Preconditioning with Near-Infrared Irradiation to Enhance the Irreversible Electroporation Efficiency in HeLa Cells

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Abstract: Irreversible electroporation (IRE) has gained attention for ablation owing to fewer side effects and fast recovery. However, a high current from the applied high voltage can cause muscle contraction. Adding cationic molecules has been introduced to lower electric field strengths and enhance IRE outcomes by inducing hyperpolarization across the cell plasma membrane. Near-infrared light (NIR) has recently been reported to induce hyperpolarization across membranes in a mode-dependent manner. In this study, we performed IRE in HeLa cells after exposure to 810 nm NIR irradiation. Preconditioning with NIR of 3 J/cm² induced changes in membrane potential, resulting in approximately two times enhancement of apoptosis by IRE. The apoptotic signals were governed by the presence of BAX and p53 and were not related to excess oxidative stress. NIR has better spatial and temporal distribution control than chemicals and, therefore, can enhance the spatial selectivity and reduce the side effects of IRE treatment. These results can be used to enhance the clinical outcomes of IRE.

Keywords: near infrared; irreversible electroporation; hyperpolarization; apoptosis

1. Introduction

Electroporation is a method in which short, direct high current square wave pulses induce transient permeation of cell membranes. Permeability is induced when the applied electric field leads to an increase in the transmembrane potential above the permeability threshold, leading to the formation of aqueous pores. With a high electric energy, the pores cannot be sealed, and cells undergo apoptosis, which is called irreversible electroporation (IRE). In addition, pores enable the delivery of foreign substances into cells [1–3]. Anticancer drugs and calcium have recently been used to increase the selectivity and efficiency of irreversible electroporation in tumor tissues [4–6]. These attempts have attracted attention due to fewer side effects and low cost compared to those of radiative cancer therapies.

However, as IRE uses a high voltage in tissues, it has limitations regarding its clinical applications. Ablation of the IRE often has three regions due to the electric field distribution: the irreversible electroporation region, the reversible electroporation region, and the intact region. This results in incomplete resection of large tumors that are over 3 cm in diameter [7]. To overcome these disadvantages, a quite high voltage should be introduced, which cannot avoid electrolysis at the electrodes and sometimes causes muscle contraction [8,9]. To address such limitations, some studies have explored more efficient ways to lower the required electric field strength. Dispersion of the ionic material around the target enhances electroporation. Kennedy et al. showed that cationic peptides surrounding electroporated tissue can reduce the required applied electric field strength [10]. Aiken et al. showed that adding ionomycin to cells yields a higher probability of electroporation, as ionomycin induces hyperpolarization of the cell before electroporation [10,11]. These chemicals used...
in electroporation are difficult to diffuse widely due to collagen fibers, and they can cause unexpected side effects.

In contrast, unlike chemicals, light as an electromagnetic wave propagates relatively uniformly toward deep tissues. Light-tissue interactions include reflection and refraction, absorption of photon energy, and multiple scattering of photons when light encounters different tissue types. Absorption and multiple scattering of photons cause the light beam to broaden and attenuate as it passes through the tissue. In particular, near infrared (NIR) propagates into deeper tissues because it is less scattered and less absorbed by water molecules. The NIR-tissue interaction induces a transient temperature increase and triggers the activation of a transmembrane ion channel, which could initiate the generation of an action potential [12,13]. The ability to change the cellular electrical properties suggests that NIR may affect cellular responses to IRE.

In this study, we aimed to enhance the efficiency of IRE by using NIR irradiation. As we observed light-induced membrane potential changes from 810 nm NIR in previous studies, we utilized 810 nm NIR light to the human cervical cancer cell line, HeLa [14,15]. HeLa cells are one of the most widely used cells for cancer research. Given that NIR effects have been reported to be dose dependent, we examined the cellular transmembrane potential and cellular reactive oxygen species (ROS) levels under different irradiation conditions [14]. We chose a NIR irradiation condition that induced hyperpolarization in the trans-membrane potential but reduced cytoplasmic ROS levels. Cellular viability was not affected by these irradiation conditions. Immediately after NIR irradiation, we applied high electric field pulses and analyzed membrane integrity and cell viability. We confirmed that pre-conditioning with 810 nm NIR irradiation of 3 J/cm² significantly enhanced IRE-induced apoptosis in HeLa cells. This result suggests the possibility of electrical modulation of cells by NIR and contributes to the clinical use of IRE at lower voltages.

