Spring 2020

Biphasic Gene Electrotransfer Enhances Gene Delivery *In Vitro*

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BIPHASIC GENE ELECTROTRANSFER ENHANCES GENE DELIVERY *IN VITRO*

by

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B.S. October 2016, Excelsior College

A Thesis Submitted to the Faculty of
Old Dominion University in Partial Fulfillment of the
Requirements for the Degree of

MASTER OF SCIENCE

BIOMEDCAL ENGINEERING

OLD DOMINION UNIVERSITY
May 2020

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ABSTRACT

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The application of short, pulsed electric fields to eukaryotic cells and tissues has been shown to permeabilize cells. This phenomenon has been used for clinical applications for irreversible electroporation of cancer cells or for molecule delivery for drug or gene therapies. Typically, a monophasic (monopolar) pulse train is used; however, recent studies have explored the possibility of using biphasic (often referred to as bipolar) pulses, primarily for irreversible electroporation (IRE), which report reduced muscle contraction during pulse train application compared to monophasic pulses. Additional studies show improved transfection efficiency using biphasic pulses, conversely, with low cell viability. The purpose of this current study is to evaluate parameters of biphasic pulses for improving gene transfer *in vitro.* B16-F10, mouse melanoma cells were cultured, suspended, and treated with microsecond pulsed electric fields in a 1mm cuvette. Various pulsing parameters were used to deliver either propidium iodide (PI) or plasmid DNA encoding green fluorescent protein (GFP) to observe cell permeabilization and transfection. Cell viability was evaluated via PrestoBlue assay. Increasing pulse trains to 8 and increasing positive pulse width to 100 us at low voltage of 40 V, both resulted in significant changes in transfection efficiency with reduced viability. On the other hand, increasing voltage to 120 V shows significantly enhanced transfection efficiency with low viability. Lastly, reducing positive pulse width to 20 us at 120 V applied, resulted in high transfection efficiency at 43% with high cell viability at 84%. This study shows that biphasic pulses enhance gene

delivery of plasmid encoding GFP into B16-F10 and maintain high cell viability *in vitro*. These results are consistent with earlier studies that gene delivery enhancement is feasible with biphasic pulses. Additional, future studies will evaluate whether such gene delivery enhancement can be maintained in excitable cells without actional potential activation.

Keywords: biphasic pulses, monophasic pulses, gene electrotransfer, transfection efficiency, cell viability.

This thesis is dedicated to my friends and
family who instill their faith into me.

ACKNOWLEDGEMENTS

This program was not an easy feat. There were various committee members, research assistants, and collaboration members involved in the completion of this thesis. I would like to thank Dr. Anna Bulysheva for her guidance throughout the program, teaching me the techniques used in laboratory work, and giving me the opportunity to work in her lab; the committee members Dr. Christian Zemlin and Dr. Claudia Muratori; members of the laboratory Carly Boye, Andrew Ojeda, Julia Pittaluga, and Alexandra Chittams.

NOMENCLATURE

BPC	Bipolar Pulse Cancellation
DOTMA	1,2-di-O-octadecenyl-3-trimethylammonium propane
ECG	Electrocardiogram
ECT	Electrochemotherapy
FBS	Fetal Bovine Serum
GET	Gene Electrotransfer
GFP	Green Fluorescent Protein
DMEM	Dulbecco's Modified Eagle Medium
IHC	Immunohistochemistry
IRE	Irreversible Electroporation
PBS	Phosphate-Buffered Saline
PAMAM	Polyaminoamine
PEI	Polyethyleneimines
PPI	Polypropyleneimine
PI	Propidium Iodide
VEGF	Vascular Endothelial Growth Factor

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CHAPTER I

INTRODUCTION

The use of short pulsed electric fields on eukaryotic cells has been shown to increase cell permeabilization. This phenomenon has numerous clinical applications such as tissue ablation or delivery of drugs and genes into the permeabilized cells. These applications often use monophasic (monopolar) pulses, but recent research has explored the possibility for biphasic (often referred to as bipolar) pulses, mainly for tumor ablation. The term monopolar and monophasic have been used interchangeably in research to describe waveforms of a single polarity. Bipolar and biphasic has been used interchangeably in numerous studies to describe waveform that uses both polarities. In a study by Chiapperino *et al*, the term monopolar and bipolar has been used to explain the pulse wave for their experiment [1]. In a study by Tovar and Tung, the term monophasic and biphasic has been used to explain the rectangular waves used for their experiments [2]. In a study by Long *et al*, they defined monopolar as a type of probe (electrode) they used and monophasic as the waveform for their tumor ablation protocol [3]. For this study, monophasic is defined as the waveform of a pulse wave that only uses one polarity. Biphasic will be defined as the waveform that uses both polarities alternating in cycles. Monopolar will be defined as the probe that induces a voltage from an applicator to a grounding pad. Lastly bipolar will be defined as the probe that has two electrodes which can function a cathode. Few studies have emerged that utilize biphasic pulses for gene electrotransfer or delivery of gene using electrical pulse. Among those studies the parameters often yielded low transfection with high cell survivability or vice versa. The purpose of this study is to determine

more efficient biphasic pulse parameters for transfection of plasmid DNA into B16-F10 murine melanoma cells while maintaining high cell viability.

Gene Therapy

Gene therapy is a technique of introducing a foreign genomic material into the cell to produce a therapeutic result [4]. This field encompasses replacing defective genes, restoring specific gene functions, or turning off or deleting defective genes [4][5]. While there is strong understanding of genetic mutations that lead to innumerable amount of diseases, target delivery to disease organs and tissue postured many difficulties [6]. Gene therapy is divided into two categories: germline which is delivery to cells during their metaphase stage, ex-vivo delivery to egg cells during *in-vitro* fertilization, or delivery to sperm cells [4][5]. Somatic therapy is delivery of gene into diploid cells of individual where genetic properties will not be passed onto offspring. Modern gene delivery preferred somatic gene delivery over germline gene delivery [4][5]. The ideal delivery system [5] is best characterized as:

- Able to include a wide array of inserted DNA
- Easy to produce and cost effective
- Able to target specific type of cells
- Does not duplicate DNA
- Will not initiate an immune response
- Maintain high cell viability

Viral methods consist of utilizing viral vectors such as adenovirus, retrovirus, poxvirus, adeno-associated virus and herpes simplex virus [6]. These viruses are manipulated by removal of the viral genes that cause disease and replaces them with therapeutic genes. Genes in the viral

vector that are kept are genes that express sequences for DNA replication and packaging [7]. The host immune system is still capable of recognizing it as a threat and initiating an immune response against the vector [4][5][6]. The viral vectors still hold some of the traits of a virus. Portion of the vector may contain proteins that bare resemblance to antigens. Once the vector has been administered into the body, it can be recognized by the host's adaptive immune system if the hosts has been previously exposed that particular virus. It can also trigger the host's innate immune system based on viral structures such as nucleic acid and cause production of INFα, which will reduce transduction and create a signal for the adaptive immune system, making subsequence application less effective [8].

Non-viral methods are gene delivery using chemical or physical means. Chemical method includes using cationic liposome and polymers and physical includes electroporation, ultrasound, magnetofection, or particle bombardment [4]. The chemical delivery system uses interaction between cationic particles, polymers, or lipid polymers, and cell surface [9]. A liposome is a positively charged lipid called 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA), that alone or with another phospholipids (from cell membrane), will form a liposome vesicle. Since DNA is negatively charged and DOTMA is positively charged, the proper ratio between the two will facilitate movement of the vesicles. With the lipid membrane being negatively charged, the positively charged DOTMA will interact with the cell membrane where it will fuse [10]. Once inside the cytoplasm of the cells, through various microtubules and motor proteins guide the vesicle to the nucleus [10]. The downside is that the delivery can be toxic and has been shown to cause low viability [9]. Additionally, polymers can be used to deliver DNA into cells. Cationic polymer uses dendrimers such as polyaminoamine (PAMAM), polyethyleneimines (PEI), and polypropyleneimine (PPI) [11]. These dendrimers have the gene

material within the branches. The polymer is delivered into the cells through endocytosis and with the positive charge of the cationic polymers and the negative charge of the DNA, it will make its path to the nucleus where the dendrimers release their materials through means such as proton sponge in which the low pH in the endolysosomal complex that results in rupture of the endolysosomal membrane that releases the gene. The advantage with cationic polymer is that it can condense DNA plasmid, with the downside involving its cytotoxicity especially with the polymers being non-biodegradable [11].

Gene gun is a form of particle bombardment that uses gold or tungsten spherical particles coated with plasmid DNA accelerated by pressurized gas or electrical shockwave into the tissue cell [12]. Magnetofections use a magnetic field to concentrate particles containing nucleic acid into the cell [13]. In magnetofections, nanoparticles are used that are coated with cationic polymers typically used in chemical method of delivery. A magnetic field is applied in the area which causes the particles to concentrate at the site where the cationic polymer will assist in delivery to the nucleus [13]. The downside with magnetofections is that it still uses cationic polymer which are toxic to cells and will affect viability [11]. Electroporation uses repetitive high voltage, short electrical pulses to form temporary pores in cells that allows for delivery of DNA plasmids [14].

Application of gene therapy can be used to replace a defective gene, to delete a gene, or to add a missing gene. Genetic disease such as diabetes was studied in rats, where a gene was inserted into the liver to manage blood glucose level and ketogenesis. The result was there was ketoacidosis was mitigated and normoglycemia was observed [5]. Non-genetic condition such as cancer has numerous studies for gene therapy. One study delivered cytokines such as IL-12, IL-2, and IL-15 into cancer cells. An immune response was initiated, resulting in tumor size

reduction at the site of the transfection and even at metastatic location in some patients [15]. Gene therapy can be applied into regenerative medicine in which genes that causes upregulation of growth factors can be used to restore cells or proteins. In a study, naked plasmid encoded with vascular endothelial growth factor (VEGF) gene was injected into the myocardium. The results show that the gene promoted neovascularization of the ischemic region of the heart, which lead to an increase in the patients' perfusion score [16]. Gene therapy has many different methods of delivery, each with benefits and risk.

