Integrative Biology

PAPER

High-frequency irreversible electroporation targets resilient tumor-initiating cells in ovarian cancer

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We explored the use of irreversible electroporation (IRE) and high-frequency irreversible electroporation (H-FIRE) to induce cell death of tumor-initiating cells using a mouse ovarian surface epithelial (MOSE) cancer model. Tumor-initiating cells (TICs) can be successfully destroyed using pulsed electric field parameters common to irreversible electroporation protocols. Additionally, high-frequency pulses seem to induce cell death of TICs at significantly lower electric fields suggesting H-FIRE can be used to selectively target TICs and malignant late-stage cells while sparing the non-malignant cells in the surrounding tissue. We evaluate the relationship between threshold for cell death from H-FIRE pulses and the capacitance of cells as well as other properties that may play a role on the differences in the response to conventional IRE versus H-FIRE treatment protocols.

Insight, innovation, integration

We make use of computational and engineering methods to evaluate the use of a non-thermal ablation modality to treat ovarian cancer. We assess the underlying mechanisms that could be driving the response at the cellular level by utilizing a syngeneic model of disease progression. We evaluate the response of early/non-malignant cells, late-stage, and highly aggressive tumor-initiating cells (TICs) to pulsed electric fields and show that with irreversible electroporation types of pulses cells can be successfully ablated with no significant difference between the energy required to ablate the early cells and TICs. Importantly, we show that with three different types of high-frequency pulses a distinct ablation of TICs and late-stage cancer cells is obtained while the benign cells maintain viability.

Introduction

Failure to successfully treat cancer nowadays is not usually due to the absence of initial remission or a primary response, but rather due to tumor recurrence or relapse after therapy; tumor-initiating cells are believed to play a major role in this negative outcome.† Tumor-initiating cells (TICs) or cancer stem cells (CSCs) are cells that are able to recapitulate the original tumor.‡ These TICs may share some characteristics with the adult stem cells from the organ of origin; this is either because they are derived from stem cells or because they have gained stem properties. They present a high capacity for self-renewal, differentiation into actively proliferating tumor cells, and resistance to radiation or chemotherapy. Over time, the genetically unstable TICs can acquire various genetic or epigenetic alterations in response to microenvironmental cues, thus different TICs can be present in a tumor to create clonal populations of cancer cells with different phenotypes (see recent reviews§,¶). The presence of TICs is associated with a worse prognosis; thus, in order to achieve long-term survival, therapies that can successfully eradicate these types of TICs need to be developed and tested.

Irreversible electroporation (IRE) is a predominately non-thermal tissue ablation modality with the capability to treat otherwise surgically inoperable tumors.∥ One of the main characteristics that sets IRE apart from the more commonly used ablation modalities such as cryoablation, radiofrequency, high intensity focused ultrasound, microwave, and laser, is its primarily non-thermal mechanism for inducing cell death. IRE uses a series of short (≈100 μs), monopolar, high-intensity pulsed electric fields (PEFs) to destabilize the cellular membrane, which in turn disrupts homeostasis and leads to cell death.¶,∥

A second generation of IRE, known as high-frequency irreversible electroporation (H-FIRE), uses a series of bursts of bipolar pulses delivered at higher frequencies (kHz to MHz) than standard clinical IRE (Hz). These higher frequency pulses

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have been shown to be better suited to penetrate epithelial layers without inducing significant Joule heating, thus causing electroporation effects deeper within the targeted tissue.\textsuperscript{7} H-FIRE can greatly reduce the muscle contractions induced by standard IRE protocols which might make it easier to deliver treatment in a clinical setting.\textsuperscript{8} In addition, H-FIRE pulses have been shown to produce a more uniform electric field distribution even when delivered to heterogeneous tissues, which makes it easier to predict through numerical modeling of treatment parameters.\textsuperscript{7,9} The particular characteristics of H-FIRE also have great impact in the clinical setting where algorithms for treatment planning can be optimized to potentiate their accuracy in predicting the lesion volume \textit{i.e.} differentiation between treated and untreated tissue.