2. Materials and Methods

2.1. Cell Culture

The human cervical cancer cell line HeLa was purchased from the Korean Cell Line Bank. The cells were maintained in the RPMI 1640 medium (Welgene, Gyeongsan, Korea) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin (100 U/mL) and streptomycin (100 µg/mL); purchased from Gibco, Life Technologies) and incubated at 37 °C in a humidified environment with 5% CO₂. Then, 3 × 10⁵ cells were seeded in 35-mm-diameter culture dishes 24 h prior to the experiment.

2.2. NIR Irradiation

For NIR irradiation of cultured cells, a device composed of an array of light-emitting diodes (LEDs) and an 8-bit-microprocessor-based controller (UM_MC95FG308_V3.20_EN, Seoul, Korea) was fabricated. The LEDs, whose wavelength was at 810 nm (PV810-3C6W-EDISAA, KAOS, Suwon, Korea), were positioned upright at 5 mm intervals for a total of 16 units (Figure 1a). For uniform light transfer through the dish, cell culture plates were positioned 19.3 mm from the end of the LED array (Figure 1a). A power meter (PM-USB-100, Thorlabs, New Jersey, USA) was used to measure the light power density at the five positions to evaluate the uniformity of light. The power density was 3627 ± 2.67 µW/cm², which was generally uniform over the entire irradiated area. The device was designed such that the light propagated through the bottom of the dish and to the cells without absorption loss from the media. Cells were irradiated at 1–5 J/cm² in the continuous wave mode. The light power density was assessed before each experiment.
Figure 1. NIR-modulated plasma membrane potential and intracellular ROS of HeLa cells in a dose-dependent manner. (a) Schematics of the NIR irradiation device. (b) Relative viability (WST-1 assay) according to NIR irradiation dose (time) after 6 h from irradiation. (c) Relative MFI of FLIPR dye right after irradiation. (d) Relative MFI of JC-1 dye (red/green) right after irradiation. (e) Relative MFI of H2DCFDA dye right after irradiation (*p < 0.05, **p < 0.01, ***p < 0.001 with students’ t-test (n = 3)).

2.3. Cell Viability

Cell viability was investigated using the water-soluble tetrazolium salt-1 (WST-1) assay (EZ-Cytox, Dogenbio, Seoul, Korea), and 1.0 × 10⁴ cells were seeded in a 96-well plate 24 h before the experiment. After 6 h from of the NIR treatment, the cell viability was assessed as previously described [14]. Absorption at 450 nm was measured using a plate reader (Tecan, USA). For the evaluation after IRE, the IRE-treated cells in cuvette were seeded in a 96-well plate as described previously, and the viability assay was performed after 6 h. To test the role of the reactive oxygen species (ROS), treatment with the ROS scavenger N-acetyl-l-cysteine (NAC: 1 mM, Sigma A7250, Sigma-Aldrich, Massachusetts, USA) was applied to cells in a culture dish for 30 min before the experiment [14,15].

2.4. Measurement of Transmembrane Potential Changes

The cytoplasmic transmembrane potential was measured using a fluorescence imaging plate reader (FLIPR; Molecular Devices, California, USA) according to the manufacturer’s protocol. A working solution was prepared by mixing the FLIPR solution with the culture medium in equal parts. After NIR irradiation at 1–5 J/cm², the cells were washed once with Dulbecco’s phosphate-buffered saline (DPBS), and the FLIPR working solution was added. The cells were incubated for 30 min at 37 °C and then washed with DPBS. Fluorescence measurements of the harvested cells were performed by flow cytometry (BD FACSVerse™; Becton, Dickinson and Company, New York, USA), and the median value of each experimental condition was analyzed using BD FACSuiteTM software. The relative intensity of each condition compared to that of the control was expressed as a fold value.

