

# Optimal Parameters for the Destruction of Prostate Cancer Using Irreversible Electroporation

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**Purpose:** Irreversible electroporation is a new tissue ablation technique that consists of applying  $\mu$ second pulses of direct current to create permanent defects in the cell membrane. Irreversible electroporation spares connective tissue in blood vessels and other tissue structures. When applied properly, it does not produce thermal damage. We determined the irreversible electroporation parameters that would reliably destroy prostate cancer cells.

**Materials and Methods:** Irreversible electroporation pulses were applied to prostate adenocarcinoma cells in vitro. Three sets of studies were performed to determine the number, length and field strength of irreversible electroporation pulses required to produce complete cancer cell ablation without inducing thermal effects. The outcome of a treatment protocol was simulated.

**Results:** We found the upper and lower limit bounds of pulse length and number in a field range of 2,000 to 250 V/cm. A total of 90 pulses at 250 V/cm for 100  $\mu$ seconds separated by 100 milliseconds could completely ablate prostate cancer cells without inducing thermal damage.

**Conclusions:** Irreversible electroporation represents a new nonthermal ablation modality. This study has produced values for prostate cancer treatment with irreversible electroporation.

*Key Words: prostate, prostatic neoplasms, adenocarcinoma, catheter ablation, electroporation*

Percutaneous prostate cryoablation has become an accepted treatment for primary prostate cancer.<sup>1</sup> However, cryoablation lesions have some distinct limitations, such as variable damage at the cryolesion margin, injury to adjacent structures such as the rectum, urethra and neurovascular bundle, and a long procedure time due to the need for multiple freeze-thaw cycles. These characteristics have limited the widespread acceptance of this modality despite certain demonstrated advantages over the more traditional treatments of radiation and radical prostatectomy.<sup>2</sup>

IRE is a new nonthermal ablation modality that uses short pulses of direct current to create irreversible pores in the cell membrane, causing cell death. This method has been shown to have significant advantages in ablating liver tissue,<sup>3</sup> such as rapid lesion creation, rapid lesion resolution, sparing of structures such as vessels and bile ducts, and uniform destruction throughout the IRE lesion with the same advantages also demonstrated in prostate tissue.<sup>4</sup> The outcome of IRE is a function of many parameters, including electrical field magnitude, pulse length, interval between pulses, number of pulses and polarity. Obviously it would be beneficial to first develop these parameters in cells.

We describe the methodology that we used to develop optimal IRE protocols for the destruction of prostate cancer

cells. In addition, we provide insight into the effects of the various parameters. It should be emphasized that the parameters developed in this study have been successfully used in the ablation of normal dog prostate<sup>4</sup> and in yet unpublished clinical results.

## MATERIALS AND METHODS

### Cell Culture

PC3 prostate adenocarcinoma cancer cells (Cell Culture Center, University of California at San Francisco, San Francisco, California) were cultured in F-12 (N4888, Sigma-Aldrich<sup>TM</sup>), a nutrient mixture medium liquid solution, supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin and 1% L-glutamine in a 5% CO<sub>2</sub> humidified incubator at 37C.

### Electroporation

PC3 cells were washed twice with phosphate buffered saline (14190-250, Invitrogen<sup>TM</sup>) removed from cell culture plates using 0.25% trypsin-ethylenediaminetetraacetic acid solution (25200-056, Invitrogen) and centrifuged at 1,800 rpm for 10 minutes at room temperature. The cell pellet was resuspended in sterile saline solution (0.9% sodium chloride solution) to a final concentration of 10<sup>6</sup> cells per ml. Electroporation was performed using an ECM830 electroporator (BTX, San Diego, California). Cells (200  $\mu$ l) in suspension at a concentration of 1 million per ml were placed in a 2 mm gap cuvette (620, BTX) and subjected to rectangular electrical pulses, as produced by this particular electroporator. The

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electrodes in this cuvette are 2 parallel flat electrodes separated by a 2 mm gap that contains the fluid with the cells. Various protocols were tested at different pulse amplitudes, lengths and numbers, and intervals between pulses.

