±38V/cm ($p=0.9997$).

Simulating lesion volumes for an *in vivo* treatment in the brain

Calculating the field threshold for an *in vitro* calcium IRE treatment enabled us to estimate what will occur during an *in vivo* treatment. Figure 6 shows the results of our numerical simulation of ECT and IRE calcium treatments in a 3D model of the brain. Here, we have accounted for dynamic changes in conductivity of the tissue due to electroporation and changes in temperature that result due to Joule heating. Figures 6A and 6D indicate the electric field distributions in the tissue after an IRE and ECT treatment respectively along a cut plane in the xz axis. For an ECT treatment, the electric field magnitude between the electrodes does not reach as high a value as an IRE treatment. This is due to the low number of pulses applied (8 instead of 80)[5]. ECT pulses are designed to reversibly electroporate tissue and therefore the majority of the tissue does not reach a large enough magnitude to kill cells. The reversible regime of electroporation also covers a smaller area than an IRE treatment does.

FIGURE6

Figure 6B shows the ablation zone for an IRE treatment without $CaCl_2$ (5mM NaCl control). The ablation zone is greatly increased when 5mM $CaCl_2$ is added (Figure6C). An ECT treatment on the other hand, only has an ablation when $CaCl_2$ is used (Figure6E). Comparing ECT and IRE ablations when 5mM $CaCl_2$ is used (Figure6F) shows that the calcium IRE lesion is larger. These results are also reflected in Table 3.

Table 3 shows lesion volumes calculated using the numerical model of the brain for applied voltages of 2000 and 3000V. For 3000V, the lesion volume for a 1mM $CaCl_2$ treatment using IRE pulses is 2.10X larger than lesion size for a 1mM NaCl treatment. The increase is even more substantial for a 5mM $CaCl_2$ treatment using IRE pulses. At an average lesion volume of $29.80\pm1.57 cm^3$, this extends well beyond the bulk tumor margin.

TABLE3

Figure 7 reveals the relationship between applied voltage and lesion volume for the 3D model of the brain. As voltage increases with constant electrode spacing, the difference between a 5 mM $CaCl_2$ treamtent using ECT and IRE pulses grows substantially. When used in combination with IRE pulses, a concentration of 5mM $CaCl_2$ results in an increase in lesion volume 1.4X an ECT treatment using the same concentration of calcium solution. This drastic increase can be attributed to the fact that both the irreversibly and reversibly electroporated regions are growing as the number of pulses delivered increases. The degree to which the tissue is electroporated increaes dramatically with increased voltage, highlighting our ability to modulate pulse parameters to optimize treatment.

FIGURE7

Discussion

The overall goal of our study was to determine if using calcium in combination with IRE resulted in larger lesions than IRE alone and ECT pulses combined with calcium. During

an IRE treatment, there will be four zones of electroporation: 1) small zone of cell death caused by thermal damage (Joule heating), 2) medium sized zone of necrotic tissue in which cells are electroporated, lose homeostasis and are unable to recover, 3) large zone of apoptotic cell death in which defects in the membrane close, but cells are unable to recover from a loss of homeostasis and 4) reversibly electroporated cells that recover and survive[27]. Use of calcium IRE intensifies this cell death phenomenon and takes advantage of the reversibly electroporated zone of cells by driving them to cell death using calcium, therefore enhancing our zone of ablation.

Lee et al., have discussed the role that calcium can play in cell death after electroporation[27]. This was evidenced when cells were electroporated with a 450 ms pulse with 150 V/cm magnitude and a sharp increase in intracellular calcium levels was observed. Instead of a rapid drop in calcium concentration after the pulse was removed, the authors saw sustained elevated levels for 10 minutes. A sudden influx of calcium ions may lead cells to consume their supply of ATP to pump excess calcium across the cell membrane. This depletion of ATP after calcium electroporation has been shown by Frandsen et al., after electroporating Chinese hamster lung fibroblast cells with ECT pulses and 1 mM calcium[14]. Intracellular ATP levels decreased markedly and remained at 10.3% of control values eight hours after treatment. Furthermore, evidence has shown that elevated levels of intracellular calcium may activate proteases and phospholipases that further damage the cell and membrane, preventing pores from resealing after electroporation[20].