2.5. Measurement of Mitochondrial Membrane Potential

Mitochondrial membrane potential (MMP) was assessed immediately after NIR treatment using a JC-1 mitochondrial membrane potential assay kit (ab113850, Abcam, Cambridge, UK). The harvested cells were washed with DPBS, incubated in 10 μM JC-1 in 1× dilution buffer for 30 min, and washed twice in 1× dilution buffer. Further, 2.0 × 10⁵ cells were transferred to 50 μL of each well within a 96-well black polystyrene
plate (CLS3603, Corning, Arizona, USA), with 50 µL of buffer. Fluorescence was assessed using a multimode microplate reader (SPARK 10M, Tecan, Grodig, Austria). MMP was calculated as the ratio of JC-1 in the RFU (red/green) [16].

2.6. Measurement of Intracellular Reactive Oxygen Species

2′,7′-Dichlorodihydrofluorescein diacetate (H2DCFDA; D399, Boston, ThermoFisher Scientific) was used to analyze the intracellular ROS levels. According to the pre-set conditions, the cells were irradiated with NIR and incubated for 5 min to prevent the probe from acting as a photosensitizer. After completion, the cells were washed, and H2DCFDA was added at a final concentration of 10 µM and diluted in DPBS. The cells were incubated in a solution for 30 min at 37 °C. After washing three times with DPBS, the cells were incubated for another 30 min, and the fluorescence per cell was measured by flow cytometry. The relative intensity of each group compared to that of the control group was expressed as a fold change.

2.7. Simulation of the Electric Field Distribution

The electric field strength applied between the electrodes was simulated using the Epo code™ (Standard Co. Ltd., Gunpo, Korea) developed by the open-source OpenFOAM. The governing equation for the electric field was determined by taking the slope of the potential (\( \Phi \)), as in Equation (1), using the electro-quasistatic approximation:

\[
0 = -\nabla \cdot (\sigma \nabla \Phi)
\]  

where \( \sigma \) is the culture conductivity. The electrical boundaries were set to \( \Phi = V \) (source) and \( \Phi = 0 \) (sink). All other boundaries were treated as electrical insulators.

2.8. IRE Procedure

Based on these simulations, eight rectangular direct current pulses with a width of 100 µs at a frequency of 1 Hz, with voltages of 300, 375, and 450 V (distance ratio: 1200, 1500, and 1800 V/cm, respectively), were applied in FBS-free media and incubated for 5 min. To output such powers, a pulse generator (Epo; The Standard, Co. Ltd., Gunpo, Korea) that generates a square wave with a width of 100 µs and an interval of 1 s was used. In addition, we used specially designed electrodes (Cuvette Plus, 4 mm gap, 800 µL, BTX, Massachusetts, USA) to deliver this energy. The harvested cells were transferred to a cuvette of 800 µL containing 1.0 \( \times \) 10⁵ cells. IRE was applied according to the conditions. For preconditioning with NIR irradiation, after applying NIR as above, cells were collected and transferred into the cuvette, and the IRE was applied.

2.9. Measurement of Annexin V and PI Staining

The cells were irradiated with NIR at 3 J/cm², and electric pulses were immediately applied, as described previously. Five minutes after IRE, the cells were washed with 0.5 mL of cold PBS. They were resuspended in the cold binding buffer, and 1.25 µL of Annexin V-FITC (EzWay Annexin V-FITC apoptosis detection kit; Komabiotech Co., Ltd., Korea) was added. After incubating the cells for 15 min, the binding buffer was removed and resuspended in a fresh, cold binding buffer after adding 10 µL of propidium iodide (PI). Fluorescence analysis was performed using FACS (BD FACSVerse™; Becton, Dickinson and Company, New York, USA). For apoptosis analysis, the cells collected after treatment with IRE were incubated for 6 h in a culture dish and then harvested again. The cells were stained with Annexin-V and PI, and the relative percentage of each condition compared to the control was expressed as fold values.