Electroporation

Electroporation is characterized as the phenomenon of inducing a high voltage, short electrical pulses to eukaryotic cells. This process will result in the cell being permeabilized, allowing for the introduction of molecules that cannot normally enter the cells [17][18]. The concept of the mechanism behind this phenomenon has been a subject for discussion since its discovery. Experts believed that the permeabilization of the membrane is due to disruption of the boundary between the lipid composition, deformation of the lipid membrane, or denaturation of the membrane proteins, but the consensus is that pores form through the lipid bilayer of the cell [15]. This permeabilization is either reversible where the membrane can be resealed with minimal damages to the cells or irreversible where the membrane cannot recover resulting in apoptosis or cell death [14].

Electrical pulses used to permeabilize cells are typically square-pulse waves. Square-pulse waves can best be defined as having very rapid charging time to a constant amplitude followed by a very rapid decay time for one cycle [19]. When using these electrical pulses, a lot of variables are taken into consideration that can determine the effect of the cell permeabilization. Field strength or voltage amplitude is often measured in voltage (V), it

determines the maximum electrical field the cells are exposed to. High voltage often leads to more irreversible results. Pulse width is the amount of time the cells are exposed to an individual pulse at the determined voltage. Pulse width can range from ps to ms. Longer pulse width means longer exposure time to the voltage for the cells, which can lead to more cell death. Pulse number is the amount of pulses applied in an experiment. A higher pulse number means more repeated exposure to the pulse, which can cause more pore formation. Period is the time in between each cycle of pulse. Longer periods often lead to higher cell viability [16].

Pore formation typically happens within microseconds of electrical pulse exposure and will continually form more pores or stay open until the pulse ceases. If the parameter is not as intense, resealing can occur, but it is normally a much slower process that can take up to minutes [20]. Resealing of the cellular membranes comes with its merits with the delivery of drugs or delivery of genes [21][22]. Electrochemotherapy (ECT) is a treatment that takes advantage of the rise in permeabilization of the membrane to introduce drugs into the cells that were considered too difficult to introduce in the cell [17]. Anti-cancer drugs such as bleomycin have been used with cells exposed to a train of high energy microsecond pulses in an experiment results in regression of tumor size when both electrical pulses and bleomycin were used in tandem [17]. Bleomycin cause multiple DNA break in the tumor and cisplatin causes intra or inter-strand DNA bond; making them a preferred drug for delivery in cancer cells [18]. Bleomycin and cisplatin are typically too large to cross the cell membrane through endocytosis or ion channels. By raising the permeability of the cell membrane through pulse electric fields, pore formation will be large enough to facilitate the transport of those drugs into the cells that are exposed to the field and not surrounding cells thus making the treatment safer than conventional chemotherapy [18].

Gene electrotransfer (GET) is a physical non-viral form of gene delivery, in which a train of high energy electrical pulses will permeabilize the cells to allow for the introduction of DNA plasmid [20]. Using the proper parameters, DNA plasmid can pass through both the lipid membrane and the cytoplasm into the cell nucleus where gene expression occurs [21][22]. Transfection efficiency is dependent on the histology and phenotype of the cells. Typically, skeletal muscles are easier to transfect due to being polynucleate and easy to access, while tumor cells are much more difficult to transfect [23]. Gene electrotransfer is similar to electrochemotherapy in concept. A set of high energy short pulses is exposed to the cells to which they will permeabilize, and plasmids that are encoding genes will pass through the lipid bilayer and the nuclear envelope to which the gene will bind to the DNA and express [16]. Applications of GET were mainly conducted with transfection of immunostimulatory cytokines such as IL-12, IL-2, IL-15, and TNF-α into the cancer to initiate an immune response [16]. The result shows that with IL-12 transfected into metastatic tumor cells, tumor size regression occurred with two patients even having complete regression of metastasized tumor without any systemic treatment [24].

Delivery of high power, short electrical pulses above a certain threshold can cause pores to be permanent, leading to cells apoptosis [25][26]. Tumor ablation through pulse electric field uses the principle of permeabilization to cause permanent pore formation, in which homeostasis in the cell cannot be maintained; this will lead to osmotic effect of the cell internal [27]. With the cell death, cell debris is left behind for the patient's innate immune system to recognize [28]. The medical application is ablation of tumors with the advantages of having a nonthermal effect resulting in sparing of blood vessel, inducing an immune response, and killing tumor cells [29]. Modern method of ablation typically relied on thermal principles in order to kill tumors.

Hyperthermal ablation utilizes probes and electrodes that use radiofrequency waves with frequency of 375-500 kHz to generate heat to raise the temperature of the cells. Between 42℃ to 45℃, vital enzymes for tissue function are disabled, leading to tissue damage [30]. Low temperature can also be used to ablate tumors through similar applications but different mechanisms [29]. Cryoablation is another form of thermal ablation that uses temperatures as low as -40℃ to induce cell death. The process uses argon gas or liquid nitrogen through the applicator to remove heat from the tissue resulting in crystal formation that will dehydrate the cell, causing the cells to shrink while the ice crystal continually forms resulting in cell damage [31]. Like hyperthermal ablation, cryoablation can also induced significant damages by removing heat from surround tissues. Irreversible electroporation is preferred as the cell damage is more intended and collateral damage is minimized due to absence of thermal effect [29].

Monophasic and Biphasic Electropermeabilization

Many studies in the field of pulse electric field involves the use of monophasic pulses. Monophasic is often referred as monopolar, but for this study, the term monophasic will be used to describe the wave form of the pulse that utilize only one polarity [32], and monopolar will be used to describe the electrode. There is a wide array of research that utilizes monophasic pulses such as tumor ablation, gene electro transfer, electrochemotherapy, and electrical stimulation of nerve [16]. The major issue with the using monophasic pulses includes sensation of pain and muscle contractions [33]. Muscle contraction has been proved to be problematic *in-vivo* as extra precaution is taken prior to administering the treatment. Muscle contraction can cause organ translocation if not properly managed, thus requiring neuromuscular blockade [34]. Before inducing any electrical pulse, the patient may need to be anesthetized or administered a paralytic agent. With regard to the patient, strict monitoring is also required and synchronization with the

cardiac cycle has to be adhered to [35]. Muscle contraction can further complicate the procedure as it is able to dislocate the position of the electrical probe used for the treatment especially when used in sensitive areas [36]. Tumor ablation near the heart with monophasic pulse has shown to induce ventricular arrythmia. During irreversible electroporation, the electric field from the applicator are set to high voltage. The magnitude of the field will dissipate throughout surrounding tissue thus potentially creating an area of reversible electroporation. The myocardium receiving electrical stimulation from this field can prematurely initiate an action potential, leading to ventricular arrythmia. ECG monitoring and synchronizing are practiced when performing tumor ablation near the heart to minimize the risk of ventricular arrythmia [37].

Biphasic pulses are often called bipolar pulses and the term has been used interchangeably in various literature. For this study, bipolar pulses are referring to the probe used, while biphasic pulses are defined as a waveform that utilize both positive and negative polarity wave to perform the treatments [38]. Biphasic pulses are typically shorter in pulse length for both polarity and uses higher frequency to achieved similar permeabilization [33]. Higher amplitude is required for biphasic pulses in order to receive the same permeabilization as long monophasic pulses [26]. For pulses to achieve membrane permeabilization, pulse amplitude is inversely proportional to pulse width [33]. This is to be taken in consideration as the longer the pulse width the more energy is deposited into the cell. Thus, for permeabilization to occur with regard to cell viability, higher amplitude requires shorter pulse and longer amplitude requires less energy.

Inter-pulse delay is the time in between the positive and negative pulse phase. The timing of the delay is especially important to consider. In biphasic pulses, the delay does not have an effect on the transmembrane potential, but it does have a substantial effect on the nuclear

envelope potential. With a short delay, the falling voltage from the positive voltage is added to the nuclear envelope as negative is rising. With delay as low as 140 ns, the nuclear envelope potential can be double. A long delay will give the positive voltage time to reach zero before the negative voltage builds up, thus reducing the nuclear envelope potential [33].

The advantage of biphasic is the reduced necessity of the pulse electric field on the pore size as compared to monophasic pulses allowing for cells to be permeabilize. With nanosecond pulse, biphasic can penetrate heterogenous tissues, allowing for more foreseeable treatment results [39]. The impedance changes in high frequency biphasic pulses is significantly minimized, which affect the distribution of the local field [40]. This reduction in impedance can reduce muscle contraction, which obviates the complications caused from the contractions [26]. Much research that uses biphasic pulses often employs them for tumor ablation. This research shows that muscle contraction does not occur, and tumor size is reduced after treatment [35][36]. Some research in the field of biphasic pulses explores use of these pulses for electrochemotherapy. The technique is similar to modern method of electrochemotherapy, but high-frequency biphasic pulses are used to permeabilize the cells [40].

Biphasic pulses require higher voltage amplitude than long monopolar pulses to achieve the same efficacy. This is due to the effect known as bipolar pulse cancellation (BPC). Bipolar pulse cancellation is a phenomenon in which permeabilization of cells is compromise due to the second pulse of opposite polarity tailing the first. The first pulse initiating the process of permeabilization, allowing for occluding molecule to leave, which affects the concentration gradient in the cells [25]. The second opposite pulse can reinsert those occluding molecules back into the pore raising the concentration gradient, affecting permeabilization for other molecules [41]. This leads to requiring higher pulse amplitude to achieve permeabilization similar to

monophasic. Since higher amplitude increases permeabilizes of the cells, bipolar pulse cancellation will therefore reduce that permeabilization down to match monophasic permeabilization [41]. Bipolar pulse cancellation can be mitigated by raising the polarity delay, which treats both waves as independent waves instead of sequential waves [41]. Electrochemotherapy can employ the use of biphasic pulse wave to introduce drugs such as cisplatin into cells. The procedure is similar to monophasic, but high-frequency biphasic pulses are used instead. Results show that higher amplitude is required for the same cytotoxicity and shows feasibility in a reversible effect [42]. Gene electrotransfer is able to utilize biphasic pulses to deliver DNA plasmid into cell. The treatment is the same as monophasic, in which the plasmid is deliver to the site and the pulse is induced. The results show biphasic pulses can transfect plasmids into cells [20][22][43].