### Cell Viability Assays

Immediately after electroporation cell viability was determined by 2 methods, that is trypan blue staining and flow cytometry live/dead assay.

For flow cytometry 100  $\mu$ l cells in solution were removed from the cuvette immediately after electroporation and plated on 24-well plates to allow the easier mixture of solution and dye for 15 minutes in a 37C 5% CO<sub>2</sub> incubator. A 2-color fluorescence assay (SYTO® live cell nucleic acid stain kit) that distinguishes metabolic activity was used. The assay kit consists of a membrane potential sensitive dye and a mitochondrial membrane potential sensitive dye. The dyes indicate activity by a fluorescence emission shift from green at 529 nm to red at 590 nm. The red-green shift indicates mitochondrial depolarization. The assay is based on the reduction of red fluorescence (red indicates viability) vs the uptake of green fluorescence (green indicates dead or compromised plasma membranes). Dyes were placed into the previously plated solution at a concentration of 10% assay to solution volume and then placed in the incubator for 15 minutes at 37C. After incubation the mixed solutions were placed into 1.5 ml microcentrifuge tubes, which in turn were placed in an ice cup. They were measured by an EPICS® XL-MCL™ flow cytometer. Percent viability was based on a sham treated control group, in which cells underwent the complete process but were not exposed to electroporation.

Analysis was done of data from the FACS device using Flowjo™, a data analysis system. Cell viability was calculated as a percent of that of the control group, which did not undergo electroporation. Analysis (gating) was based on a reading of green at 529 nm and red at 590 nm.

Trypan blue staining was done in the remaining 100  $\mu$ l in the electroporated cuvette. Cells were removed from the cuvette, 10  $\mu$ l cell suspension were mixed with 10  $\mu$ l trypan blue solution (0.4% trypan blue, T8154, Sigma-Aldrich) and placed in a Bright-Line™ hemocytometer cell counting chamber. Viable cells were detected under light microscopy using an Olympus™ Model BHS light microscope. In this technique trypan blue can penetrate only dead cells and, therefore, every cell with dye (blue) is dead. In addition, in several experiments we noted changes in cell morphology under microscopy as another way to evaluate the effects of electroporation pulses on the cells.

### Temperature Calculations

The overall goal of our research is to develop a systematic methodology for designing IRE protocols that can cause complete cell necrosis by IRE alone with minimal thermal damage.

Electrical pulses produce heating as a function of field magnitude and pulse duration. The described analysis can be used to determine a correlation among field magnitude, pulse length and the temperature increase. The analysis is for 2 parallel plate electrodes, similar to those in this experiment. This is the worst case in terms of a temperature increase among any electrode configurations and, therefore, the results can serve as an upper limit.

The general equation for determining the relation between pulse length and the temperature increase due to application of an electrical field between 2 parallel plates is  $t = (r \times \rho \times c_p \times \delta T)/E^2$ , where  $t$  represents the pulse length that increases the temperature of the solution by  $\Delta T$  degrees C,  $r$  represents the solution resistivity, which for saline was considered to be 0.7 Ohm-m,  $\rho$  represents solution density, which for saline was considered to be 1 gm/cm<sup>3</sup>,  $c_p$  represents the specific heat of the solution, which for saline was considered to be 4,200 J/kg-K, and  $E$  represents the electroporation field imposed on the cells, which in the cuvette configuration could be calculated by dividing the voltage across the cuvette by the width of the cuvette, that is 2 mm in our case.

### Experimental Protocol

The study was designed to produce insight into the effects of IRE electrical fields on prostate cancer cells during electroporation and illustrate a methodology for designing optimal IRE tissue ablation protocols. The results of such an in vitro study can be used in combination with treatment planning<sup>3</sup> to design optimal IRE protocols.

