Published in final edited form as:

Ann Biomed Eng. 2018 January ; 46(1): 25–36. doi:10.1007/s10439-017-1932-3.

Physical and chemical enhancement of and adaptive resistance to irreversible electroporation of pancreatic cancer

Qi Shao^{1,2}, Feng Liu¹, Connie Chung¹, Kianna Elahi-Gedwillo³, Paolo P. Provenzano^{2,3,4,5,6}, Bruce Forsyth⁷, and John C. Bischof^{1,2,3,*}

¹Department of Mechanical Engineering, University of Minnesota, Minneapolis, MN, USA

²Institute for Engineering in Medicine, University of Minnesota, Minneapolis, MN, USA

³Department of Biomedical Engineering, University of Minnesota, Minneapolis, MN, USA

⁴Physical Sciences in Oncology Center, University of Minnesota, Minneapolis, MN, USA

⁵Masonic Cancer Center, University of Minnesota, Minneapolis, MN, USA

⁶Stem Cell Institute, University of Minnesota, Minneapolis, MN, USA

⁷Boston Scientific Corporation, Maple Grove, MN, USA

Abstract

Irreversible electroporation (IRE) can be used to treat cancer by electrical pulses, with advantages over traditional thermal approaches. Here we assess for the first time the IRE response of pancreatic cancer, one of the deadliest forms of cancer, both *in vitro* and *in vivo*. We demonstrate that both established and primary cancer cell lines can be destroyed by IRE, but with differential susceptibility and thresholds. We further demonstrate *in vitro* that viability for a given IRE dose can vary with the local chemistry as outcomes were shown to depend on suspending medium and reduction of glucose in the media significantly improved IRE destruction. Data here also demonstrate that repeated IRE treatments can lead to adaptive resistance in pancreatic carcinoma cells thereby reducing subsequent treatment efficacy. In addition, we demonstrate that physical enhancement of IRE, by re-arranging the pulse sequences without increasing the electrical energy delivered, achieve reduced viability *in vitro* and decreased tumor growth in an *in vivo* xenograft model. Together, these results show that IRE can destroy pancreatic cancer *in vitro* and *in vivo*, that there are both chemical and physical enhancements that can improve tumor destruction, and that one should guard against adaptive resistance when performing repeated treatments.

Key terms

Irreversible electroporation; pancreatic cancer; cancer treatment; enhancement; resistance; adaptation; glucose; *in vitro*; *in vivo*

* Address correspondence to John Bischof, Department of Mechanical Engineering, University of Minnesota, 111 Church St. SE, Minneapolis, MN 55455, USA. bischof@umn.edu.

INTRODUCTION

As pancreatic cancer remains one of the deadliest forms of cancer, there is an ongoing search for new and improved treatments [1]. While surgical resection remains the most likely curative option for pancreatic ductal adenocarcinoma (PDA), only ~80% of PDA patients present with surgically unresectable disease, resulting in a lower 5-year survival compared to patients that undergo resection [2, 3]. Approaches to treat established primary or metastatic disease, and perhaps increase the potential for resection, include the use of focal therapies, including endoscopic delivery of chemotherapy, cryotherapy, photodynamic therapy, and radiofrequency (RF) ablation. However, these approaches need to either provide a unique treatment or be at least comparable to standard treatments [4]. However, many of these approaches are currently limited. For instance, implementation of heat approaches (i.e. radiofrequency, RF) can be limited by concerns about thermal injury to adjacent organs and vessels leading to bleeding [5] or bile duct fistulae or leakage [6]. In addition, the success of thermal diffusion based approaches (i.e. cryotherapy and heat) can be impacted by local perfusion (i.e. blood flow source or sink) [7]. In short, the use of focal thermal therapies in pancreatic cancer has largely been avoided due to the possibility of under treatment due to perfusion effects in adjacent vessels [8], or the possibility of over-treatment which can induce pancreatitis [9].

Nevertheless, one focal therapy, irreversible electroporation (IRE), avoids many of the pitfalls of thermal focal therapy by delivering micro to millisecond electrical pulses which, when properly designed, can permeabilize and locally destroy cancer cells. Further, IRE can be used to non-thermally destroy large volumes of tissue in a controlled manner with several groups reporting sharp boundaries between affected and unaffected tissues [10, 11]. Finally, IRE leads to cell death but does not injure the extracellular matrix or compromising the structural integrity of large vessels; thus preserving structural components and potentially avoiding complications of traditional thermal therapy [10, 12]. For these reasons, IRE is being investigated as a focal therapy for locally advanced pancreatic cancer [13].

The outcome of IRE on pancreatic cancer will depend on IRE parameters as well as cancer biological characteristics (e.g. susceptibility, size, shape and degree of local invasion) and local chemistry (i.e. chemical environment the cells are directly exposed to). For instance, the IRE parameters including pulse amplitude, duration, and the number of pulses needed to destroy pancreatic cancer cells are not yet known [14]. Further, it has been estimated that ideally the cancer tumor size should be ≤ 3.0 cm, and that the procedure can take up to 60 minutes because of the need for multiple probe pairing and placement sessions [15]. Thus, there remain important questions about IRE dosing in pancreatic cancer including: (1) baseline *in vitro* and *in vivo* thresholds of IRE in pancreatic cancer, (2) conditions for physical enhancement (i.e. pulse design), (3) chemical enhancement (i.e. local chemistry and/or glucose deprivation), and (4) the impact of repeated treatments (i.e. adaptation).

In this paper, we report on pancreatic cancer response to IRE for the first time. Two different types of pancreatic cancer cells (AsPC-1 and *KPC*) are treated with IRE over a range of clinically relevant electric fields and pulse parameters *in vitro*. AsPC-1 is taken forward for extensive enhancement tests. Specifically, we investigate and evaluate physical approaches

for IRE enhancement. We change the electrical parameters (pulse timing and frequency) while keeping the total energy constant to achieve both *in vitro* and *in vivo* enhancements. Further, we demonstrate that the chemical environment (i.e. culture media and glucose concentration) can influence IRE outcomes. Finally, we demonstrate the first evidence of pancreatic cancer cells developing adaptive resistance to IRE, in which cells are less susceptible to a new IRE treatment after a previous IRE treatment. Together, these results begin to frame the appropriate physical and chemical conditions of IRE use for treatment of pancreatic cancer.