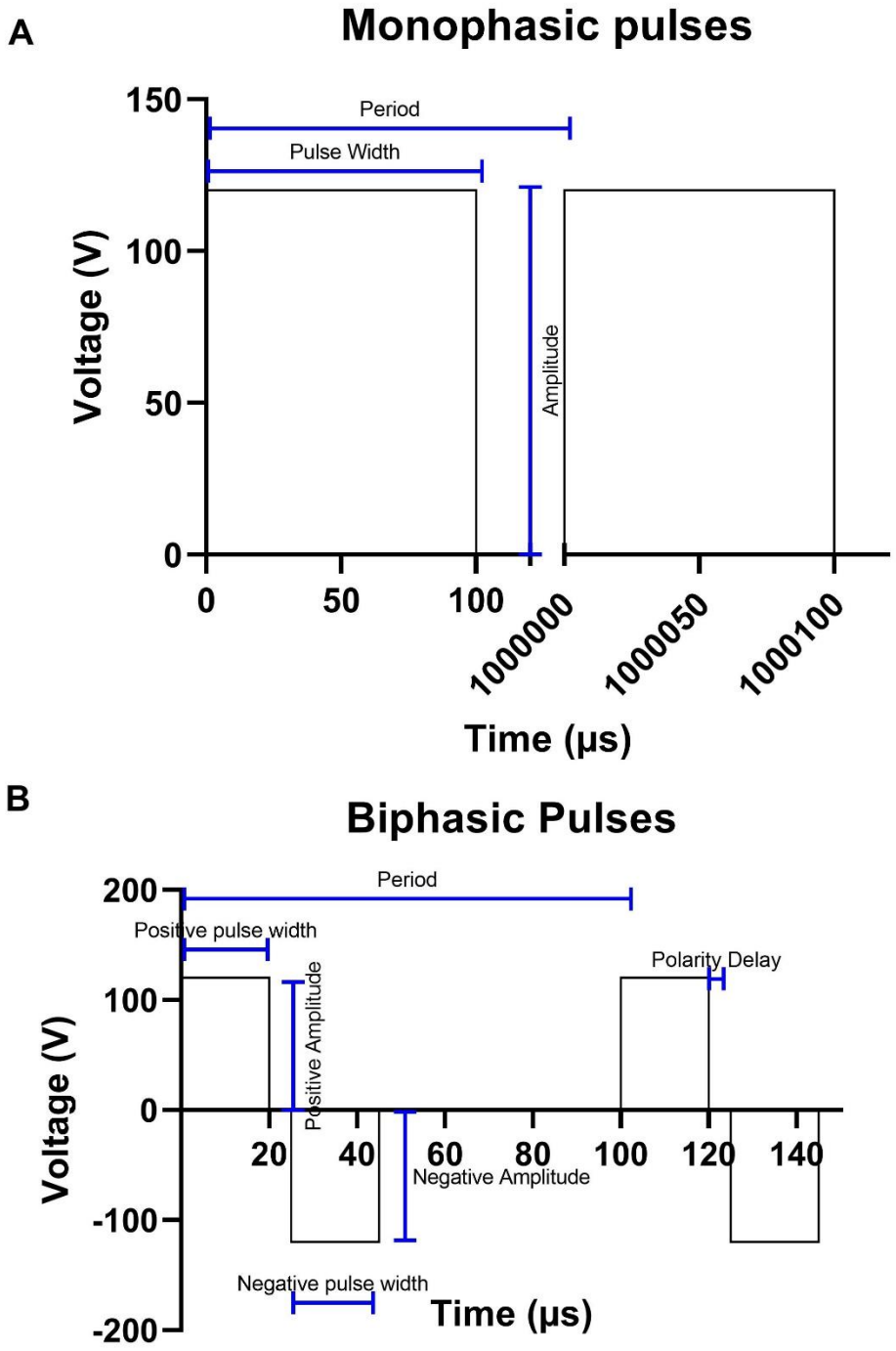


Figure 1: Graph presenting the waveform of biphasic and monophasic pulses (A) Monophasic pulse waveform (120 V amplitude, 100 us pulse width, 1 second period). (B) Biphasic pulse waveform (+120 V/-120 V amplitude, +20 us/-20 us pulse width, 5 us polarity delay, 100 us period).

Project Aim

The purpose of this project is to investigate the use of biphasic pulses for gene electrotransfer. Many different forms of gene transfer have shown success in literature. Viral based vectors have high transfection efficacy but have been shown to initiate an immune response and caused patient morbidity [8]. Chemical forms of delivery have been used in which cationic liposome and polymers were used to deliver plasmid into the cell. The results yielded good transfection efficiency, but low cell viability to the cytotoxic nature of the polymer and compound [10][11]. Many physical methods of transfection have low transfection efficiency. Gene electrotransfer has shown to have high transfection efficiency and high viability [14].

In a previous study, biphasic pulses were used to transfect plasmid DNA into NIH 3T3 fibroblast cells. The results show that biphasic pulses transfect 1.7x better than monophasic, but cell viability was exceptionally low at 44%-62% due to the condition being too extreme with high voltage, long pulse width, and low delay [22]. To this day, gene electro transfer with biphasic pulses has not resulted in both high transfection efficiency of plasmid and high cell viability. The aim for this project is to determine if high transfection efficiency and high cell viability can be achieved using biphasic pulses in comparison to monophasic pulses. The consideration taken is to keep the delay high enough to allow for the cells to recover, but low enough to minimize muscle contractions.

CHAPTER II

METHODS AND MATERIALS

Cell Culture

B16-F10 Cells (ATCC, Manassas, Virginia) were cultured in a 75 cm^3 flask in 15 ml McCoy Media (supplemented with L-glutamine, Corning, Manassas, Virginia) containing 10% fetal bovine serum (FBS)(R&D Systems, Flowery Branch, Georgia) and 1% of final concentration of gentamicin (R&D Systems, Flowery Branch, Georgia) at 37 °C in 5% CO_2 in an incubator. All cells were harvested for experiments by trypsinization at 80% confluency using 3 mL of 0.25% EDTA (R&D Systems, Flowery Branch, Georgia) for 5 minutes. Trypsinization was blocked by adding 7 mL of media containing 10% FBS and 1% gentamicin. Suspended cells were centrifuged for 5 minutes. Cells were counted using the Cellometer (Nexcelom Bioscience, Lawrence, Massachusetts). Trypsin-media solution was removed and cells were resuspended with new media. Cells were placed into individual 0.1 cm gap sterile cuvettes (Biosmith, Vandergrift, Pennsylvania) at a density of $6x10^5$ cell/100µl.

Electrotransfer Protocol

Gwiz-GFP plasmid DNA (2mg/ml, Aldevron, Fargo, North Dakota) are stored in a freeze at -20°C, thawed to room temperature, and mixed at a volume of 1.25 uL with 100 uL of media containing $6x10^5$ cells in each cuvette for group pulsed with GFP. Concentration of 200ug for every 100 uL of plasmid DNA was used.

Propidium iodide (PI, Millipore Sigma, St. Louis, Missouri) were warmed up and mixed at a volume of 5 uL containing 100 uL of media containing $6x10^5$ cells in each cuvette for group pulsed with PI. Electrotransfer experiments were conducted using cuvettes (Biosmith,

Vandergrift, Pennsylvania) with a .1 cm gap in between the electrodes, with volumes of 100 uL. Stock concentration is 1mg/mL.

The generator used is an Electrocell B-10 pulse generator (Leroy, Saint-Orens-de-Gameville, France), which is capable of generating monophasic and biphasic pulses. The parameters available are positive voltage, negative voltage, period, pulse number, positive pulse length, negative pulse length, and polarity delay shown in Fig. 1. The positive voltage sets the maximum positive amplitude of the square-wave. The negative voltage sets the maximum negative amplitude of the square-wave. The period set the time between each cycle of pulses. The number of pulses sets how many pulses the B16-F10 cells will experience. The positive pulse width determines how long the positive electrical field is exposed to the cells for each cycle of pulse. The negative pulse width determines how long the negative electrical field is exposed to the cells for each cycle. The polarity delay set the time distance between positive and negative pulse width, which affects how the biphasic pulses interact with the B16-F10 cells. The parameters used for the experimental group are as shown in Table 1. After electrotransfer for each cuvette, cells were then pipetted into a 24-well plate containing 500ml of media in each well. Plate were then incubated at 37 °C in 5% CO_2 in a humidified atmosphere incubator for 48 hours.