In typical IRE the electrical pulses are delivered between 2 needle-like electrodes inserted in the tissue and placed on the outer margins of undesirable tissue. Two different voltages are applied to the electrodes in the form of an electrical pulse. One voltage is usually ground. Application of the electrical pulse produces electrical fields in the tissue. These fields are not uniform. They have the highest values near the electrodes and they taper down with distance from the electrodes. The range of values of the electrical field in the tissue changes as a function of the distance from the electrodes as well as the value of the voltages that are applied to the electrodes. The electrical fields that develop in the tissue can be calculated from knowledge of the voltage applied to the electrodes and the distance between the electrodes.<sup>3,5,6</sup>

In the design of the IRE protocol the voltages on the electrodes and the distances between the electrodes are chosen in such a way as to produce a range of IRE fields that encompasses the entire undesirable area of tissue. The effects of the electrical pulses are a function of several parameters, including the electrical field, pulse length, pulse shape, interval between pulses and number of pulses. To completely ablate the undesirable area it is important to know the effect of the various parameters and design the protocol in terms of all design parameters in such a way as to ensure complete ablation. In addition, electrical pulses produce heat, which increases tissue temperature. Damage due to thermal effects is completely different from damage due to IRE. While IRE affects only the cell membrane and does not affect connective tissue, collagen or proteins, if the temperature during the application of IRE is sufficiently increased to induce thermal damage, it can cause the denaturation of proteins and collagen, and the destruction of connective tissue and blood vessel, urethra or bile duct structures. Therefore, it is desirable when designing an IRE protocol to choose such parameters that, while inducing IRE damage, do not produce thermal modes of damage.<sup>5</sup> This study presents a methodology for developing IRE protocols.

The experimental design methodology uses the three steps described. Step 1 of the study addressed the issue of avoiding thermal damage. Because thermal damage is a

function of field and pulse length, the aim of step 1 was to provide information on bounds for the length of the pulses used for ablating cells with IRE that would not produce damaging thermal effects. The amplitude of the electrical field produced by the pulses was chosen to encompass the range of electrical fields of interest in a particular application, ie the range of electrical fields that treatment planning showed would encompass the region to be ablated. Pulse length was determined from mathematical analysis, as described. Analysis shows the pulse length that would produce no thermal damage for a given electrical field. In this series we calculated the pulse length that would cause a maximal temperature increase of 50C<sup>5</sup> in physiological saline solution for the experimental configuration of the cuvette (2 parallel electroporation plates). In these experiments we assumed that the initial temperature was 22C (room temperature), the final temperature was 50C and, therefore,  $\Delta T$  in C = 28C. Obviously different parameters would be used for calculations representing different tissues and different initial temperatures.

In this study we tested the effects of electrical fields in the range of 2,000 to 250 V/cm.<sup>7</sup> For each field the pulse was applied for the maximal duration that would not induce thermal effects, as calculated. To simulate typical electroporation protocols pulses were applied as a series of 100- $\mu$ second pulses separated by 100 milliseconds for a total application length that did not exceed the allowable non-thermal effect time. It should be noted that the 100-millisecond intervals allowed the solution to cool, and, therefore, the anticipated temperature was lower than the designed 50C. Viability of the cells exposed to these electroporation parameters was evaluated with the trypan blue test and the 2 fluorescent dye method.

Step 2 of the study was to evaluate the lower limit of electrical pulse field amplitude and length required for complete IRE ablation without thermal effects. This delineates the outer margin of the IRE affected region in treatment planning. In this study we chose 250 V/cm as the lowest effective field for ablation. We applied various numbers of pulses of 250 V/cm amplitude at 100  $\mu$ second length at 100-millisecond intervals in increments of 15 pulses and evaluated the cell viability for the various numbers of pulses. In this series of experiments we used FACS and the 2 fluorescent dye methods to evaluate viability.