MATERIALS AND METHODS

Cell Culture

Validated human (AsPC-1) pancreatic adenocarcinoma cells were obtained from ATCC. Primary *KPC* cells were isolated from a pancreatic adenocarcinoma in the genetically engineered *Kras*^{G12D/+}; *Tp53*^{R172H/+}; *Pdx1-Cre* (*KPC*) murine model of human cancer (approved by the Institutional Animal Care and Use Committee (IACUC), University of Minnesota) and previously described [16]. This model is faithful for the genetic, histologic, and clinical evolution of PDA [16, 17]. AsPC-1 cells were cultured in a medium consisting of Roswell Park Memorial Institute (RPMI) medium 1640, 2 mM Glutamine, 1 mM Sodium Pyruvate, 10% Fetal Bovine Serum (FBS), 100 U/ml Penicillin and 100 µg/ml Streptomycin. *KPC* cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 4.5 g/L glucose, 10% FBS, 100 U/ml Penicillin and 100 µg/ml Streptomycin. Human dermal fibroblasts (HDFs) were cultured as previously described [18]. All cells are cultured in T flasks incubated at 37 °C with 5% CO₂. Once reaching 70–85% confluency the flasks were exposed to Trypsin-EDTA (0.05% and 0.53 mM) for 5 min and split 1:3 to 1:6 to continue culture, or (at 85% confluency) used immediately for experiments.

In vitro assessment and enhancements

After culture and harvest as above, the total number of cells from each flask were counted by a hemocytometer. Cell pellets were made from suspensions centrifuged at 200 g for 5 min and the supernatant was removed. Cells were re-suspended in their original cell culture medium as described above to final cell density of 0.2–0.6×10⁶ cells/ml, unless otherwise stated. While in suspension, the cell sizes were measured by an automated cell counter (Countess II, Invitrogen). The brightfield microscopic images of the cells were captured, processed and analyzed by the built-in software (v1.0.247).

The *in vitro* experimental set-up is shown in Figure 1(a). For each IRE test, 400 µL of the prepared cell suspension was pipetted into an electroporation cuvette (FB102, Fisher Scientific) between the two plate electrodes (2 mm apart). The cuvette was then placed in an external electric field created by an electric pulse generator (BTX ECM 830, Harvard apparatus). The electrical parameters, shown in Figure 1(b), were set from the pulse generator, with electric field determined by voltage applied/distance between electrodes. The electrical pulse duration and pulse interval were set to 50 µs and 100 ms (i.e. pulse frequency of 10 Hz), unless otherwise stated.

Post-IRE cell viability was evaluated using a Tetrazolium-based Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Technologies), which has been widely used in assessing viability of a variety of pancreatic cancer cells [19, 20]. While numerous viability assays exist with various strengths and weaknesses [21], we have elected to use CCK-8 as a simple and effective approach to assess metabolic activity after IRE treatment. Following IRE, 100 μ L aliquots of the electroporated cell suspensions were mixed with 10 μ L of CCK-8 reagent in a flat-bottomed 96-well plate and incubated for 4 h at 37 °C in the incubator with 5% CO₂. The optical absorption of the sample was then read in a spectrophotometer (Synergy, BioTek) at a wavelength of 450 nm. The viabilities of a specific sample were determined by normalizing the absorption to a calibration curve, which is made of untreated cells in suspension from the same culture condition (control=100% viability), the same solution containing no cell (0% viability) and half of the amount untreated cells (50% viability). The viability is shown as average $\pm$ standard deviation from n = 6 replicates. Single factor ANOVA tests were performed on experimental groups where p value was taken to delineate statistical significance among groups. P value less than 0.05 is considered to be statistically significant.

i. Physical Enhancement—*In vitro* IRE physical enhancement was accomplished by arranging the electrical pulses while keeping the total number of pulses and waveform of individual pulse (i.e. total energy) unchanged. The effect of physical enhancements was tested on AsPC-1 cell suspensions to assess the viability after different IRE treatments, as shown in Figure 1(c). Here, the baseline of IRE parameters (51 pulses, 50 μ s duration) was chosen to keep consistent with previous published ones [22, 23]. The electric field was set between 1250 and 1750 V/cm to achieve 50–75% cell death after IRE to leave room for more cell death after enhancement.

ii. Chemical Enhancements—Chemical enhancement was investigated by comparing the effects of IRE on AsPC-1 pancreatic cells suspended in different solutions and through glucose deprivation, as shown in Figure 1(d). AsPC-1 cells were cultured, grown and harvested as above and equally divided into different treatment groups. Cell suspensions were centrifuged and then resuspended in different media (original basal medium recipe without additives) to final cell density of 0.4×10^6 cells/ml. 99 IRE pulses were applied to different cells suspension with electric field of 750, 1000 and 1250 V/cm. Another chemical enhancement with different glucose concentration in the media was prepared with final glucose concentration ranging from 80 to 380 mg/dL. After harvest, the same batch of AsPC-1 cells were rinsed with phosphate-buffered saline (PBS) twice and re-suspended at the same density in a series of solutions. This set of solutions is made up of the original recipe of cell culture medium (described previously) and only differs in the concentration of glucose. All cell suspensions were exposed to the same IRE parameters (1750 V/cm, 50 μ s pulse duration, 1Hz and 51 pulses). 4 sets of experiments were conducted and results are reported as the average viability $\pm$ standard deviation.

iii. Adaptive Resistance—We designed a test for adaptive resistance post IRE as shown in Figure 1(e). Here AsPC-1 cells were cultured normally with the media changed the day before harvest. On the day of testing the cells were detached, rinsed and re-suspended in

PBS at a density of 1×10^6 cells/ml which was used for control and treatment groups. In the first control group, 1/5 of the prepared cell suspension was transferred to sterile electroporation cuvettes without applying an electric field. In the second treatment group, 4/5 of the cell suspension was transferred to cuvettes where they were exposed to IRE (1000 V/cm, 50 pulses, 50 μ s duration and 1 Hz). These parameters have shown roughly 25% survival previously. The cells from both groups were then transferred to new flasks filled with fresh cell culture medium. In both groups, culture media was further changed day 1 and 3 after the treatment, which also removed dead (unattached) cells from the flasks. At day 5, the cells from flasks of both control and treatment groups were harvested, suspended at a density of 0.4×10^6 cells /ml in PBS and tested for viability after IRE with the same initial parameters (1000 V/cm, 50 pulses, 50 μ s duration and 1 Hz). The simple composition of PBS eliminates interference of other organic and inorganic compounds during the electroporation process.