Viability Assay

Viability was assessed through PrestoBlue Cell Viability Assay (Life Technologies Corporatio1n, Eugene, Oregon) according to manufacturer's instructions. After 24 hours of incubation at 37 ℃ and 5% CO_2 for the B16-F10 cells, media was prepared in volume of 12 mL. Working PrestoBlue solution was made by mixing PrestoBlue with media at a 1:10 dilution of reagent to media, and pipetted to thoroughly mixed the solution. Old cell media was removed

from the plate and washed with 500 µL of PBS (Corning, Manassas, Virginia) per well. PBS was removed and replaced with 500 µL of PrestoBlue working solution and incubated at 37 ℃ and 5% CO_2 for 45 minutes. PrestoBlue solution was then transferred to a new sterile 24-well plate to be read. Absorbance was read under a Spectra Max I3 (Molecular Device, San Jose, California) at 570 nm with lid removed, and no shaking. Absorbance result were normalized to the average of positive control group (group that received no pulse) and set to a viability percentage by the equation:

$$\text{Cell Viability (\%)} = \frac{\text{Absorbance of each Well}}{\text{Average Absorbance form Positive Control Wells}} \text{ x } 100\%$$

Microscopy

After cells in 24-well plate were incubated for 48 hours, cells were imaged at brightfield, blue excitation B1E, and green excitation CY3 HYQ filter with using an inverted epifluorescence microscope. Setting was set to 10x magnification and camera images were taken at 50 msec. The brightfield setting is to view the cells by illuminating the sample with bright light. B-1E operates with a 470-490 nm blue excitation light and will excite the sample to fluoresce green light at an emission of 525 nm max. This setting is used to view cells expressing GFP. With the blue light exciting the green fluorescent protein in the cells causing them to emit a green light that can be view with the microscope. GFP from the Gwiz-GFP plasmid has an excitation peak at 470-480 nm and an emission peak of 510 nm, which met the bandwidth of the filters used. CY3 filter operates with a green light excitation of 512 to 550 nm and will excite the sample to fluoresce red light at an emission of 570 to 615 nm. CY3 setting was used to view cells expressing PI. With the green light exciting the PI chemical in the cell nucleus causing them to

emit a red light. PI has an excitation peak at 535 nm and an emission peak of 617 nm max therefore it falls into the CY3 emission and excitation band.

Transfection Efficiency

Once images were taken, cells were counted using ImageJ (National Institute of Health, Bethesda, Maryland). Transfection Efficiency was based on cells counted in brightfield for particular image and cell that fluorescence green color with the following equation:

$$\text{Transfection Efficiency (\%)} = \frac{\#\ of\ GFP\ positive\ Cells}{Total\ number\ of\ Cells} \text{ x } 100\%$$

Permeability Measurement

Once images were taken, cells were counted using ImageJ (National Institute of Health, Bethesda, Maryland). Permeabilization was based on cells counted in brightfield for particular image and cell that fluorescence red color with the following equation:

$$\text{Permeability (\%)} = \frac{\#\ of\ PI\ positive\ Cells}{Total\ number\ of\ Cells} \text{ x } 100\%$$

Statistical Analysis

All quantitative data were analyzed by performing a one-way ANOVA using Graphpad Prism 8 (Graphpad Software, San Diego, California), with p-value <0.05 being statistically significant. All quantitative data are presented with Standard Error of the Mean (SEM). Tukey test was conducted on one-way ANOVA analysis to determine significant difference between individual groups. One-way ANOVA is often used to determine the significant different across an entire group with one independent variable such as changes in one parameter for each group.

Group	Positive Voltage (V)	Negative Voltage (V)	Period (µs)	Number of Pulses	Positive Pulse Width (µs)	Negative Pulse Width (µs)	Polarity Delay (µs)	Agent added
1	20	75	200	1579x1	75	20	2	GFP
2	20	75	200	1579x2	75	20	2	GFP
3	20	75	200	1579x8	75	20	2	GFP
4	40	75	190	1579x8	75	20	2	GFP
5	40	75	210	1579x8	85	20	2	GFP
6	40	75	240	1579x8	100	20	2	GFP
7	50	75	210	1579x8	85	20	2	GFP
8	90	75	210	1579x8	85	20	2	GFP
9	120	75	210	1579x8	85	20	2	GFP
10	120	75	100	1579x8	20	20	2	GFP
11	120	75	100	1579x8	20	20	2	PI
12	120	75	150	1579x8	50	20	2	GFP
13	120	75	150	1579x8	50	20	2	PI
14	120	75	190	1579x8	75	20	2	GFP
15	120	75	190	1579x8	75	20	2	PI
16	120	0	10^6	16	100	1	1	GFP
17	0	0	0	0	0	0	0	GFP

Table 1: Experimental Parameters of GET of B16-F10 cells. Group 1-3 were treated at different number of pulses with GFP. Group 4-6 were treated with different positive pulse width at low voltage with GFP. Group 7-9 were treated with different voltages with GFP. Group 10, 12, & 14 were treated with various pulse width at high voltage with GFP. Group 11, 13, & 15 were treated with various pulse width at high voltage with PI. Group 16 was treated with trains of monophasic pulses with GFP. Group 17 was mixed with GFP but receive no pulse treatment.

CHAPTER III

RESULTS

Pulse Number Effects on Transfection Efficiency

The first set of groups was used to investigate the effect of biphasic pulses with changes in pulse number. The parameter with voltage of +20 V/-75 V, 200 us period, pulse width of +75 us/-20 us, 1579x8 pulses, and 2 us polarity delay, was used in another project within in the lab, that transfected plasmid encoding GFP through GET into rat skin. The project was conducted *in vivo* (unpublished data), thus the experiment for this study determined the effect of using less pulses *in-vitro*. Group 1 was treated with 1579 pulses. Group 2 was treated with 1579x2 pulses. Group 3 was treated with 1579x8 pulses. Based on the images shown in Fig 2A-C, it can be inferred that viability is high, but transfection is low. As shown in Fig 2D, there is a significant difference in transfection efficiency (p-value=0.0040). Tukey test shows that there is significant difference between Group 1 and the DNA only group with p-value of 0.190 and significant difference between Group 3 and DNA only group with p-value of 0.0033. Cell viability assay was used to calculate viability of group 1, 2, & 3. From Fig 2E, the viability trends downward as the number of pulses increases. One-way ANOVA showed a significant difference between all groups for viability (p-value=0.0002). Tukey Test shows the viability significant difference is between Group 1 and 3 with p-value of 0.0017. The viability significant difference is between Group 2 and 3 with p-value at 0.0049. The significant difference is between Group 2 and DNA only with p-value of 0.0437. Lastly the viability significant difference is between Group 3 and DNA only with p-value of 0.0002.

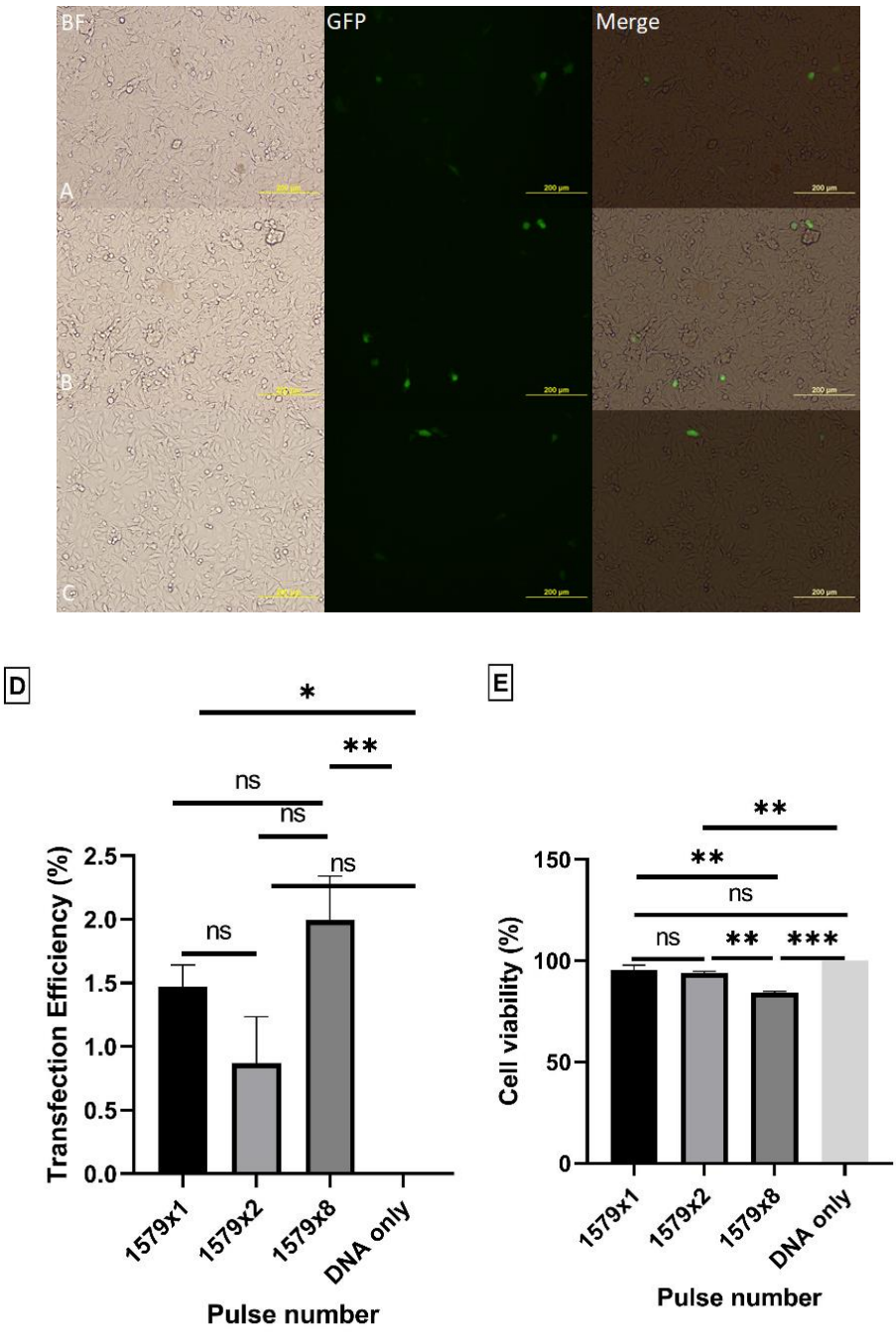


Figure 2: Increasing train of biphasic pulses has significant increase on transfection efficiency but reduces cell viability**.** All groups were treated with voltage of +20 V/-75 V, period of 200 us, pulse width of +75 us/-20 us and polarity delay of 2 us with GFP. A) Brightfield and fluorescence microscopy of Group 1. Group 1 was treated with 1579 pulses. B) Brightfield and fluorescence microscopy of Group 2. Group 2 was treated with 1579x2 pulses. C) Brightfield and fluorescence microscopy of Group 3. Group 3 was treated with 1579x8 pulses. D) Transfection efficiency was significantly difference between treatment. (One-way ANOVA p-value = 0.0.0040). E) Cell viability between group shows significant difference. Cell viability does decrease with increase in pulse number. (One-way ANOVA p-value =0.0002)

Pulse Width Effects on Transfection Efficiency

Groups 4, 5, and 6 were used to investigate the effect with changes in biphasic pulse width while at lower voltage. All groups mentioned were treated with voltage of +40 V/-75 V, negative pulse width of 20 us, 1579x8 pulses, and 2 us polarity delay. Group 4 was treated with +75 us pulse width and period of 190 us. Group 5 was treated with +85 us pulse width, and period of 210 us. Group 6 was treated with +100 us pulse width, and period of 240 us. As shown from the images in Fig 3A-C, cell viability is very high showing that increase in voltage affect cell viability with the remaining parameters being the same as the GET of rat skin project (unpublished data). Fig 3D shows significant difference in transfection efficiency with p-value of 0.0499 from an ANOVA analysis. Significant difference was determined with the Tukey test and it shows the significant difference is between Group 3 and DNA only with p-value of 0.0347. In Fig 3E, cell viability shows significant difference across the groups (p-value =0.0002). Using the Tukey Test, the significant difference is between Group 1 and 2 with (p-value = 0.0050), Group 1 and 3 with (p-value 0.0008), Group 2 and DNA (p-value = 0.0020), and Group 3 and DNA only (p-value = 0.0004).