2.10. Transmission Electron Microscopy (TEM)

Six hours after pulsing, the cells were fixed in 0.15 M sodium cacodylate (pH = 7.4) at 37 °C, with 2% paraformaldehyde and 2.5% glutaraldehyde (Ted Pella, Redding, CA, USA) and placed in a pre-cooled fixative on ice for 1 h to optimize the mitochondrial structural
preservation and the membrane contrast. The cells were postfixed with 1% osmium tetroxide, 0.8% potassium ferrocyanide, and 3 mM calcium chloride in 0.1 M sodium cacodylate (pH = 7.4) for 1 h; washed with ice-cold distilled water; stained with 2% uranyl acetate at 4 °C; dehydrated using graded ethanol; and embedded in Durcupan resin (Fluka, St. Louis, MO, USA). Ultrathin sections of 70 nm were post-stained with uranyl acetate and lead salts and observed using a JEOL 1200FX (JEOL, Japan) at 80 kV. Further, the images were digitized at 1800 dpi using a Nikon Cool Scan system (Nikon Instruments, New York, USA), giving an image pixel array of 4033 × 6010 and a pixel resolution of 1.77 nm.

2.11. Measurement of mRNA Amounts Related to Apoptosis

Six hours after pulsing, the cells were harvested and lysed, and the total ribonucleic acid (RNA) was extracted using an RNeasy Mini Kit (Qiagen). The total RNA was converted to complementary deoxyribonucleic acid (cDNA) using reverse transcriptase and random primers (cDNA synthesis kit, Toyobo) according to the manufacturer’s protocol. The same amount of extracted total RNA from each sample was used for cDNA synthesis. The synthesized cDNA was used for real-time polymerase chain reaction using a CFX96TM Real-Time System (Bio-Rad). The relative gene expression was evaluated using the comparative cycle threshold method. The relative amount of mRNA expression was normalized to that of RPL13a and expressed as a fold change compared to the control. The primer sequences for apoptosis and necrosis were as follows:

- **GAPDH** (F: GGCATCCTCACCTGAAGTA, R: AGGTGTGGTGCCAGATTTC)
- **p53** (F: TAAACGTTCCCTGATGGGCGGC, R: AGGACAGGCACAAACACGCACC)
- **BAX** (F: AACATGGAGCTGCAGAGGAT, R: CAGTTGAGGTGGCAGTCA)
- **SOD1** (F: GGCAAAAGGGTGAGTGAAGA, R: GGGCCTCAGACTACATCCAA)
- **GSR** (F: GTTGCGCCAGGGGAGATGTGA, R: TAGGGCTGAGGTCCAG)

2.12. Measurement of Capacitance and Conductance

To investigate the electrical response after the NIR irradiation, we used an indium tin oxide (ITO)-coated glass slide, provided by Samsung Electronics (Samsung Electronics, Suwon, Korea), as a transparent electrode with a resistivity of 8–10 Ω/square. The glasses were cut into an area of 25 × 76 mm. The ITO surface was etched using HCl (1:1 v/v dilution, concentrated acid/water) with FeCl₃ (2–5% (wt/wt)) to create a parallel electrode with a distance of 5 mm. Completion of the etching process was confirmed by measuring the resistance (Protek 608, Protek, Incheon, Korea). Polymethylsiloxane (PDMS; Sigma Aldrich, Massachusetts, USA) and silicone elastomer curing agent (9:1 w/w) were mixed and cured overnight. Circular holes were punched with a diameter of 10 mm, and then the PDMS block was bonded to the ITO glass substrate after oxygen plasma for 10 min. It was then disinfected with ethyl alcohol and thoroughly washed with PBS, and 2.0 × 10⁶ cells were seeded into a PDMS well and incubated for 12 h. Capacitance and conductivity was measured in parallel over a 1.0 V level ranging from 50 Hz to 2000 Hz in 50 Hz increments, using an LCR meter (LCR-6002, GWINSTEK, California, USA) as shown in Figure A1. Measurements were performed in a CO₂ incubator to maintain temperature.