In Vivo Assessment and Enhancement

All animal usage and experimental procedure protocols were reviewed and approved by IACUC. Male nude mice (6–8 weeks, Athymic Nude-Foxn1nu, Harlan Laboratories) were injected with 1×10^6 AsPC-1 cells in 100 μ L suspension (50% Matrigel and 50% PBS) subcutaneously over the dorsal flanks near the hind limbs under a brief anesthesia by inhaling 5% Isoflurane.

An IRE probe was constructed and used *in vivo* based on a previous probe design [23]. The geometry of the probe is shown Figure 2(a). The IRE electric field distributions in the hindlimb tumor were modeled using COMSOL Multiphysics (Version 4.4). The modeling was done on a plane at the center of the tumor and perpendicular to the electrodes, using the actual geometry of a typical tumor and the IRE probe. Simulations were conducted with settings and parameters described in [23].

The probe was comprised of two needle-type electrodes, each with a conductive tip (i.e. exposed without insulation). The needle electrodes were made from 304 fully hardened stainless steel and the tips of the needle electrodes are made of 1 mm trocar ground down for easier penetration through the skin and into a tumor. The base was machined from white Acetal/Delrin with a 10–24 UNC pinch screw for precise needle fixation. 24 AWG stranded electrical wires were terminated to the proximal ends of the needle electrodes and to the banana connectors.

Experiments were performed 12 to 24 days after tumor seeding when spherically shaped tumors had reached total volume of roughly 100 mm³ (major axis 8–10 mm, minor axis 6–8 mm), as shown in Figure 2(a). Mice with irregular shaped tumors were not included in the experiments. The resulting 47 mice were randomized into the 4 groups for experiments. Animals were placed under general anesthesia (by Ketamine and Xylazine) before the procedure. Directly prior to each IRE probe insertion, the applicator was wiped with ethanol pads to avoid any infection. The needle electrodes were then inserted symmetrically to the center of the hindlimb tumor through the skin. Sham procedure was performed on 17 mice that received no other treatment than needle penetration (i.e. no IRE).

i. Physical Enhancement—Baseline parameters were selected to destroy part of the tumor (based on the electric field distribution simulation), to allow comparison to enhanced treatments in enhancement groups. Besides a group of sham procedures, all 3 treatment groups all receive 51 IRE pulses (600 V between 2 electrodes, 50 μ s pulse duration), except the pulses were arranged differently as shown in Figure 2(c). The overall treatment time was less than 2 minutes in all cases. After IRE treatment, the mice were returned to their cages, allowed to recover and monitored for 40 days after the IRE treatment. Tumor growth measurements were taken every 5 days using a caliper. The length of the major axis (L), minor axis (S), were measured three times and the averaged values were recorded and used to calculate tumor volumes (V) by an established modified ellipsoidal formula $V=0.5 \times L \times S^2$ [24, 25].

RESULTS

In Vitro Assessment and Enhancement

The responses of primary *KPC* (murine) and AsPC-1 (human) PDA cells to IRE are shown in Figure 3. Results show that both *KPC* and AsPC-1 can be destroyed by IRE and that increased pulse number and higher electric field led to lower viability after IRE. However, the *KPC* are more susceptible to IRE than AsPC-1 under the same conditions of testing. For example, when comparing 50 pulses at 750 V/cm, IRE resulted in 14.24% viability drop in *KPC* vs. 2.60% in AsPC-1 ($p < 0.05$). Further, at 1000 V/cm and *KPC* drop was 52.81% vs. 30.12% in AsPC-1 ($p < 0.05$). Finally, with 10 pulses, substantially less *KPC* cells survived compared to AsPC-1. For instance, 85.39% vs. 97.34% at 750 V/cm, and 64.05% vs. 90.39% at 1000 V/cm, both $p < 0.05$ for *KPC* vs. AsPC-1 respectively. In comparison, non-cancerous HDF cells can also be destroyed by IRE as shown in the appendix, where the HDF susceptibility lies between the two cancer cell lines tested.

i. Physical Enhancement—Physical enhancements of IRE on AsPC-1 were taken forward into physical and chemical enhancement studies. Here, physical enhancement was investigated by keeping the total energy delivered the same (51 pulses, 50 μ s per pulse) but using different pulse timing and frequency of pulsing at a given electric field. Specifically, pulse timing, defined as the total pulses spread out by a delay between trains, and decreased frequency from 10 Hz to 1 Hz were evaluated as potential enhancements at a given electric field over baseline parameters (51 pulses, 50 μ s per pulse, 10 Hz). Indeed, pulse timing and reduced frequency can enhance cell destruction by IRE at a given electric field (Figure 4a). Pulse timing led to a roughly 5 – 10 % drop in viability over baseline at each electric field tested. Moreover, the 1-Hz pulsing arrangement led to a 15 – 25% drop in all tested electric fields.

ii. Chemical Enhancements—Switching the media of cells in suspension prior to electroporation was shown to enhance IRE cell destruction (Figure 4b). At 750 V/cm, the cell viability in PBS, DMEM and RPMI medium were 50%, 74% and 97% respectively ($p < 0.01$). This trend continued at higher electric fields including 1000 V/cm (18%, 22% and 86%) and 1250 V/cm (10%, 10% and 74%) for PBS, DMEM and RPMI viability, respectively. At all three electric field levels (750, 1000 and 1250 V/cm), cancer cell

destruction in PBS was the most successful. Further, the gap between PBS and RPMI was much larger ($p < 0.01$ at 750, 1000 and 1250 V/cm) than that between DMEM and RPMI ($p > 0.05$ at 1000 and 1250 V/cm). However, even DMEM substantially decreased the post-IRE viability of AsPC-1 cells ($p < 0.01$) for 750, 1000 and 1250 V/cm over control. Thus, the suspending medium has a dramatic impact on the efficacy of IRE when all other physical conditions are held constant, suggesting that the local cell microenvironment could impact the efficacy of IRE.