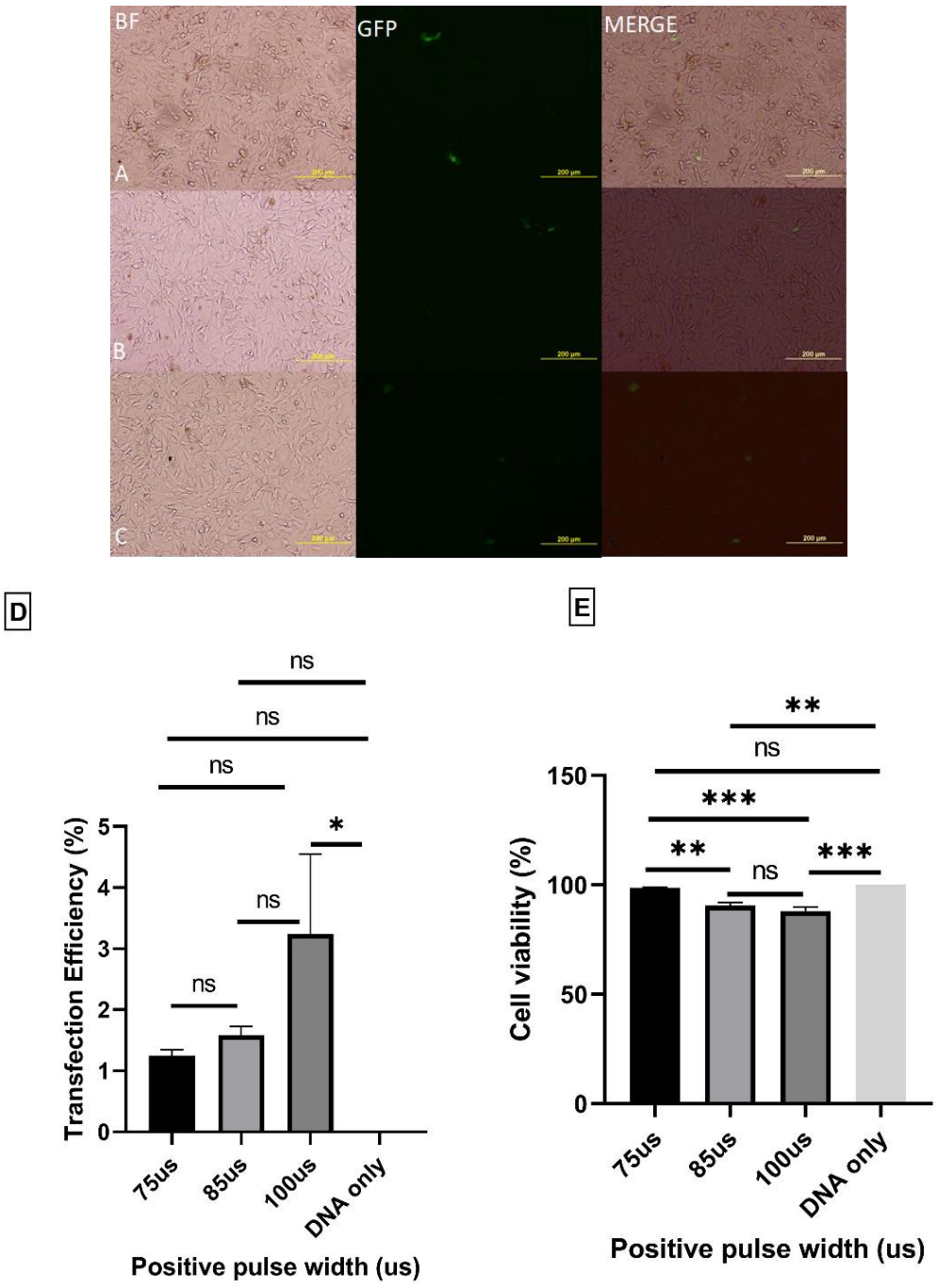


Figure 3: Increasing positive pulse width has significant change on transfection efficiency but reduces cell viability. All groups were treated with voltage of +40 V/-75 V, negative pulse width of 20 us, 1579x8 pulses and polarity delay of 2 us with GFP. A) Brightfield and fluorescence microscopy of Group 4. Group 4 was treated with +75 us pulse width and 190 us period. B) Brightfield and fluorescence microscopy of Group 5. Group 5 was treated with +85 us pulse width and 210 us period. C) Brightfield and fluorescence microscopy of Group 6. Group 6 was treated with +100 us pulse width and 240 us period. D) Transfection efficiency was significantly different between groups. (One-way ANOVA p-value = 0.0499). E) Cell viability between group shows significant difference. Cell viability decreases with increased positive pulse width. (One-way ANOVA p-value =0.0002)

Applied Voltage Effects on Transfection Efficiency

Groups 7, 8, and 9 were used to observe the effect of raising positive voltages of biphasic pulses. Each group were treated with negative voltage of -75 V, pulse width of +75 us/-20 us, 190 us period, and 2 us polarity delay. Group 7 was treated with +50 V amplitude. Group 8 was treated with +90 V amplitude. Group 9 was treated with +120 V amplitude. Amplitude was started off at +50 V vice +40 V since it was shown from previous experiment that it did not significantly affect transfection efficiency. From images in Fig 4A-C, it can be assessed that cell viability greatly diminish with increase voltages. In Fig 4C it is shown that the cell sample has decrease to an extremely low amount. Fig 4D, shows transfection efficiency of the groups. As presented, there are significant between the groups as transfection efficiency is significantly improving with increases in voltages (p-value=0.0002). Tukey test show the significant difference is between Group 7 and 9 (p-value = 0.0006), Group 8 and 9 with (p-value = 0.0093), Group 8 and DNA only (p-value = 0.0197), and Group 9 and DNA only (0.0002). In Fig 4E, cell viability is shown to be very significant (p-value <0.0001) with cell viability decreasing as voltage increases. Tukey test shows that the both comparison between Group 7 and 8 (p-value = 0.0050), Group 7 and 9 (p-value<0.0001), Group 8 and 9 (p-value<0.0001), Group 7 and DNA only (p-value = 0.0006), Group 8 and DNA only (p-value<0.0001), and Group 9 and DNA only (p-value<0.0001).