Even though IRE has been gaining attention due to its success in treating pancreatic,\textsuperscript{10} liver,\textsuperscript{11} kidney,\textsuperscript{12} and prostate\textsuperscript{13} tumors, the potential of this technique to treat ovarian cancer has not been fully investigated. Ovarian cancer is the fifth leading cause of cancer-related deaths in women in the United States. An estimated 22,440 women will receive a new diagnosis of ovarian cancer and approximately 14,080 patients will die from the disease in 2017 in the United States alone, according to the American Cancer Society. Similar to other TICs, ovarian cancer stem cells can recapitulate the original tumor, and have an increased resistance to traditional chemotherapy.\textsuperscript{14,15} Due to the genetically and histologically diverse nature of ovarian cancer, the expression of stem markers (CD44, CD133, c-kit/CD117, TLR-4+/MyD88+, EZH2, ABCG2)\textsuperscript{16–20} also varies among reports.

We hypothesize that H-FIRE can be particularly suitable for treating ovarian cancer by preferentially targeting TICs that may be susceptible to H-FIRE pulses. To explore our hypothesis, we used mouse ovarian surface epithelial (MOSE) cells, which are representative of different stages of ovarian cancer and tumor-initiating variants (MOSE-L\textsubscript{TICV}).\textsuperscript{21–23} The unique characteristics and advantages of the syngeneic model of disease progression used in this study include the ability to study the cell properties and responses to various stimuli at the different stages, without having to consider differences originating from inter-individual variability from cells originating from different hosts. The MOSE model has been proven to undergo similar changes in gene expression levels as those seen in the disease progression in humans;\textsuperscript{24} this makes it a suitable platform to study the mechanisms of ovarian cancer, from early to malignant stages, and to test the response of these to various treatment modalities.

The work presented here comprises the first study to evaluate the effectiveness of H-FIRE to treat ovarian cancer by determining the different thresholds for cell death in benign, tumorigenic, and tumor-initiating cells by pulsed electric fields. We found that there was no differential response of the cells to IRE, indicating that IRE does indeed kill the treatment-resilient cells within the targeted area. Of particular interest, we found that MOSE-L\textsubscript{TICV} and malignant, late-stage (MOSE-L) cells displayed a higher susceptibility to H-FIRE as compared to the non-tumorigenic (MOSE-E) cells. These results may have clinical implications for treating ovarian cancer and preventing disease recurrence after treatment with H-FIRE in which it may be possible to target the TICs and non-stem, late-stage, malignant cancer cells while preserving the benign epithelial cells and maintaining a viable healthy tissue that can aid recovery.

### Experimental

#### Materials and methods

**Cell culture.** The mouse ovarian surface epithelial (MOSE) cell model was previously developed from female C57BL/6 mice \textit{via in vitro} passaging and represents different stages of disease.\textsuperscript{21,24} For the studies here we used the benign (MOSE-E) and tumorigenic (MOSE-L) cell lines. The MOSE-L\textsubscript{TICV} were generated by intra-peritoneal injection of tumorigenic MOSE-L cells into syngeneic mice and subsequent recovery of cells from the ascites. These cells represent fast-developing disease: the injection of $1 \times 10^7$ MOSE-L\textsubscript{TICV} develops lethal disease in 23 days in contrast to the MOSE-L that represent a slow-developing disease (lethal disease is induced after 100 days after the injection of $1 \times 10^6$ cells).\textsuperscript{21,23,25} All cell lines were grown in high-glucose Dulbecco’s Modified Eagle’s Medium (Sigma Aldrich, St. Louis, MO), supplemented with 4% fetal bovine serum (Atlanta Biological, Atlanta, GA), and 1% penicillin streptomycin (Invitrogen, Carlsbad, CA). All cell lines were stably transfected with enhanced green fluorescent protein (EGFP) and 4 $\mu$g mL$^{-1}$ of puromycin was added to maintain EGFP-expressing cells. All ovarian cancer cell lines were grown to 75% confluency before being trypsinized and embedded in collagen gels for electroporation cell death threshold characterization experiments.

**Collagen I extraction and polymerization.** Collagen I extraction was performed as previously described.\textsuperscript{26,27} Specifically, rat tail tendons from Sprague Dawley rats were extracted and dissolved overnight in 0.1 M hydrochloric acid (pH 2.0) with agitation. After centrifugation at 30,000g, 4 $^\circ$C for 45 minutes, the supernatant was decanted and the final concentration of stock solution was determined by weighing volumes of 0.25, 0.5, and 1 mL before and after being dried at 75 $^\circ$C overnight. The collagen was sterilized by layering over chloroform overnight, and the final collagen was stored at 4 $^\circ$C until time of use. A neutralizing buffer consisting of 10 $\times$ DMEM, 1 N NaOH, and dH$_2$O (final concentration of collagen: 4 mg mL$^{-1}$) was used to re-suspend a cell pellet (harvested by trypsination and counted) to achieve a final concentration of $1 \times 10^6$ cells per mL. 235 $\mu$L of the collagen plus cells mixture were pipetted into 10 mm diameter cylindrical PDMS molds in six-well tissue culture plates. After polymerization for 20 minutes at 37 $^\circ$C, molds were removed from the collagen I hydrogels and 3 mL of complete media were added to each well to maintain cell viability. Gels were incubated overnight before treatment with IRE or H-FIRE. Rat tails were discarded tissue from experiments carried out in accordance with the guidelines approved by the Virginia Tech Institutional Animal Care and Use Committee.