As many patients with pancreatic cancer are, or can become, diabetic [26], a relationship between glucose concentration on IRE pancreatic cell destruction was also studied. Increasing glucose concentration from 80 to 380 mg/dL, led to a significant increase in the survival of AsPC-1 cells from 16.5% to 30.4% ($p < 0.05$) after treatment with same set of electrical parameters (Figure 4c), demonstrating that IRE is less effective in inducing cell death in high glucose conditions.

iii. Adaptive Resistance—To assess the ability of pancreatic cancer to adapt to IRE, the viability post IRE of naïve cells (untreated) was compared to that of cells that had already experienced IRE treatment 5 days before. Cells for all groups were derived from the same original flask, and were divided into different group receiving same culture condition and were tested viability after IRE. At all field strengths (1000, 1250 and 1500 V/cm) a statistically higher viability resulted in the previously treated cells (Figure 4d). For instance, at 1000 V/cm, the cellular viability is 8% higher in previously treated vs. untreated cells ($p < 0.01$). At higher electric field strengths (1250 and 1500 V/cm), the viabilities of previously treated cells were more than 2.8 times higher than their untreated naïve counterparts (8.7% vs. 25.3% and 5.7% vs. 16.3%, both $p < 0.01$). Thus, cell derived from PDA can acquire some resistance to repeated exposure to the same IRE treatment. Interestingly, we did not observe a significant difference in overall cell sizes before and 5 days after IRE treatment ($14.90 \pm 0.57 \mu\text{m}$ vs. $15.03 \pm 0.61 \mu\text{m}$, $p > 0.5$).

In Vivo Assessment and Physical Enhancement

Based on the simulation of electric field during IRE (Figure 5a), the electric field reaches more than 1250 V/cm within 2×4 mm area (circled by grey dashed line) at the center the tumor when 600 V is applied between the two electrodes, which made up about 1/4 of the tumor volume (circled region: $2 \times 4 \times 3 \text{ mm}^3$, tumor volume: 100 mm^3). To provide an initial treatment estimate we assume that cell injury will respond the same way *in vivo* as *in vitro*. Thus, knowing that the AsPC-1 viability is 41 % at 1250 V/cm we can predict that at least 60% of the cells are dead within the region circled by dashed line (1250 V/cm or 1/4 of tumor volume). Therefore, the simulation predicts that roughly 15% of the cells are dead given the baseline IRE parameters (51 pulses, 50 μs and 10 Hz). The large portion of “less-than-lethal” regions within the tumor will allow us to test for physical enhancements.

This modeling approach was complemented by experimental *in vivo* assessment of sham, baseline IRE (600 V between probes, 51 pulses, 50 μs and 10 Hz) and physical enhancements (pulse timing and low frequency IRE). The Sham group showed the most rapid tumor growth curve over 40 days (Figure 5b). In contrast, the average volume of

baseline IRE treated tumors was statistically consistently 8–25% below Sham throughout the tumor growth curve at day 5, 10 and 20 ($p < 0.05$). In addition to the baseline IRE treatment, two other treatment groups, namely “pulse-timing” and “low frequency” were assessed by comparing tumor volumes to control group (sham) at same days after the treatment, as shown in Figure 5b. Compared to sham, pulse-timing led to tumor volumes 32–45% less than that observed in the sham group between day 10 and the end of monitoring of day 40 ($p < 0.01$). The overall reduction in tumor growth by pulse timing is substantially better than the baseline IRE treatment. Additionally, the low-frequency treatment outperformed both the baseline and pulse-timing groups by a large margin: tumor volumes were 52–64% smaller than those in the sham group during the first 40 days of IRE treatment ($p < 0.01$).

DISCUSSION

In this study, we have demonstrated baseline and enhanced protocols for IRE destruction of pancreatic carcinoma cells both *in vitro* and *in vivo*. As demonstrated in Figure 3, IRE parameters (electric field, number of pulses, pulse duration and pulse interval) can be selected to achieve pancreatic cancer cell destruction consistent with other cancer models [27]. In general, higher electric field and pulse number for a given set of electrical parameters in IRE contribute to lower cell viability. Nevertheless, the response of cancer cells to electric pulse *in vitro* (e.g. in cell suspension) and *in vivo* can be different and further studies under more physiologically relevant experimental conditions are warranted. Furthermore, cell properties (size and shape) have a significant effect on the electroporation outcome [28]. Yet, the *in vitro* tests we present provide a simple and high-throughput platform for testing response of cancer cells to IRE under a variety of conditions.

A wide range of cells have been tested for viability after IRE both *in vitro* and *in vivo*, including normal cells and cancerous cells [27, 29]. In our *in vitro* studies, both pancreatic cancer cells (AsPC-1 and KPC) and normal cells (HDF) were shown to be susceptible to IRE destruction. The KPC cells were found to be somewhat more susceptible than the AsPC-1 cells to IRE with HDFs susceptibility lying between the two as shown in Figure 3 and Appendix. This can also be appreciated more directly through a comparison of an LE50, or median electric field needed to achieve 50% cell viability (medium lethal dose) given at 50 pulses with 50 μ s pulse duration and 10 Hz pulsing frequency for cells in their native cell culture medium as shown in Table 1.

Table 1 demonstrates that different cells have different susceptibility to IRE, with LE50 ranging from 400 to 1300 V/cm. We also see that human cancer cell lines generally have higher LE50 than those from mouse, which suggests that a higher electric field is required for translating IRE treatment from mouse cancer models to a human cancer (e.g. prostate cancer TRAMP-C2 vs. LNCaP and pancreatic cancer KPC vs. AsPC-1). For comparison, even from the same species (e.g. human), cells of different cancer have different response to IRE, which suggests the need for careful characterization of IRE response in the given cancer or tissue of interest. There is no clear correlation between IRE susceptibility and size of cells, by comparing different cell lines listed in table 1.

Physical Enhancements

Physical enhancements by arrangement of the electrical pulses in Figure 2(c) is an attractive and readily translatable approach to improve IRE outcomes. We have shown that low frequency (1 Hz) IRE pulsing is advantageous over conventional 10-Hz pulsing and that pulse-timing (i.e. adding a 30 s delay between pulse trains) can also enhance outcomes as recently shown for prostate cancer [23]. Compared to IRE treatment with higher pulsing rate, pulsing at the rate of the heart (roughly 1 Hz for human) is desirable as high voltage can induce cardiac arrhythmias and defibrillation, which pose a significant safety concern over application of IRE, especially when applied close to heart [30]. However, the risk can be mitigated by synchronization of IRE pulse delivery with the cardiac cycle [30–32]. In addition to the physiological reasoning for using lower frequency, our data now also shows that IRE around 1 Hz can be more efficient in destroying cancerous cells although the precise mechanism is not completely understood. We were able to repeat these studies both *in vitro* and *in vivo* (Figure 4a and 5) and demonstrate that the lower frequency pulsing is the most important effective, although pulse timing is also helpful.