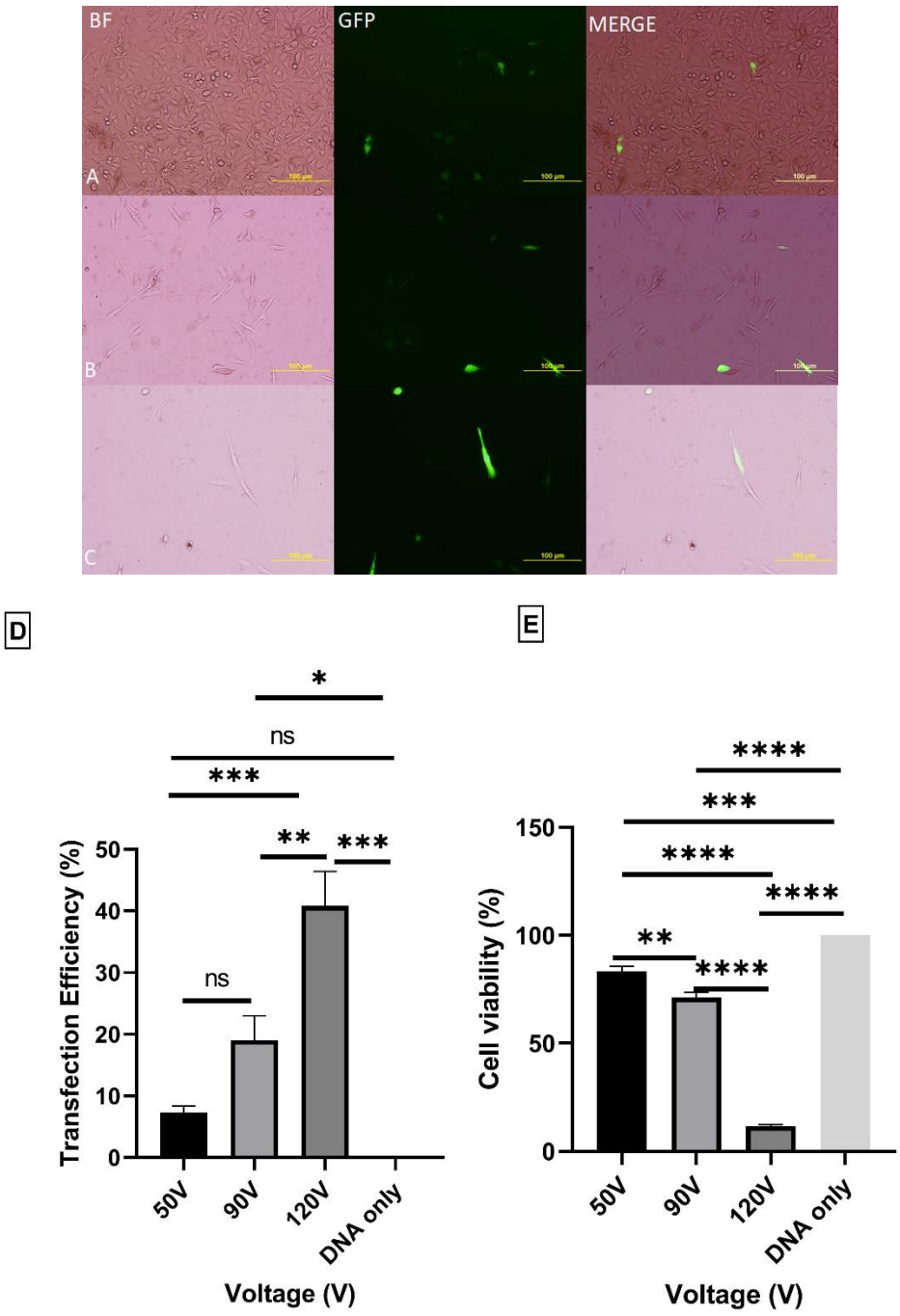


Figure 4: Increasing positive voltage increases transfection efficiency and reduces cell viability. All groups were treated with negative voltage of -20 V, pulse width of +85 us/-20 us, 1579x8 pulses, period of 210 us and polarity delay of 2 us with GFP. A) Brightfield and fluorescence microscopy of Group 7. Group 7 was treated with +50 V. B) Brightfield and fluorescence microscopy of Group 8. Group 8 was treated with +90 V. C) Brightfield and fluorescence microscopy of Group 9. Group 9 was treated with +120 V. D) Transfection efficiency significantly increase with raises in positive voltage. (One-way ANOVA p-value<0.0001). E) Cell viability between group shows significant difference. Cell viability decreases with increase in positive voltage, at 120 V cell viability is significantly low. (One-way ANOVA p-value <0.0001).

Pulse width Effects on Membrane Permeabilization

Group 11, 13, and 15 were used to determine if permeabilization does occur with determined parameters. PI is the agent used to determine if pore formation occurs. All groups were treated with voltage of +120 V/-75 V, negative pulse width of 20 us, 1579x8 pulses, and polarity delay of 2 us. Group 11 was treated with positive pulse width of 20 us and period of 100 us. Group 13 was treated with positive pulse width of 50 us and period of 150 us. Group 15 was treated with positive pulse width of 75 us and period of 190 us. The parameter was determined from previous experiment that yielded no transfection efficiency or low cell viability. With the previous experiments showing that high voltage yielded better transfection, but very low viability, pulse width was chosen to be tested. With high voltage, lower pulses were considered with respect to biphasic pulses. Images from Fig 5A-C shows that permeabilization does occur throughout all groups. Data from Fig 5D shows that permeability does decrease with pulses width, but the difference between the group is not significant (p-value=0.4519). Data from Fig 5E present significant difference among the groups for cell viability (p-value=0.0289). With the Tukey test, the significant difference was between group 11 and group 15 with a p-value of 0.372.

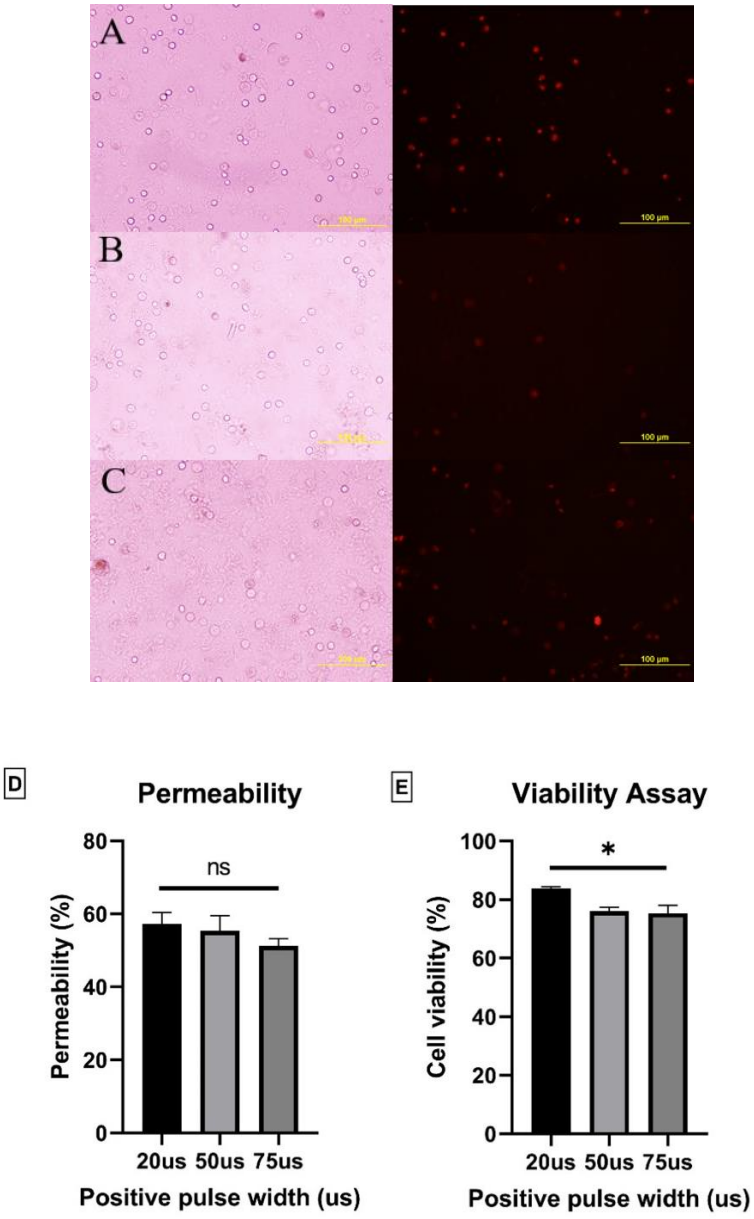
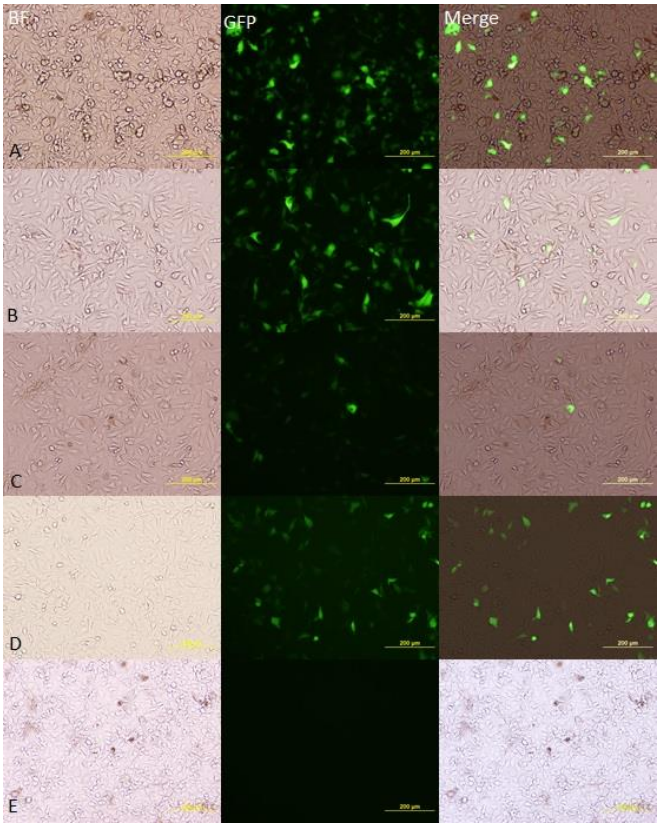


Figure 5: Increasing positive pulse width of biphasic pulses had no change on transfection efficiency but reduced cell viability**.** All groups were treated with voltage of +120 V/-75 V, negative pulse width of 20 us, 1579x8 pulses, and polarity delay of 2 us with PI. A) Brightfield and fluorescence microscopy of Group 10. Group 10 was treated with +20 us and 100 us. B) Brightfield and fluorescence microscopy of Group 11. Group 11 was treated with +50 us and 150 us period. C) Brightfield and fluorescence microscopy of Group 12. Group 12 was treated with +75 us and 190 us period. D) Permeability changes was not significant between all group with increasing positive pulse width. (One-way ANOVA p-value = 0.4519). E) Cell viability between group shows significant difference. Cell viability decreases with increase in positive pulse width. (One-way ANOVA p-value =0.0289).