2.13. Statistical Analysis

All experiments were repeated at least three times, and the data are expressed as the mean and standard deviation (SD). Statistical significance was evaluated using the unpaired Student’s t-tests (two-tailed, equal SD) with Microsoft Excel, where *; **, and *** indicate p-values of < 0.05, < 0.01, and < 0.001, respectively, compared to the control value, and # represents a p-value as compared with the other group.
3. Results
3.1. Conditioning the NIR Irradiation Time

The irradiation time was modulated to increase the irradiation intensity from 1 to 5 J/cm², based on previous studies [17]. We examined cell viability, cytoplasmic transmembrane potential (CMP), mitochondrial transmembrane potential (MMP), and intracellular ROS levels, immediately after NIR irradiation. Cell viability was assessed using the WST-1 assay, which was used for quantification of DNA synthesis by colorimetry. Cell viability was not affected when irradiation was below 4 J/cm² and was slightly reduced at 5 J/cm² (Figure 1b). The CMP was assessed with FLIPR dye, which indicates hyperpolarization as the dye bleaches and depolarizes as the dye intensifies. The cells subjected to 2–4 J/cm² showed hyperpolarization compared to the control (Figure 1c). MMP was assessed with JC-1 dye, which aggregates in the inner mitochondrial membrane to exhibit red fluorescence in polarized mitochondria and green fluorescence in other places. The ratio of red to green fluorescence intensity was used to determine the degree of MMP. Cells subjected to 1–2 J/cm² showed hyperpolarization as compared to the control (Figure 1d). ROS production was not linearly dependent on the NIR dose. The cells subjected to NIR irradiation of 2 J/cm² exhibited increased amounts of ROS, while those subjected to NIR irradiation of 3–5 J/cm² exhibited a reduction in ROS as compared to the control (Figure 1e). We chose 3 J/cm² NIR irradiation for subsequent experiments with IRE, which had no harmful effects on proliferation and induced CMP hyperpolarization, induced no effects on MMP, and reduced ROS levels.

3.2. Plasma Membrane Disintegration Right after IRE

The electric field distribution was simulated, as shown in Figure 2a. The distribution was uniformly distributed between electrodes for the applied voltages of 300, 375, 450, and 500 V. Based on the simulation results, it was investigated whether the applied voltage caused an arc due to the overcurrent between the electrodes. Five hundred volts were excluded from the experiment. The efficiency of the IRE was estimated immediately after the electric pulses. Membrane translocation and negatively charged molecular transportation were analyzed by staining phosphatidylserine (PS) and measuring PI permeation. PS is localized exclusively in the inner leaflet of the cell membrane, where it forms part of the protein docking sites necessary for the activation of several key signaling pathways. It is exposed to the outer leaflet when the cell loses its membrane integrity or apoptosis begins. In our experiment, the PS signal increased with the applied electric field strength, especially when pre-treated with NIR (Figure 2b). The enhancement was significant when the field strength exceeded 1500 V/cm. PI is a widely used membrane-impermeable, negatively charged dye to test membrane integrity. The PI intensity also increased with the applied electric field strength, especially when pre-treated with NIR (Figure 2c). These results confirm that pre-treatment with NIR accelerates the distortion of the cell membrane by high electric pulses.
Figure 2. The 3 J/cm² NIR pre-treatment enhanced plasma membrane disintegration, right after IRE in HeLa cells. (a) Simulations of electric fields between two electrodes. (b) Relative MFI of Annexin-V dye according to the electric field intensity w/or w/o NIR pre-treatment. (c) Relative MFI of PI dye cells according to electric field intensity w/or w/o NIR pre-treatment. (** p < 0.01, and *** p < 0.001 with the Student’s t-test to the control, # p < 0.05 and ## p < 0.01 with the Student’s t-test to the non-irradiated group (n = 3)).

