Healing in 3D Skin Equivalent by the Induction of a Regenerative Response through the Application of External Stimuli

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ABSTRACT

Wound healing in mammals is a complex biological process whose main goals are to close the wound quickly, inhibit blood loss, and fight infection. The skin left after a wound healing process will never achieve the same level of functionality or tensile strength as undamaged tissue. In contrast to mammalian models, the wound response observed in some amphibian species results in the replication of fully functional tissue. The goal of this study was to induce, through the application of external stimuli, a regenerative response in mammals to mimic that of regenerative species and provide a new technology for wound care. This study developed a 3D *in vitro* skin model in which the effects of applied electric fields and membrane potential altering solutions on improved wound healing were assessed. An electrode system was designed to measure relevant electrical properties of the model and histology and wound closure rates were analyzed as parameters for improved wound healing after treatment. Preliminary results proved the model to be effective for the testing of these parameters. Further optimization of these treatments could provide an effective new wound care technology that uses the body's own machinery to provide a simple, yet complete healing of the wound.

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1. SIGNIFICANCE

Foot ulcerations are the most common cause of hospitalization in diabetic patients, as diminished circulation and nerve function in extremities result in increased susceptibility to chronic, non-healing wounds. A recent study reports that the United Sates healthcare system spends an estimated 3 billion dollars annually on venous ulcers related to diabetes (Bergan *et al*. 2006). Chronic wounds like these ulcers require extensive medical treatment and patient compliance in that treatment and can lead to life-threatening infections and amputations; they can significantly affect the patient's quality of life. Treatment of chronic wounds presents a large burden on the American healthcare system and improving wound care treatments is an increasing area of medical research. New techniques must be developed to help decrease this economic burden and increase the quality of life of patients who suffer from chronic wounds.

Extensive wound damage leaves exposed tissue susceptible to infections, decreasing wound closure time and adding strain to the healing system. One of the most common and successful treatments today for non-healing wounds is the replacement of the tissue through skin grafts. Autologous grafting and allografts are used to replace damaged tissue with replacement skin either donated from healthy skin on the patient or from another donor. Although often a successful method when non-healing wounds require tissue implantation, autologous grafts use a sample of the patient's skin elsewhere on the body, resulting in additional wound sites. Allografts require the availability of a donor and the use of immunosuppressants. Both are expensive, susceptible to rejection, and report long healing times (Fonder, 2008). More recent studies have developed an artificial skin graft composed of two layers of non-living tissue that are seeded with the

patient's endothelial cells to grow new tissue (Integra, 2010). Although these treatments are of great aid to patients with severe tissue damage in improving their quality of life, they are also very expensive, often rely on foreign cells or proteins (i.e. bovine collagen) and are still unable to restore full functionality of the tissue (Fonder, 2008).

In addition, these treatments provide a resulting tissue with compromised functionality. After significant tissue damage as a result of infection, burns, or amputation, the body begins a wound healing process to close off the injured tissue from the external environment. The result of this process is the deposition of fibrous scar tissue (disordered collagen) at the wound site, leaving tissue with compromised functionality and decreased levels of tensile strength as compared to intact tissue. The collagen left after the wound healing process lacks a necessary order of organization seen in undamaged tissue (Levin, 2007). In order to recreate undamaged tissue, one would ideally induce a regeneration process modeled after the regenerative capacity of some amphibian species. Previous studies have begun to examine the impact of applied current flow on the induction of a regeneration pathway in normally non-regenerative species (Levin *et al*. 2010). This study will design a treatment that provides a unique combination of depolarization agents in conjunction with an applied electric field to a mammalian full-thickness wound site in order to induce and examine a regenerative response in the injured tissue. Using the body's own machinery to regenerate native tissue through the application of external stimuli would provide a simple, yet complete healing of the wound. This approach would result in a significant step forward in wound healing, as it would provide an inexpensive and efficient treatment, requiring no foreign proteins, and restoring complete functionality of injured tissue.

2. BACKGROUND

2.1 - Mammalian Wound Repair: In mammals, after an injury is sustained, the surrounding tissue begins the wound healing process. This is a complicated, multi-step process that results in the replacement of healthy skin tissue with less functional scar tissue. When an injury occurs, blood vessels are broken, triggering platelets to begin releasing growth factors associated with wound healing including serotonin, platelet derived growth factor (PDGF), and transforming growth factor-β (TGF-β), which help to increase angiogenesis and cell proliferation (Lise *et al*. 1998). Fibronectin and fibrin clot together at the wound site to inhibit blood loss and trap proteins. An increase of blood flow to the wound site brings monocytes, which then differentiate into macrophages and remove bacteria and dead tissue through phagocytosis. Increased angiogenesis also brings epithelial cells to the wound site, which adhere to the fibrin clot along with collagen produced by the fibroblasts. Originally, the clot is made of mostly fibronectin and collagen type III and these proteins are later replaced by collagen type I and epithelial cells. Fibroblasts at the wound site differentiate into myofibroblasts, which induce wound contraction and closure (Lise *et al*. 1998). The myofibroblasts attach to the wound edges and contract similarly to smooth muscle cells in order to pull the wound closed.
Cells that are no longer needed undergo apoptosis and collagen is realigned. This collagen, however, will never reach the degree of organization seen in undamaged tissue and much of the skin's structural integrity is lost (Levin, 2007). In order to recreate undamaged tissue, the skin would require instead, a biological regenerative process.

2.2 - Regenerative Capacity of Amphibian Models: Inherently, mammals do not possess the regenerative abilities of some other amphibian species. Many amphibian models have been studied and although a lot is yet to be understood about the specifics of their regeneration pathways, similarities among these organisms have provided a baseline understanding of the components necessary for regeneration (Levin, 2007).

In general, the first step towards regeneration is migration of fibroblasts to close the wound and form a clot. While this is occurring, tissues at the wound site begin to differentiate and cells form the apical epithelial cap (AEC) and blastula (Figure 2.1). The apical epithelial cap is a mass of epithelial cells containing fibronectin and controlled by fibroblast growth factors that forms directly below the wound closure and above the clot. The AEC helps in directing cell growth outwards to reform the injured tissue or limb. As cells in the surrounding tissues continue to dedifferentiate they migrate towards the forming blastula. The blastula is made of many different types of cells including fibroblasts along with cells from the dermis and muscle tissue that dedifferentiates into blastula cells with the help of enzymes such as collagenase and matrixmetalloproteinase-9 (Han *et al*. 2005). Blastula cells then redifferentiate to form functional tissue completing the regeneration process. The result is fully functional tissue containing all components of native tissue. The goal of this study will be to induce a regenerative response in order to restore full functionality to injured tissue.