The possibility of enhancing ablation size by using calcium to drive cells in the reversibly electroporated zone to undergo cell death is supported by our experimental results and numerical modeling. In Figure 2, ECT treatments using NaCl solutions resulted in no cell death. Cells exposed to this ECT treatment are reversibly electroporated and are therefore able to recover and maintain viability. As evidenced by both ECT and sham treatments, the saline solution does not lead to cell death. Scaffolds exposed to IRE pulses in NaCl solutions, on the other hand, form a small region of cell death extending beyond the immediate vicinity of the electrodes (13.2±3.6 mm^2 for 5mM NaCl). It can be concluded that these cells experience a loss of homeostasis due to electroporation resulting in cell death (zone 2 described above). For both ECT and IRE treatments using $CaCl_2$, a large zone of cell death formed in the scaffold. Since a comparable lesion was not observed for NaCl treatments using the same applied pulses, it can be concluded that the region of cell death extending beyond what was seen with NaCl is due to the action of calcium and not the buffer or additional heating effects due to changes in conductivity of the scaffold. A 1mM $CaCl_2$ treatment results in a lesion area of 25.1 ± 3.9mm^2 for IRE and 23.8 ± 2.5mm^2 for ECT. A 5mM $CaCl_2$ treatment resulted in much larger lesion areas for both IRE (32.5 ± 2.0mm^2) and ECT (26.7 ± 2.1mm^2). The fact that 1mM $CaCl_2$ results in a smaller lesion area than 5mM $CaCl_2$ suggests that some cells in the periphery of the reversibly electroporated zone can recover from treatment. Using a higher concentration (5mM $CaCl_2$) further exacerbates the effects of calcium, rendering the cells unable to recover from the treatment, causing additional cell death. The lesion area of our treatments may be influenced by the lower conductivity of our medium, as shown previously by Pucihar et al[33]. Our lesion area results are consistent with the findings of Frandsen et al., when comparing calcium ECT treatment

to NaCl controls *in vitro*[14] using three cell lines. The half maximal effective concentration of calcium (EC50) was found to be 0.57 mM. At 1mM calcium, viability for all three cell lines ranged from 10-30% and at 3 mM calcium, viability for all three cell lines was below 20%. Cell death saturated at 5 mM calcium with viability for all three cell lines being below 10%. We have extended the implications of that study to provide further evidence that calcium can also be used to enhance lesion boundaries when used in combination with IRE pulses.

Calcium treatments decreased the required electric field threshold for cell death. When treating with IRE alone, we obtained a field threshold of 745 ± 139V/cm whereas for 5mM $CaCl_2$, we obtained 377 ± 19V/cm, just over half of its control. This result is similar to findings of Hansen et al., where SW780 human bladder cancer cell lines were treated using ECT pulses with varying applied electric field magnitudes[22]. This suggests that it may be possible to increase lesion size without increasing the applied voltage, mitigating concern of additional thermal damage with higher energy IRE treatment.

Our numerical model that simulates a treatment in the brain, predicts an efficacy enhancement for IRE and calcium therapy that may be expected in a clinically relevant *in vivo* setting. A 5mM $CaCl_2$ IRE treatment resulted in an increase in lesion volume three times that of its NaCl control. Comparable results were found in a mouse model, when tumors treated with 168mM $CaCl_2$ and electroporation dramatically decreased in volume immediately after treatment and continued to decrease in size up to 28 days after treatment[14]. We have previously demonstrated the safety and efficacy of IRE treatment for brain cancer in canine patients that presented with spontaneous gliomas[12,15,17]. Rossmeisl et al., demonstrated that IRE did not lead to neurotoxicity in six out of seven canine patients, increasing Karnofsky Performance Scale in all patients[34]. It has also been previously reported that non-thermal IRE treatment spares critical structures such as nerves[28,32,37], therefore we do not have reason to believe that neuron function will be impaired during IRE treatment. In future studies, it will be necessary to better characterize the effect of treatment on neurons and the increase in conductivity due to electroporation in human brain tissue. Treatments will also need to be simulated using 3D models reconstructed from patient MR images in order to ensure maximum accuracy and efficacy.

It should be noted that despite most tissues having extracellular calcium concentrations around 1mM, calcium ions are often bound by other macromolecules and only a small fraction are free in the extracellular fluid. Therefore, when treating tumors *in vivo* using calcium electroporation, administration of exogenous calcium will be required for effect. Ensuring uniform distribution of calcium in the tissue to be treated may be difficult to achieve due to leaky vasculature, high interstitial pressure and convective forces that drive fluid out of the tumor. The potential challenges associated with delivery of calcium needs to be investigated in future research. Previously, Frandsen et al., has demonstrated that direct tumor injection in combination with ECT has been successful in treating mice that were injected with human small cell lung cancer cells[14]. $CaCl_2$ solutions were injected into the tumor at a volume that is 50% of tumor volume and the needle was moved around the tumor to ensure uniform injection. Using this technique, 89% of treated tumors (~1cm^3) were eliminated. It may also be possible to have co-

delivery of the treatment with a needle/electrode system, although this may present a challenge in and of itself and will be left for future work.

Calcium IRE may provide an advantage over calcium ECT for *in vivo* treatment of tumors since IRE treatment ensures that the bulk of the tumor will be killed whereas ECT does not. While the bulk tumor is destroyed, the reversibly electroporated region extending beyond the tumor margin will experience an influx of calcium, exacerbating cell death and enhancing the ablation margin.