Importantly, the concept that physical “pulsing” parameters can be optimized for achieving improved IRE treatment efficacy has been proposed previously [27]. Briefly, membrane defects typically last longer (up to seconds) than the exertion of the electric field (less than 1 ms). Therefore, it is hypothesized that temporally distributed pulses extend the effort of recovery, thus causing more irreversible damage than reversible pulses on the membrane. Note that the IRE parameters including waveform, amplitude and repetition rate will be ultimately constrained by electrical hardware and clinical concerns such as treatment time, muscle contraction and localized heating. Further work in this area would greatly benefit from a model of the cell or tissue that could predict IRE treatment outcomes (IRE dose) at any given electrical parameters would be very beneficial.

Chemical Enhancements

The chemical microenvironment in IRE treatment volume can enhance destruction as shown in low glucose and PBS media *in vitro* (Figure 4). It has been shown that a variety of chemical substances are able to enhance IRE treatment, including chemotherapeutic drugs or cytotoxic agents for electrochemotherapy [33, 34] and calcium [35]. Other studies have shown that modifying membrane properties by surfactants, impeding the resealing process are effective in enhancing the IRE destructive potential. Such chemicals include DMSO [22], C12E8 [36, 37] and SDS [38, 39]. Here, by comparing the IRE effect on cells suspending in PBS, DMEM and RPMI, we have shown that inorganic compounds and common essential molecules in media can also play a crucial role determining the treatment outcome. For instance, the absence of amino acids, vitamins and glucose in PBS compared to cell culture media (DMEM and RPMI) may ultimately contribute the dramatic enhancement of IRE. The addition of a few vitamins (e.g. Biotin, Para-Aminobenzoic Acid and Vitamin B12) in RPMI medium compared to DMEM may also contribute to cell resistance to IRE. Although the exact underlying mechanism is not yet understood, medium conductivity [40] or electroporation-induced cell sensitization [41] have been proposed. It has been shown that patients with diabetes are more likely to have a poor pancreatic cancer prognosis [42]. Additionally, a lower glucose level is likely to improve pancreatic cancer cell

destruction by IRE treatment. Thus, we believe a reduction in blood glucose levels in pancreatic cancer patients during IRE treatment may improve pancreatic cancer outcomes, given the facts that patients are 5 times more likely to have impaired fasting glucose and 26 times more likely to be diabetic compared to general population [26]. Although the mechanism behind glucose deprivation and enhanced IRE treatment outcome is not fully understood, treatment strategies that target metabolic pathways during hyperthermia in cancer are well known [43]. For instance, most cancer cells depend predominantly on glycolysis for energy production [44], therefore cellular stress due to IRE or heat is likely to be augmented at low glucose concentrations whereas the metabolic function of the cell is compromised. In the case of IRE, this stress is perhaps exacerbated by loss of intracellular enzymes needed for glycolysis [45]. A more rigorous study on the role of individual components in their enhancement or impediment of IRE treatment is needed. The method this study presented provides a platform to quickly identify molecules affecting IRE, which is in huge need for further investigation. The understanding of *in vitro* chemical enhancements and its underlying mechanism can be helpful in the development of *in vivo* adjuvant chemical approaches for enhancing IRE.

Adaptive Resistance

Our work highlights what appears to be a new phenomenon, that multiple treatments of the same cancer with IRE may lead to adaptive resistance and reduced efficacy of the IRE treatment. Specifically, surviving cells after initial IRE were shown to be substantially less susceptible to IRE destruction (Figure 4(d)). This seems similar to adaptive resistance observed in chemotherapy [46], radiotherapy [47] and thermal therapy [48, 49]. The underlying mechanism may be related to physical changes to the membrane as the cells went through about 2 divisions between 2 treatments over 5 days. While this work is very preliminary, it does suggest that adaptive resistance of pancreatic cancer over IRE should be studied if repeated IRE treatments are likely, as secondary treatment may be less effective based on our results.

In Vivo Translation

While both chemical and physical methods of enhancement are likely to benefit IRE treatments in the future, we chose to initially demonstrate physical enhancement for *in vivo* translation. Importantly, adjuvant chemical approaches for enhancing IRE have already demonstrated great promise, as reviewed in [27]. However, controlled delivery or manipulation of drugs or chemicals *in vivo* to achieve the same conditions *in vitro* is challenging largely due to the physical barrier posed by stroma of pancreatic cancer [16]. Fortunately, physical enhancements based on pulse design can be directly implemented *in vivo* with just a few caveats. For instance, in the cuvette the electrical field strength is mostly uniform, whereas in the *in vivo* setting the field will vary spatially. However, the overall response from tumor growth delay can be used as a general metric of the impact of pulse design (i.e. physical enhancement) on treatment outcome, if everything else in model and protocol remain unchanged. Further, we can argue that IRE physical enhancement is unlikely to be interfered by resistive mechanism of cancer cells received prior biochemical treatment, and that the distribution of electric field within the tumor should remain relatively constant. Thus, physical enhancement by pulse timing, and low frequency pulsing were

shown to yield statistically significant increases in pancreatic cancer destruction both *in vitro* and *in vivo* and suggest a possible path forward for clinical translation.

Acknowledgments

This study was supported by Boston Scientific and the University of Minnesota Institute for Engineering in Medicine (IEM) Cancer Animal Core. QS was partially supported by Doctoral Dissertation Fellowship, University of Minnesota. JB was supported by the Carl and Janet Kuhrmeyer Chair in the Department of Mechanical Engineering. PPP was supported by the NIH (R01CA181385 and U54CA210190) and the Randy Shaver Research and Community Fund. KEG was supported by the CSE fellowship and the ARCS foundation. The content of this work is solely the responsibility of the authors and does not necessarily represent the official views of the NIH or other funding agencies. We thank Marjorie Carlson for assistance with the *KPC* mouse models. We also thank Navid Manuchehrabadi, Hong Cao, Kanav Khosla and Tim Ostroot for helpful discussions and technical support.

