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Effects of Aspirin and its Derivatives in Combination with Electroporation for Drug Delivery in Cultured Cells

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by

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EFFECTS OF ASPIRIN AND ITS DERIVATIVES IN COMBINATION WITH ELECTROPORATION FOR DRUG DELIVERY IN CULTURED CELLS

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ABSTRACT

The purpose of this research was to investigate the effects that aspirin (ASA) and its metabolites, salicylic acid (SA) and acetic acid (AA), have on the delivery of drugs across biological barriers when used in conjunction with electroporation. Electroporation is a technique used to enhance drug delivery across bio-membranes in which a transmembrane potential is induced into cellular membranes, resulting in the creation of aqueous pores that allow molecules to pass through the otherwise impermeable barrier. Aspirin is a widely used drug that has been used for over a century and has been proven relatively safe at normal doses as indicated by the low number of reports of poisoning cases it has been involved in. Components of aspirin are known to soften the cellular membranes by solubilizing the cell's surface proteins.

B16F10 murine melanoma cancer cells were used in this investigation and treated with a 120μM buffered solution of calcein, a fluorescent indicator, in which the amount of delivered tracer molecules was measured using fluorescence. Identical concentrations of ASA and SA were investigated (1mM, 5mM, and 10mM) separately, focusing the effects concentration has electroporation delivery. Diluted acetic acid was also

investigated at pH values of 6.42, 5.36, and 4.40. The concentration of acetic acid that had the lowest pH and ASA with the highest concentration had the greatest impacts on the augmentation of calcein delivery. Therefore, this demonstrates that aspirin and acetic acid have the potential to improve targeted molecular delivery in combination with electroporation.

CHAPTER 1: INTRODUCTION

1.1 Introduction to Drug Delivery by Electroporation

Electroporation (E.P.) is a phenomenon in which biomembranes can enter a state of reversible electrical breakdown (REB) which allows external molecules to pass through the otherwise impermeable lipid phase of the cell membrane. REB is typically induced by applying an electrical potential to the cells which results in a temporary destabilization of the cell membrane and permeation through aqueous-filled pore openings [1, 2]. The transmembrane potential needed to rupture through a membrane bilayer is referred to as the electroporation threshold [3]. Although the mechanism by which this phenomenon occurs is not understood in its entirety, it is known that E.P. is the direct result of an induced transmembrane potential that is caused by the applied electric field [4]. Figure 1.1 shows the induced transmembrane potential in a cell exposed to electroporation. Pores form in regions of the membrane where the induced potential is large. Membrane resealing is a crucial phenomenon of electroporation. The level of membrane recovery is dependent on the amplitude and length of the applied pulses because there is a critical range in which the cell can undergo lysis, often referred to as irreversible membrane permeabilization [5]. The transient aqueous pore theory describes the combined effects of the electric field across a membrane and the associated thermal fluctuations [1, 2]. During the destabilization period, the cell membrane is highly permeable to exogenous molecules present in the surrounding media.

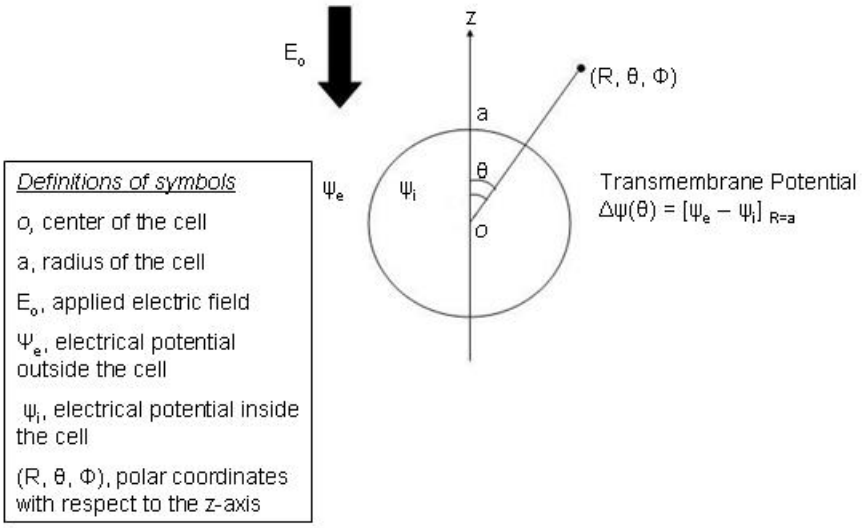

Figure 1.1 Transmembrane Potential Induced in a Spherical Cell by an External Electric Field [4]

There are several parameters that affect electroporation as well as the recovery of the membrane such as temperature [6], waveform parameters, the composition of the bilayer membrane and ionic and osmotic conditions [7, 8]. One of the most important parameters is the electric field strength, E, which is inversely proportional to the distance between the electrodes, *d,* with an applied voltage, V, (E = V/*d*). As the voltage is increased, the induced transmembrane potential is raised [4]. The result is the creation of pores and pore expansion due to structural alterations. In the case of an isolated spherical membrane, this phenomenon is described by the derived equation [9, 10]:

$$\Delta\psi(t) = 1.5E(t)a\cos\theta,$$

where $\Delta\psi$ is the induced transmembrane potential, θ is the angle between the applied electric field and the site on the membrane at which the potential is measured, and *a* is the radius of the cell. Once the membrane becomes porous, further increase in $\Delta\psi$ is counteracted by the ionic current through the pores [4].

Another critical parameter is the pulse time τ, which is derived from the total circuit resistance, R, and the capacitance, C, between the electrodes ($\tau = R \times C$). The pulse time is a major indication of how long the pores will stay opened. For mammalian cells, optimal pulse times have been found to range from tens of microseconds to milliseconds [11, 12]. Joule heating occurs as electrical current is applied and is linearly related to the square of the field intensity and pulse duration [13]. This condition is easier to control in *in vitro* studies by using a low ionic content pulsing buffer, whereas in *in vivo* studies, local heating is more difficult to avoid; therefore, by applying shorter pulse lengths, this effect can be minimized.

Electroporation has the potential to be used in biotechnological applications both *in vivo* and *in vitro*. Though many *in vivo* applications originate from *in vitro* experimentation, there are a variety of *in vitro* examples that capitalize on the effects of electroporation. These include loading genetic material and pharmaceutical drugs into cells, membrane protein insertion, and clinical applications such as cell-cell and cell-tissue fusion. Electroporation of tissue has recently become popular in the medical field due to its many possible applications such as cancer tumor therapy [14, 15], localized gene therapy [11, 16-18], and transdermal drug delivery [19, 20].

Electrochemotherapy is a novel therapy approach to enhance delivery of non-permeant chemotherapeutic agents to the cytosol of cells, with the assistance of locally applied electric fields to temporarily destabilize cell membranes. This form of therapy has shown to be effective in clinical trials for the treatment of tumors including melanoma, head and neck squamous cell carcinoma, basal cell carcinoma, Kaposi's sarcoma and adenocarcinoma [14, 15].

Gene therapy is a rapidly growing biotechnological field in which a gene delivery problem exists. The development of gene transfer methods (both *in vivo* and *in vitro*) include either the biological delivery approach of viral vectors or non-viral methods that involve chemical techniques such as lipofection [21], or physical techniques such as electroporation and gene gun transfection. Viral vectors are considered the most widely used method for transfection [9] but are often associated with immune responses; therefore, continued research in non-viral techniques is essential in order to enhance efficiency. In one study designed to compare the efficiency of *in vivo,* non-viral gene transfer methods, electroporation was found to be as successful and promising as lipofection, gene gun and direct DNA injection methods [11].

Transdermal delivery of drugs is yet another widely explored topic of in vivo electroporation applications. This route of delivery is a useful alternative to the several conventional routes of administration such as orals or injectables because it avoids degradation in the gastrointestinal tract and first-pass hepatic metabolism. Metoprolol, (a beta blocker) used in the treatment of angina, has shown successful results in a study investigating the mechanisms behind improved transdermal drug delivery by electroporation [19]. Reversible skin permeability, electrophoretic movement of the drug into the skin with applied pulses, and drug release from the skin reservoir as a result of electroporation are examples of mechanisms that link the linear correlation between pulse voltage and the quantity of drug delivered [4, 20].

Examples of *in vivo* electroporation applications include clinical treatments not only for humans, but for animals also. In a recent study [22], plasmid DNA encoding mycobacterial antigens was injected into the muscles of farm animals that are frequently

infected with *Mycobacterium bovis*. This bacterium causes bovine tuberculosis. It has been estimated that the economic losses due to tuberculosis in infected cattle alone is $3 billion annually. The DNA vaccine delivered by electroporation was reported to protect against bovine TB. The results from the study revealed an increased humoral immune response in goats and improved T-cell responses in cattle after the DNA vaccine was delivered using E.P. When compared to animals that did not receive electroporation, the vaccine was much more successful.

Unfortunately, there are undesirable side effects that are contributed to high-voltage permeation such as irreversible cellular damage [23]. Troiano et al [24] showed that by altering the cell's membrane, excessive damage to the cell may be prevented. The authors of the study added the nonionic surfactant, octaethyleneglycol mono *n*-dodecyl ether ($C_{12}E_8$) to absorb into a lipid bilayer membrane and found that using various concentrations of the surface active agent allowed electroporation at lower intensities and/or shorter pulse durations to reduce the electroporation threshold.

Surfactants are typically used in pharmaceuticals to provide increasing distribution and penetration to the cell's membrane by influencing the rate and extent of absorption of certain drugs. Though it has been shown that high concentrations of surfactants promote toxicity to the cell's structure by causing lysis and other irreversible damage to the membrane, Lee et al [25] has provided evidence that supports the use of the nonionic surface active agent poloxamer 188 (P188) in restoring electrical damage and resealing of electropermeabilized cells. Another group of scientists [26] concluded that co-injection of P188 with plasmid DNA helped facilitate gene transfer in skeletal

muscles, which supports one application of using surfactants as an excipient for intramuscular delivery of ions or water soluble species including drugs or naked DNA.

Just as surface-active agents are capable of solubilizing cell membranes, many pharmaceutical agents share the same phenomena by interacting with membranes due to their physiochemical properties. Amphiphilic, or hydrophobic, molecules undergo various mechanisms of self-association whose main site of action is by rapidly permeating the plasma membrane and/or accumulating in the hydrophobic interior of the lipid bilayer. They have been reported to self-associate and bind to the membranes first, causing disruption and solubilization, very similar to the action of detergents [27].

Amphiphilic compounds contain either an ionic or non-ionic polar head group with a hydrophobic portion. These properties allow the compounds to organize themselves as micelles or bilayers. Their molecular shape and hydrophilic-lipophilic balance (HLB) are factors that determine their tendency to form other structures which often is a function of pH, temperature, ionic strength, and concentration.

1.2 Aspirin and Its Derivatives

The structure of aspirin and two of its derivatives, acetic acid and salicylic acid, are shown in Figure 1.2. Aspirin and salicylic acid are hydrophobic compounds. When aspirin is exposed to water or moisture it will begin to hydrolyze, resulting in salicylic acid and acetic acid. The rate of hydrolysis that aspirin may undergo once dissolved in a basic solution is second order because it is dependent not only on the aspirin concentration, but on the pH of the solution [28].