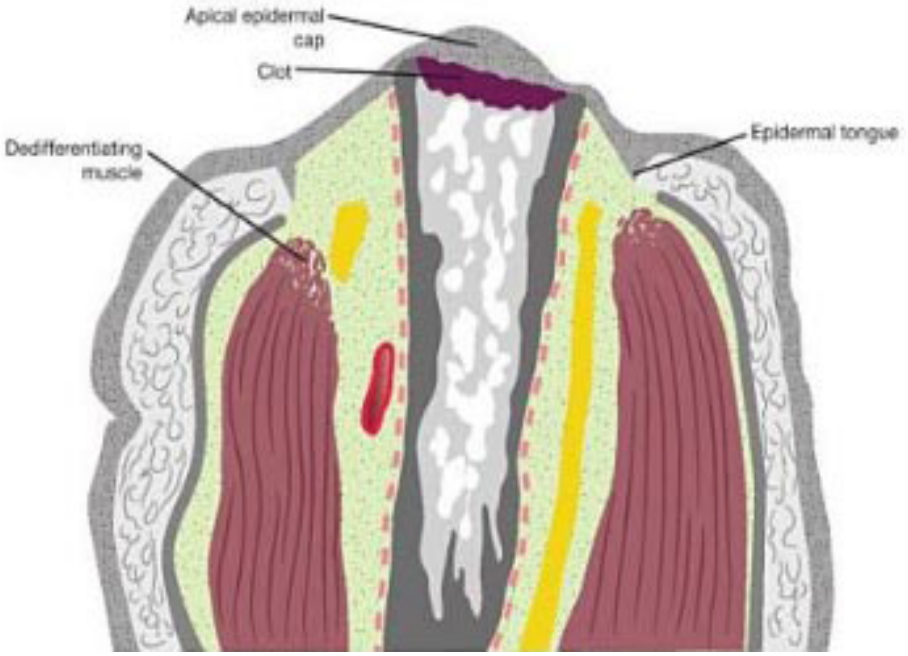

Figure 2.1. Components of the desired regeneration process including apical epithelial cap and dedifferentiation muscle tissue. (Carlson, 2007).

2.3 - Induced Current Flow in Wound Sites: Ion channels and pumps in somatic cells produce directional flow of positive and negative charges, inducing potential differences throughout both healthy and injured tissue. Although little is understood about the relationship between current flow through wound sites and regenerative capacities of the tissues, studies have shown that the polarization and therefore induced current flow in the wounded tissue is necessary for regeneration processes (Carlson, 2007). One study examined the levels of current at limb amputation sites of regenerative and non-regenerative amphibians and found that the current levels through the wound site in the non-regenerative species (frogs) slowly decreased down to normal levels for the frogs as the wound healed. Contrastingly, the cells at the wound site in the regenerative species (newts) maintain an increased potential difference across tissues for an extended period of time. These polarities induced a current flow that remained measureable in the tissue

for weeks or months after injury (Borgens *et al*. 1979). Another study has shown that the inhibition of the excess current flow through shunting or the blocking of ion channels inhibits regeneration in the normally regenerative species (Levin, 2007). These discoveries have lead to further experimentation in which external electric fields were used to induce current flow to wound sites of non-regenerative species in an effort to induce regeneration. These experiments have been successful in some normally non-regenerative species including adult frogs, chicks, and rodents (Levin *et al*. 2010).

Different hypotheses exist describing the mechanism through which current flow works to induce regeneration. Experimental results showing the necessity of nervous tissue for regeneration processes lead some investigators to hypothesize that the currents induce regeneration by simply attracting neural cells to the regeneration site (Kumar *et al.* 2007). Other hypotheses suggest that the current is crucial to regeneration because of its ability to induce dedifferentiation of mature cells. It has been shown that mitosis can be induced in mature nerve cells simply through the depolarization of the surrounding cells (Watt, 1998). This ability to induce stem-cell characteristics in mature somatic cells is an important component in the regeneration process as it induces the creation of non-terminally differentiated cells that have the potential to form the variety of cells required of fully functional, native tissue. (Kumar *et al*. 2007).

2.4 - Transepithelial Potential (TEP) and Wound Potential: The development of potential differences and current flow between tissues in the body help regulate many important biological functions including embryo development and the healing of wounds in mammals or regrowth of wounded tissue in regenerative species (McCaig *et al.* 2005).

Like other organs, the skin regulates potential differences across its surface to maintain specific values that aid function. This TEP varies in value from species to species and according to location on the body. TEP values have proven difficult to measure without disturbing the epithelia but average values have been determined to be in the range of tens to one-hundred mV in humans (Nuccitelli *et al.* 2008, McCaig *et al.* 2005). This fairly constant electric field across the skin is developed via the asymmetric pumping of positive and negative ions. The tissue segregates Na^+ and Cl^- channels to the apical side of epithelial cells and K^+ ion transporters to their basal side. Na^+/K^+ ATPase pumps decrease intracellular Na^+ concentration and increase intracellular K^+ concentration. This diminished intracellular Na^+ concentration causes a net influx of Na^+ ions on the apical side of the cells where Na^+ channels are localized. The net effects of this carefully regulated ions flux is the pumping of positive Na^+ ions from the apical to basal side and negative Cl^- ions from basal to apical resulting in a transepitehlial potential with a positive pole on the inside of the skin. (Figure 2.2A, B).

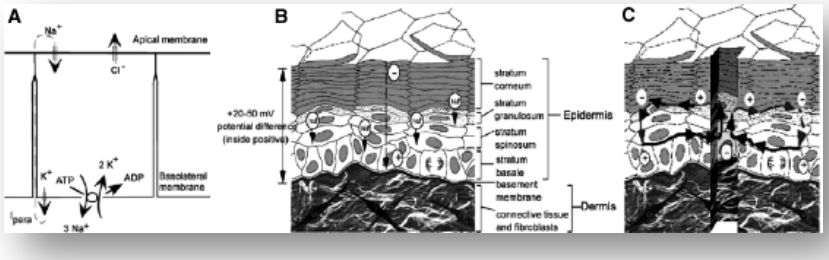

Figure 1. Generation of skin wound electric fields. (A) Diagram of a typical epithelial cell in a monolayer with Na^+ and Cl^- channels localized on the apical plasma membrane and K+ channels localized on the basolateral membranes along with the Na^+/K^+-ATPase. This asymmetric distribution of ion channels generates a transcellular flow of positive current that must flow back between the cells through the paracellular pathway (Ipara). This current flow generates a transepithelial potential that is positive on the basolateral side of the monolayer. (B) Unbroken skin maintains this ''skin battery'' or transepidermal potential of 20–50 mV. (C) When wounded this potential drives current flow through the newly formed low resistance pathway, generating a lateral electric field whose negative vector points toward the wound center at the lower portion of the epidermis and away from the wound on the upper portion just beneath the stratum corneum (B and C reprinted with permission from Figure 2 of *Current Topics in Developmental Biology* (2003 58:1–26).

Figure 2.2. Depicts the cellular mechanisms responsible for the formation of the TEP (2A and B) and the current flow induced upon wounding (2C). [Nuccitelli *et al.* 2008]

High resistivity of cornified skin cells helps prevent bulk ion flow within the tissue and the diminishing of this TEP. Thus, the potential remains constant until the disruption of the resistive barrier that occurs with wounding. Skin wounds provide a low-resistance path from basal to apical side of the tissue allowing for the free flow of ions across tissue layers and the reorganization of the skin potential. This newly structured electric field, called the wound potential, is characterized by an increase in potential difference across the skin with increasing distance from the wound site (Figure 2C). In an effort to understand the patterns of TEP values during wounding, one study measured the TEP across skin wounds made on the backs of pigs (Figure 2.3). This previous study reported an increase in TEP values from days 4-6 followed by a decrease in this potential difference down to a constant value. The research attributed this increase in TEP to the continued differentiation of the epidermis of the skin model and noted the decrease down to a constant value as the initially expected results. Throughout differentiation of the epidermis, expression of Na^+/K^+ ATPase pumps was shown to increase then decrease after wounding, likely resulting in the observed variation in TEP values (Dubé *et al.* 2010).