3.3. Apoptosis 6 h after IRE

The efficiency of IRE was estimated by the induction of cell death or apoptosis 6 h after IRE. Cell viability, membrane integrity, mitochondrial morphology, and transcription of apoptosis-related genes were examined. Cell viability was reduced according to the applied electric field strength, and it reduced more significantly with pre-treatment from NIR (Figure 3a). We confirmed that this reduction is related to apoptosis. Apoptotic cells were classified with positively stained Annexin-V FITC, and the apoptotic rate was significantly enhanced by pre-treatment with NIR (Figure 3b). TEM images showed that mitochondria in NIR+IRE cells were severely distorted, which implies accelerated apoptosis (Figure 3c). Mitochondrial degeneration occurs with the rupture of the mitochondrial outer membrane, as indicated by the white arrows. The rupture of the mitochondria was observed partially and entirely in HeLa cells that were under IRE and NIR-IRE. Entire ruptures were much more likely with NIR-IRE than with IRE.
Figure 3. The NIR pre-treatment enhanced apoptosis ratio after 6 h from IRE in Hela cells. 3 J/cm² NIR was irradiated, and then 1200, 1500, and 1800 V/cm electric pulses were applied. (a) WST-1 assay according to electric field intensity w/or w/o NIR pre-treatment. (b) Apoptosis rate according to electric field intensity w/or w/o NIR pre-treatment. (c) Representative TEM images of mitochondria in IRE-treated HeLa cells w/or w/o NIR pre-treatment. (d) Relative mRNA expression levels of BAX, p53, SOD1, and GSR. (e) WST-1 assay w/or w/o NAC. (* p < 0.05, ** p < 0.01, and *** p < 0.001 with the Student’s t-test to the control, ### p < 0.01 with the Student’s t-test to the non-irradiated group, (n = 3)).

Such apoptotic phenomena also appears at the gene level. We confirmed the increased transcription levels of apoptosis-related genes, BAX (Bcl-2-associated X protein), and p53 (Figure 3d). BAX is a pro-apoptotic protein that induces the opening of the mitochondrial membrane ion channel, and the release of cytochrome c. p53 is activated in response to many stress stimuli and directly regulates the transcription of apoptosis-related genes, including BAX [18]. HeLa cells have no mutations in p53 [19]. Additionally, we examined the transcription levels of oxidative stress-related genes: SOD1 (superoxide dismutase 1) and glutathione reductase (GSR). SOD1 is responsible for destroying excessive free...
superoxide radicals by converting them to molecular oxygen and hydrogen peroxide in the outer mitochondrial membrane, and GSR is a key member of the glutathione antioxidant defense system. The reduction in the expression of these genes implies that the increase in apoptosis was not initiated by high oxidative stress in NIR pre-treated samples. This was confirmed by the addition of an antioxidant, which is widely used as a pharmacological antioxidant. The decline in cell viability by NIR+IRE was not recovered by treatment with NAC (Figure 3e). These results suggest that enhanced IRE-induced apoptosis was not due to enhanced oxidative stress.

3.4. Capacitance and Conductance Changes with NIR Irradiation

The capacitance and conductance were measured in real time with NIR irradiation, as shown in Figure 4a. To maximize the contribution of cellular capacitance in the impedance measurement, the full coverage of electrodes with the cells was confirmed before the experiment. Data with a 1 kHz alternating current (AC) were used to investigate the electrical changes in the cells with NIR irradiation [20]. The parallel relative capacitance and relative conductivity were the values divided by the control value before the NIR irradiation. Figure 4b shows that the capacitance started to decrease when the NIR energy density increased above 2 J/cm². In contrast, the conductance increased immediately upon NIR irradiation, and the value remained almost constant (Figure 4c). The conductance dropped slightly at approximately 1.3 J/cm² and then increased again, which might be related to the function of membrane proteins, as shown in Figure 1c. However, the change was within the error range. At the end of 3 J/cm² NIR irradiation, the capacitance decreased by 0.4% from the beginning, and the conductance increased by 0.6%. This implies that NIR induces alterations in the electrical properties of the cells. The cell-free capacitance decreased and conductivity slightly increased, which may be due to photoreceptors in FBS-containing media. These reference values were subtracted from the measured data of the cells.

4. Discussion

We successfully demonstrated that NIR pre-treatment enhanced IRE-induced apoptosis in vitro. The cell membrane was easily disturbed by electric pulses, which was shown by increased PS translocation and PI transportation with NIR irradiation (Figure 2). The apoptosis signal was enhanced, which was shown by decreased DNA synthesis, increased PS translocation, mitochondrial condensation, and increased apoptosis-related mRNA synthesis after 6 h from IRE (Figure 3a–d).