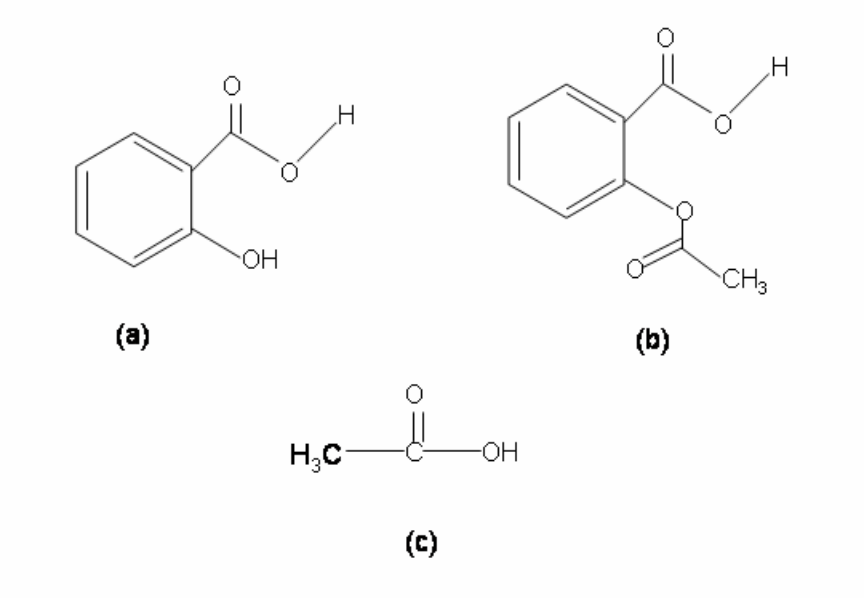

Figure 1.2 Chemical Structures of (a) Salicylic Acid (b) Acetylsalicylic Acid and (c) Acetic Acid [29]

1.2.1 Properties of Salicylic Acid

Salicylic acid is a crystalline organic carboxylic acid that is derived from the bark of the willow tree. Willow bark had been used for centuries in folk medicine in certain parts of the world as a pain relief treatment. It is soluble in alcohol, but only slightly soluble in water. It was thought to be too tough on the stomach, therefore it was reacted with acetic anhydryde as a buffering agent. In 1897, the German chemist and employee of Friedrich Bayer & Co., Felix Hoffman, was the first to market the buffered form of salicylic acid called acetylsalicylic acid [30].

Salicylic acid is thought to act by solubilizing the cell proteins that keep the stratum corneum intact, resulting in desquamation. Salicylic acid and its derivatives are toxic when consumed in large amounts, but is a popular choice as a beta hydroxy acid for acne-prone skin care, especially those with sensitive skin. Mixtures composed of 1-2%, aids in effectively penetrating and exfoliating the pores in the skin due to its larger

molecular size compared to its cousin, alpha hydroxy acid. At concentrations of 3-6%, it can begin to promote the skin to scale or peel off. A 50% concentration is used to eradicate warts and calluses, due to its tendency to destroy tissue at higher strengths [31].

1.2.2 Properties of Acetylsalicylic Acid

Acetylsalicylic acid, commonly known as aspirin is a derivative of salicylic acid. In the formation of ASA, the hydroxyl group of SA reacts with acetic anhydride, with sulfuric acid as a catalyst. In this condensation reaction, aspirin and acetic acid are the products. Repeated recrystallization is an effective purification method for impurities such as unreacted salicylic acid [29].

Aspirin is the acetate ester of salicylic acid, therefore it will hydrolyze to form acetic acid and salicylic acid. Both products are present to some extent in aspirin and a measure of their concentration can be indicative of the extent of hydrolysis, which will occur when aspirin is added to basic solutions with a pH of greater than 7.4 [20]. Its solubility in PBS (pH 7.2) is at least 3 mg/mL and is recommended to use within 30 minutes of preparation [28].

There is a wide variety of uses for aspirin, and is continuously investigated for more uses. Some indications include, but not limited to, the use as an analgesic, anti-inflammatory, antipyretic, anticoagulant and antirheumatic. It reduces fever by causing the blood vessels in the skin to dilate, allowing heat from the body to leave more rapidly [30]. It is also used as an additive in food, animal feed, drugs and cosmetics, and is now the active ingredient in more than 50 over-the-counter preparations.

1.2.3 Properties of Acetic Acid

Acetic acid is a clear, colorless chemical compound that is responsible for the characteristic odor and sour taste of vinegar. Glacial, or ice-like, acetic acid has a boiling point of 118°C, with a density of 1.049 g/ml at 25°C, and a flash point of 39°C. Its freezing point is 16.7°C, slightly lower than room temperature. It is classified as a weak acid due to its ability to not dissociate into its component ions when dissolved in aqueous solutions. Highly concentrated solutions of acetic acid are extremely corrosive, which can result in burns if contacted with skin surfaces [32].

Acetic acid is a derivative of aspirin, as mentioned earlier, and has several other pharmaceutical indications for both adults and children. When used topically, it can be useful for treating fungal infections, wound care (0.25% to 20% concentrations), iontophoreisis (2% to 5% concentrations), in diagnostic testing (3% to 5% concentrations) such as during a colposcopy or cervicoscopy, and for the use of otitis externa (for adults and pediatrics). At concentrations of 2%, acetic acid is often used as a household disinfectant, and is an effective method for cleaning and disinfecting respiratory equipment used in the care of cystic fibrosis. Acetic acid is also often used for urinary tract irrigations at a concentration of 0.25% [32].

A high-dose intra-tumoral acetic acid injection, for the treatment of hepatocellular carcinoma (HCC), is another form of acetic acid therapy. This method of injecting 3 to 5 ml of a 50% concentration solution has shown to effectively treat fewer than 4 small (< 3cm diameter) lesions. This treatment has been investigated for over a decade and when compared to a similar method, percutaneous ethanol injection (PEI) therapy, the acetic

acid injection has shown to be superior in that it has a lower recurrence rate of lesions (8% vs. 37%) and a higher 2-year survival rate of patients (92% vs. 63%) [33, 34].

CHAPTER 2: RESEARCH GOALS

It is clear that electroporation is an established and effective method of delivering molecules to cells. E.P. has proven to affect cellular membranes in many *in vivo* and *in vitro* biotechnological applications, which include treatments in humans and animals alike. The need for research to enhance the delivery of drugs, genetic material, vaccines, and other molecules beyond the established capabilities of electroporation is essential in the medical field. The benefits of electroporation come with some undesirable side effects. Irreversible damage to tissues and cells occur as a result of electrical stimuli.

The use of surfactants in combination with electroporation delivery has been explored because it can allow for lower electrical thresholds and shorter pulse durations. The addition of low concentrations of surfactants has also shown to enhance the permeation of molecules through cellular membranes. It has even been reported that certain surfactants can help restore electrical damage to membranes. Similar properties of surfactants exist in some pharmaceutical drugs categorized as amphiphilic, or hydrophobic compounds. These drugs have the potential to assist in the treatment of electroporation by enhancing the disruption of membranes and the formation of aqueous pathways, resulting in more efficient molecule delivery.

The purpose of this study was to determine the effects that aspirin and two of its derived components have on the delivery of molecules through the cell membrane alone

and in combination with electroporation treatment. Concentration and pH are factors that were investigated as well as the extent of recovery and swelling of cell membranes. The results are intended to provide insight to the potential of improving molecular delivery by electroporation. This study was designed specifically to determine if:

- ASA or 2 of its derivatives could facilitate molecular uptake through the cell membrane
- ASA and its derivatives could augment electroporation mediated delivery of non-permeant molecules
- cell membranes recover from treatment with ASA and its derivatives alone and when coupled with electroporation.

CHAPTER 3: MATERIALS & METHODS

3.1 Cell Preparation

3.1.1 Cell Line and Growth

B16-F10 mouse melanoma cells (ATCC #: CRL-6475) were obtained and grown in McCoy's Media (Cellgro Mediatech, Inc., Herndon, VA), adjusted to contain 10% fetal bovine serum (Cellgro Mediatech, Inc.) and (25mg) gentamicin 50mg/mL (Mediatech, Inc.), and were incubated in 5% CO_2 at 37°C. Serum was essential for many reasons. It provided hormonal factors that stimulated cell growth and function. It also contains essential proteins, amino acids, minerals, and lipids [35]. Gentamicin is an antibiotic used as a preventative measure to help reduce microbial growth and contamination.

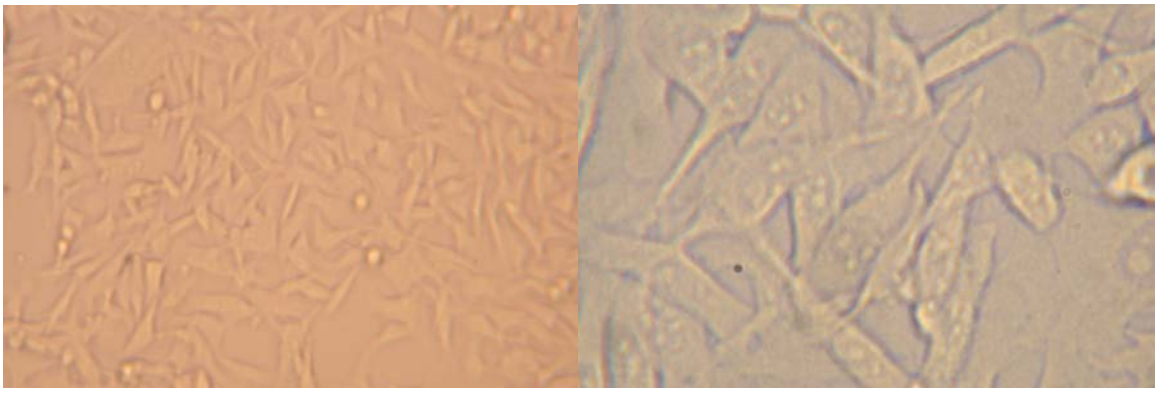

Figure 3.1 Untreated B16-F10 Cells in McCoy's Media (100X) (Left) and in PBS (200X) (Right)

B16F10 cells grow exponentially as adherent monolayers (Figure 3.1), and required fluid renewal and/or sub-culturing every 2 to 3 days using 0.25% trypsin-EDTA (Sigma Chemical Co., St. Louis, MO) for detachment.

3.1.2 Counting

After harvesting, the cells were washed 3 times by centrifugation. Cells were centrifuged (5810R, Eppendorf, Westbury, NY) at 220 × g for 5 minutes at 37°C and resuspended in approximately 2.5 ml of PBS for each wash. A sample of cells was then diluted in 0.9% sodium chloride (APP, Schaumburg, IL) and 0.4% trypan blue stain (Cellgro Mediatech, Inc.). Trypan blue is an indicator in which the membranes of non-viable cells are penetrated and can be distinguished from the viable cells. Using a hemacytometer (Hausser Scientific, Horsham, PA), the viable and nonviable cells were counted. The number of cells per milliliter was computed by multiplying the number of cells counted per square millimeter × the dilution (when used) × 10,000 (conversion factor). Only those cultures that resulted in 80% to 100% viability were used for experimentation.