Step 3 of the study was to illustrate the evaluation of the outcome of a particular IRE protocol. For illustration purpose we chose to evaluate a protocol in which tissue was destroyed by IRE ablation only without any thermal effect in the field range of 1,000 to 250 V/cm. We chose to apply 90,

100- $\mu$ second pulses, which were found in step 2, as described, to be the minimal number of pulses required to ablate cells with an electrical field of 250 V/cm. To more precisely simulate a nonthermal IRE protocol in tissue we applied pulses with longer intervals between pulses of 200 milliseconds and stopped for 10 seconds between each 15 pulses. Following electroporation cell viability was determined using trypan blue staining immediately after electroporation. Cells were removed from the cuvette, 10  $\mu$ l cell suspension were mixed with 10  $\mu$ l 0.4% trypan blue solution and placed under an Olympus upright light microscope. In each experiment cells were examined at 3 locations on the slide that were chosen at random. Intake of trypan blue and/or loss of morphology were used to evaluate cell viability. Controls were performed with cells that experienced the protocol described without electroporation. It was found that the experimental protocol itself did not affect cell viability.

## RESULTS

Tables 1 and 2 shows the results of tests in which the effects of electrical pulses on PC3 cells were evaluated with trypan blue and fluorescence tests, respectively. Data were obtained from 12 repeat experiments. Tables 1 and 2 show the electrical field that was applied and the number of pulses applied for each field. These values were chosen by the described calculations, so as not to cause thermal damage. We also measured the temperature of the solution in sham experiments immediately after electroporation protocols with a thermocouple and noted values lower than the 50C. Table 1 shows the mean  $\pm$  SD percent of cells alive and as a percent of the viability of sham treated controls. These values were calculated from the results of 12 repeats with the standard Student t test. Table 2 shows the gated range of cell viability obtained with FACS measurement for the different electrical pulses as a percent of sham treated control viability.

While it is most likely superfluous, we provide a brief explanation of this reading. The value of flow cytometry is that it can examine an extremely large number of cells. However, the resolution of the reading is inherently poor. Flow cytometry uses 2 measures for viability, that is flow cytometry of an antibody against apoptotic protein (eg the Bcl-2 family, Apaf-1, caspase-3 and 9, cytochrome C, etc) and cell morphology. FACS contains 2 or 3 lasers and the blue laser is usually the main laser from which 2 parameters are obtained, that is 1) forward scattered light (passed through the particles) and 2) side scattered light (reflected at a 90-degree angle from the particles). From these 2 parameters the FACS machine calculates certain parameters, that is

TABLE 1. Trypan blue test results of PC3 cell viability after nonthermal IRE at range of fields and number of pulses

	No. Alive/No. Dead (% alive)			Mean $\pm$ SD % Alive
	Trial 1	Trial 2	Trial 3	
	Control	80/0 (100.0)	90/0 (100.0)	
IRE:				
2,000 V/cm, 10, 100 $\mu$ sec pulses	65/25 (72.2)	74/25 (74.7)	33/19 (63.5)	70 $\pm$ 6
1,500 V/cm, 17, 100 $\mu$ sec pulses	53/30 (63.9)	35/50 (41.2)	32/19 (62.7)	56 $\pm$ 12
1,000 V/cm, 60, 100 $\mu$ sec pulses	35/60 (36.8)	23/60 (27.7)	10/45 (18.2)	27 $\pm$ 9
750 V/cm, 106, 100 $\mu$ sec pulses	7/50 (12.3)	7/83 (7.8)	11/60 (15.5)	12 $\pm$ 4
500 V/cm, 240, 100 $\mu$ sec pulses	0/80	0/90	0/50	0.0
250 V/cm, 960, 100 $\mu$ sec pulses	0/80	0/90	0/50	0.0
125 V/cm, 3,840, 100 $\mu$ sec pulses	0/80	0/90	0/50	0.0

TABLE 2. Flow cytometry results of PC3 cell viability after nonthermal IRE at range of fields and number of pulses