Conclusion

We have demonstrated that $CaCl_2$ treatment used in combination with IRE therapy leads to larger lesions than IRE alone. Consequently, the electric field threshold needed to kill the cancer cells is reduced by nearly half, suggesting that larger lesions are attainable without an increase in applied energy. Results from our numerical models indicate calcium IRE treatment in vivo can lead to treatment volumes that are 1.4X larger than calcium ECT treatments and 3.2X larger than IRE alone. While this study focused on calcium IRE treatment of brain cancer cells, the implications of these results are applicable to many types of cancer that are unresectable and invasive. Since calcium buffer solutions are regularly used in clinical settings and are known to be nontoxic to cells with intact cell membranes, calcium IRE may provide a safe and effective way to increase treatment volumes without inducing additional thermal damage.

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Figure Captions

Figure 1: Experimental Setup. A) Custom made electrodes inserted into the collagen scaffold platform prior to pulsing with electrode spacing of 4.0mm (scale bar 4mm). **B)** Geometry of the finite element model used to simulate treatment of the hydrogel platform (scale bar 2mm). The extra fine mesh consisted of 103,297 tetrahedral elements. **C)** Electric field distribution in the scaffold after an IRE treatment (450V, 1Hz, 100μs pulse width, 80 pulses) showing the region of collagen affected by different electric field thresholds (scale bar 2mm).

Figure 2: $CaCl_2$ treatments produce larger cell death lesions than NaCl controls for both IRE and ECT pulses (scale bar 1mm). Collagen scaffold experiments consisted of 4.0mm electrode spacing, 100-μs pulse width, 1Hz frequency and 450V. IRE treatments were delivered with a total of 80 pulses (n = 11) while ECT treatments were delivered with 8 pulses (n = 11, n =10). 24hr after treatment, hydrogels were stained with Calcein AM (stains live cells green) and propidium iodide (stains dead cells red).

Figure 3: Lesion sizes measured in collagen scaffold experiments demonstrate that $CaCl_2$ treatments lead to significantly larger lesion sizes than their NaCl controls. Collagen scaffold experiments consisted of 4.0mm electrode spacing, 100μs pulse width, 1Hz frequency and 450V. For IRE treatments, 80 pulses were delivered while for ECT treatments, 8 pulses were delivered. Scaffolds had a total area of 78.54mm^2 and thickness of 1mm. U251 MG cells were seeded at a concentration of $1x10^6$ cells/mL. ECT treatments with NaCl solutions are not shown due to no lesion present. (***$p<0.0001$).

Figure 4: The numerical model of the experimental setup is used to determine the area of collagen exposed to various electric field thresholds for applied voltages of 450V and 800V. A) Numerically integrating the finite element model of the collagen scaffold for different electric field thresholds allows us to construct a plot that relates the two variables. **B)** Overlay of electric field contour lines taken from numerical model with an image of a 5mM $CaCl_2$ treatment demonstrates the accuracy of the finite element model (scale bar 1mm). The scaffold has a total area of 78.54mm^2.

Figure 5: Calculated electric field thresholds for collagen scaffold experiments demonstrate that $CaCl_2$ treatments lead to significantly lower cell death thresholds than their NaCl controls. 3D collagen experiments consisted of 4.0-mm electrode spacing, 100µs pulse width, 1Hz frequency and 450V. For IRE, 80 pulses were delivered while for ECT, 8 pulses were delivered. Collagen scaffolds had a total area of 78.54 mm^2 and thickness of 1mm. U251 MG cells were seeded at a concentration of $1x10^6$ cells/mL. ECT treatments with NaCl solutions are not shown due to no lesion being present. (***$p<0.0001$).

Figure 6: Numerical model indicates IRE has a larger increase in lesion size than ECT when used in combination with 5mM $CaCl_2$ (xz plane, labels indicate V/cm). A) Electric field distribution after IRE treatment (3000V, 2.0cm electrode spacing, 80 pulses). **B)** Temperature distribution after IRE treatment **C)** Lesion size after IRE with 5mM NaCl control (white) **D)** Comparing lesion size for 5mM NaCl control (white) and 5mM $CaCl_2$ (gray). **E)** Electric field distribution after ECT treatment (3000V, 2.0cm electrode spacing, 8 pulses). **F)** Temperature distribution after ECT treatment **G)** Lesion size after ECT treatment with 5mM $CaCl_2$. **H)** Comparing lesion size for ECT (white) and IRE (gray) treatments in combination with 5mM $CaCl_2$.

Figure 7: Numerical modeling predicts larger lesion volume *in vivo* for an IRE calcium treatment when compared to an ECT calcium treatment or IRE alone. The model consisted of two electrodes spaced 2.0cm apart in a 3D domain of brain tissue. Three different applied voltages were tested (1000V, 2000V, 3000V).