3.1.3 Well Seeding

Experiments in this study were conducted using 48-well tissue culture plates (BD Falcon, Franklin Lakes, NJ) made from polystyrene. Each well held approximately 1.4 ml of liquid with a surface area of 0.75 cm^2. The depth and diameter of each well were 18 mm and 10 mm, respectively.

From experimentation, it was found that pre-treatment of the wells with a 0.1% gelatin (Acros, NJ, USA; Geel, Belgium) film coat reduced loosening of the cells after electrical stimulation. Under aseptic conditions, 150µl of the sterile gelatin solution was added to each well and let stand for 1 hour. The gelatin was then aspirated from each of the wells and air dried for 5 minutes. 500µl of McCoy's Media was added to the treated wells in addition to 7.5×10^4 viable cells and incubated at 37°C for 18 hours before treatment.

3.2 Electrode

Gilbert et al [36] designed and compared several innovative electrodes for use in electrochemotherapy treatment of murine B16 melanoma tumors. They found that a 6-needle array was the most successful when tested *in vivo*, measured statistically by a 97.1% complete response rate.

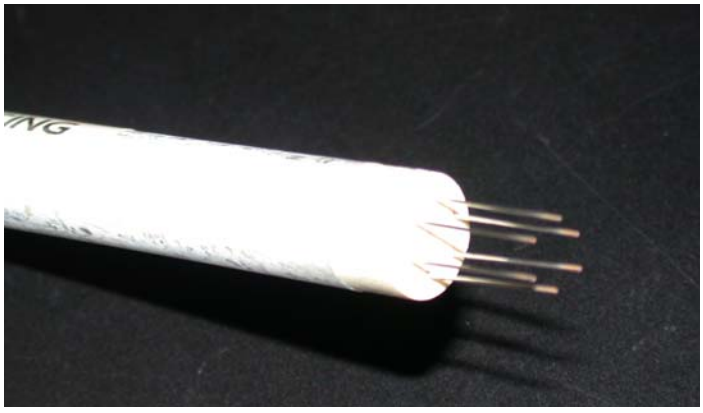

Figure 3.2 6-Prong Electrode Design

The electrode shown in Figure 3.2 was specially designed for all experimentation in this thesis and was similar to the design mentioned above. The electrode consisted of

6 stainless steel electrodes, equally spaced at 60° intervals around a 0.7 cm diameter circle. The needles extended 1.8 cm from the electrode body to fit precisely in the wells in order to set flush against the treatment/cell growth surface.

Cells in each well were treated with a total of 12 DC pulses. Figure 3.3 illustrates the sequence pattern used to energize specific needles, where each small circle represents the location of each needle. Pulses were directed to each needle by a rotary switch. Needles 1 & 2 were negative (represented as dark circles) for the first two consecutive pulses, whereas opposing needles 4 & 5 (represented as lightly shaded circles) were positive. This pattern was repeated for the next two pulses except the pattern was rotated one-needle clockwise. This clockwise rotation preceded each set of two pulses and was repeated until 12 pulses were delivered. This sequence was designed to treat 360° of the cell growth surface.

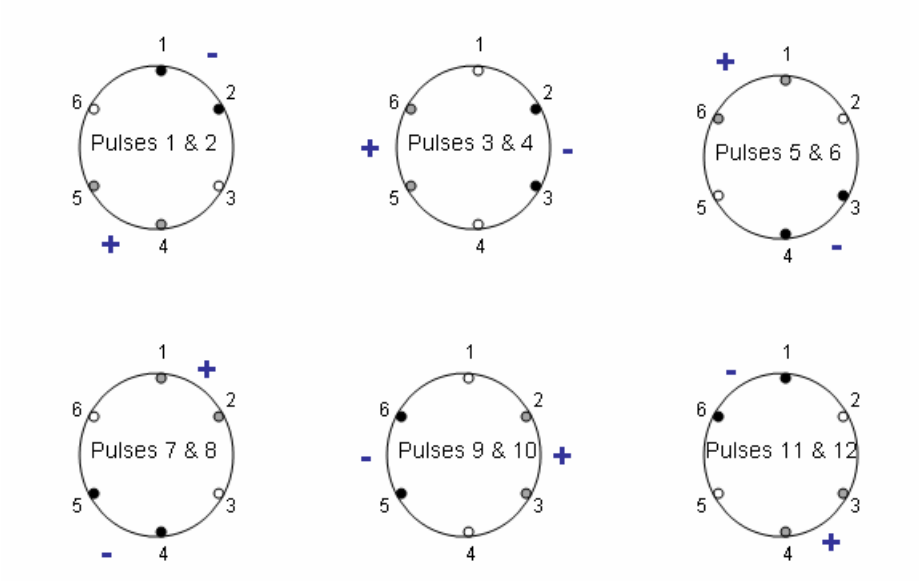

Figure 3.3 6-Needle Pulsing Sequence Used for Energizing Cells [36]

3.3 Testing Conditions & Experimental Protocol

A hot water bath (Isotemp 105, Fisher Scientific, Hampton, NH) was used during the treatment of the cultures to maintain cell viability and was set at 50°C. A sheet of Plexiglas was cut to fit over the water bath and was used as a surface for maintaining an approximate temperature of 37°C. All solutions used were allowed to reach room temperature (~22°C) to prevent thermal shock to the cells.

The absolute amplitude for the DC pulse generator (Transfector 800; BTX, Inc., San Diego, CA) ranged from 0-970 V, which corresponds to electric field intensities delivered between 0-1385 V/cm. The pulse width was set within optimal conditions at 99 μsec (maximum). Using short electrical pulses for cellular manipulation has the advantage of resulting in negligible thermal heating [12].

Each set of treatment conditions was tested in triplicate wells. 18 hours after seeding plates, each well was treated individually by aspirating the media, and quickly adding 150 μl of solution, just enough to coat the bottom of the well. For those conditions that required electric current, the electrode was placed down in the well so that the prongs set flush on the bottom to minimize any movement of the electrode (Figure 3.4). Electrical current was then applied in the manner that was described in Section 3.2.

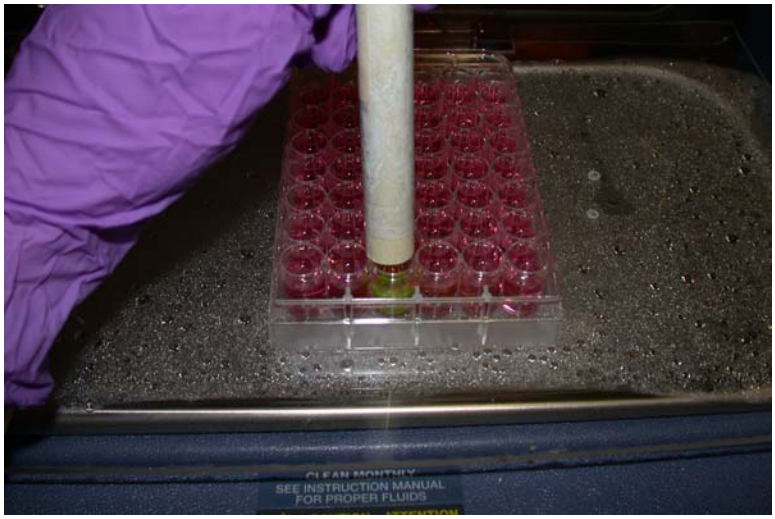

Figure 3.4 Demonstration of Electrical Treatment

After application of the pulses, cells were incubated for 1 hour at 37°C. This time allowed membrane resealing before the cells were carefully washed with 500 µl aliquots of PBS three times. Liquid from each wash was carefully and thoroughly aspirated. Then, each well was filled with 500 µl of PBS.

The presence of delivered calcein was observed under the microscope with the aid of a fluorescence microscope. A 0.9% sodium dodecyl sulfate (SDS) solution was then used to lyse the cells after the completion of the washing. SDS is a detergent that dissolves hydrophobic molecules, therefore when cells are incubated with an SDS solution, the membrane proteins and lipids denature and solubilize. 250 µl of the SDS solution was added to each of the wells containing PBS to yield a final concentration of 0.3%.

The contents of each well were transferred to a 5 ml FACS tube (BD Falcon). An additional 500 µl of PBS was added to each tube to increase sample volume. All samples were then centrifuged at 220 × g for 5 minutes at 37°C.

3.4 Fluorescence Measurements

Individual readings of each sample were taken using a fluorescence spectrometer (Perkin-Elmer LS-3B, Oakbrook, IL). The optimal excitation and emission wavelengths for calcein were found to be 488 and 515, respectively. The readings were made in a 1 cm^2 quartz cuvette, using a sample volume of 1 ml.

3.5 Membrane Recovery

The membrane integrity of the cells after electroporation treatment is an essential factor in determining the applications in which this procedure will be utilized. It is typically desired to preserve, as much as possible, the original cellular structures of these living membranes in order to obtain optimal post-treatment results. As mentioned earlier, cellular membranes can undergo REB in which the biological structures will eventually return to their normal state. Twenty four hours post-treatment, the fate of the cells can typically be determined. For the purpose of this study, optimal testing conditions were used to treat B16F10 cells to conclude the extent of reversible permeation.

Due to the large surface area of the wells, the cells were physically counted by observing 7 out of 21 fields of view along the horizontal diameter of each well at a magnification of 400× (see Figure 3.6). The recovery of the membranes after exposure to each treating condition was expressed as a percentage of live cells remaining after each hour.

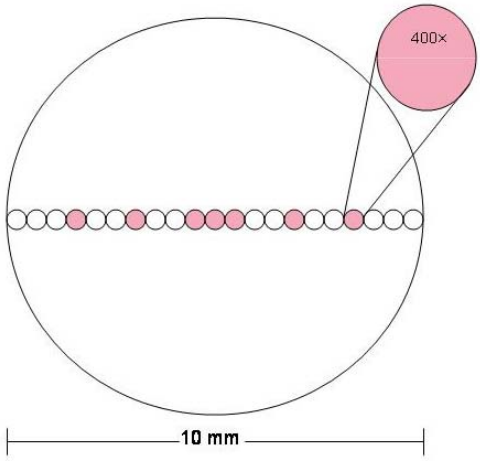

Figure 3.5 Fields of View for One Well at 400× Magnification. The shaded circles represent the fields of view selected for counting

In order to distinguish the viable cells from the dead, a 0.4% trypan blue solution in PBS was used. 150µl of the solution was added to each well for 1 minute, and then the cells were counted using the described method. Each test condition was studied at 1 hour intervals ranging from 1 hour to 4 hours. In addition, membrane recovery was examined 24 hours post treatment.

3.6 B16F10 Cell Size Measurements

The size and shape of the cells may alter when exposed to solutions that will penetrate the membranes during electroporation. Therefore some samples in this study were further investigated in order to determine the extent of change that occurred due to membrane swelling. Due to the variety of shapes the cells can take under the conditions of E.P., sizes of two axis were collected from cells for each treatment condition, the long axis (horizontal) and short axis (vertical). A stage micrometer was used to calibrate the

reticle in the eyepiece of the microscope to a standardized measurement scale at each magnification under a white light, where each unit on the reticle is configured to measure in micrometers.