	No. Alive/No. Dead (% alive)			Mean % Alive
	Trial 1	Trial 2	Trial 3	
	Control	10/0 (100)	10/0 (100)	
IRE:				
2,000 V/cm, 10, 100 $\mu$ sec pulses	8/2 (80)	8/2 (80)	8/2 (80)	80
1,500 V/cm, 17, 100 $\mu$ sec pulses	6/4 (60)	6/4 (60)	6/4 (60)	60
1,000 V/cm, 60, 100 $\mu$ sec pulses	3/7 (30)	3/7 (30)	3/7 (30)	30
750 V/cm, 106, 100 $\mu$ sec pulses	1/9 (10)	1/9 (10)	1/9 (10)	10
500 V/cm, 240, 100 $\mu$ sec pulses	0/10	0/10	0/10	0
250 V/cm, 960, 100 $\mu$ sec pulses	0/10	0/10	0/10	0
125 V/cm, 3,840, 100 $\mu$ sec pulses	0/10	0/10	0/10	0

1) particle size and 2) how much the particle is granulized. It then produces a chart in which each particle it measures is shown as a dot defined by these 2 parameters. Because apoptotic and necrotic cells are small and granulized, they appear on the left bottom corner of the FACS chart. The percent of dead cells of the total number is determined on these charts through a region bounded by threshold values. The percent of data points below that threshold on the FACS charts is considered to be the percent of dead cells.

The FACS reading cannot provide values of 100% cell death because 1) the user defines the thresholds, 2) most of the time the FACS program cannot calculate the threshold by itself, 3) there is variability in the same group of cells and each time a small sample from the same group of cells is taken it is possible to obtain different values, which can vary by about 5%, and d) when necrotic cells or apoptotic cells create aggregates, which are almost impossible to avoid, they appear as a large cell in the FACS reading. Therefore, because of the inherent uncertainty and low resolution of the FACS measurement, it is common to divide reading thresholds into typical gated ranges of values. For instance, the outcome of electroporation with 500, 250 and 125 V/cm, and the particular number of pulses used was in the gated threshold range of between 0% and 10% viability (table 2). This means that for these electroporation parameters the percent of cells alive, ie above the defined threshold, was between 0% and 10%. This range represents the resolution of the FACS that we used, meaning that if 0% of the cells were alive, or 5% or 10%, the FACS would produce the same indication. It is common to believe that more than 95% apoptotic cells on flow cytometry means almost 100%.

As indicated, the value of our FACS measurements was the high number of cells examined and the detriment was the low resolution. However, comparing the results of the trypan blue and 2-dye fluorescent tests showed that the results of the 2 tests were consistent. It can be appreciated from table 2 that for the voltage field parameters of 2,000 through 750 Vs/cm the number of pulses allowable before achieving thermal effects was not enough to ensure 100% cell death. Only within the parameters of 500 V/cm and below were we able to stay within nonthermal parameters and still produce what according to the trypan blue test was 100% cell destruction and according to the FACS test could have been 100% cell destruction.

T3 Table 3 shows the results of step 2 of the study. In each experiment cells were electroporated with pulses of 250 V/cm that were 100  $\mu$ seconds long and separated by 100 milliseconds. We increased the number of pulses in 15 pulse increments. Three repeats were done with identical results.

In this series of experiments we used FACS and the 2 fluorescent dye method to evaluate viability. Consistent with the way of reading FACS data, this table indicates that 15, 250 V/cm pulses left between 80% and 90% of the cells alive and killed between 10% and 0%. Table 3 shows the effect of increasing the number of pulses in increments of 15 pulses. Table 3 also shows that the percent of cells alive decreased with the number of pulses and the percent of cells alive after 90 pulses was between 0% and 10%. It should be emphasized that this range represents the resolution of the FACS that we used, meaning that if 0% of the cells were alive, or 5% or 10%, the FACS would produce the same value.

Three repeats of the third study revealed that all cells were ablated in the field range of 1,000 to 250 V/cm. Tests with a similar application of 90 pulses and a field of 125 V/cm showed that cells survived these pulses. Figures 1 to 3 F1-3 show the results. Figure 1, A shows controls that were subjected to the protocol without electroporation. Figures 1, B, 2 and 3, which were obtained under microscopy during trypan blue staining tests, are typical and show results for 1,000, 750, 500, 250 and 125 V/cm, respectively.