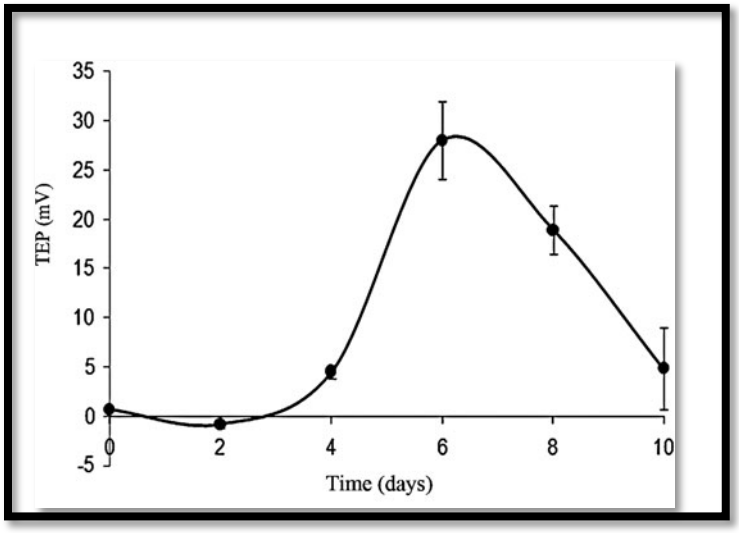

Figure 2.3. Shows the trend in transepithelial potential about a wound site in pigs for 10 days after wounding [Dubé *et al.* 2010].

Much is yet to be understood about the role and regulation of these skin potentials but the characterization and alteration of the electric fields at wound sites is an increasing field of study as new potential treatments are developed. The focus of this study is the alteration of TEP and wound potential through membrane voltage altering solutions and externally applied electric field to guide the wound-healing pathway. The goal is to promote the mimicking, in mammals, of the regenerative currents observed in amphibian models to hopefully induce regenerative cell pathways.

2.5 - Current Wound Care Treatments and their Limitations: The enormous amount of healthcare costs and time required of wound care demands significant improvement in wound care technology. Most current technology surrounds the improvement of existing wound dressing techniques (Harding *et al*, 2002). Compression dressings and stockings aim to direct blood flow in weakened veins and arteries surrounding wound sites and have demonstrated improved healing in shallow skin wounds as well as some chronic

wounds. However, wounds with extensive ischemia and necrotic are often unable to heal themselves simply with the application of external dressing (Morgan, 2002). Biological and biodegradable dressings have been developed to supply proteins and structure to wounds that are unable to close. These dressings are formed from biologically active molecules that contribute to the reconstruction of the damaged tissue or degrade into the wound site without harm. For example, collagen products derived from human, bovine, or porcine sources have been applied to wounds to integrate into the wound site and lay scaffolding for tissue regeneration. Cross-linked hyaluronic acid (a naturally occurring glycosaimnoglyan) has also proven successful at providing favorable wound healing conditions for many wound types (Vercruysse, 1998). Biological dressings have become a promising area of research as they provide biologically active molecules to aid in the wound healing process.

Autologous skin grafts have been effective in wound treatment when replacement tissue is required due to location and severity of wound. However, these grafts are susceptible to rejection and require the creation of a second wound site on the patient with chance of infection and failure to heal (Fonder, 2008). Bioengineered skin alternatives have seen limited success mainly due to the long time period required to culture the grafts, their susceptibility to infection, and poor root uptake upon implantation (Harding *et al*, 2002). New bioengineered skin implants are increasingly commercially available and their technology is improving but their prices remain high and they often use foreign proteins.

Using the body's carefully designed systems through applications of stimuli such as electric fields to induce a regenerative response would reduce complications and costs

of current treatments. Chronic wound care is a big area of medical research in which an improved technology is evidently necessary.

2.6 - Previous Studies: Wound care has recently become a focus of research as it presents a large clinical burden on the United States healthcare system and is especially prevalent within the rising diabetic population. The treatment of chronic wounds with external electric fields presents a potential treatment to improve healing of these chronic wounds. Numerous studies have tested the effects of applied external electric fields on cell migration, orientation, and the rate of wound closure and it has been found that various cell phenotypes exhibit different directionality and degrees of migration when subjected to particular electric field polarities. Upon application of DC electric fields, cathodal migration was reported in lens epithelial cells by McCaig *et al.* (2003), and in bovine vascular endothelial cells by Li and Kolega (2002), while Silman *et al.* (2003) found that human dermal fibroblasts exhibit no directional migration applied DC fields. These studies examined the DC electric field influence on migration of distinct cell types but failed to provide insight into the migrational effects in the wound bed environment as a whole. In order to optimize the application of external electric fields to further improve wound closure rates, migrational effects must be measured in a controlled system that properly models the cell phenotype and orientation of the *in vivo* wound environment.

This study aimed to develop an *in vitro* model to allow for the study of wound currents as well as optimization of electric field application and depolarization solution treatments. The creation of an *in vitro* model was proposed in order minimize the variables observed in our previous *in vivo* studies and provide a more controlled system.

Many previous studies, as listed above, have developed controlled *in vitro* models in order to assess electric field effects on cell migration in cell monolayers. However, the design of full thickness skin models for the test of these parameters will provide a much more relevant model. One recent study measured the transepithelial potential of a tissue engineered skin equivalent model throughout a wounding period and found trends similar to those observed *in vivo* (Figure 2.4). This previously designed skin model used external mechanical supports to culture the tissue at a liquid air interface and $Ag/AgCl_2$ electrodes interface with the tissue. The goal of this research is to design for a more simple tissue engineered skin model and measure TEP values comparable to *in vivo* values to verify the relevancy of our model. Then, further, to use this model for the optimization of the effects of membrane altering biochemical solutions and externally applied electric fields.

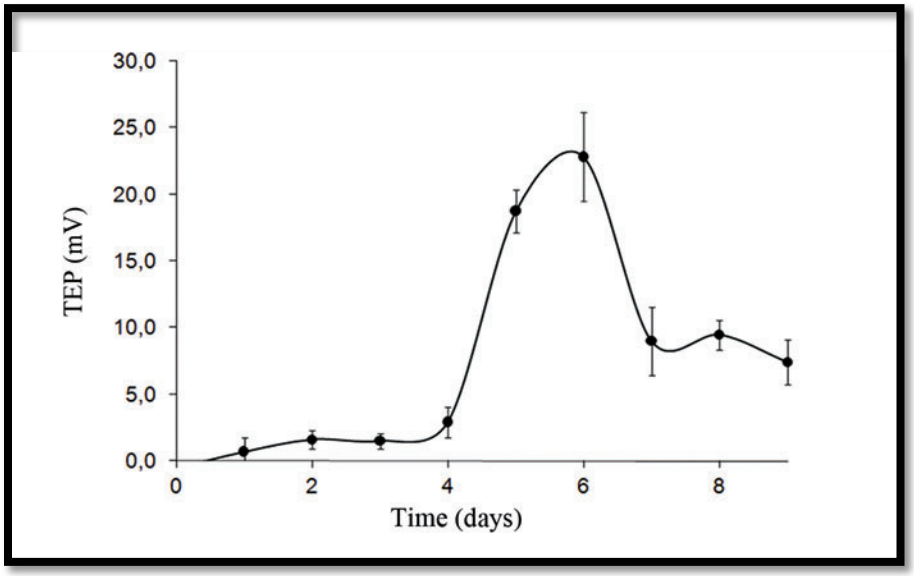

Figure 2.4. TEP measured in a tissue-engineered skin equivalent 9 days after wounding. [Dubé *et al.* 2010].