3.7 pH Measurements

The three concentrations of ASA, SA, and AA had pH values that were determined. The pH measurements were made using a pH meter (Colloidal Dynamics, AZR2, Sydney, Australia), which was calibrated using 3 solutions with known pH values. The pH values of each solution were 4, 7, and 10.

3.8 Statistical Methods

To determine the statistical relevance of the data, a criterion for considering the mean data of one treatment condition as more successful than another had to be established. The null hypothesis used was that no change took place when considering one set of treatment parameters over another, and the alternative hypothesis used was that a significant change took place that resulted in different mean fluorescence values. The method used to test the null hypothesis was a two-tailed paired sample *t* test, with a level of significance of $\alpha = 0.05$. If the computed *t*-score was in between the critical values, then the null hypothesis would be accepted, whereas if the *t*-score was a value that lied outside of the critical value parameters, there would be significant evidence to allow for a conclusion that the treatments differed in their effectiveness.

CHAPTER 4: RESULTS & CONCLUSIONS

4.1 Effects of Salicylic Acid on Calcein Delivered by E.P.

The first set of experiments conducted in this investigation focused on the effects of salicylic acid on the delivery of calcein to B16F10 cells using electroporation. Three identical experiments were carried out. Figure 4.1 shows mean spectrofluorometric data for these experiments. All cells were exposed to 120 μM calcein. In addition, cells in some samples were exposed to one of three different concentrations of SA (1 mM, 5 mM, or 10 mM) with pH values of 6.70, 6.32, and 3.85, respectively. Pulses with one of four different field strengths were also applied (500, 750, 1000, or 1385 V/cm) to certain samples. SA concentration and field strength were experimental variables; however, some cells were not exposed to applied fields or SA for comparison/control purposes.

The results demonstrated that when cells were exposed to calcein and the three concentrations of SA (no applied electric field), SA did not augment the delivery when compared to samples exposed to calcein alone. Overall, the data for samples treated with calcein and either 1, 5, or 10 mM SA and electrical pulses were not significantly higher in fluorescent magnitude than any of the samples that were treated with calcein and pulses (no SA). Therefore, this suggests that SA is not a viable candidate for enhancing electroporative delivery of calcein.

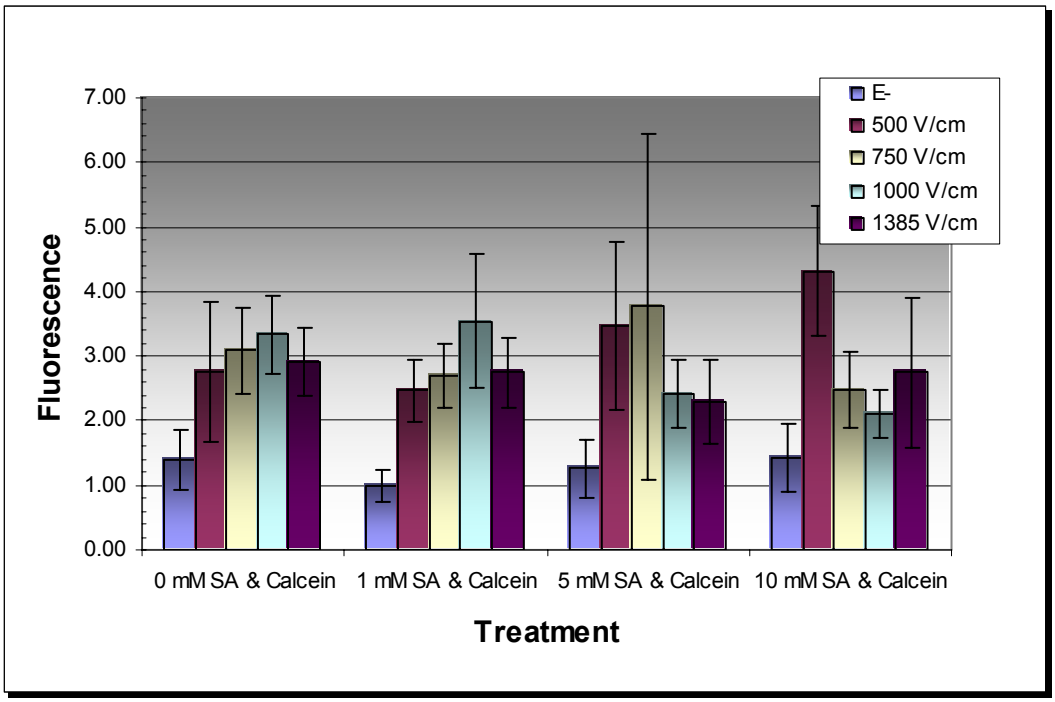

Figure 4.1 Electroporated Delivery with Salicylic Acid: Mean and S.E.M. of 3 Experiments

4.2 Effects of Acetylsalicylic Acid on Calcein Delivered by E.P.

The second set of experiments conducted in this investigation focused on the delivery of calcein using aspirin. The treatments were similar to those in section 4.1 in that three concentrations of aspirin were used (1 mM, 5 mM, or 10 mM) with pH values of 6.39, 5.40, and 4.40, respectively. The same range of pulsing conditions was used and the variables were identical for the sample treatment. Figure 4.2 shows the mean data from three replicate experiments. The data indicates that exposure to 1 mM, or 5 mM ASA (alone) did not result in a significant increase in fluorescence magnitude when compared to the treatment of 120 μM calcein alone (no ASA). In addition, samples treated with 1 and 5 mM ASA that were also exposed to electric fields had mean fluorescence magnitude that were not significantly different from those samples that were

exposed to electric fields alone (no ASA). In contrast, samples that were exposed to calcein in 10mM ASA (no pulses) had an average fluorescent magnitude of 4.28 (SEM = 0.63) and samples that were exposed to calcein (no ASA or pulses) had mean fluorescent magnitude 0.83 (SEM = 0.05). This 5-fold increase in internalized calcein that resulted from exposure to 10mM ASA was statistically significant when compared to the control sample ($p < 0.0001$). Figure 4.2 also demonstrates that 10 mM ASA assisted in the delivery of calcein at each of the four electric fields. As the applied field was increased, the fluorescence remained at an elevated magnitude relative to samples that were exposed to 10mM ASA (alone) and to samples that were exposed to same electric fields alone. At 500 V/cm (and 10mM ASA), there was a 3.7-fold increase in fluorescence magnitude relative to samples treated with calcein at 500 V/cm (no ASA). These same increases at 750, 1000, and 1385 V/cm were 3.82, 3.57, and 3.18-fold, respectively. The mean data with samples treated at each field were significantly different from analogous samples that did not include ASA. The p-values of these comparisons were < 0.0001 (500 V/cm), 0.0011 (750 V/cm), 0.0014 (1000 V/cm), and 0.0066 (1385 V/cm). Therefore, these data clearly show that 10 mM ASA alone can augment calcein delivery alone to the exterior of cells and also enhance delivery as a result of electroporation.

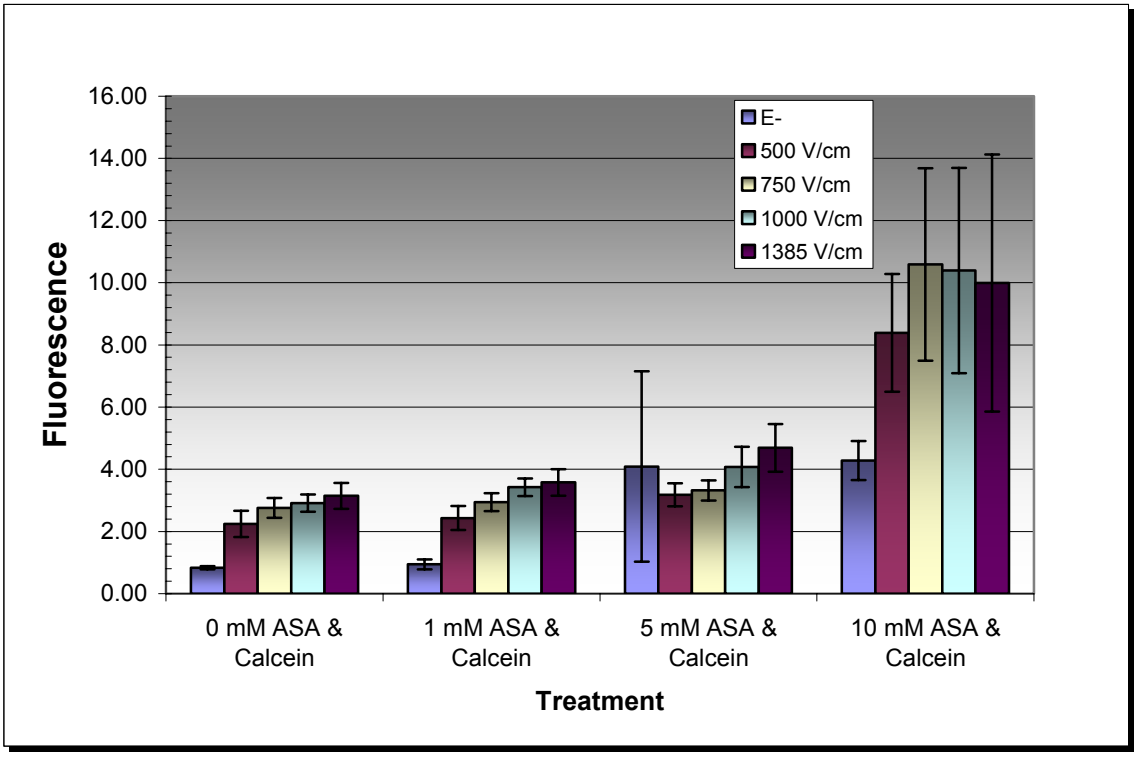

Figure 4.2 Electroporated Delivery with Acetylsalicylic Acid: Mean and S.E.M of 3 Experiments

4.3 Effects of Acetic Acid on Calcein Delivered by E.P.

The third set of experiments included the investigation of calcein delivery by E.P. using acetic acid as an adjuvant. The set of 3 replicate experiments included three concentrations of AA, each with a different pH (4.40, 5.36, or 6.42). The pH values of the three solutions of acetic acid were matched to those of ASA for the purpose of determining if AA is the derivative in ASA that enhances the delivery of calcein. The three concentrations of the AA solutions in PBS were similar, which was about 0.15%, and varied slightly in order to establish the desired pH value. In addition, one of three electric field strengths were applied to cells (500, 750, or 1000 V/cm). Some cells were

not exposed to applied fields or any concentration of AA for purposes of comparison and control.

Samples treated with acetic acid failed to show a significant increase in fluorescence magnitude at higher pH (5.36 or 6.42) regardless of whether or not pulses were applied, when compared to samples not exposed to AA. However, Figure 4.3 shows that acetic acid had a tremendous effect on the delivery of calcein, using a low pH solution (pH 4.40). The data for samples treated with AA alone (no pulses with a mean fluorescence magnitude of 1.04) was significantly different in mean fluorescence when compared to control samples that were not treated with AA or pulses (mean fluorescence magnitude of 8.57). This was an 8.2-fold increase. When electric fields were used in combination with the lowest pH solution of AA, fluorescent data was even higher than when this concentration of AA was used alone (no pulses).