DISCUSSION

We evaluated the effects of IRE pulses on prostate cancer cells in the context of IRE treatment planning. It has been known since the early 1970s that an increase in cell membrane permeability occurs when  $\mu$ second electrical fields are applied across the cell, presumably by the formation of nano scale defects in the cell membrane.<sup>5</sup> This phenomenon is named electroporation. Defects that do not reseal or IRE cause eventual cell death due to loss of cell homeostatic mechanisms. The membrane ability to reseal or reversible electroporation has been used for gene transfection in cells.<sup>8</sup> Reversible electroporation has important applications in tissue as a method of gene insertion in cells (electrogene-therapy) and for the introduction of potent but normally impermeable anticancer drugs, such as bleomycin, in cells to

TABLE 3.

No. Pulses	% Alive	% Dead
Control	90-100	10-0
IRE:		
15	80-90	20-10
30	60-70	40-30
45	40-50	50-60
60	30-40	60-70
75	10-20	80-90
90	0-10	90-100

COLOR

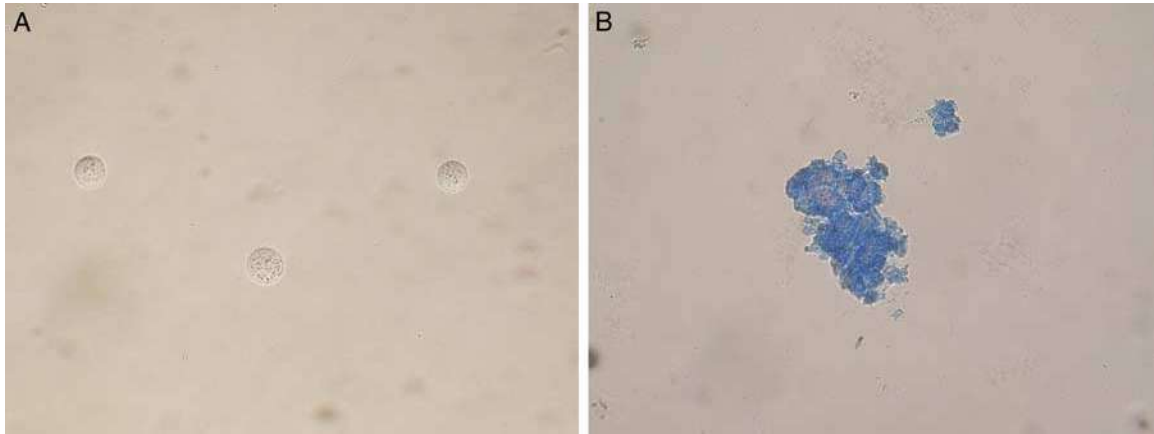


FIG. 1. *A*, sham treated PC3 cells without electroporation. *B*, aggregate of disrupted PC3 cells electroporated at 1,000 V/cm according to step 3 protocol. Reduced from  $\times XXX$ .

AQ: 1

ablate tissue (electrochemotherapy).<sup>9</sup> These procedures are accomplished by injecting drugs or macromolecules into a targeted area of tissue. Electrodes are placed in tissue to generate a reversible permeabilizing electric field, thereby introducing drugs or macromolecules into the cells of the affected area. In the described applications electroporation must be reversible and IRE is consciously avoided. Therefore, the electrical parameters that induce IRE were studied only as an upper limit to the range of electrical parameters that induce reversible electroporation.

When our group began developing IRE in tissue as a tissue ablation method, we thought that it could be used for surgical tissue ablation without the inherent limitations of chemotherapeutic injection or the narrow window of parameters associated with reversible electroporation. We also speculated that IRE would have advantages over currently used thermal ablation methods. However, according to the data gathered in reversible electroporation studies IRE uses larger electrical fields. Therefore, we were concerned that the Joule heating effect of IRE may cause thermal ablation and IRE may not exist as a substantial ablation modality. In our first published study in this field we addressed this issue and noted through analysis that IRE can ablate a substantial amount of tissue in a domain of electrical fields that do not cause thermal damage and it is a real ablation modality.<sup>5</sup>