Pulse Width with Higher Applied Voltage

Groups 10, 12, and 14 were used to investigate if transfection efficiency is improved with increasing pulse width at a high voltage. Each group had the same treatment as the previous experiment except GFP was used instead of PI. The groups were all treated with +120 V/-75 V voltage, negative pulse of 20 us, 1579x8 pulses, and polarity delay of 2 us. Group 10 was treated with +20 us and 100 us period. Group 12 was treated with +50 us and 150 us period. Group 14 was treated with +75 us and 190 us period. Group 16 was treated with monophasic pulses at +120 V/0 V voltage, 16 pulses, +100 us/-1 us pulse width, 10^6 us period, and polarity delay of 1 us. Group 16 served to be a positive control. Group 17 received no treatment and just had plasmid encoding GFP added. From the images in Fig 6A-C, it can be visually inferred that transfection has improved with lowering pulse width. Fig 6D shows images of Group 16 and that the positive control did provide a baseline of suitable transfection. Fig 6E shows Group 17, which is the negative control to provide that the cells with plasmid encoding GFP should not be fluorescing unless it was properly delivered, for this case through pulse electric field. Fig 6F shows how that increases in pulse width will significantly reduce GFP expression for the group treated with biphasic pulses (p-value<0.0001). Using the Tukey Test, there are significant difference between Group 10 and 14 (p-value<0.0001), Group 12 and 14 (p-value<0.0001), Group 10 and Monophasic (p-value<0.0001), Group 10 and DNA only (p-value<0.0001), Group 12 and Monophasic (p-value $= 0.0001$), Group 12 and DNA only (<0.0001), Group 14 and Monophasic (0.0041), Group 14 and DNA only (p-value $= 0.0277$), and Monophasic and DNA only (p-value<0.0001). In Fig 6G, cell viability is shown to significantly decrease with increase in pulse width (p-value<0.0001). Tukey Test shows significant difference between Group 10 and DNA only (p-value $= 0.0003$), Group 12 and DNA only (p-value<0.0001), Group 14 and DNA only (p-value<0.0001), and Monophasic and DNA only (p-value<0.0001).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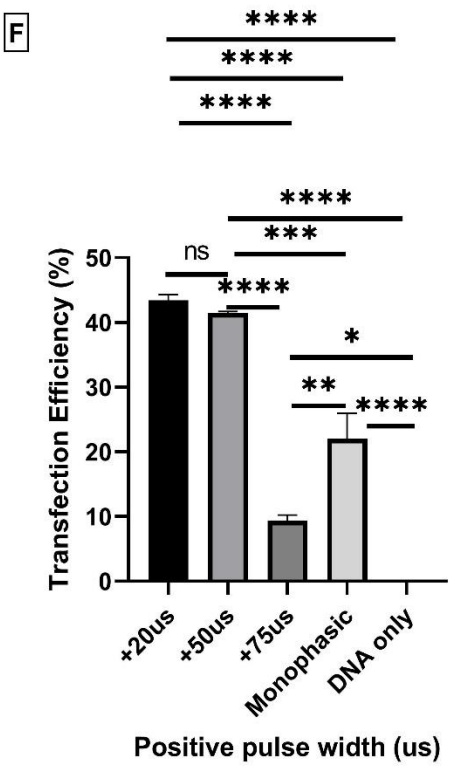
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Transfection Efficiency (%)
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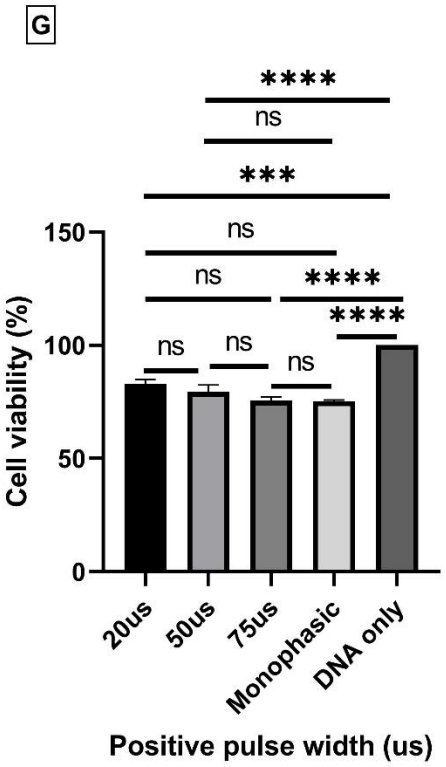
DNA only
Positive pulse width (us)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Cell viability (%)
150
100
50
0
20us
50us
75us
Monophasic
DNA only
Positive pulse width (us)

Figure 6: Increasing positive pulse width of biphasic pulses decreases transfection efficiency and decrease cell viability. All groups were treated with voltage of +120 V/-75 V, negative pulse width of 20 us, 1579x8 pulses, and polarity delay of 2 us with GFP. A) Brightfield and fluorescence microscopy of Group 13. Group 13 was treated with +20 us and 100 us. B) Brightfield and fluorescence microscopy of Group 14. Group 14 was treated with +50 us and 150 us period. C) Brightfield and fluorescence microscopy of Group 15. Group 15 was treated with +75 us and 190 us period. D) Brightfield and fluorescence microscopy of Group 16. Group 16 is induced with monophasic pulse with +120 V/0 V voltage, 16 pulses, pulse width of +100 us/-1 us, 10^6 us period, and 1 us polarity delay. Group 16 served as a positive control for the study. E) Brightfield and fluorescent microscopy of Group 17. Group 17 received no treatment and served as a negative control. F) Transfection efficiency changes significantly changed between all group with treated with biphasic pulses. As positive pulse width increases, transfection efficiency decrease (One-way ANOVA p-value < 0.0001). Comparison between the groups with monophasic pulses shows significant difference between the three biphasic group and monophasic group (One-way ANOVA p-value <0.0001) G) Cell viability between all group treated with biphasic pulses shows no significant with raises in positive pulse width (One-way ANOVA p-value = 0.1438). Comparison between the three biphasic group with the monophasic group shows no significant in viability with increasing pulse width (One-way ANOVA p-value<0.0001).

CHAPTER IV

DISCUSSION

The aim of this project is to determine if high transfection and high viability can be achieved using biphasic pulses as compared to monophasic. For this experiment, high transfection is considered high if transfection is higher than 30% transfection efficiency and cell viability is higher than 80%. Each group utilizes three cuvettes and three wells to test consistency of parameters. The experiment uses a monophasic pulse with parameters set with: voltage at +120 V and -0 V, +100 us and -1 us pulse width, period at 1 second, 16 pulses, and 1 us polarity delay as a positive control. The setting of negative pulse width and polarity delay is set to a value due to the B-10 Electrocell pulse generator requiring a minimum of 1 us for those setpoint, but without any negative voltage amplitude set, those values are negligible. In other studies, the biphasic pulses are typically conducted in burst meaning that a series of positive and negative alternating pulse in short succession, followed by a short break to the next series based on the settings. Due to the limitation of the B-10 Electrocell pulse generator, burst is not feasible. Therefore, for this study, the pulse is generated with a +20 us wave, a 2 us delay, a -20 us wave, and then followed by a 58 us delay until the next pulse for a period of 100 us.

Fig 2 shows that transfection efficiency does significantly improve with increasing pulse train and it does show that raising pulse train decrease the viability. The significant difference is only at the x1 train and the x8 train when comparing each to the DNA only which shows no transfection as expected. Therefore, transfection efficiency is enhanced compared to a naked DNA injection, but transfection efficiency is still very low with x8 train only being at 2%. Fig 3 shows that transfection efficiency does significantly improved with raising pulse width, but

viability does decrease with raising pulse width. Although the significant difference is only at 100us and DNA only. Since DNA only resulted in a transfection efficiency of 0, there is significant difference with 100 us yielding 3%, but the transfection is still considered too low. The pulses for groups shown in Fig 3 were decided based on data from the previous set of groups. From those data, voltage needed to increase to raise permeability to potentially improve transfection efficiency [19]. For this group the voltage was increase to 40 V, but the experiment to determine the effect of changes with positive pulse width with an increase in voltage. The results show very minimal increase in efficiency but a slight improvement from the previous conditions.

Fig 4 show that transfection efficiency does significantly improve with increase in voltage, but it shows that voltages do significantly decrease cell viability. This information shows that voltage plays a crucial role in transfection and play a role in reducing cell viability. The transfection efficiency increase is attributed to high power, but due to low population of cells, the results can be represented a lot better with an improvement in sample size. Fig 5 shows that the parameters being test induced permeabilization for all groups in that figure. The purpose of using PI is to determine if permeabilization occurs with the condition tested. Fig 6 shows that at high voltage, lowering pulse width does significantly improve transfection efficiency with improvement in cell viability. The parameter of +120 V/-75 V voltage, +20 us/-20 us pulse width, 100 us period, 1579x8 pulses, and 2 us polarity delay shows to yield the best result of high transfection efficiency and high viability. The condition from the biphasic group in Fig 6 shared the same parameter as the group with the condition using PI. The significant difference shown is between both the group that has pulse width of 20 us and 50 us as they both resulted in high transfection efficiency at 43% and 42% respectively.

In the study conducted by Tekle *et al*, they transfected NIH 3T3 fibroblast cells with plasmid DNA SV2-neo, which makes mammalian cells resistance to the antibiotic G418 [22]. From their data, the parameter that had best transfection for biphasic pulses compared to monophasic had cell viability of 62% for biphasic pulses. The fibroblast cells were exposed to voltage amplitude at +5.5 kV and -5.5 kV, 400 us pulse duration, at 60 kHz frequency, and no polarity delay, with electrode gap at 5 cm. Many studies have biphasic pulses parameter set to 100us pulse width and much lower voltage for tumor ablation [26][34][34][38]. The voltage density is very high at +1.1 kV/cm and –1.1 kV/cm and high pulse during at 400 us. But in this study, the parameter with high expression and viability has the voltage density at +1.2 kV/cm and -.75 kV/cm and pulse duration is 1.26 seconds total. The reason for this difference in cell viability is due to the lack of delay in Tekle's parameter. For biphasic pulses, transmembrane potential is not affected by the delay, but the nuclear envelope potential is. When the positive wave decays, a lack of decay means that the nucleus is still charged as the negative wave potential increase, thus potentially doubling the nuclear envelope potential in that one cycle [33]. Since there is still no delay between negative to positive wave, the nuclear envelope potential is still affected. With the current research, the delay between polarity is set to 2 us, which gives time for the nuclear envelope potential to zero out and the delay between the negative to positive wave allows the nuclear envelope to zero out before the next cycle.