The data for samples treated with AA and electrical pulses was significantly higher than any of the samples that were treated with calcein and pulses (no AA). At the lowest applied field, 500 V/cm, acetic acid augmented the resulting fluorescence magnitude by 3-fold. The mean fluorescence magnitudes were 12.77 for samples treated with AA (pH 4.40) and 4.19 for the samples that were not exposed to AA. This difference was significant ($p < 0.0001$). For the 750 V/cm samples, this same increase was 2.25-fold and was statistically significant ($p = 0.0002$). Similarily, there was a 3.5-fold increase for the 1000 V/cm samples. This increase was also significant ($p < 0.0001$).

As a result of this data, a low pH (4.40) solution of acetic acid is clearly a candidate to augment calcein for optimal E.P. delivery of calcein in B16F10 cells at any

of the three electric fields, though it is often desired to apply lower field strengths to minimize the possibility of cellular damage.

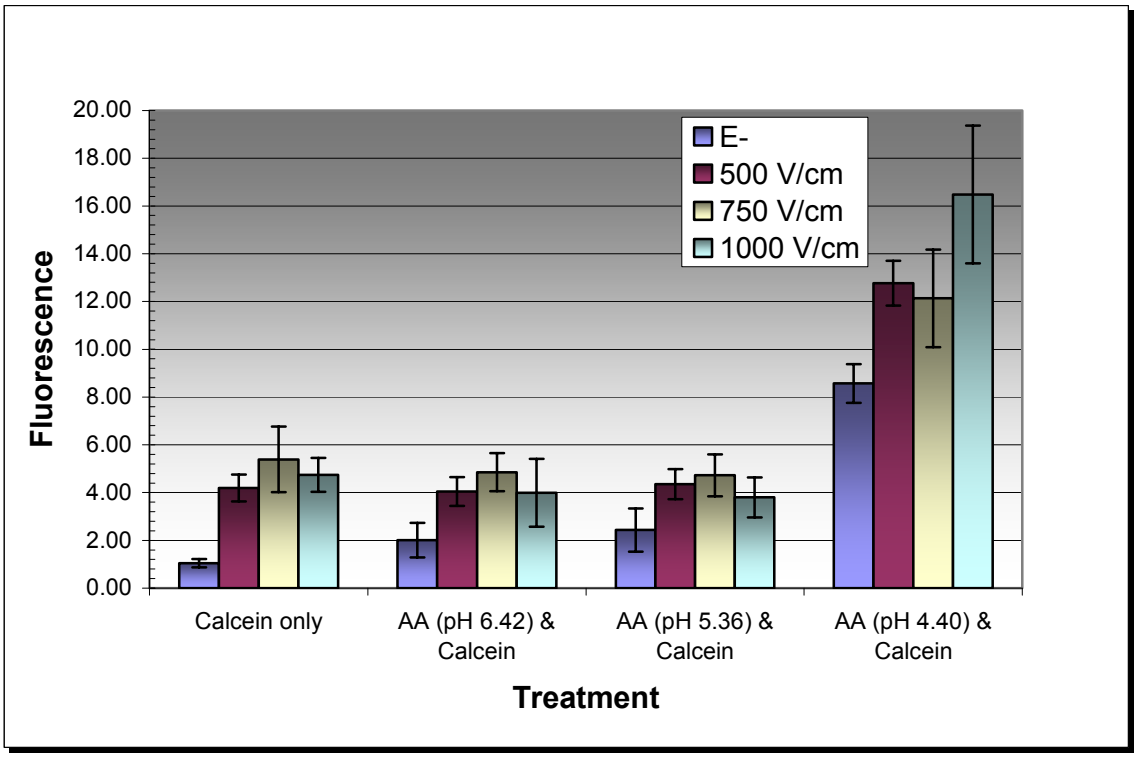

Figure 4.3 Electroporated Delivery with Acetic Acid: Mean and S.E.M of 3 Experiments

4.4 Membrane Recovery

Acetic acid was chosen as the optimal for this study, therefore, further investigation of the effects it had on cell membranes was carried out. An ideal method of delivery would result in cell membranes that are intact after the method was performed. The trypan blue test was used to determine membrane integrity as trypan blue dye will only penetrate into cells that have porous membranes. The electric field strength of 750 V/cm was applied to triplicate wells containing PBS alone as well as acetic acid (pH.4.40) alone. In addition, for comparison, individual treatments of PBS and acetic

acid (pH 4.40) without electrical pulses were investigated. The protocol used was similar to the methods described for the delivery of calcein, after one hour of exposure at 37°, the cells were washed three times with 500 µl aliquots of PBS. Liquid from the last wash was carefully aspirated, and then filled with 500 µl of growth media.

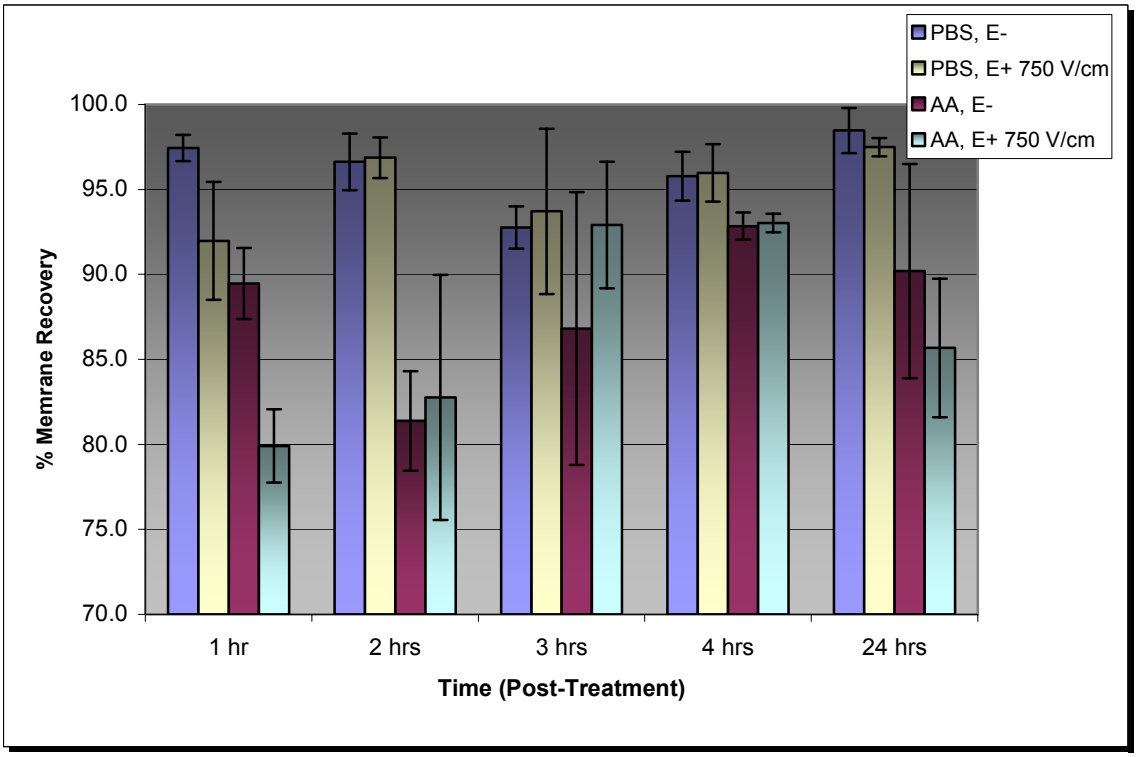

Figure 4.4 Membrane Recovery of Cells after Exposure: Mean and S.D. of 3 Experiments

Figure 4.4 illustrates the time duration of membrane recovery after exposure. Treatments with PBS alone (no pulses) and with 750 V/cm applied electric field yielded between 90-100% membrane recovery during the 24 hours of observation. However, acetic acid alone had a greater impact on the recovery of the cells, ranging from an average of 80-95% membrane integrity. At a time of 1 hour post-treatment for samples treated with acetic acid at 750 V/cm, there was a 17% decline in the number of cells with

intact membranes, implying that some of the cells either did not survive after exposure or was permeabilized. The number of cells that excluded the trypan blue dye then increased as the hours proceeded to a value of 86% after 24 hours. 90% of the cells exposed to AA alone had intact membranes 24 hours after treatment.

4.5 B16F10 Cell Sizes Post Treatment

After the cells had been exposed to any of the treatment conditions mentioned earlier, they varied slightly in shape and size due to membrane swelling as determined by direct observation. This phenomenon is an indication that fluid was being delivered through the cell's membrane. An investigation as to how much more swelling occurred at various conditions, compared to normal B16F10 cells, was conducted. Due to the nature of the shape and size changes, both horizontal (long axis) and vertical (short axis) dimensions were collected for 30 cells for each treatment condition at a magnification of 400×. The treatment conditions investigated consisted of PBS (no pulses), 10mM ASA (no pulses), 10mM ASA and pulses at 750 V/cm, 10mM SA (no pulses), 10mM SA and pulses at 750 V/cm, AA (pH 4.40) (no pulses), and AA (pH 4.40) and pulses at 750 V/cm. For comparison and control purposes, measurements of 30 untreated cells were taken.

The vertical axis of the untreated cells averaged 15μm, whereas the horizontal axis averaged 44.1μm. The samples of cells investigated at any of the other treatment conditions were exposed to the solutions and pulses (when applied) between approximately 1-10 minutes before the measurements were taken. Figure 3.5 shows the mean results of the measurements as well as the corresponding standard deviations. It

can clearly be seen that there is no significant difference in the measured dimensions at any condition, compared to the untreated cells, and is confirmed using the two-tailed paired sample *t* test at the 0.05 significance level.