The specific pathways by which applied electric fields affect migration, orientation, and phenotype and how these fields interact with inherent electric fields and ion flux of injured tissue are being researched. Recent studies conducted by our collaborator, Dr. Mike Levin, have demonstrated the necessity of intracellular ion flux in regeneration pathways and have suggested the potential of biochemical alterations to these ion currents to induce regeneration of injured tissue in normally non-regenerative species (Levin *et al.* 2010). In order to improve electric field treatment options in chronic wounds, we need to better understand the mechanisms by which these currents increase wound closure rates and how they affect the naturally existing wound currents created by ion flux in tissues.

3. EXPERIMENTAL DESIGN

3.1 – Objectives and Hypothesis: The long-term goal of this project is to provide a new technique for the treatment of chronic wounds. This treatment will combine the application of external electric fields and membrane potential altering biochemical solutions to induce a regenerative response in wounded tissue in order to provide a faster and more complete healing of the wound. The goal of this study was to characterize the effects of combined application of external electric fields and chemical alterations of the cell membrane potential in a 3D epithelial wound environment. As such, the objectives were to individually assess the application of electric fields and biochemical membrane potential alterations on a 3D epithelial tissue model and study the effects of combined treatments on the regenerative response in the tissue measured by the rate of wound closure. ***The research hypothesis was that the combination of migrational effects induced by electric field application and increased plasticity and proliferation due to alterations in cell membrane potential would provide a faster and more complete healing of a full thickness skin wound in 3D tissue models.*** In order to meet these objectives and test the hypothesis, two specific aims were designed.

3.2 - Specific Aim 1: Grow a 3D *in vitro* epithelial model in which a relevant transdermal potential can be recorded.

Rationale: Many studies (McCaig *et al.* 2003, Li and Kolega 2002, Silman *et al.* 2003) have examined the migrational effects of applied electric fields on various skin cell phenotypes in an effort to understand cell migration effects as a result of current strength and directionality at the wound site. However, these single cell type results do not

provide insight into cell migration in the healing process as a whole system. In order to understand how cell migration at a wound site will aid in wound healing we need to understand these migrational effects in an environment that accurately represents the wound bed. The designed model provides a full thickness skin model comprised of human dermal fibroblasts, and keratinocytes in a collagen matrix, stratified to a liquid-air interface representative of native skin.

Approach: Human fibroblasts and keratinocytes in a collagen matrix formed the dermis and epidermis of 3D wound model. Flexible platinum electrodes were used to measure potential differences across the tissue to ensure comparable potentials to those found *in vivo*. A data acquisition system was designed to amplify and measure the signal to compare to previous *in vivo* values reported in the literature.

3.3 - Specific Aim 2: Deduce the efficacy of combined cathodal electric field application and membrane potential altering solution treatment in 3D wound model.

Rationale: The system designed in **Specific Aim 1** provided a model to perform relevant testing of membrane altering solutions and electric field strengths on closure rates of a full thickness skin wound. The skin model and electrode design allowed for treatment of tissues with both the application of a cathodal electric field and depolarization solution. After individual treatment application, the two treatments were combined to test the hypothesis that the addition of a depolarizing media as well as cathodal external electric field would increase wound closure rates and allow for a more complete healing of the wound.

Approach: The optimized *in vitro* epithelial tissue provided the wound healing model for the testing of our two treatments: 1 - the efficacy of cathodal electric field application on cell migration through application via platinum electrodes and 2- the influence of exposure to membrane altering solutions.

Using the same flexible platinum electrodes described in **Specific Aim 1**, a cathodal electric field was applied to the wound model with a 500 mV potential difference in an effort to induce cell migration at electric field strengths proven to be required for both human dermal fibroblasts and keratinocytes (Guo *et al*. 2011). Cell migration was measured in the form of histological analysis of wound closure. The tissue was wounded and treated for 5 days with the electrode system applying electric field orientation shown in Figure 3.1. It was hypothesized that the application of a cathodal electric field to the wounded skin equivalent would provide a faster wound closure.

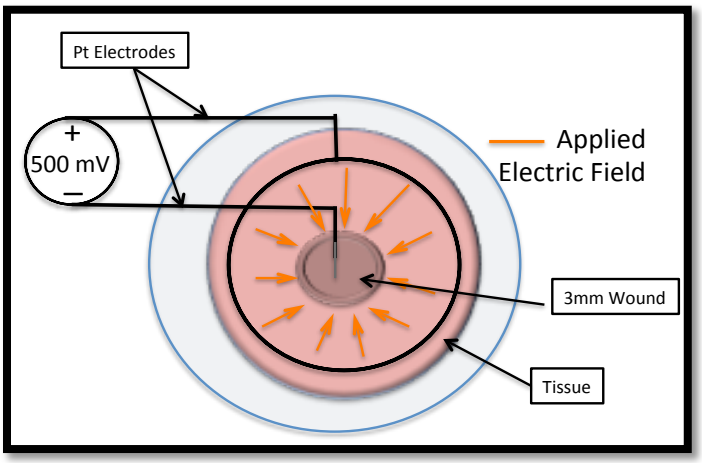

Figure 3.1. Design of the applied electric field.

Also, during the 5-day wounding period, an experimental group was treated with depolarization solutions containing a high concentration of positive ions and a sodium and potassium ionophore to increase cell membrane permeability to these positive ions.

The goal was that these solutions would increase depolarization of cells at the wound site, mimicking wound sites of regenerative species, and lead to the induction of a regenerative response in the wounded tissue model.

3.4 – Overview of Design: In this research, the development of a relevant full thickness *in vitro* skin tissue model (**Specific Aim 1**) was proposed for the optimization of wound healing through the application of external electric fields and membrane potential altering solutions. It was hypothesized that these treatments, when applied to the developed skin model, would increase wound closure rates and provide a more complete healing of the wound as observed via histological analysis (**Specific Aim 2**). Figure 7 shows a schematic of the experimental design.

Figure 3.2. Flow chart outlining the experimental design of the research.

In order to first record a relevant transepithelial potential in the skin model and then assess the effects of the previously described treatments the experimental groups listed in Table 3.1 were devised.

Table 3.1. Shows the sample size of each experimental group, their treatments, and the purpose of those treatments

No. of Tissues	*Action*	*Purpose*
Controls		
1	Take down on wound day	Control to check fully grown tissue
3	No wound No treatment Measure TEP throughout	Be sure inherent TEP exists
3	Wound No treatment Measure TEP throughout	Be sure inherent TEP exists, assess how wounding affects TEP in the absence of any treatment
Voltage		
3	Wound Apply E field	Assess how applied E field affect wound closure rates
Depolarization Solution		
3	No wound Apply soln Measure TEP throughout	Assess how depolarization solution affects TEP of unwounded tissue
3	Wound Apply soln Measure TEP throughout	Assess how depolarization solution affects TEP of wounded tissue
Both		
2	Wound Apply E field Apply soln	Assess how combining treatments affects wound closure rate

4. MATERIALS AND METHODS

Specific Aim 1: Grow a 3D *in vitro* epithelial model in which relevant transdermal potentials can be recorded.