**IRE treatment.** Media was aspirated from the wells and the hydrogels were treated with IRE. Pulses were delivered using 1.3 mm diameter stainless steel hollow needle electrodes with a 3.35 mm center-to-center spacing. A total of 80 monopolar,
rectangular-wave pulses with 100 μs duration at a frequency of 1 Hz were delivered using an ECM 830 electromedical system (BTX-Harvard Apparatus, Holliston, MA). Pulses were applied at 300, 375, and 450 volts based on existing IRE protocols. Needle electrodes were inserted into the center of the gels for pulse delivery, cleaned with 70% ethanol in between each treatment, and completely dried before following treatment. Electrodes were inserted into control gels without pulsing to validate that no observed effects resulted from IRE treatment alone. Post-treatment, media was added to the well plates and collagen gels were incubated from a range of 24 hours to 7 days before fluorescence microscopy images were taken to examine the death region and surrounding live cells.

**H-FIRE treatment.** A procedure similar to the one explained above for IRE treatment was followed for treatments with H-FIRE type of pulses. A custom-built electrical stimulator, capable of withstanding the desired pulse parameters within a specific energy regime, was used to deliver the treatment. It was controlled by a function generator (AFG 3011, Tektronix, Inc., Beaverton, OR). An oscilloscope (DPO 2012, Tektronix, Inc., Beaverton, OR) was used to evaluate the waveforms after voltage attenuation with a 1000× high voltage probe (BTX Enhancer™ High Voltage Probe, Harvard Apparatus, Holliston, MA) and the current was measured using a current probe (TCP312A, Tektronix, Inc., Beaverton, OR) connected to the AC/DC oscilloscope module current probe amplifier (TCPA300, Tektronix, Inc., Beaverton, OR). Three different treatment types consisting of rectangular, bipolar pulses: a burst of 25 cycles of 2 μs pulses with a 5 μs inter-pulse delay (2-5-2), a burst of 25 cycles of 2 μs pulses with a 2 μs inter-pulse delay (2-2-2), and a burst of 50 cycles of 1 μs pulses with a 2 μs inter-pulse delay (1-2-1) were all delivered with a 1 s interval between bursts for a total of 80 bursts in order to correlate to IRE treatments with 80 pulses of 100 μs duration delivered with 1 s interval. This means the ‘on’ time of energy delivery was the same for both IRE and H-FIRE types of treatments. Fig. 1 shows a schematic of pulse parameters for each type of treatment used in this study.

**Viability analysis.** Post-delivery of pulsed electric fields, hydrogels were incubated between 24 hours and 7 days prior to viability assessment to determine that no recurrence of cells appeared inside the death region. Viability was assessed using a Leica DMI 6000 fluorescent microscope (Leica Microsystems, Buffalo Grove, IL) and Zeiss Observer Z1 microscope (Carl Zeiss Microscopy LLC, Thornwood, NY), tiling a set of images to reconstruct the entire gel surface. The death region was imaged by fluorescence microscopy where only live cells express EGFP.

**Numerical modeling.** Calculations of the electric field threshold for cell death were determined using finite element analysis (COMSOL Multiphysics 4.3a, Stockholm, Sweden). The model reconstructed the 3D geometry of the gel and electrode setup where the hollow electrodes have a diameter of 1.3 mm and thickness (inner to outer rim) of 0.335 mm, and the hydrogel cylinder has a diameter of 9.94 mm with a height of 2.25 mm, Fig. 2. The electrical conductivity for the hydrogel and electrodes was 1.2 S m⁻¹ and 2.22 × 10⁶ S m⁻¹, respectively. The electric field was calculated at points from the center to the edge along the hydrogel; this distribution is numerically solved from the Laplace’s equation \( \nabla^2 \phi(z) = 0 \) where \( \phi \) is the electric potential. Boundary conditions were set to specify electric potentials in which one electrode is set as ground with \( \phi = 0 \) V while the other is charged to a specified voltage, \( \phi = V_0 \), matching the different voltages applied experimentally. The remainder of the boundaries in the model including the hydrogel were set as insulation. The lesion size was measured and compared to the numerical model to correlate the lesion size to the electric field induced in that region as described by Arena et al., and as shown in Fig. 2. The length of the axis in between the electrodes inside the lesion area is the most variable and thus was used to determine the electric field threshold at the transition between live and dead cells as previously described. An extremely fine user-controlled mesh was used resulting in a < 0.01% difference in electric field calculations with successive refinements. A parametric study on voltage was performed to determine