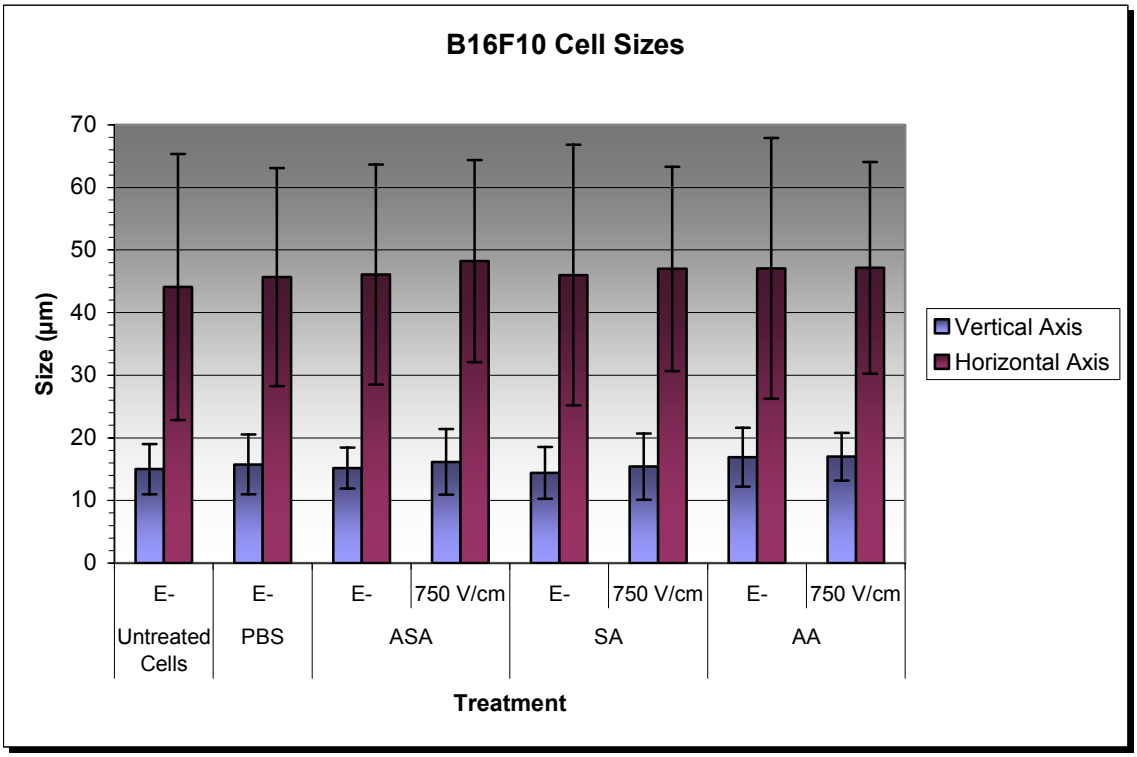

Figure 4.5 Vertical and Horizontal Axis of B16F10 Cells

4.6 Conclusions and Discussion

Aspirin and two of its derivatives were tested in this study, salicylic acid and acetic acid. Two of the three succeeded in delivering calcein to cells. ASA (10mM) and AA (pH 4.40) augmented the delivery of calcein at all 4 chosen electrical field strengths, but AA (pH4.40) was clearly a better candidate. The low pH solution of AA alone (no pulses) augmented delivery, indicating a strong effect on the membranes, and improved with the presence of pulses. When compared to any other treatment condition, including

those using ASA and SA, with or without the presence of electrical pulses, AA (pH 4.40) clearly resulted in higher mean fluorescence data. Surprisingly, SA did not show a great effect in calcein delivery, given by the significance of statistical *t* tests.

The fact that ASA and AA augmented delivery to the cells over SA may not be a coincidence. As mentioned earlier, ASA will hydrolyze to AA and SA derivatives. This is why acetic acid, in addition to SA, was chosen to be investigated. After ASA showed to be successful in experimentation, it had to be established whether or not the presence of AA was the reason of such a promising result.

A comparison of the data can help establish if pH alone is a determining factor of calcein delivery. As an outcome, acetic acid (pH 4.40) resulted in higher mean spectrofluorometric data than that of ASA and/or SA at all treatment conditions. The 0.15% concentration of AA (pH 4.40) had a higher pH value than 10 mM SA (pH 3.86), and was equal to the pH of 10 mM ASA (pH 4.40). This indicates that the successful delivery of calcein was not the direct effect of a low pH solution, and that 0.15% acetic acid with a pH of 4.40 inherently is the most optimal in delivering calcein to B16F10 cells by electroporation.

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APPENDICES

Appendix A: Data Collected for Salicylic Acid Trials

Table A.1 Salicylic Acid Delivery: Experiment 1

		Experiment 1				
	Treatment	*1*	*2*	*3*	*Average*	*Standard Deviation*
E-	Control	0.4	0.1	0.1	0.20	0.17
	CALCEIN	1.2	2.2	0.6	1.33	0.81
	1mM SA&CAL	0.6	0.6	1.3	0.83	0.40
	5mM SA&CAL	0.5	0.5	0.9	0.63	0.23
	10mM SA&CAL	3.2	2.4	2.1	2.57	0.57
500 V/cm	CALCEIN	0.8	1.8	1	1.20	0.53
	1mM SA&CAL	3.2	1.5	1.5	2.07	0.98
	5mM SA&CAL	2.8	8.8	4.3	5.30	3.12
	10mM SA&CAL	5.4	7	5.3	5.90	0.95
750 V/cm	CALCEIN	4.5	3.4	5.2	4.37	0.91
	1mM SA&CAL	1.7	2.3	4	2.67	1.19
	5mM SA&CAL	1.6	2.6	15.9	6.70	7.98
	10mM SA&CAL	0.9	1.2	2.3	1.47	0.74
1000 V/cm	CALCEIN	3	3.9	4.4	3.77	0.71
	1mM SA&CAL	2.9	3.5	8.2	4.87	2.90
	5mM SA&CAL	2.5	1.5	1.2	1.73	0.68
	10mM SA&CAL	3.3	1.7	1.1	2.03	1.14
1385 V/cm	CALCEIN	4.6	3.2	3	3.60	0.87
	1mM SA&CAL	4	4.1	3.4	3.83	0.38
	5mM SA&CAL	3.1	3.1	4.4	3.53	0.75
	10mM SA&CAL	2.9	1	3.8	2.57	1.43

Appendix A: (Continued)

Table A.2 Salicylic Acid Delivery: Experiment 2

		Experiment 2				
	Treatment	*1*	*2*	*3*	*Average*	*Standard Deviation*
E-	Control	0.2	0.1	0.1	0.13	0.06
	CALCEIN	2.3	1.1	2.7	2.03	0.83
	1mM SA&CAL	1.1	0.6	1.4	1.03	0.40
	5mM SA&CAL	1.5	2.7	2.2	2.13	0.60
	10mM SA&CAL	0.9	1	1	0.97	0.06
500 V/cm	CALCEIN	2.9	2.2	1.3	2.13	0.80
	1mM SA&CAL	2.9	3.1	2.6	2.87	0.25
	5mM SA&CAL	3.1	2.7	2.1	2.63	0.50
	10mM SA&CAL	4.3	2.4	6.1	4.27	1.85
750 V/cm	CALCEIN	1.9	1.7	2.9	2.17	0.64
	1mM SA&CAL	1.9	3.5	3.8	3.07	1.02
	5mM SA&CAL	1.8	1.1	2.5	1.80	0.70
	10mM SA&CAL	3.3	4.2	2.4	3.30	0.90
1000 V/cm	CALCEIN	2.4	2.2	2.3	2.30	0.10
	1mM SA&CAL	3	2.5	3.7	3.07	0.60
	5mM SA&CAL	1.3	3.8	3.2	2.77	1.31
	10mM SA&CAL	1.6	2.4	2.6	2.20	0.53
1385 V/cm	CALCEIN	3.5	1.2	2.2	2.30	1.15
	1mM SA&CAL	1.8	1.7	1.9	1.80	0.10
	5mM SA&CAL	2.5	1.3	1.6	1.80	0.62
	10mM SA&CAL	1.6	1.6	0.6	1.27	0.58

Appendix A: (Continued)

Table A.3 Salicylic Acid Delivery: Experiment 3

		Experiment 3				
	Treatment	1	2	3	*Average*	*Standard Deviation*
E-	Control	0.3	0.5	0.5	0.43	0.12
	CALCEIN	1.1	0.6	0.8	0.83	0.25
	1mM SA&CAL	0.6	1.8	0.9	1.10	0.62
	5mM SA&CAL	1.4	0.9	0.7	1.00	0.36
	10mM SA&CAL	0.7	0.5	1	0.73	0.25
500 V/cm	CALCEIN	6.3	4.6	3.9	4.93	1.23
	1mM SA&CAL	1.2	2.9	3.3	2.47	1.12
	5mM SA&CAL	2.5	4	0.9	2.47	1.55
	10mM SA&CAL	3.5	2.4	2.4	2.77	0.64
750 V/cm	CALCEIN	2.6	2.5	3.1	2.73	0.32
	1mM SA&CAL	2.4	2.8	1.9	2.37	0.45
	5mM SA&CAL	4.4	1.8	2.3	2.83	1.38
	10mM SA&CAL	3.1	2.4	2.6	2.70	0.36
1000 V/cm	CALCEIN	3.8	5.2	2.8	3.93	1.21
	1mM SA&CAL	3.2	2	2.9	2.70	0.62
	5mM SA&CAL	2.8	2.8	2.6	2.73	0.12
	10mM SA&CAL	1.9	1.9	2.4	2.07	0.29
1385 V/cm	CALCEIN	2.9	2.8	2.8	2.83	0.06
	1mM SA&CAL	2.9	2.6	2.3	2.60	0.30
	5mM SA&CAL	0.8	1.9	1.9	1.53	0.64
	10mM SA&CAL	7.1	2.2	3.9	4.40	2.49

Appendix A: (Continued)

Table A.4 Salicylic Acid Delivery: Average of 3 Experiments

		Experiments 1, 2 & 3		
	Treatment	Average	Standard Deviation	Standard Error Mean
E-	Control, PBS	0.26	0.17	0.10
	CALCEIN	1.40	0.79	0.46
	1mM SA&CAL	0.99	0.44	0.25
	5mM SA&CAL	1.26	0.77	0.45
	10mM SA&CAL	1.42	0.92	0.53
500 V/cm	CALCEIN	2.76	1.86	1.07
	1mM SA&CAL	2.47	0.83	0.48
	5mM SA&CAL	3.47	2.24	1.29
	10mM SA&CAL	4.31	1.74	1.00
750 V/cm	CALCEIN	3.09	1.15	0.66
	1mM SA&CAL	2.70	0.87	0.50
	5mM SA&CAL	3.78	4.64	2.68
	10mM SA&CAL	2.49	1.01	0.58
1000 V/cm	CALCEIN	3.33	1.05	0.60
	1mM SA&CAL	3.54	1.82	1.05
	5mM SA&CAL	2.41	0.90	0.52
	10mM SA&CAL	2.10	0.65	0.37
1385 V/cm	CALCEIN	2.91	0.92	0.53
	1mM SA&CAL	2.74	0.92	0.53
	5mM SA&CAL	2.29	1.11	0.64
	10mM SA&CAL	2.74	2.00	1.15

Appendix B: Data Collected for Acetylsalicylic Acid Experiments

Table B.1 Acetylsalicylic Acid Delivery: Experiment 1

		Experiment 1				
	Treatment	*1*	*2*	*3*	*Average*	*Standard Deviation*
E-	Control, PBS	0.4	0	0	0.13	0.23
	CALCEIN	0.3	0.7	0.5	0.50	0.20
	1mM ASA&CAL	0.5	0.6	0.8	0.63	0.15
	5mM ASA&CAL	0.7	28.5	1.4	10.20	15.85
	10mM ASA&CAL	3.9	5.2	6.4	5.17	1.25
500 V/cm	CALCEIN	1.9	1.2	1.4	1.50	0.36
	1mM ASA&CAL	2.3	2.7	2.8	2.60	0.26
	5mM ASA&CAL	2.3	4.4	4.1	3.60	1.14
	10mM ASA&CAL	9.9	12.7	13.9	12.17	2.05
750 V/cm	CALCEIN	3.2	3.3	3.5	3.33	0.15
	1mM ASA&CAL	3.7	2.8	3.4	3.30	0.46
	5mM ASA&CAL	1.8	3.3	4.1	3.07	1.17
	10mM ASA&CAL	13.8	15.1	20.9	16.60	3.78
1000 V/cm	CALCEIN	6.1	2.4	1.9	3.47	2.29
	1mM ASA&CAL	3.2	4.1	4.5	3.93	0.67
	5mM ASA&CAL	5.8	3.8	5.8	5.13	1.15
	10mM ASA&CAL	19.4	11.6	16.9	15.97	3.98
1385 V/cm	CALCEIN	4	4.1	3.8	3.97	0.15
	1mM ASA&CAL	3.5	3.9	3.9	3.77	0.23
	5mM ASA&CAL	8.1	3.5	5.6	5.73	2.30
	10mM ASA&CAL	16.1	17.6	18.5	17.40	1.21