4.1 - Development of Tissue Model and Wound Induction*:* A full thickness skin model was grown following protocols developed and optimized by collaborators in Johnathan Garlick's Lab at the Tufts School of Dental Medicine (Figure 4.1). In the development of this tissue, human dermal fibroblasts were cultured and seeded in 6 well transwell plates. Similarly, human keratinocytes were cultured and then seeded on top of the fibroblasts to replicate the layering within the epidermis and dermis of normal human skin tissue (Garlick *et al.* 2009). These tissues were grown together for 21 days.

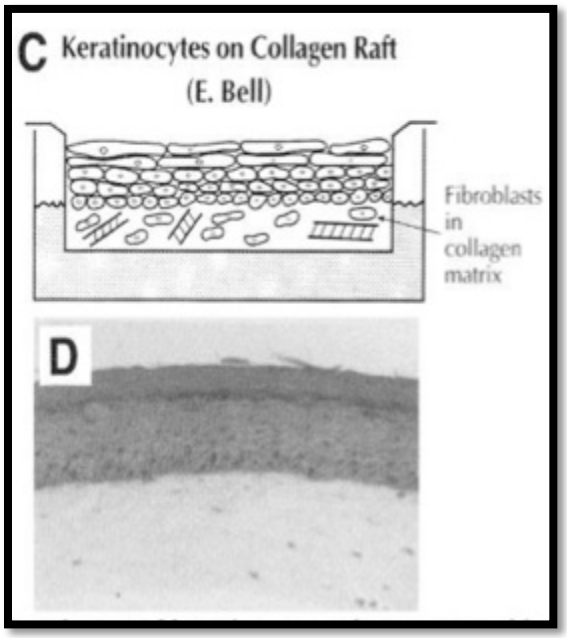

Figure 4.1. Schematic of tissue components as well as image of completed tissue. (Garlick et al. 1999).

4.1.1 – Fibroblast layer. Neonatal Human Dermal Fibroblasts (NHDF) were purchased from Lonza Biologics and grown to confluence in Fibroblast growth media (FGM-2) also purchased from Lonza. Note: FGM-2 was made in the lab after all Lonza media was used since formulation was very standard (10% FBS, 1% penstrep in DMEM). Once confluent, the fibroblasts were split 9:1 in order to stimulate active replication before

seeding onto the collagen matrix on the following day. On the day of dermis seeding, two matrices were made following the component lists in Table 4.1 and Table 4.2 (amounts are calculated for several different numbers of tissues per batch). All components were stored on ice in the tissue culture hood in order to prevent premature gelling of the collagen. Matrix 1 components were combined in transwell culture inserts purchased from BD, placed in sterile 6-well trays. 1 mL of matrix 1 was added to each transwell and allowed to set for 20 minutes while matrix 2 was prepared.

Table 4.1. Matrix 1 components [T. DesRochers]

Materials	**Number of Inserts**						
	6	**12**	**18**	**24**	**30**	**36**	**66**
10x EMEM	0.6 mL	1.2 mL	1.8 mL	2.4 mL	3.0 mL	3.6 mL	6.6 mL
L-Glutamine	54 µL	108 µL	163 µL	217 µL	271 µL	326 µL	594 µL
FBS	680 µL	1.35 mL	2.02 mL	2.7 mL	3.38 mL	4.04 mL	7.48 mL
Na Bicarb	187 µL	374 µL	561 µL	748 µL	935 µL	1.12 mL	2.06 mL
Collagen I	5 mL	10 mL	15 mL	20 mL	25 mL	30 mL	55 mL

Fibroblasts were trypsinized, counted, and added to matrix 2 components in the concentration of $3x10^5$ cells/mL in the amount listed in Table 4.2. Once matrix 1 had solidified, 3 mL of matrix 2 were added on top of the 1 mL of matrix 1 in each transwell cup.

Table 4.2. Matrix 2 Components [T. DesRochers]

Materials	**Number of Inserts**						
	6	**12**	**18**	**24**	**30**	**36**	**66**
10x EMEM	1.78 mL	3.56 mL	5.34 mL	7.12 mL	8.9 mL	10.7 mL	19.6 mL
L-Glutamine	163 µL	325 µL	488 µL	650 µL	813 µL	975 µL	1.8 mL
FBS	2.02 ml	4.04 mL	6.06 mL	8.08 mL	10.1 mL	12.1 mL	22.2 mL
Na Bicarbonate	560 µL	1.12 mL	1.68 mL	2.24 mL	2.8 mL	3.36 mL	6.16 mL
Collagen I	15 mL	30 mL	45 mL	60 mL	75 mL	90 mL	165 mL
NHDF ($3x10^5$/mL)	1.65 mL	3.3 mL	4.95 mL	6.6 mL	8.25 mL	9.9 mL	18.2 mL

The trays were then incubated at 37ºC for 30 minutes and fed with 2 mL of FGM-2 on the outside of each cup and 1 mL of FGM-2 to the inside of each cup. Dermis trays were fed (i.e. the FGM-2 was replaced outside and inside the cup) 3 times per week for one week until epidermis was seeded on top of pre-formed dermis layer.

4.1.2 – Keratinocyte layer. On the same day that the dermis matrices were seeded in the transwell cups as described in section 1.1, Neonatal Human Epidermal Keratinocytes from Lonza Biologics were seeded in T-175 flask with KGM-Gold (for NHEK), also purchased from Lonza. Cells were fed every other day until 1 week after seeding and then resuspended in Epi1 Media at a concentration of 500,000 cells/50uL media (See Table 9.1 in Appendix for media formulation). Media was removed from previously prepared dermis trays and 50uL of the NHEK cell suspension was added directly to the top of each dermis tissue. Trays were allowed to sit in the hood for 15 minutes then fed with 1 mL Epi1 media to the inside of the transwell cup and 2 mL Epi1 media to the outside of the transwell cup.

4.1.3 – Tissue maintenance, growth, and wounding. Two days after seeding the epidermis layer, the Epi1 media was removed from the trays and replaced with Epi2 media (formulation found in Table 9.1 in Appendix). Two days following media change to Epi2, media was again removed and replaced with Cornification media (formulation found in Table 9.1 of Appendix), this time only on the outside of the transwell cup in order to promote growth in a liquid air interface. In this environment, the cells from the bottom of the tissues were in contact with the media through the porous membrane of the transwell and fed nutrients up to the top layer of the tissues, which began to cornify as in native human skin tissue. Cornification media was replaced 3 times per week for 8 days

before wounding and once on day 3 after wounding. To induce wounding in this model, a 4mm biopsy punch was used to make a circular wound through all tissue layers. The wounded tissue was then placed on a previously prepared fibroblast skin layer in a transwell plate. In planning for this, the fibroblast layer was seeded one week prior to wounding day following the protocol described in section 1.1.