![Fig. 1 Schematic of pulse waveforms used to treat MOSE cell hydrogels.](https://example.com/fig1)

IRE treatments comprise monopolar pulses of 100 microseconds (μs) delivered at 1 second intervals (A). H-FIRE treatments deliver a set of bursts with alternating polarity (bipolar) pulses of various durations and inter-pulse delay such as 2 μs positive, 2 μs delay, 2 μs negative (B); 1 μs positive, 2 μs delay, 1 μs negative (C); and 2 μs positive, 5 μs delay, 2 μs negative (D).

![Fig. 2 Schematic of experimental setup (left) for treatment delivery in collagen I gels embedded with ovarian cancer cells and the corresponding finite element model (right) used to simulate the electric field distribution.](https://example.com/fig2)
the electric fields along points across the gel for 300, 375, and 450 V for IRE, and 480 and 500 V for H-FIRE.

**Determination of cell death threshold from lesion area.** Fluorescent microscopy images were exported as JPEG and analyzed using ImageJ (NIH, Bethesda, MD).\(^{30}\) Contrast was enhanced by 0.2% before converting to binary image and creating a selection delineating the lesion area. An ellipse was fitted to highlight the lesion area with clean margins and the minor and major axis lengths were exported and tabulated. The electric field magnitude corresponding to a lesion of the same size from the numerical model was assigned as the threshold for cell death for the particular case.

**Cell and nucleus size measurements.** Collagen gels were washed three times with phosphate-buffered saline (PBS), fixed in 10% formalin for 45 minutes, washed three more times with PBS, permeabilized with solution containing 10 mL PBS plus 5 μL Triton-X and 200 mg BSA (bovine serum albumin) for 30 minutes before washing three times with PBS and adding DAPI (4',6-diamidino-2-phenylindole) in a 1 : 5000 dilution. Gels were washed three more times with PBS before imaging. ImageJ\(^ {30}\) was used to analyze 63 × fluorescent images of cells embedded in collagen I hydrogels. Blue (nucleus stained with DAPI) and green (cytoplasm with EGFP) channels of each image were analyzed separately. The images were first converted to 8-bit and the threshold was adjusted to delineate the nucleus and cytoplasm area; the area was reported using the “analyze particles” feature and tabulated for subsequent analysis.

**Statistical analysis.** A one-way ANOVA was used to calculate the variances between the different treatments for each particular type of cell and for each treatment type between the different cells. Minitab 17 Statistical Software (2010). [Computer software]. State College, PA: Minitab, Inc. was used for all statistical calculations. Results were tabulated with a 95% confidence interval where the F value showed statistically significant variances in results. Post-test analysis was performed using Tukey’s test to determine which results were significantly different from one another. They are further discussed below.

**Results and discussion**

**Determination of electric field threshold for cell death**

Applied voltages of 300, 375, and 450 V were used to determine the electric field threshold for cell death from standard IRE pulse parameters. As shown in Fig. 3, results show a close agreement in the electric field threshold for cell death even for the values with highest variance as calculated by comparison of experimental measurements of lesion width (y-axis perpendicular to axis created by the pair of electrodes) with the corresponding contour of the electric field magnitude for that same measurement from the computational model at each of the three different voltages applied. The results corroborate the behavior observed by Arena et al.\(^ {48}\) where comparisons of lesion area and length along the height present a lower variability than that along the width. The results presented serve as further validation of this in vitro model for determination of electric field thresholds for cell death from electroporation protocols. There is no statistically significant difference between the means of the treatments at all three voltages for each stage of disease as shown in Fig. 4. Moreover, there is no statistically significant difference between the threshold for cell death for MOSE-E and MOSE-L-TICv, indicating that these two types of cells—representative of non-malignant and highly aggressive tumor-initiating disease stage—require a similar amount of energy from IRE type of pulses to induce cell death. The IRE threshold for cell death for MOSE-L cells was significantly different than that for MOSE-E and MOSE-L-TICv. Also shown in Fig. 4 is the combined average from treatments at all three voltages which was used as the threshold for cell death for IRE treatment with 80 monopolar 100 μs rectangular-wave pulses delivered at 1 Hz.