In this study, we used 810 nm NIR light, which has demonstrated mitochondrial activation or intracellular ROS enhancement in previous reports [14,15]. However, our results showed that cellular responses to 810 nm NIR were dependent on the irradiation intensity. The CMP was hyperpolarized at 2–4 J/cm², and the MMP was hyperpolarized.
at a lower intensity of 1–2 J/cm² (Figure 1c,d). Figure 1e shows a significant reduction in intracellular ROS at 3–5 J/cm². We used 3 J/cm² NIR irradiation for the experiments with IRE, which had no harmful effects on proliferation, induced CMP hyperpolarization, induced no effects on MMP, and reduced the ROS levels. Under these conditions, we hypothesized that NIR pre-stimulation promotes IRE-induced cell death through membrane hyperpolarization, rather than through ROS generation. Irrelevance of ROS in the enhanced apoptosis was confirmed with reduced antioxidant gene expression and with no recovery from antioxidant chemicals (Figure 3d,e).

Therefore, we hypothesize that CMP hyperpolarization may be one of the reasons for the higher IRE-induced apoptosis. As the induced transmembrane potential caused by externally applied electric pulses is superimposed on the resting CMP of the cell, the side of the cell facing the anode is hyperpolarized, while the side facing the cathode is depolarized [21]. Previous studies reported that hyperpolarization yielded a more statistically significant electroporation efficiency than depolarization [14]. The addition of cationic peptides makes the anode-facing cell membrane more negative, and cations interact electrostatically with the plasma membrane, producing a greater negative charge, thereby increasing the electrostatic potential across the membrane. Kanduser et al. showed that CMP can affect the threshold voltage that triggers electroporation [22]. Kim et al. showed that CMP is positively correlated with electroporation-induced molecular transfer through the membrane [23,24].

Although photobiomodulation is widely used in research and clinical fields, the mechanism by which NIR modulates cellular behavior is not yet clearly understood. Cytochrome c oxidase in the mitochondrial respiratory chain has been considered the primary photo-acceptor for photobiomodulation [14,25,26]. However, cellular complex responses cannot be explained simply by one chromophore [27,28], and other potential mechanisms (e.g., interfacial water layer) and chromophores (e.g., light-sensitive ion channels) have been suggested [29,30]. Shapiro et al. suggested that pulsed NIR increased membrane capacitance and capacitive currents through temperature changes in a short time [13]. Our impedance measurements showed a slight decrease in capacitance and a slight increase in conductance with NIR irradiation. This indicates a change in the plasma membrane structure or an increase in charge. We speculate that this is another cause for the enhanced IRE efficiency, as well as the increased CMP. This phenomenon may include biological responses rather than physical responses.

Our results also show that there are windows for hyperpolarization in the cytoplasmic and mitochondrial membranes and the latter seems more vulnerable to NIR stimulation. This cannot be explained simply as a physical phenomenon. According to previous studies, NIR irradiation can cause either depolarization or hyperpolarization across the cytoplasmic membrane based on the wavelength, irradiation intensity, or irradiation mode (continuous wave (CW) or pulsed wave (PW)) [15,25,31]. Sanderson et al. showed that NIR can modulate mitochondrial activity with wavelength [25]. Nguyen et al. showed that NIR can activate the mitochondrial function in an intensity window [31]. Kim et al. showed that NIR can modulate CMP and ROS under different pulsing conditions [15]. Intensity dependency was also observed for intracellular ROS levels. Harmonized work by diverse cellular components should be studied in the future to understand these dose-dependent cellular physiological events.

In summary, NIR-induced hyperpolarization can result in increased IRE efficiency, potentially reducing the required applied voltage. The dose-dependent physiological modulation by NIR irradiance can be used to enhance the spatial selectivity of IRE treatment. These results may contribute to the clinical use of IRE to reduce the risk of high voltage.

Author Contributions: H.B.K. designed and performed the experiments and wrote the original manuscript. S.J. performed simulations of the electric field and the temperature of the electric pulses. K.Y.B. performed the experiments, reviewed, and edited the manuscript. All authors have read and agreed to the published version of the manuscript.
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Appendix A

Figure A1. (a) An equivalent circuit diagram to measure the impedance of cells cultured on ITO glasses in culture media. (b) Capacitance and conductance spectroscopies according to frequency.

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