4.2 - Transepithelial Potential Measurements: In order to assure that the previously described *in vitro* tissue engineered skin model showed relevant electrical properties, a data acquisition and electrode system was designed to measure voltage potentials across the tissues. The final design of the system included a platinum electrode as the interface with the tissue to record potential values. These electrodes fed the potential values to a breadboard circuit set to amplify the signal to a value within the recommended input range of a data acquisition card, which fed signals to be read out in TracerDAQ software.

4.2.1 – Electrodes. Perfluoroalcoxy (PFA) coated platinum wires with a 0.003" diameter were inserted into the bottom of the dermis skin layer and top of epidermis to serve as electrodes to provide TEP values (Figure 4.2). These platinum wires served as a functional electrode model as they were thin enough to bend under the transwell cup and insert into the underside of the tissue as well as bend under the lid of the 6 well plate as shown in Figure 4.3. The ability to take electrical measurements each day without removal of the lid from the 6-well plate was crucial in order to avoid contamination of the tissues. The PFA coating was removed at the skin contact end of the electrode as well as the breadboard-end connection using a candle flame, which provided the correct temperature between the listed melting point of PFA and Platinum. Electrodes were

inserted on Day 1 when tissues were wounded and used 1 time per day to collect voltage values across the skin (insertion of electrodes shown in Figure 4.4).

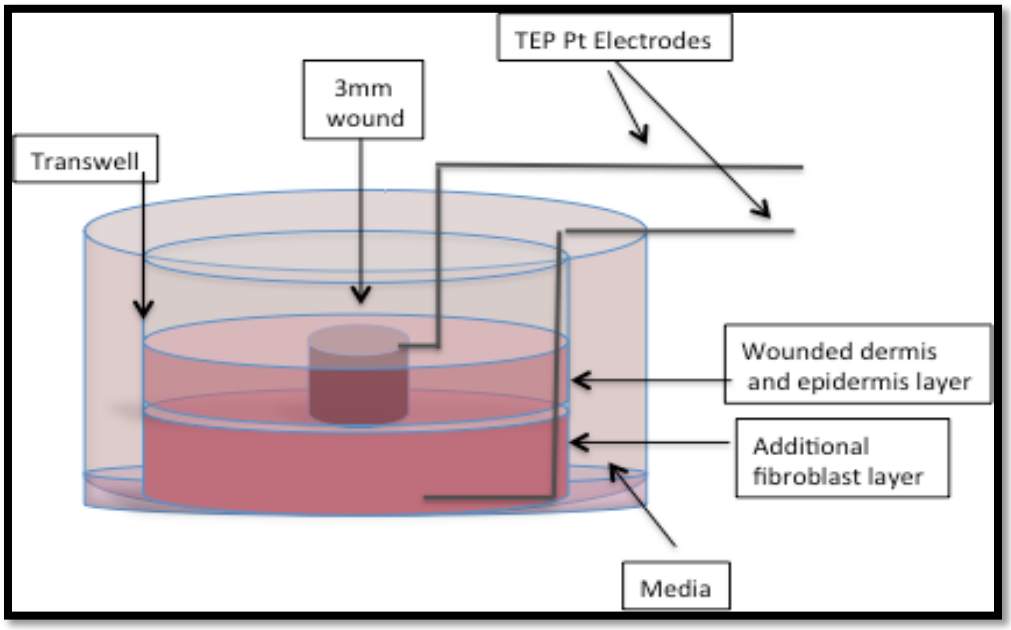

Figure 4.2. Diagram of electrode placement in wound model.

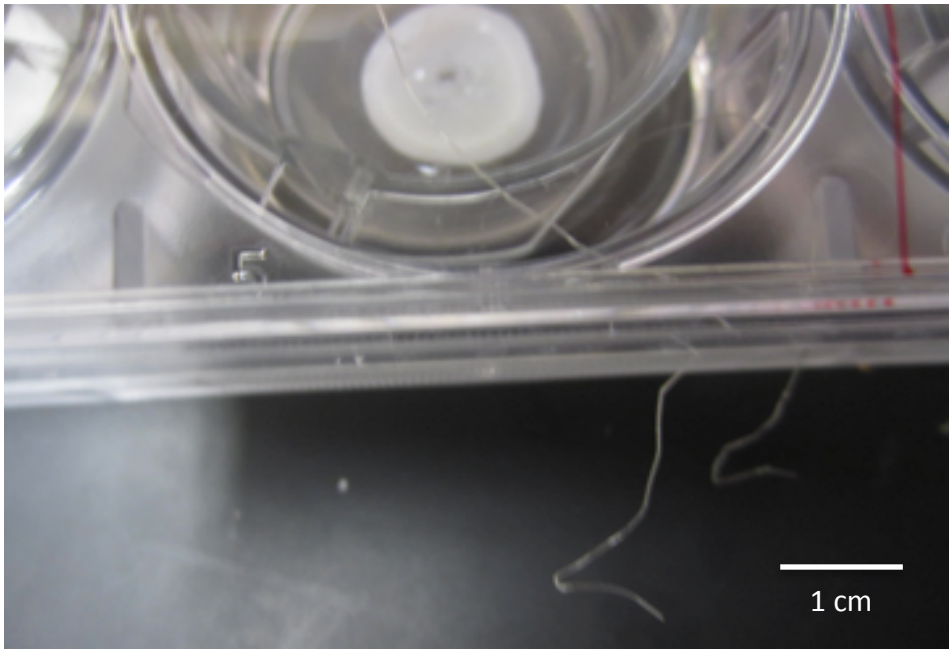

Figure 4.3. Platinum electrodes under 6-well plate with closed lid.

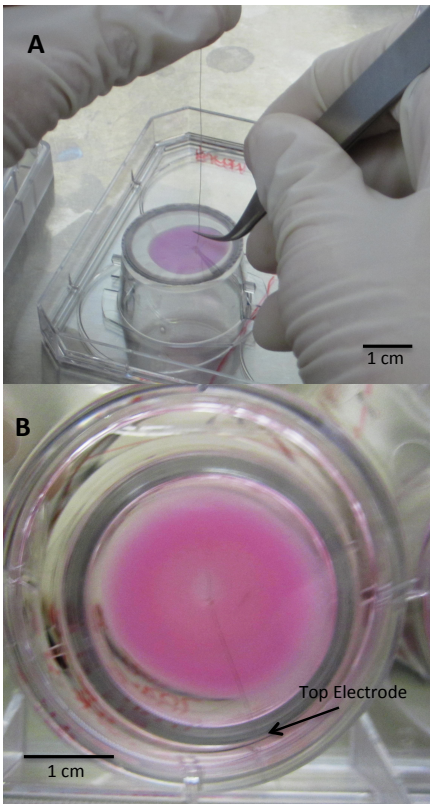

Figure 4.4. A. Shows insertion of bottom electrode through the membrane of the transwell plate. **B.** Shows the placement of top electrode into the tissue.

4.2.2 – DAQ system. Voltage values were collected using a 12-bit DAQ device with 4 differential analog inputs purchased from Measurement Computing (Product # USB-1208LS). Values were amplified using an AD620 amplifier purchased from Analog Devices. The amplifier gain was adjustable by varying the resistance across port 1 and 8, shown in Figure 4.5 and the resistance required was calculated using the gain equation provided by Analog Devices shown in Figure 4.6. Initial testing of TEP values in preliminary tissues gave values in the hundred of millivolts range, so amplification was set to a gain of 100 in order to amplify signal to the volt range specified as optimal for the data acquisition card. Figure 4.7 shows a schematic of all components of the data acquisition system.