In the study by Vuyst *et al*, the author references Tekle's result of having cell viability of 1-97% depending on the condition [20]. In Vuyst's study, ECV304 human bladder carcinoma cells were exposed to bipolar pulse of 50 kHz frequency, 2 ms pulse duration, and no delay followed by 10 kHz frequency, 1 second pulse duration, and no delay with varying voltages to transfect 6-CF fluorescent reporter dye [20]. Their best result with voltage density set to 1.2

kV/cm has 98% viability but their transfection of 6-CF is at 20%. Vuyst's study uses two different frequencies and two different durations to transfect their cells and those repeated with each pulse duration counted as a pulse for a total of 15 pulses [20]. The region of the short pulses uses 100 bipolar pulses at +10 us and -10 us pulse width and the long pulses uses 10 bipolar pulses at +50 ms and -50 ms pulse width. The contributing factor for their transfection efficiency is possibly due to the type of cells used, since B16-F10 murine melanoma cells have different morphology and impedance as ECV304 human bladder carcinoma cells. Vuyst's study has lower pulse number and high voltage density. Mainly in Vuyst's study they did not have a delay in between polarity, which allows for bipolar pulse cancellation to occur. At delay less than 50 us, biphasic pulses can cause bipolar pulse cancellation which will hinder uptake of molecules [41]. In this study, polarity delay is only 2 us, which makes the experiment subjected to bipolar cancellation as well. The power density between this study and Vuyst's study are the same, but the applied voltages are different. In this experiment the applied voltage is 120 V while in Vuyst's it is 60 V, which affects the cells closest to the electrodes. The electric field strength is a very important variable for permeabilization for transfection [19].

CHAPTER V

CONCLUSION

Biphasic pulses are typically used in tumor ablation due to the benefits of not causing muscle contractions during treatment, alleviating the need for paralytic agent and anesthetic [34]. Recent research has explored the possibility of using biphasic pulses for gene electrotransfer. Research conducted by Tekle *et al* has yielded high transfection efficiency with low cell viability in NIH 3T3 Fibroblast cells of 62% [22] and research conducted by Vuyst *et al* has yielded high cell viability of 98% and low transfection in ECV304 Human bladder carcinoma cells of only 20% [20]. The purpose of this study was to determine if high transfection and viability can be achieved with biphasic pulses as compared to monophasic. Initially the transfection efficiency was low with high viability both the pulse width and pulse train changes, but with extreme changes from the voltage change experiment, transfection was high with very low viability. The experiment with various pulse width at high voltage, shows that lower pulse width yielded high transfection at 43% with viability at 84%. Those results meet the goal of this study in which high transfection efficiency and viability was both achieved using biphasic pulses. In this current study, biphasic pulses have shown improved transfection efficiency with high viability for B16-F10 Murine melanoma cells. Further study of biphasic pulses can be conducted to evaluate the possibility of gene electrotransfer without eliciting an action potential.

CHAPTER VI

FUTURE WORK

From this study, setting the parameter to +120 V/-75 V amplitude, +20 us/-20 us pulse width, 2 us polarity delay, 100 us period, and 1579x8 pulses showed enhanced transfection efficiency of plasmid encoding GFP with high viability for B16-F10 cells using a B10 Bipolar pulse generator. Future studies can test these parameters on skeletal muscle cells with GFP to determine if high transfection and high cell viability will enhance with these parameters. In a study by Andre *et al*, they conducted a study using high voltage pulses followed by low voltage pulses for gene electrotransfer in B16-F10 cells, skeletal muscle cells, liver cells, and skin. The conclusion they drew was that DNA can easily allocate in muscle and reaches a plateau of luciferase expression at lower voltage (600 V/cm) than B16-F10 (800 V/cm) [23]. Muscle also has high longevity and can serve to provide long-term secretion of proteins for systemic effect [23].

Research involving biphasic pulses for irreversible electroporation finds therapeutic results similar to monophasic pulse without muscle contraction. C2C12 Murine skeletal muscle cells can be cultured in DMEM with 10% FBS until confluency at 40-50% confluency. At 90-100% confluency, these cells will differentiate on their own. Differentiation can be determined through immunohistochemistry. Immunohistochemistry for myosin can be done using anti-Fast Myosin skeletal heavy chain antibody. Myosin is a protein that is found in muscle that is responsible for muscle contraction. It is found in muscle cells once they are differentiated [44]. Transfection would be conducted *in-vitro* in a monolayer to observe transfection efficiency and cell viability. Adjustment have to be made for parameters as transfection to muscle cells is easier than tumor cells [23]. Once transfection efficiency and high viability is high, further

studies can determine if muscle contraction can be avoided with high transfection and high viability with C2C12 skeletal muscle progenitor cells seeded in a collagen sponge. Video microscopy can be used to determine if notable contraction occurs in the collagen sponge. In research it was determined that the polarity delay has a significant effect on the occurrence of muscle contraction on a swine model [32]. The experiment had the same positive and negative pulse width, positive and negative voltage, and pulse number. Raises in polarity voltage from 2 us to 5 us, or to 10 us yielded significant increase in muscle contraction [32]. The experiment had voltage a lot higher due to conducting irreversible electroporation. Bipolar pulse cancellation can be considered as the delay does affect bipolar pulse cancellation [41]. Bipolar pulse cancellation is the phenomenon where the second wave which is reverse polarity follows the first wave closely and lower the reduce the change in cell permeability. This is due to occlusion molecules being pushed back into the pore from the second wave, lowering the change in concentration inside the cell, restricting outside molecules from entering [41]. The study concluded that <50 us is when bipolar pulse cancellation is to occur [41].

Once contraction of skeletal muscle is determined to be avoided, gene delivery of plasmid *in-vivo*. The delivery can be done in a small animal model such as a rat. The rat model should switch from plasmid encoding GFP to plasmid encoding luciferase. GFP detection *in vivo* is difficult. Luciferase expression detection can be done on a live animal and can be done over a period of several months, thus reducing the number of animals needed for these experiments. Unlike fluorescence, luminescence is measured through a luminometer where the light that is emitted by the gene that reacts with an enzyme that can be administer on site. The luminometer contains photomultiplier tubes used to read the light signal and quantify using a software program [45]. During the experiment an accelerometer can be implemented at the site

of the pulsing to determine if any muscle contraction occurs. An accelerometer is used in various studies of biphasic irreversible electroporation to determine muscle contraction [38]. Once the plasmid encoding luciferase is delivered to the rat model, 24 hours should be given for the gene to express. After 24 hours, the rat model can be taken to IVIS luminometer where the reactive enzyme is administered for the luciferase protein to activate. After approximately 5 minutes have elapsed, the luminescence reading can be used to determine if the treatment is successful. The luminescence intensity will tell if the GET is a success. If the intensity is weak or does not exist, the reactive enzyme does not trigger the protein in the cell.

Once *in-vivo* experiment in the rat model concludes high transfection and no contraction, the experiment can be move into cardiomyocytes. Cardiomyocytes have limited cell renewal; therefore, high cell viability is very crucial [46]. After a myocardial infarction, the ischemic region of the heart is remodeled with fibroblast which deposits collagen in place of the cardiomyocyte, that results in scar formation that does not contract [47]. When cardiomyocytes are injured, they release an amount of cardiac troponin that can be found in the blood stream of a person suffering myocardial infarction [45]. ELISA can be used to detect level of Troponin T in the bloodstream. How ELISA works is it uses antigen and antibody interaction of the target proteins. The antibody used to bind directly or indirectly (binds to another conjugate that binds to the target) to the target will contain alkaline phosphatase or horseradish peroxidase where its signal can be read. Troponin T is the cardiac regulatory protein that governs the calcium facilitated interaction between the myosin and actin in the heart [48]. Like the previously mentioned procedure, luciferase should be delivered to the heart through GET. For this protocol, consideration should be made to the heartbeat of the rat model. When delivering the plasmid encoding luciferase to the rat model's heart, pulsing should be conducted while syncing to the R-

Wave of the electrocardiography (ECG) [47]. Research on pulse electric field has been conducted on or near the heart with respect to syncing to the R-wave of the ECG and has shown little to no animal model morbidity [37][47]. Blood can be checked for troponin T using ELISA to determine if cardiac injuries has occurred [47]. Luciferase will be measured like the skeletal muscle protocol.

If transfection is high, and troponin T level is low [47], the experiment can move towards pulsing during the T phase of the ECG to determine if an action potential occurs at the ventricle. The T-phase is the portion of the cardiac cycle where the ventricle hyperpolarized or relax, in which inducing an action potential at this phase can result in ventricular fibrillation [49]. If the rat model shows no sign of muscle contraction of the heart, a larger animal model can be utilize using bovines or porcine models.

Bovine and porcine models have hearts that can be comparable to humans. Using the parameters from this study, plasmid encoding luciferase should initially be transfected during the R-wave of the heart, while monitoring the ECG. If transfection is high, the cell viability is high, and the level of troponin in the blood is low, pulse with the parameters from this study during the T-phase of the ECG to determine if an action potential occurs. If large animal morbidity is low and unrelated to the pulsing parameters, further research can be conducted using other genes. In a study, gene transfer of phVEGF can be achieved using injection of naked DNA plasmid encoding with Vascular endothelial growth factor (VEGF). Vascular endothelial growth factor is a growth factor that promotes the formation of new blood vessels. The results were promising as the perfusion score has improved [16]. The issue is that naked DNA is known for having low efficiency and requires multiple application [4]. In another study, gene electrotransfer was used to transfect pVEGF-A into an ischemic heart. VEGF-A is a growth factor that promote

formation of new blood vessels, promotes stem cell mobilization and differentiation into cardiomyocytes, promote cardiomyocyte proliferation, and promotes embryonic stem cell differentiation in mouse [47]. The study induced trains of electrical pulses to the heart in sync with the R-wave of an ECG while administering the VEGF-A to the ischemic area. The result from that study shows that delivery of VEGF-A will reduce the size of the infract for an ischemic heart that received the treatment and that pulsing the heart in sync with the R-wave will safely deliver the gene [47]. Further research in this study can lead to gene delivery into skeletal muscle and cardiac muscle without the concern for muscle contraction.

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VITA

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