**Comparison of electric field threshold for cell death from H-FIRE and IRE**

The results from standard IRE treatments show a similar threshold for cell death for both MOSE-E and MOSE-L-TICv at 436 ± 20 and 432 ± 16 V cm\(^{-1}\), respectively. MOSE-L cells presented a higher electric field threshold for cell death at

![Fig. 3](https://academic.oup.com/ib/article-abstract/9/12/979/5115343/12970515343) Fluorescent microscopy of MOSE-L cells tagged with eGFP embedded in collagen hydrogel after IRE treatment at 450 V with superimposed electric field contour lines (a). Numerical model of the experimental setup from COMSOL for the treatment with 450 V showing selected contour fields in white (b). Legend shows color map of corresponding electric field magnitude in V cm\(^{-1}\).

![Fig. 4](https://academic.oup.com/ib/article-abstract/9/12/979/5115343/12970515343) No statistically significant difference was found for IRE treatments at 300, 375, and 450 V (p = 0.05) for each type of cell except for a slightly significant difference for MOSE-L at 450 V compared to 300 V and 375 V (p = 0.02). The threshold for cell death for MOSE-L is significantly different than that for MOSE-E and MOSE-L-TICv, but there is no statistically significant difference for the thresholds of the latter two. (N = 3) *Statistical significance at p < 0.05.
546 ± 28 V cm⁻¹, roughly 20.1% and 20.9% increase over the IRE threshold for MOSE-E and MOSE-L TICv, respectively as shown in Fig. 4. In contrast, H-FIRE treatments (Fig. 5) required a lower electric field threshold for cell death for the MOSE-L TICv across all three different sets of pulse parameters used in this study. The difference in thresholds for cell death for MOSE-L TICv and MOSE-L cells was consistently lower for the 2-5-2 (95% CI; p = 0.02 and p < 0.001, respectively) and 2-2-2 (95% CI; p = 0.001 and p < 0.001, respectively) sets of pulses compared to those for the 1-2-1 set. The threshold for cell death for the MOSE-L cells after H-FIRE treatment with the 2-5-2 pulse set was 816 ± 29 V cm⁻¹ and 855 ± 17 V cm⁻¹ for 2-2-2 pulses compared to 1045 ± 34 V cm⁻¹ for 1-2-1 type of pulse. H-FIRE threshold for cell death for MOSE-L TICv was found to be 722 ± 25 V cm⁻¹ for 2-2-2, 751 ± 24 V cm⁻¹ for 2-5-2, and 835 ± 23 V cm⁻¹ for 1-2-1 types of pulses. There is a statistically significant difference between the thresholds for cell death for 1-2-1 H-FIRE treatment and those for 2-2-2 and 2-5-2, and between all three of these and IRE threshold for cell death. There is no statistically significant difference between the thresholds for cell death at 2-5-2 and 2-2-2 sets of pulses for each cell type which is not surprising since they both consist of 2 μs square waves of alternating polarity with the only difference being an inter-pulse delay of 5 μs versus 2 μs for the time between the changes in polarity. Additionally, there is no significant difference in the mean thresholds for cell death for both late cells and TICs from 2-5-2 pulses which could indicate that this is the ideal type of pulse to successfully treat both late-stage malignant and tumor-initiating cells with a single treatment protocol while sparing healthy cells.

Furthermore, all three sets of H-FIRE treatments failed to elicit any considerable cell death in the MOSE-E cells; specifically, no lesion formation was observed for any of the different sets of pulses used in this study. Fig. 6 shows representative images for each H-FIRE treatment used on all three stages of disease progression. In the hydrogels with MOSE-E cells, only the mechanical damage produced by the insertion of needle electrodes into the gel was observed. From the results obtained an electric field of 2000 V cm⁻¹, which would produce a small detectable lesion on the gels in the area in between the electrodes, was still insufficient to induce cell death in the MOSE-E cells. This indicates that an electric field higher than 2000 V cm⁻¹ would be required to induce cell death in MOSE-E cells using the pulse parameters evaluated in this study.