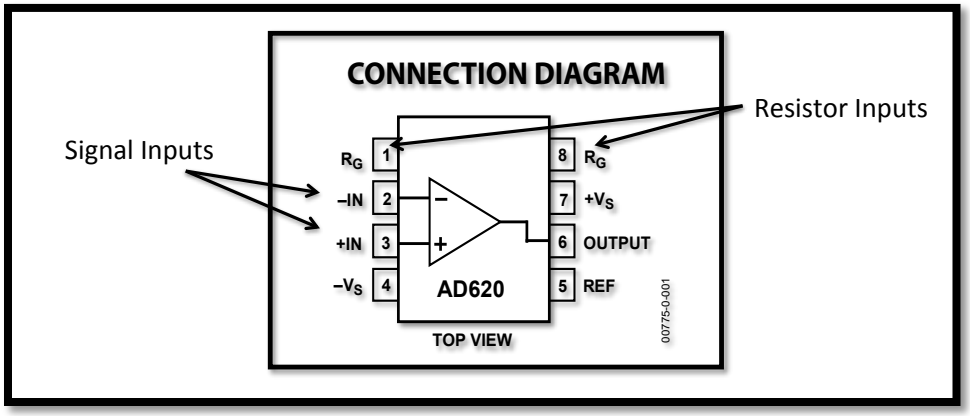

Figure 4.5. Shows the connection diagram of the AD620 amplifier used in the DAQ system.

$$G = \frac{49.4k\Omega}{R_G} + 1$$

$$R_G = \frac{49.4k\Omega}{G-1}$$

Figure 4.6. Equations used to calculate the resistance required to provide the desired gain.

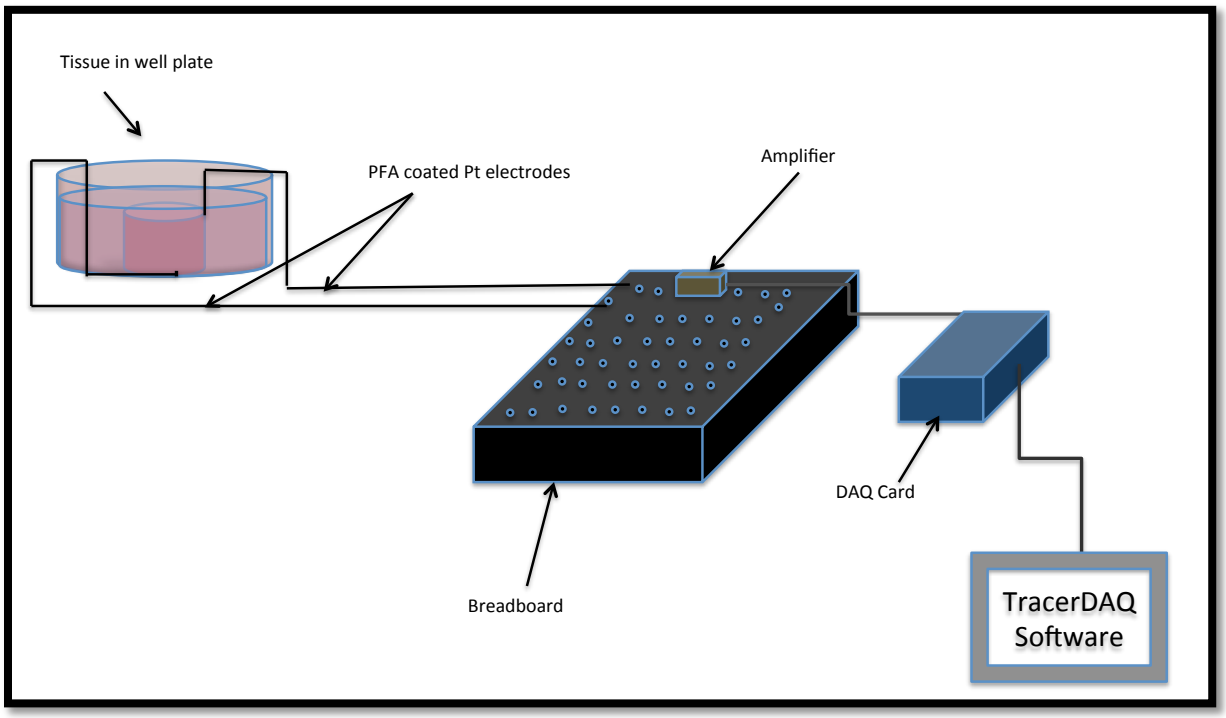

Figure 4.7. Components of the data acquisition system for the collection of TEP values.

The DAQ system was designed to measure the difference in potential between the two electrodes on the tissue, therefore outputting the TEP to the amplification circuit which then relayed the amplified signal to the DAQ card to be recorded and stored using TracerDAQ software. The data acquisition card was set to record values at a sampling rate of 100 Hz for one minute and sample points were averaged. Measurements were taken one time per day throughout the 5-day data-collection period.

As shown in Table 3 of the experimental design, four different groups were fixed with electrodes to measure TEP throughout the 5-day data collection period. One group, plated only in the cornification media, served as the control group and the TEP was measured throughout data collection period on three unwounded samples and three wounded samples.

Another group, also of three wounded and three unwounded samples, was plated in the depolarization solution in order to note any differences in TEP induced by the depolarization solution. These experimental groups allowed baseline TEP values to be recorded to verify that the *in vitro* model exhibited TEP values relevant to native tissue and allowed comparisons to be made in the analysis of effects of wounding and depolarization solutions on TEP values.

<u>Specific Aim 2:</u> Deduce the efficacy of combined cathodal electric field application and membrane potential altering solution treatment in 3D wound model.

<u>4.3 - Application of Electric Field:</u> For experimental groups shown in Table 3.1 of the Experimental design, a cathodal electric field was applied for 30 minutes each day throughout the 5-day data collection period.

4.3.1 – Electrodes. As in specific aim 1, PEF coated Platinum electrodes with a diameter of 0.003" were used as electrodes to interface with the tissue to apply voltage. In order to apply a cathodal electric field about the wound site, one wire was formed into a ring shape that was placed between the wounded tissue and the prepared fibroblast layer described in materials and methods section 1.3 (Figure 4.8A). This ring electrode served as the anode in the application of a 500 mV difference between electrodes. The cathodal electrode was uncoated at the point of interface with the tissue and inserted directly into the wound site through the layers of wounded tissue (Figure 4.8B). Figure 4.9 shows a schematic of electrode placement within the tissue layers of the wound model.