Appendix

Non-cancerous cells such as human dermal fibroblasts (HDFs), can also be destroyed by IRE in a similar manner to cancer cells. Lower electric field (i.e. 500 V/cm) appear to do no damage to the cells with nearly 100% viability after IRE. The viabilities drop with both increased electric field (500 to 1250 V/cm) and number of pulses (10 to 99 pulses). Compared to pancreatic cancer cells (*KPC* and *AsPC-1*) studied, HDF appears to be slightly more resistant to IRE than *KPC*, with difference less 20% than on all conditions tested. However, HDF is more susceptible than *AsPC-1*, showing more than 20% decrease in viability after IRE with 1000–1250 V/cm and 50 pulses.

Figure 1.

The *in vitro* assessment IRE outcome on a non-cancerous cancer cell line (human dermal fibroblasts, HDFs) to irreversible electroporation. Viabilities were assessed at a range of electric field (500 to 1250 V/cm) and different number of pulses (10, 50 and 99). All data points are the result of $n = 4$ trials and are reported as the average $\pm$ standard deviation.

References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global Cancer Statistics. *Ca-a Cancer Journal for Clinicians*. 2011; 61(2):69–90. [PubMed: 21296855]
2. Siegel RL, Miller KD, Jemal A. Cancer Statistics, 2016. *Ca-a Cancer Journal for Clinicians*. 2016; 66(1):7–30. [PubMed: 26742998]
3. Li DH, Xie KP, Wolff R, Abbruzzese JL. Pancreatic cancer. *Lancet*. 2004; 363(9414):1049–1057. [PubMed: 15051286]

4. Vincent A, Herman J, Schulick R, Hruban RH, Goggins M. Pancreatic cancer. *Lancet*. 2011; 378(9791):607–620. [PubMed: 21620466]
5. Varshney S, Sewkani A, Sharma S, Kapoor S, Naik S, Sharma A, Patel K. Radiofrequency ablation of unresectable pancreatic carcinoma: feasibility, efficacy and safety. *Jop*. 2006; 7(1):74–78. [PubMed: 16407624]
6. Moir J, White SA, French JJ, Littler P, Manas DM. Systematic review of irreversible electroporation in the treatment of advanced pancreatic cancer. *Ejso*. 2014; 40(12):1598–1604. [PubMed: 25307210]
7. Chu KF, Dupuy DE. Thermal ablation of tumours: biological mechanisms and advances in therapy. *Nature Reviews Cancer*. 2014; 14(3):199–208. [PubMed: 24561446]
8. Patterson EJ, Scudamore CH, Nagy AG, Buczkowski AK. Radiofrequency ablation of porcine liver in vivo - Effects of blood flow and treatment time on lesion size. *Annals of Surgery*. 1998; 227(4): 559–565. [PubMed: 9563546]
9. Date RS, McMahon RF, Siriwardena AK. Radiofrequency ablation of the pancreas. I: Definition of optimal thermal kinetic parameters and the effect of simulated portal venous circulation in an ex-vivo porcine model. *JOP*. 2005; 6(6):581–587. [PubMed: 16286709]
10. Davalos RV, Mir LM, Rubinsky B. Tissue ablation with irreversible electroporation. *Annals of Biomedical Engineering*. 2005; 33(2):223–231. [PubMed: 15771276]
11. Davalos RV, Rubinsky B, Mir LM. Theoretical analysis of the thermal effects during in vivo tissue electroporation. *Bioelectrochemistry*. 2003; 61(1):99–107. [PubMed: 14642915]
12. Maor E, Ivorra A, Leor J, Rubinsky B. The effect of irreversible electroporation on blood vessels. *Technology in Cancer Research & Treatment*. 2007; 6(4):307–312. [PubMed: 17668938]
13. Latouche EL, Sano MB, Lorenzo MF, Davalos RV, Martin RCG. Irreversible electroporation for the ablation of pancreatic malignancies: A patient-specific methodology. *Journal of Surgical Oncology*. 2017; 115(6):711–717. [PubMed: 28185295]
14. Bower M, Sherwood L, Li Y, Martin R. Irreversible Electroporation of the Pancreas: Definitive Local Therapy Without Systemic Effects. *Journal of Surgical Oncology*. 2011; 104(1):22–28. [PubMed: 21360714]
15. Martin RCG. Use of irreversible electroporation in unresectable pancreatic cancer. *Hepatobiliary Surgery and Nutrition*. 2015; 4(3):211–215. [PubMed: 26151062]
16. Provenzano PP, Cuevas C, Chang AE, Goel VK, Von Hoff DD, Hingorani SR. Enzymatic Targeting of the Stroma Ablates Physical Barriers to Treatment of Pancreatic Ductal Adenocarcinoma. *Cancer Cell*. 2012; 21(3):418–429. [PubMed: 22439937]
17. Hingorani SR, Wang LF, Multani AS, Combs C, Deramaudt TB, Hruban RH, Rustgi AK, Chang S, Tuveson DA. Trp53(R172H) and Kras(G12D) cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice. *Cancer Cell*. 2005; 7(5):469–483. [PubMed: 15894267]
18. Manuchehrabadi N, Gao Z, Zhang JJ, Ring HL, Shao Q, Liu F, McDermott M, Fok A, Rabin Y, Brockbank KGM, Garwood M, Haynes CL, Bischof JC. Improved tissue cryopreservation using inductive heating of magnetic nanoparticles. *Science Translational Medicine*. 2017; 9(379)
19. Awale S, Lu J, Kalauni SK, Kurashima Y, Tezuka Y, Kadota S, Esumi H. Identification of arctigenin as an antitumor agent having the ability to eliminate the tolerance of cancer cells to nutrient starvation. *Cancer Research*. 2006; 66(3):1751–1757. [PubMed: 16452235]
20. Phillips PA, Dudeja V, McCarroll JA, Borja-Cacho D, Dawra RK, Grizzle WE, Vickers SM, Saluja AK. Triptolide induces pancreatic cancer cell death via inhibition of heat shock protein 70. *Cancer Research*. 2007; 67(19):9407–9416. [PubMed: 17909050]
21. Pegg DE. VIABILITY ASSAYS FOR PRESERVED CELLS, TISSUES, AND ORGANS. *Cryobiology*. 1989; 26(3):212–231. [PubMed: 2743785]
22. Jiang CL, Qin ZP, Bischof J. Membrane-Targeting Approaches for Enhanced Cancer Cell Destruction with Irreversible Electroporation. *Annals of Biomedical Engineering*. 2014; 42(1): 193–204. [PubMed: 23949655]
23. Jiang CL, Shao Q, Bischof J. Pulse Timing During Irreversible Electroporation Achieves Enhanced Destruction in a Hindlimb Model of Cancer. *Annals of Biomedical Engineering*. 2015; 43(4):887–895. [PubMed: 25269611]