### Determining a relationship from treatment response based on cell and nucleus size

Ivey et al. postulated that the response to H-FIRE treatments is directly proportional to the size of a cell’s nucleus; our results seem to follow a similar trend but the high variability in cell and nucleus size, especially for MOSE-L TICv, can hinder a direct relationship based on size alone. There is no significant difference between means for nucleus size of MOSE-E and MOSE-L cells (95% CI). The mean nucleus size of TICs is significantly larger than both MOSE-E and MOSE-L cells for a 95% confidence interval with p < 0.001 for both cases. The mean for cytoplasm (or overall cell size) of MOSE-L TICv is significantly larger than both MOSE-E and MOSE-L cells.

### Table 1 Electric field thresholds for cell death from IRE and H-FIRE treatments on all three stages of disease progression

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Ovarian cancer stage</th>
<th>Electric field threshold for cell death mean (st. dev.) in V cm⁻¹, N = 9</th>
</tr>
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<tbody>
<tr>
<td>MOSE-E</td>
<td>Early</td>
<td>H-FIRE (2-2-2) No visible lesion</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H-FIRE (1-2-1) No visible lesion</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H-FIRE (2-5-2) No visible lesion</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IRE 436 (20)</td>
</tr>
<tr>
<td>MOSE-L</td>
<td>Late</td>
<td>855 (17)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1045 (34)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>816 (29)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>546 (28)</td>
</tr>
<tr>
<td>MOSE-L TICv</td>
<td>Highly aggressive, stem-like properties</td>
<td>722 (25)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>835 (23)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>751 (24)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>432 (16)</td>
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</tbody>
</table>

722–5–2 (95% CI; 835/C6) difference being an inter-pulse delay of 5 μs for both cell types which is not surprising since they both consist of 2 μs square waves of alternating polarity with the only difference being an inter-pulse delay of 5 μs versus 2 μs for the time between the changes in polarity. Additionally, there is no significant difference in the mean thresholds for cell death for both late cells and TICs from 2-5-2 pulses which could indicate that this is the ideal type of pulse to successfully treat both late-stage malignant and tumor-initiating cells with a single treatment protocol while sparing healthy cells.
larger than MOSE-E (95% CI, $p < 0.001$), MOSE-L is significantly larger than MOSE-E (95% CI, $p = 0.032$), and there is no statistically significant difference for MOSE-L cells compared to MOSE-LTICv (95% CI, $p = 0.2$). There is no statistically significant difference between N/C ratio of MOSE-E compared to both MOSE-L and MOSE-LTICv, as calculated by Tukey simultaneous 95% CIs. The nucleus to cytoplasm (N/C) ratio of MOSE-LTICv is significantly larger than the N/C ratio of MOSE-E (95% CI, $p = 0.008$). Comparing these results to the response observed for IRE and H-FIRE, there is a relationship between IRE and N/C ratio where cells with a bigger nucleus area compared to its cytoplasm area are more susceptible to IRE type of pulses than cells with a lower N/C ratio. In the case of H-FIRE, nucleus size can play a role where larger nuclei are more susceptible to H-FIRE types of pulses but this might not be the only factor driving the response as our results would not explain the lack of lesion formation for MOSE-E cells after treatment with H-FIRE; some MOSE-L or MOSE-LTICv of the same nucleus size could have succumbed to the types of pulses used in this study. From theoretical studies, we know that cell size plays a role in electroporation protocols where larger cells are more susceptible to electric fields than smaller cells but as with everything dealing with biological organisms, there is rarely a direct relationship from treatment response based on a single factor. Some groups argue a linear relationship of decreasing IRE threshold for cell death with increasing cell size, and some have found there is no strong relationship between electroporation and cell size. It could be the case that larger cells are more easily electroporated but there could be another mechanism that allows some cells to more rapidly recover homeostasis and avoid cell death. Below we present another explanation that could be driving the observed effects.

Fig. 8 shows representative images of cell and nucleus size for each particular stage of disease. Even though the cell morphology is more circular than what would be expected in vivo, the results from this study are very promising especially for H-FIRE treatments where we would expect a more homogenous response to the applied electric fields despite differences in the spatial conformation of cells.