This was followed by an acute study in a rat model showing that a substantial amount of tissue can be ablated with IRE without thermal effects and the extent of tissue ablation is predictable by mathematical analysis.<sup>6</sup> An *in vitro* study in liver cancer cells demonstrated that IRE has the ability to completely ablate cancer cells in the nonthermal regimen and other electrical parameters than those used in reversible electroporation may be needed for IRE.<sup>10</sup> We followed with studies in large animal models in the pig liver<sup>3</sup> and the dog prostate.<sup>4</sup> These studies showed the possibility of using treatment planning to deliver IRE precisely. We noted that most data on the effects of electroporation pulses on cells were produced for reversible electroporation and there were no IRE cell data on cancer treatment. To our knowledge this report represents the first systematic study to determine the parameters needed for IRE in prostate cancer cells and their use in designing optimal IRE tissue ablation protocols.

Our study elucidated the parameters needed to destroy prostate cancer cells but the results are somewhat surprising (tables 1 and 2). It appears that within the constraint of applying electroporation pulses that produce no thermal damage lower electrical fields are actually more effective for cell ablation with IRE alone than higher electrical fields. Previous studies of electroporation focused on reversible electroporation and used 8 pulses.<sup>11</sup> In fact, 8 pulses have

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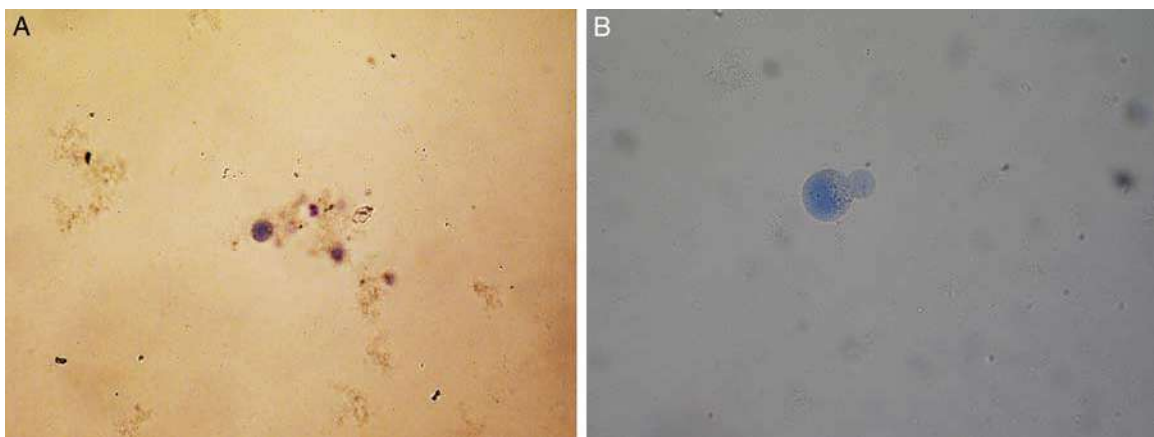


FIG. 2. PC3 cells electroporated according to step 3 protocol. *A*, at 750 V/cm some cells were disrupted and others stained with trypan blue. *B*, at 500 V/cm cells stained with trypan blue. Reduced from  $\times XXX$ .