24. Tomayko MM, Reynolds CP. DETERMINATION OF SUBCUTANEOUS TUMOR SIZE IN ATHYMIC (NUDE) MICE. *Cancer Chemotherapy and Pharmacology*. 1989; 24(3):148–154. [PubMed: 2544306]
25. Euhus DM, Hudd C, Laregina M, Johnson FE. TUMOR MEASUREMENT IN THE NUDE-MOUSE. *Journal of Surgical Oncology*. 1986; 31(4):229–234. [PubMed: 3724177]
26. Pannala R, Leirness JB, Bamlet WR, Easu A, Petersen GM, Chari ST. Prevalence and clinical profile of pancreatic cancer-associated diabetes mellitus. *Gastroenterology*. 2008; 134(4):981–987. [PubMed: 18395079]
27. Jiang CL, Davalos RV, Bischof JC. A Review of Basic to Clinical Studies of Irreversible Electroporation Therapy. *Ieee Transactions on Biomedical Engineering*. 2015; 62(1):4–20. [PubMed: 25389236]
28. Agarwal A, Zudans I, Weber EA, Olofsson J, Orwar O, Weber SG. Effect of cell size and shape on single-cell electroporation. *Analytical Chemistry*. 2007; 79(10):3589–3596. [PubMed: 17444611]
29. Vogel JA, van Veldhuisen E, Agnass P, Crezee J, Dijk F, Verheij J, van Gulik TM, Meijerink MR, Vroomen LG, van Lienden KP, Besselink MG. Time-Dependent Impact of Irreversible Electroporation on Pancreas, Liver, Blood Vessels and Nerves: A Systematic Review of Experimental Studies (vol 11, e0166987, 2016). *Plos One*. 2017; 12(3)
30. Mali B, Jarm T, Corovic S, Paulin-Kosir MS, Cemazar M, Sersa G, Miklavcic D. The effect of electroporation pulses on functioning of the heart. *Medical & Biological Engineering & Computing*. 2008; 46(8):745–757. [PubMed: 18415132]
31. Deodhar A, Dickfeld T, Single GW, Hamilton WC, Thornton RH, Sofocleous CT, Maybody M, Gonen M, Rubinsky B, Solomon SB. Irreversible Electroporation Near the Heart: Ventricular Arrhythmias Can Be Prevented With ECG Synchronization. *American Journal of Roentgenology*. 2011; 196(3):W330–W335. [PubMed: 21343484]
32. Mali B, Jarm T, Jager F, Miklavcic D. An algorithm for synchronization of in vivo electroporation with ECG. *J Med Eng Technol*. 2005; 29(6):288–296. [PubMed: 16287678]
33. Gothelf A, Mir LM, Gehl J. Electrochemotherapy: results of cancer treatment using enhanced delivery of bleomycin by electroporation. *Cancer Treatment Reviews*. 2003; 29(5):371–387. [PubMed: 12972356]
34. Sersa G, Jarm T, Kotnik T, Coer A, Podkrajsek M, Sentjunc M, Miklavcic D, Kadivec M, Kranjc S, Secerov A, Cemazar M. Vascular disrupting action of electroporation and electrochemotherapy with bleomycin in murine sarcoma. *British Journal of Cancer*. 2008; 98(2):388–398. [PubMed: 18182988]
35. Frandsen SK, Gissel H, Hojman P, Tramm T, Eriksen J, Gehl J. Direct Therapeutic Applications of Calcium Electroporation to Effectively Induce Tumor Necrosis. *Cancer Research*. 2012; 72(6):1336–1341. [PubMed: 22282658]
36. Kanduser M, Fosnaric M, Sentjunc M, Kralj-Iglic V, Hagerstrand H, Iglic A, Miklavcic D. Effect of surfactant polyoxyethylene glycol (C12E8) on electroporation of cell line DC3F. *Colloids and Surfaces a-Physicochemical and Engineering Aspects*. 2003; 214(1–3):205–217.
37. Troiano GC, Tung L, Sharma V, Stebe KJ. The reduction in electroporation voltages by the addition of a surfactant to planar lipid bilayers. *Biophysical Journal*. 1998; 75(2):880–888. [PubMed: 9675188]
38. Murthy SN, Zhao YL, Sen A, Hui SW. Cyclodextrin enhanced transdermal delivery of piroxicam and carboxyfluorescein by electroporation. *Journal of Controlled Release*. 2004; 99(3):393–402. [PubMed: 15451597]
39. Murthy SN, Sen A, Hui SW. Surfactant-enhanced transdermal delivery by electroporation. *Journal of Controlled Release*. 2004; 98(2):307–315. [PubMed: 15262421]
40. Silve A, Leray I, Pognard C, Mir LM. Impact of external medium conductivity on cell membrane electroporabilization by microsecond and nanosecond electric pulses. *Scientific Reports*. 2016; 6
41. Dermol J, Pakhomova ON, Pakhomov AG, Miklavcic D. Cell Electrosensitization Exists Only in Certain Electroporation Buffers. *Plos One*. 2016; 11(7)