**H-FIRE protocols may induce cell death based on the bioelectromechanical properties of cells**

It has been previously postulated that when a cell is exposed to conventional IRE unipolar pulses, these have a strong direct-current (DC) component which renders the membrane capacitance negligible in the circuit model representing a cell exposed to this type of pulsed electric fields. On the other hand, there is a significant contribution from the alternating current (AC) components when high-frequency bipolar bursts are used in the PEF treatment. Due to the capacitive nature of the cell membrane, the effective membrane impedance is reduced at these high-frequency pulses as opposed to exposure to standard IRE pulses. Previously, the biomechanical properties of MOSE cells were measured using atomic force microscopy (AFM) where the elastic modulus was measured to be decreasing as a function of disease aggressiveness. It was reported to be lower for highly aggressive stem-like cells, with a slight increase for the malignant late-stage cells, and an even sharper increase for the non-malignant early cells. A lower elastic modulus corresponds to a higher compliance in these cells; results indicate that early cells are stiffer than the malignant types. Furthermore, an increased capacitance for the MOSE-LTICv cells is corroborated by another study in which the crossover frequency was found to be lower than that for MOSE-L, and the one for MOSE-E cells was found to be lower than the one for MOSE-E cells. At low frequencies, the membrane bulk conductivity prevents applied electric fields from penetrating the interior of a cell; with high frequencies, the membrane effective resistance drops and electric fields penetrate the cell. The specific membrane capacitance can be estimated from the ratio of crossover frequency when a cell is exposed to dielectrophoresis (DEP). The changes in capacitance for the early cells could explain why they were not affected by any combination of H-FIRE pulses used in this study.

If a relationship is found between the susceptibility to high-frequency pulses and cellular compliance or capacitance, this could be a very promising treatment modality for the treatment of various types of cancer which have all been investigated and consistently report that malignant cancer cells are more compliant than their healthy counterparts for each tissue type including breast, prostate, thyroid, bladder, and kidney. It would be of particular interest in the treatment of tumor-initiating cells which are highly responsible for tumor recurrence and metastasis and have been reported to have a high compliance which allows them to translocate across different kinds of tissues.

**Modeling the cell as a spherical capacitor when exposed to high-frequency bipolar pulses**

If we consider the nucleus of a cell as the inner sphere of a spherical capacitor and the cell membrane as the outer sphere, then a charge $Q$ on the inner sphere with radius $a$ (radius of the nucleus) and a charge $-Q$ on the outer sphere with radius $b$ (radius of the cell) will be created when exposed to an electric field, $E$. By definition, the capacitance, $C$, will be given by the charge divided by the potential difference between the two spheres:

$$C = \frac{Q}{\varepsilon E}$$

### Table 2: Overall cell and nucleus size measurements of cells embedded in collagen hydrogels

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Nucleus size (st. dev.) in μm²</th>
<th>Cell size (st. dev.) in μm²</th>
<th>Nucleus-to-cytoplasm ratio (st. dev.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOSE-E</td>
<td>66.08 (21)</td>
<td>159.86 (73)</td>
<td>0.47 (0.2)</td>
</tr>
<tr>
<td>MOSE-L</td>
<td>81.23 (14)</td>
<td>195.48 (31)</td>
<td>0.42 (0.1)</td>
</tr>
<tr>
<td>MOSE-LTICv</td>
<td>113.36 (38)</td>
<td>220.33 (77)</td>
<td>0.53 (0.1)</td>
</tr>
</tbody>
</table>
To find the potential difference, $V$, between the two spheres, we can start with the electric field outside the inner sphere which is given by $k_e(Q/KR^2)$; recall that $k_e$ is Coulomb’s constant defined as $1/(4\pi\varepsilon_0)$ where $\varepsilon_0$ is the permittivity of free space, $Q$ is the magnitude of electric charge, $K$ is the dielectric constant of the space between the inner and outer spheres, and $R$ is any distance between the nucleus and the cell membrane. For a constant electric field, $E = V/R$. Since the electric field in our case is not constant, but the electro quasistatic approximation still holds and we only consider changes in space and not time, the potential difference is given by $dV = EdR$ and thus

$$\Delta V_{b\rightarrow a} = V = -\int_a^b \frac{k_eQ}{KR^2}dR \quad (2)$$

$$\Delta V = V = \frac{k_eQ}{K} \left( \frac{1}{a} - \frac{1}{b} \right) = \frac{k_eQ}{K} \left( \frac{b - a}{ab} \right) \quad (3)$$

Therefore, combining eqn (1) and (3), the capacitance is given by:

$$C = 4\pi K_\varepsilon_0 \left( \frac{ab}{b-a} \right) \quad (4)$$

From eqn (4) above, we can see that the capacitance of a spherical capacitor is dependent on the radii of the inner and

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**Fig. 7** Overall cell and nucleus size, and size contribution to capacitance (product of nucleus and cytoplasm over their difference), increases with increasing malignancy for MOSE cells based on the average across measurements. A high variability is observed for cell and nucleus size of MOSE-L-TICv and for cell size of MOSE-E cells. $N = 40$ for all measurements.