Appendix B: (Continued)

Table B.2 Acetylsalicylic Acid Delivery: Experiment 2

		Experiment 2				
	Treatment	*1*	*2*	*3*	*Average*	*Standard Deviation*
E-	Control, PBS	0.3	0.3	0.2	0.27	0.06
	CALCEIN	0.7	1.3	0.6	0.87	0.38
	1mM ASA&CAL	0.8	1.8	0.5	1.03	0.68
	5mM ASA&CAL	1.8	1.1	0.8	1.23	0.51
	10mM ASA&CAL	2.7	2.5	4	3.07	0.81
500 V/cm	CALCEIN	2.7	1.3	2.8	2.27	0.84
	1mM ASA&CAL	3.1	2.9	3	3.00	0.10
	5mM ASA&CAL	1.9	1.8	3.6	2.43	1.01
	10mM ASA&CAL	4.6	7.2	7	6.27	1.45
750 V/cm	CALCEIN	2.4	3.2	2.5	2.70	0.44
	1mM ASA&CAL	1.6	3.4	4.5	3.17	1.46
	5mM ASA&CAL	5.1	4.3	2.5	3.97	1.33
	10mM ASA&CAL	11.1	7.2	8.3	8.87	2.01
1000 V/cm	CALCEIN	1.9	3.5	2.3	2.57	0.83
	1mM ASA&CAL	2.4	3	3.5	2.97	0.55
	5mM ASA&CAL	2.2	2.8	3.7	2.90	0.75
	10mM ASA&CAL	11.4	10.8	9.8	10.67	0.81
1385 V/cm	CALCEIN	1.9	2.4	4.2	2.83	1.21
	1mM ASA&CAL	3.1	7.1	2.4	4.20	2.54
	5mM ASA&CAL	2.5	3.1	4	3.20	0.75
	10mM ASA&CAL	12.1	6.1	10.2	9.47	3.07

Appendix B: (Continued)

Table B.3 Acetylsalicylic Acid Delivery: Experiment 3

		Experiment 3				
	Treatment	1	2	3	*Average*	*Standard Deviation*
E-	Control, PBS	0.1	0.3	0.1	0.17	0.12
	CALCEIN	0.4	0.6	0.6	0.53	0.12
	1mM ASA&CAL	1.3	0.5	1.7	1.17	0.61
	5mM ASA&CAL	0.6	1	0.9	0.83	0.21
	10mM ASA&CAL	2.5	4.3	7	4.60	2.26
500 V/cm	CALCEIN	5.6	2	1.3	2.97	2.31
	1mM ASA&CAL	1.1	2	2	1.70	0.52
	5mM ASA&CAL	4.5	2.8	3.2	3.50	0.89
	10mM ASA&CAL	6.1	7.9	6.2	6.73	1.01
750 V/cm	CALCEIN	2.4	1.9	2.4	2.23	0.29
	1mM ASA&CAL	2.2	3.4	1.5	2.37	0.96
	5mM ASA&CAL	2.6	3.7	2.5	2.93	0.67
	10mM ASA&CAL	4.8	4.2	9.9	6.30	3.13
1000 V/cm	CALCEIN	3.2	2.8	2.1	2.70	0.56
	1mM ASA&CAL	4.5	3.1	2.5	3.37	1.03
	5mM ASA&CAL	4.5	3.9	4.2	4.20	0.30
	10mM ASA&CAL	4	4.3	5.3	4.53	0.68
1385 V/cm	CALCEIN	3	2.5	2.4	2.63	0.32
	1mM ASA&CAL	2.5	2.6	3.2	2.77	0.38
	5mM ASA&CAL	4.4	3.4	7.6	5.13	2.19
	10mM ASA&CAL	3.4	1.2	4.7	3.10	1.77

Appendix B: (Continued)

Table B.4 Acetylsalicylic Acid Delivery: Average of 3 Experiments

		Experiments 1, 2 & 3		
	Treatment	Average	Standard Deviation	Standard Error Mean
E-	Control, PBS	0.19	0.07	0.04
	CALCEIN	0.63	0.20	0.12
	1mM ASA&CAL	0.94	0.28	0.16
	5mM ASA&CAL	4.09	5.30	3.06
	10mM ASA&CAL	4.28	1.09	0.63
500 V/cm	CALCEIN	2.24	0.73	0.42
	1mM ASA&CAL	2.43	0.67	0.38
	5mM ASA&CAL	3.18	0.65	0.37
	10mM ASA&CAL	8.39	3.28	1.89
750 V/cm	CALCEIN	2.76	0.55	0.32
	1mM ASA&CAL	2.94	0.50	0.29
	5mM ASA&CAL	3.32	0.56	0.32
	10mM ASA&CAL	10.59	5.36	3.10
1000 V/cm	CALCEIN	2.91	0.49	0.28
	1mM ASA&CAL	3.42	0.49	0.28
	5mM ASA&CAL	4.08	1.12	0.65
	10mM ASA&CAL	10.39	5.72	3.30
1385 V/cm	CALCEIN	3.14	0.72	0.42
	1mM ASA&CAL	3.58	0.74	0.42
	5mM ASA&CAL	4.69	1.32	0.76
	10mM ASA&CAL	9.99	7.16	4.14

Appendix C: Data Collected for Acetic Acid Experiments

Table C.1 Acetic Acid Delivery: Experiment 1

		Experiment 1				
	Treatment	*1*	*2*	*3*	*Average*	*Standard Deviation*
E-	Control	0.2	0.1	0.2	0.17	0.06
	CALCEIN	1.1	1.6	1.4	1.37	0.25
	Acetic Acid pH 6.42	3.3	2.4	4.7	3.47	1.16
	Acetic Acid pH 5.36	4.8	4.7	2.4	3.97	1.36
	Acetic Acid pH 4.40	7.5	10	8.1	8.53	1.31
500 V/cm	CALCEIN	4.9	4	4.5	4.47	0.45
	Acetic Acid pH 6.42	3.5	4.7	4.8	4.33	0.72
	Acetic Acid pH 5.36	4.7	3.5	4	4.07	0.60
	Acetic Acid pH 4.40	12.1	13.1	10.8	12.00	1.15
750 V/cm	CALCEIN	4.7	2.3	2.8	3.27	1.27
	Acetic Acid pH 6.42	3.5	4.1	4.7	4.10	0.60
	Acetic Acid pH 5.36	5.7	3.8	4.7	4.73	0.95
	Acetic Acid pH 4.40	18.3	17.4	12.8	16.17	2.95
1000 V/cm	CALCEIN	6.1	4.7	4.9	5.23	0.76
	Acetic Acid pH 6.42	4.2	9.8	5.5	6.50	2.93
	Acetic Acid pH 5.36	5.6	5.5	4.4	5.17	0.67
	Acetic Acid pH 4.40	17.5	17	21.3	18.60	2.35

Appendix C: (Continued)

Table C.2 Acetic Acid Delivery: Experiment 2

		Experiment 2				
	Treatment	*1*	*2*	*3*	*Average*	*Standard Deviation*
E-	Control	0.4	0.2	0.2	0.27	0.12
	CALCEIN	0.8	1	0.8	0.87	0.12
	Acetic Acid pH 6.42	0.8	1.2	1.2	1.07	0.23
	Acetic Acid pH 5.36	1.5	3.5	2.2	2.40	1.01
	Acetic Acid pH 4.40	8.4	8.8	11.1	9.43	1.46
500 V/cm	CALCEIN	4.6	3.1	2.7	3.47	1.00
	Acetic Acid pH 6.42	2	4.9	5.1	4.00	1.73
	Acetic Acid pH 5.36	4.9	4.8	5.3	5.00	0.26
	Acetic Acid pH 4.40	12.9	13.7	12.6	13.07	0.57
750 V/cm	CALCEIN	7.4	9.5	7.8	8.23	1.12
	Acetic Acid pH 6.42	5.2	3.8	5.7	4.90	0.98
	Acetic Acid pH 5.36	6.8	5.9	5.4	6.03	0.71
	Acetic Acid pH 4.40	9.7	8.5	11	9.73	1.25
1000 V/cm	CALCEIN	4.8	4.4	2.6	3.93	1.17
	Acetic Acid pH 6.42	3.6	2	2.4	2.67	0.83
	Acetic Acid pH 5.36	3.5	0.7	3.4	2.53	1.59
	Acetic Acid pH 4.40	21.1	20.7	20.3	20.70	0.40

Appendix C: (Continued)

Table C.3 Acetic Acid Delivery: Experiment 3

		Experiment 3				
	Treatment	1	2	3	*Average*	*Standard Deviation*
E-	Control	0.3	0.5	0.5	0.43	0.12
	CALCEIN	1.1	0.7	0.9	0.90	0.20
	Acetic Acid pH 6.42	1.5	1.6	1.4	1.50	0.10
	Acetic Acid pH 5.36	0.8	1.2	0.8	0.93	0.23
	Acetic Acid pH 4.40	6.2	8	9	7.73	1.42
500 V/cm	CALCEIN	5.6	3.3	5	4.63	1.19
	Acetic Acid pH 6.42	2.9	4	4.5	3.80	0.82
	Acetic Acid pH 5.36	2.4	3.6	6	4.00	1.83
	Acetic Acid pH 4.40	10.6	16.1	13	13.23	2.76
750 V/cm	CALCEIN	4.4	4.8	4.8	4.67	0.23
	Acetic Acid pH 6.42	5.2	3.6	7.9	5.57	2.17
	Acetic Acid pH 5.36	5.3	2.6	2.3	3.40	1.65
	Acetic Acid pH 4.40	8.7	10.9	11.9	10.50	1.64
1000 V/cm	CALCEIN	6	6	3.2	5.07	1.62
	Acetic Acid pH 6.42	3.5	3	1.9	2.80	0.82
	Acetic Acid pH 5.36	3.6	3.2	4.3	3.70	0.56
	Acetic Acid pH 4.40	10.5	9.4	10.5	10.13	0.64

Appendix C: (Continued)