AQ: 2

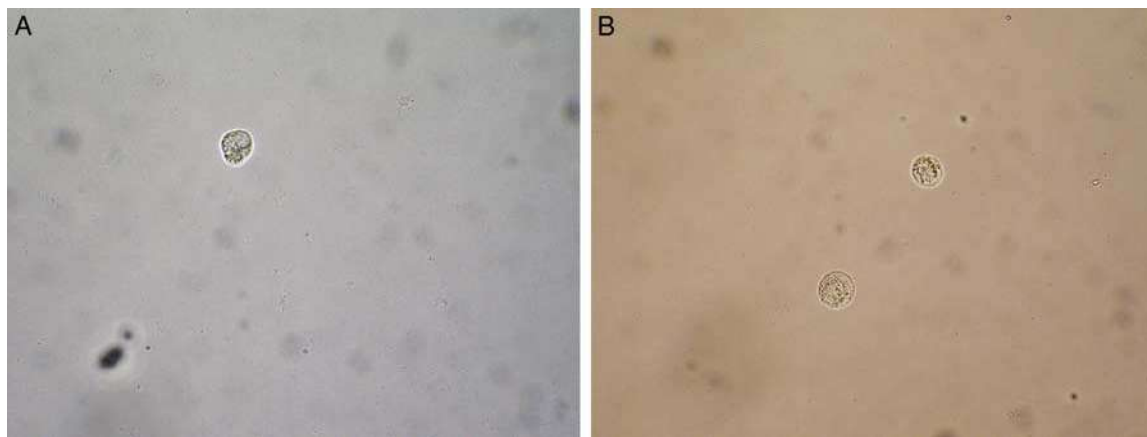


FIG. 3. PC3 cells electroporated according to step 3 protocol. *A*, at 250 V/cm cells were small and granulated, indicating necrosis. *B*, at 125 V/cm cells did not take up trypan blue and appeared intact. Reduced from  $\times XXX$ .

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COLOR

become the standard in reversible electroporation. The studies using 8 pulses demonstrated that IRE occurs only with electrical fields that are on the order of 500 V/cm and higher.<sup>12</sup> This study shows that with lower fields a substantially larger number of pulses can be used without causing any thermal effects. The study also shows that the pulses produced by these lower fields cause IRE ablation. This finding is of great importance in the practical use of IRE for cancer treatment. It suggests that a large volume of tissue can be ablated with low electrical fields by simply increasing the number of pulses beyond the traditional 8. The increase in the number of pulses can be designed in such a way as to not produce thermal effects.

The finding that lower electrical fields allow the application of longer pulses without damage is a consequence of the Joule effect and evident from the temperature calculation formula, as shown. The formula shows that the allowable time is a direct function of tissue properties, although it is an inverse function of the field squared. Therefore, allowable pulse time increases with a decreasing electrical field, not in a linear way, but rather in a squared way. However, the surprising finding, which to our knowledge was not known before our studies, is that increasing the number of pulses is an important parameter that promotes tissue ablation with IRE.

Table 3 results confirm the findings in table 1. They show that for prostate cancer cells and a field of 250 V/cm it is sufficient to use 90 pulses. This suggests that treatment planning in which the tissue to be ablated is between the electrodes and the 250 V/cm isofield line requires only 90 pulses to ensure complete ablation of that region.

From our microscopic visualization of treated cells it is evident that the nature of damage changes with the electrical field. At 1,000 V/cm all cells appeared completely fragmented. At 750 V/cm a large number of cells became completely fragmented and, while others were not completely fragmented, they were dead, as evident on trypan blue staining. At 500 V/cm fewer cells appeared fragmented (fig. 2, *B*), while all others stained with trypan blue. At 250 V/cm we did not observe fragmented cells (fig. 3, *A*), although all cells stained with trypan blue or had the morphologically distorted appearance indicated. It is interesting that at 125 V/cm cells remained intact with preserved membrane integrity and without trypan blue staining (fig. 3, *B*), which indicates what happens outside the treated zone.

These findings are encouraging and raise the expectation that IRE can successfully be translated clinically into a viable low morbidity prostate cancer treatment. Based on our previous experience with cryosurgery the procedure would probably be best applied using a transperineal approach by individual needles bounding the area to be destroyed. However, it must be appreciated that in vitro cells may not completely reflect a clinical situation, in which there is a lack of tissue homogeneity, which could affect the IRE parameters needed to successfully treat prostate cancer. Further animal and clinical studies are needed to confirm our findings.

## CONCLUSIONS

The data in this study show a methodology and a mode of thinking for designing IRE protocols for various tissues and cancers. The ability to do in vitro testing for various cell types should allow the development of optimal procedure protocols from the outset of patient treatment. IRE treatment parameters that are clinically feasible for prostate cancer treatment were confirmed.

### Abbreviations and Acronyms

FACS	=	fluorescence activated cell sorting
IRE	=	irreversible electroporation

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## AUTHOR QUERIES

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