**Fig. 8** Representative images of all three cell types showing the nuclei in blue (left column), cytoplasm in green (middle), and merged image of the previous two (right column). Scale bar = 25 µm.
outer spheres and the dielectric constant of the medium between the two. For a cell exposed to high frequency AC pulses as in the case of H-FIRE treatments, we can think of the cell behaving as a spherical capacitor where its capacitance is determined by the radius of the nucleus and that of the whole cell as well as by the dielectric constant of the space between the two membranes also known as the cytoplasm.

Using the equation for capacitance shown above (eqn (4)) to determine the corresponding values for all three stages of disease progression: MOSE-E, MOSE-L, and MOSE-LTIC, the average capacitance of MOSE-L cells is 1.1 times larger than MOSE-E cells and that of TICs is 1.7 times larger than MOSE-E cells if we assume they all have the same value for cytoplasm dielectric constant. This is an arbitrary assumption since we do not have specific values measured for each of the different cells studied. Fig. 7 shows the size contribution from eqn (4) to the overall cell capacitance for all three cell types. It may be possible, or even probable, that these cells present different dielectric constants and that this is another factor that may be driving the difference in capacitance and overall response to the same PEF treatment parameters. Furthermore, Gascoyne et al.4 found a correlation between the dielectric properties of a cell and its morphology by dielectrophoretic field-flow fractionation studies. They found that total cell capacitance varied with both cell size and plasma membrane folding which, based on the MOSE model used in this study, once again fits the criteria for malignant, late-stage cells presenting a higher capacitance than their non-malignant counterparts since they have been shown to present a higher degree of membrane folding.21 Similarly to how biophysical properties of cells are used in DEP experiments to selectively target and isolate cancer cells from non-malignant cells, these properties could be exploited by AC pulses (such as those employed in H-FIRE) to target cancer cells over healthy cells within the same region.

It has been widely postulated that for electroporation to occur, a critical transmembrane potential (TMP) must be exceeded. TMP is calculated as the potential difference across the membrane: $\text{TMP} = V_e - V_i$, where the subscripts e and i correspond to the outside and inside of the cell. From eqn (4) we can see that for a cell with higher capacitance, a lower potential would be required to accumulate the same magnitude of electric charge. Thus a lower potential can give rise to the same critical TMP for cells with a higher capacitance than those with low capacitance. This would explain the behavior observed in our study for the H-FIRE treatments where the low capacitance, MOSE-E cells, might not have reached the critical transmembrane potential to induce irreversible electroporation; whereas their malignant and tumor-initiating counterparts did reach their threshold for cell death. Since this behavior of spherical capacitors can only be applied to biological cells exposed to high-frequency pulses of AC current and not to DC pulses as IRE are usually considered to be based on the long pulse duration compared to the membrane charging time (<1 µs).44 Thus the same behavior would not hold true for IRE treatments which is consistent with the observed results in this study.

**Conclusions**

The results gathered in this study show a promising outlook on the use of high-frequency irreversible electroporation types of pulses for the selective treatment of both malignant, late-stage cancer cells and tumor-initiating cells over their non-malignant counterparts in the healthy surrounding tissue. Future work should consider using different pulse parameters to evaluate their effect on cell death thresholds while still maintaining the non-thermal characteristic of electroporation modalities. In addition, the use of flexible planar electrodes should be investigated in the clinical application of IRE and H-FIRE protocols for the treatment of ovarian cancer. These would prevent physical puncture to the tissues in the peritoneal cavity which would decrease the chance of complications from the procedure and speed up recovery. The planar electrodes could be placed on the epithelial layer without puncturing and the higher frequency of H-FIRE may enable the electric field to further penetrate the epithelial surface as compared to IRE. The particular characteristics of H-FIRE protocols such as decreased muscle contractions and malignant cell selectivity can be exploited to successfully treat small tumor masses without damage to healthy cells and surrounding critical structures. Variations in the pulse parameters may induce some interesting responses in different types of cells and, as was recently reported for asymmetric H-FIRE pulses,45 be able to decrease the threshold for cell death which means less energy would be required to eradicate undesired tissue. Promising observations from the use of high-frequency pulses in treatments with irreversible electroporation warrant further investigation to realize the full potential of this modality to successfully treat cancer as well as to elucidate the underlying mechanisms driving this response in biological tissues.

**Conflicts of interest**

RVD is an inventor on pending and issued patents related to the work. Authors declare no other conflicts of interest.

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