Table C.4 Acetic Acid Delivery: Average of 3 Experiments

		Experiments 1, 2 & 3		
	Treatment	Average	Standard Deviation	Standard Error Mean
E-	Control, PBS	0.29	0.15	0.08
	CALCEIN	1.04	0.30	0.17
	Acetic Acid pH 6.42	2.01	1.26	0.73
	Acetic Acid pH 5.36	2.43	1.57	0.91
	Acetic Acid pH 4.40	8.57	1.42	0.82
500 V/cm	CALCEIN	4.19	0.98	0.56
	Acetic Acid pH 6.42	4.04	1.05	0.61
	Acetic Acid pH 5.36	4.36	1.09	0.63
	Acetic Acid pH 4.40	12.77	1.63	0.94
750 V/cm	CALCEIN	5.39	2.38	1.37
	Acetic Acid pH 6.42	4.86	1.38	0.80
	Acetic Acid pH 5.36	4.72	1.53	0.88
	Acetic Acid pH 4.40	12.13	3.54	2.04
1000 V/cm	CALCEIN	4.74	1.23	0.71
	Acetic Acid pH 6.42	3.99	2.46	1.42
	Acetic Acid pH 5.36	3.80	1.46	0.84
	Acetic Acid pH 4.40	16.48	5.00	2.89

Appendix D: Data Collected for Membrane Recovery

Table D.1 Membrane Recovery after 1 Hour

1 HOUR POST-EXPOSURE

		Live	Dead	Total Count	% Dead	% Viable	SD
PBS (no pulses)	1	59	1	60	1.7	98.3	
	2	64	2	66	3.0	97.0	
	3	65	2	67	3.0	97.0	
	average	*62.7*	*1.7*	*64.3*	*2.6*	*97.4*	*0.8*
AA (no pulses)	1	43	4	47	8.5	91.5	
	2	62	9	71	12.7	87.3	
	3	103	12	115	10.4	89.6	
	average	*69.3*	*8.3*	*77.7*	*10.5*	*89.5*	*2.1*
PBS, 750 V/cm	1	54	7	61	11.5	88.5	
	2	57	5	62	8.1	91.9	
	3	63	3	66	4.5	95.5	
	average	*58.0*	*5.0*	*63.0*	*8.0*	*92.0*	*3.5*
AA, 750 V/cm	1	53	12	65	18.5	81.5	
	2	67	16	83	19.3	80.7	
	3	55	16	71	22.5	77.5	
	average	*58.3*	*14.7*	*73.0*	*20.1*	*79.9*	*2.2*

Table D.2 Membrane Recovery after 2 Hours

2 HOURS POST-EXPOSURE

		Live	Dead	Total Count	% Dead	% Viable	SD
PBS (no pulses)	1	56	2	58	3.4	96.6	
	2	59	1	60	1.7	98.3	
	3	57	3	60	5.0	95.0	
	average	*57.3*	*2.0*	*59.3*	*3.4*	*96.6*	*1.7*
AA (no pulses)	1	36	10	46	21.7	78.3	
	2	63	14	77	18.2	81.8	
	3	58	11	69	15.9	84.1	
	average	*52.3*	*11.7*	*64.0*	*18.6*	*81.4*	*2.9*
PBS, 750 V/cm	1	44	2	46	4.3	95.7	
	2	50	1	51	2.0	98.0	
	3	63	2	65	3.1	96.9	
	average	*52.3*	*1.7*	*54.0*	*3.1*	*96.9*	*1.2*
AA, 750 V/cm	1	58	7	65	10.8	89.2	
	2	45	15	60	25.0	75.0	
	3	58	11	69	15.9	84.1	
	average	*53.7*	*11.0*	*64.7*	*17.2*	*82.8*	*7.2*

Appendix D: (Continued)

Table D.3 Membrane Recovery after 3 Hours

3 HOURS POST-EXPOSURE							
		Live	Dead	Total Count	% Dead	% Viable	SD
PBS (no pulses)	1	51	4	55	7.3	92.7	
	2	54	5	59	8.5	91.5	
	3	47	3	50	6.0	94.0	
	average	*50.7*	*4.0*	*54.7*	*7.2*	*92.8*	*1.2*
AA (no pulses)	1	95	5	100	5.0	95.0	
	2	64	10	74	13.5	86.5	
	3	45	12	57	21.1	78.9	
	average	*68.0*	*9.0*	*77.0*	*13.2*	*86.8*	*8.0*
PBS, 750 V/cm	1	56	2	58	3.4	96.6	
	2	55	2	57	3.5	96.5	
	3	37	5	42	11.9	88.1	
	average	*49.3*	*3.0*	*52.3*	*6.3*	*93.7*	*4.9*
AA, 750 V/cm	1	57	2	59	3.4	96.6	
	2	74	9	83	10.8	89.2	
	3	66	5	71	7.0	93.0	
	average	*65.7*	*5.3*	*71.0*	*7.1*	*92.9*	*3.7*

Table D.4 Membrane Recovery after 4 Hours

4 HOURS POST-EXPOSURE							
		Live	Dead	Total Count	% Dead	% Viable	SD
PBS (no pulses)	1	64	4	68	5.9	94.1	
	2	59	2	61	3.3	96.7	
	3	55	2	57	3.5	96.5	
	average	*59.3*	*2.7*	*62.0*	*4.2*	*95.8*	*1.4*
AA (no pulses)	1	45	3	48	6.3	93.8	
	2	72	6	78	7.7	92.3	
	3	49	4	53	7.5	92.5	
	average	*55.3*	*4.3*	*59.7*	*7.2*	*92.8*	*0.8*
PBS, 750 V/cm	1	58	3	61	4.9	95.1	
	2	47	1	48	2.1	97.9	
	3	56	3	59	5.1	94.9	
	average	*53.7*	*2.3*	*56.0*	*4.0*	*96.0*	*1.7*
AA, 750 V/cm	1	55	4	59	6.8	93.2	
	2	57	4	61	6.6	93.4	
	3	73	6	79	7.6	92.4	
	average	*61.7*	*4.7*	*66.3*	*7.0*	*93.0*	*0.5*

Appendix D: (Continued)

Table D.5 Membrane Recovery after 24 Hours

24 HOURS POST-EXPOSURE

		Live	Dead	Total Count	% Dead	% Viable	SD
PBS (no pulses)	1	137	0	137	0.0	100.0	
	2	122	3	125	2.4	97.6	
	3	89	2	91	2.2	97.8	
	average	*116.0*	*1.7*	*117.7*	*1.5*	*98.5*	*1.3*
AA (no pulses)	1	49	10	59	16.9	83.1	
	2	62	5	67	7.5	92.5	
	3	95	5	100	5.0	95.0	
	average	*68.7*	*6.7*	*75.3*	*9.8*	*90.2*	*6.3*
PBS, 750 V/cm	1	52	1	53	1.9	98.1	
	2	68	2	70	2.9	97.1	
	3	70	2	72	2.8	97.2	
	average	*63.3*	*1.7*	*65.0*	*2.5*	*97.5*	*0.5*
AA, 750 V/cm	1	60	7	67	10.4	89.6	
	2	74	12	86	14.0	86.0	
	3	57	13	70	18.6	81.4	
	average	*63.7*	*10.7*	*74.3*	*14.3*	*85.7*	*4.1*

Appendix E: B16F10 Cell Sizes Post Treatment

Table E.1 B16F10 Cell Sizes

	Untreated Cells		PBS (no pulses)		10mM ASA (no pulses)		10mM ASA 750 V/cm		10mM SA (no pulses)		10mM SA 750 V/cm		AA (pH4.40) (no pulses)		AA (pH4.40) 750 V/cm	
	V	*H*	*V*	*H*	*V*	*H*	*V*	*H*	*V*	*H*	*V*	*H*	*V*	*H*	*V*	*H*
1	12.5	57.5	12.5	50	12.5	50	12.5	45	10	82.5	12.5	45	20	25	20	40
2	22.5	25	15	62.5	20	37.5	15	47.5	12.5	40	10	35	22.5	30	12.5	37.5
3	15	25	12.5	50	17.5	60	10	60	12.5	50	12.5	60	22.5	40	17.5	62.5
4	12.5	57.5	17.5	65	12.5	30	12.5	50	12.5	75	15	52.5	20	55	20	75
5	15	50	10	60	20	55	15	42.5	10	35	12.5	42.5	12.5	60	12.5	22.5
6	20	25	10	17.5	15	30	15	52.5	15	80	12.5	47.5	20	40	17.5	40
7	12.5	70	15	42.5	12.5	55	12.5	45	15	52.5	10	45	12.5	77.5	12.5	55
8	12.5	15	25	45	12.5	17.5	12.5	40	12.5	35	12.5	40	10	80	20	42.5
9	22.5	82.5	17.5	65	25	37.5	10	75	10	55	10	75	12.5	65	12.5	37.5
10	15	65	25	55	15	35	22.5	37.5	12.5	65	22.5	37.5	15	57.5	17.5	25
11	15	17.5	15	72.5	17.5	45	15	57.5	10	15	15	55	12.5	70	17.5	75
12	17.5	52.5	30	47.5	12.5	50	25	77.5	12.5	62.5	22.5	77.5	12.5	20	12.5	52.5
13	15	57.5	12.5	40	17.5	77.5	12.5	65	12.5	60	15	65	12.5	40	25	42.5
14	20	35	15	40	12.5	52.5	22.5	45	20	25	20	45	12.5	25	17.5	75
15	12.5	15	20	52.5	17.5	45	10	25	20	65	12.5	25	17.5	72.5	15	37.5
16	25	70	12.5	20	15	30	20	47.5	10	20	17.5	47.5	17.5	35	12.5	45
17	15	22.5	12.5	52.5	12.5	25	17.5	22.5	12.5	35	10	30	25	85	17.5	57.5
18	12.5	30	10	65	15	22.5	15	50	12.5	20	12.5	55	15	22.5	22.5	55
19	17.5	30	15	10	15	65	15	80	25	25	15	80	12.5	20	15	75
20	12.5	65	17.5	40	15	25	20	72.5	22.5	70	17.5	67.5	17.5	52.5	12.5	65
21	12.5	47.5	15	20	12.5	75	15	40	20	60	15	22.5	15	22.5	17.5	25
22	20	25	12.5	35	12.5	60	22.5	65	15	40	17.5	65	27.5	35	20	30
23	10	80	15	65	22.5	35	12.5	45	12.5	25	12.5	30	25	25	20	60
24	15	27.5	12.5	40	12.5	37.5	10	35	12.5	50	10	37.5	20	65	17.5	70
25	15	60	12.5	45	15	25	10	42.5	12.5	17.5	15	47.5	15	22.5	15	47.5
26	12.5	75	20	80	15	77.5	15	20	15	62.5	12.5	20	15	60	15	22.5
27	10	30	17.5	30	12.5	72.5	25	37.5	22.5	25	30	42.5	20	62.5	25	35
28	10	55	10	40	12.5	62.5	20	22.5	12.5	35	20	22.5	12.5	70	15	25
29	12.5	40	20	17.5	12.5	35	30	62.5	17.5	22.5	30	55	12.5	52.5	12.5	47.5
30	10	15	17.5	45	15	57.5	15	40	12.5	75	12.5	40	22.5	25	22.5	35
Avg.	15.00	44.08	15.75	45.67	15.17	46.08	16.17	48.25	14.42	46.00	15.42	47.00	16.92	47.08	17.00	47.17
S.D.	3.99	21.26	4.79	17.43	3.28	17.58	5.24	16.15	4.14	20.81	5.30	16.33	4.72	20.80	3.79	16.93