42. Calle EE, Murphy TK, Rodriguez C, Thun MJ, Heath CW. Diabetes mellitus and pancreatic cancer mortality in a prospective cohort of United States adults. *Cancer Causes & Control*. 1998; 9(4): 403–410. [PubMed: 9794172]
43. Tennant DA, Duran RV, Gottlieb E. Targeting metabolic transformation for cancer therapy. *Nature Reviews Cancer*. 2010; 10(4):267–277. [PubMed: 20300106]
44. Gatenby RA, Gillies RJ. Why do cancers have high aerobic glycolysis? *Nature Reviews Cancer*. 2004; 4(11):891–899. [PubMed: 15516961]
45. Liu F, Roy P, Shao Q, Jiang C, Choi J, Chung C, Mehra D, Bischof J. The Role of Protein Loss and Denaturation in Determining Outcomes of Heat, Cryotherapy and Irreversible Electroporation on Cardiomyocytes. *Journal of Biomechanical Engineering*. 2017
46. Holohan C, Van Schaeybroeck S, Longley DB, Johnston PG. Cancer drug resistance: an evolving paradigm. *Nature Reviews Cancer*. 2013; 13(10):714–726. [PubMed: 24060863]
47. Cao N, Li SY, Wang ZQ, Ahmed KM, Degnan ME, Fan M, Dynlacht JR, Li JJ. NF-kappa B-Mediated HER2 Overexpression in Radiation-Adaptive Resistance. *Radiation Research*. 2009; 171(1):9–21. [PubMed: 19138055]
48. Li GC, Werb Z. CORRELATION BETWEEN SYNTHESIS OF HEAT-SHOCK PROTEINS AND DEVELOPMENT OF THERMOTOLERANCE IN CHINESE-HAMSTER FIBROBLASTS. *Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences*. 1982; 79(10):3218–3222.
49. Dewhirst MW, Viglianti BL, Lora-Michiels M, Hanson M, Hoopes PJ. Basic principles of thermal dosimetry and thermal thresholds for tissue damage from hyperthermia. *International Journal of Hyperthermia*. 2003; 19(3):267–294. [PubMed: 12745972]

Figure 1.

Schematic of *in vitro* IRE experiments. (a) Cells in suspensions are loaded into the cuvette between two electrodes. The electrodes are connected to a pulse power supply where the electrical parameters are set. Voltage is applied between two electrodes to generate an evenly distributed electric field through the cell suspension. (b) The electrical parameters of IRE treatments. (c) IRE enhancement by arranging the pulses while keeping the waveform of each pulses (50 μ s square wave) and the number of pulses (51) the same. The parameters are (I) "Baseline", all pulses were continuously at 10 Hz pulsing frequency; (II) "Pulse timing", all pulses were delivered in three trains of 17 pulses at 10 Hz but with delays of 30 s in between trains and (III) "Low frequency", all pulses are given at a frequency of 1 Hz. (d) Experimental design for chemical enhancement by changing the cell suspension medium. For comparing medium effect, cells were suspended in phosphate buffered saline (PBS), DMEM or RPMI to the same density. For comparison, the effect of glucose level, cells were suspended to the same medium except for different glucose concentration. IRE treatments

were performed immediately after the cells were exposed to new medium. (e) Experimental design for adaptive resistance. Cells were derived from the same flask. Following resuspension and distribution into cuvettes, cells in both groups (control vs. IRE treatment) were replated and cultured for 5 days under the same conditions. Lastly, the cells in each group were tested for viability after IRE under a same set of parameters.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 2. Schematic of *in vivo* IRE treatments. (a) Top and side view of the IRE applicator when inserted into a selected tumor. (b) A photo of the *in vivo* IRE applicator. (c) Illustration of IRE enhancement by pulse sequencing as (I) Baseline, (II) Pulse timing and (III) Low frequency. (d) *In vivo* physical enhancement study by tumor growth delay.

Figure 3.

The *in vitro* assessment IRE outcome on two types of pancreatic cancer cell lines, *KPC* and AsPC-1. IRE causes more cell death (lower viability) by increased electric field and the number of pulses in both cell lines. *KPC* (left) is more responsive than AsPC-1 (right) to IRE treatments. Both cell types were tested at 750 V/cm and 1000 V/cm at 10, 50 and 99 pulses. Due to the differential susceptibility of these cells to IRE, the *KPC* were tested as low as 500 V/cm and AsPC-1 as high as 1250 V/cm. All data points are the result of $n = 6$ trials and are reported as the average $\pm$ standard deviation.

Figure 4.

In vitro assessment of (a) physical, (b and c) chemical enhancement and (d) adaptive resistance to irreversible electroporation of pancreatic cancer cells (AsPC-1). (a) Comparison of IRE treatments of AsPC-1 cells in suspension after pulse sequencing (baseline, pulse timing, or low frequency). (b) Comparison of IRE outcome of AsPC-1 cells in PBS, DMEM and RPMI 1640. (c) Glucose concentration is positively correlated with post-IRE viability. (d) Adaptive resistance of cells shown by comparison of post-IRE cellular viability between originally untreated and IRE-treated cells. All data points are the result reported as the average $\pm$ standard deviation. Statistical significance is indicated with asterisks: * $P < 0.01$.

Figure 5.

Hindlimb pancreatic tumor response after IRE treatments. (a) Modeling of electric field distribution during the application of baseline IRE treatment. (b) Tumor growth delay after baseline and pulse modifications (pulse timing and low frequency). Compared to control (sham), Low-frequency IRE achieved the largest tumor volume reduction when compared at the same day after treatment, followed by pulse timing and baseline IRE. All tumor growth delay data points results are the result of $n = 9$ mice (see legend) and presented as average $\pm$ standard error as a quantitative comparison between treatment outcomes.

Comparison of median lethal electric field (LE50) necessary to destroy 50% of a cancer cell population with 50 pulses, 50 μ s pulse duration and 10 Hz pulsing frequency. Linear interpolation was used to derived LE50 from data source. The cell size is shown as average $\pm$ standard deviation from 4 replicates. No clear correlation between cell size and IRE susceptibility is observed.

Table 1

Cell line	Organism	Type	LE50 (V/cm)	Cell size (μ m)	Reference
MDA-MB-231	Human	Breast cancer	1200–1300	17.30 $\pm$ 0.37	
AsPC-1	Human	Pancreatic cancer	1100–1200	14.90 $\pm$ 0.57	
LNCaP	Human	Prostate cancer	1050–1150	19.05 $\pm$ 0.48	[27]
HL-1	Mouse	Cardiomyocyte	1000–1100	15.03 $\pm$ 0.31	[27]
HDF	Human	Dermal fibroblasts	950–1050	19.90 $\pm$ 1.08	
KPC	Mouse	Pancreatic cancer	900–1000	12.80 $\pm$ 0.25	
CLU-172	Mouse	Hypothalamus	700–800	19.17 $\pm$ 1.03	[27]
TRAMP-C2	Mouse	Prostate cancer	600–700	16.92 $\pm$ 0.40	
B16	Mouse	Melanoma	400–500	18.17 $\pm$ 1.25	