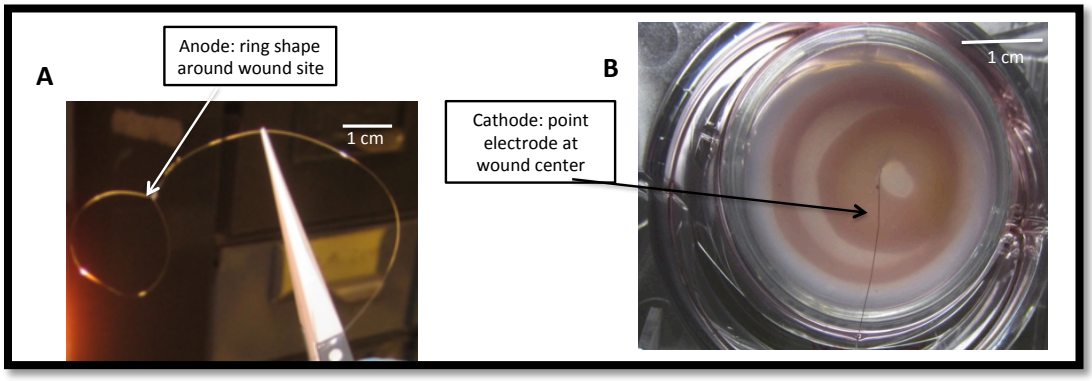

Figure 4.8. A. Anodal electrode before insertion between fibroblast layers. **B.** Placement of cathodal electrode at wound center.

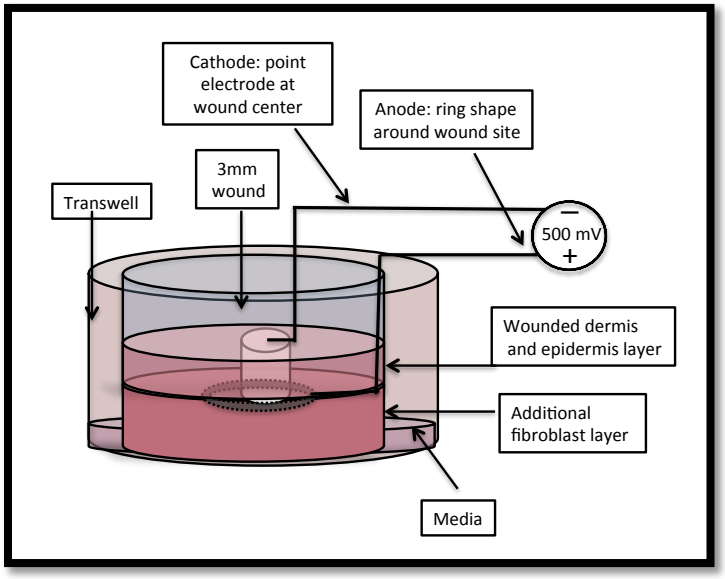

Figure 4.9. Schematic of electrode placement into tissue for application of electric field.

4.3.2 – Voltage Source. The flexibility of the platinum wire allowed for the electrodes to be place into the tissues on Day 1 when the tissues were wounded and voltage applied across these electrodes each day without removing the lid of the well plates. The platinum electrodes remained in the tissue for the 5-day period and connections using alligator clips were made to the exterior portion of the electrode in order to apply voltage (Figure 4.10).

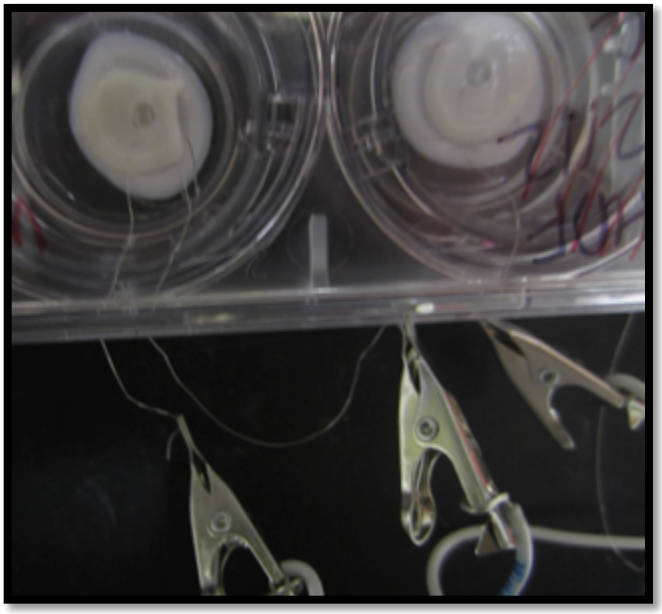

Figure 4.10. Flexible platinum electrode remained in tissues throughout 5-day data collection period. Exterior portion of the electrode was connected to the DAQ system without removing the lid of the tissues in order to prevent contamination.

A standard, available voltage source was used to supply a potential difference of 500 mV across the tissues from anode ring electrode to central cathode. Voltage was supplied to a circuit on the breadboard that split the current and allowed the 500 mV voltage difference to be supplied simultaneously to three tissues in order to minimize the use of well plates and time for tissues to be out of the incubators. Figure 4.11 shows the power source set up as well as connection from breadboard to Platinum electrodes via

alligator clips.

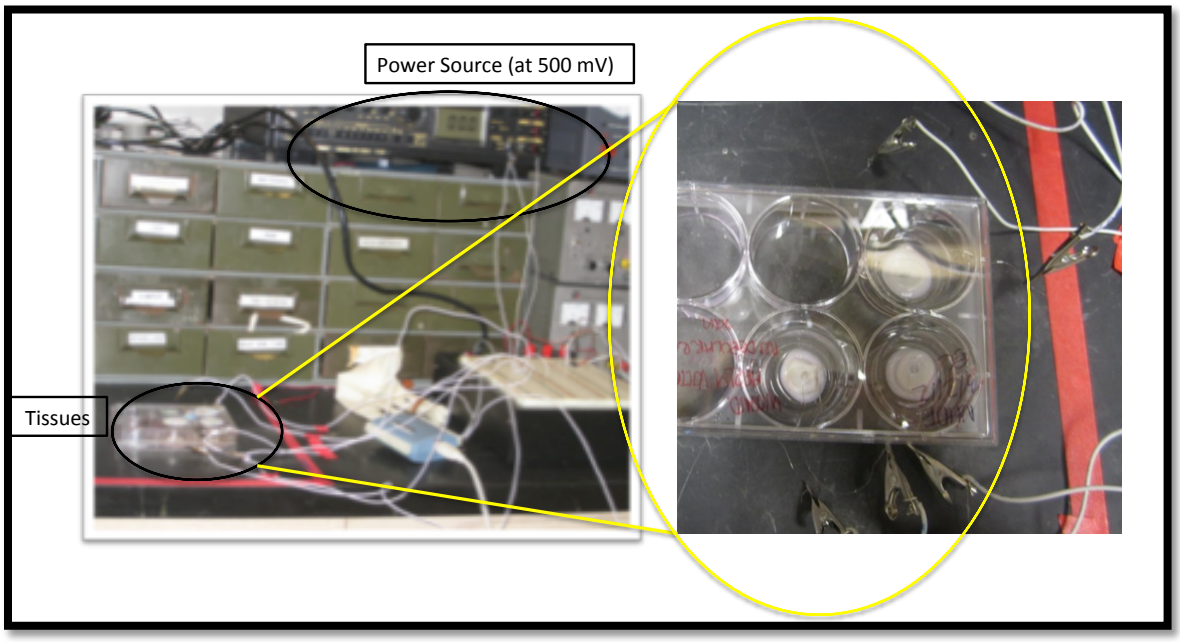

Figure 4.11. Power source supplying voltage to the breadboard circuit which was then distributed the 500mV potential across three wounded tissues simultaneously.

The cathodal electric field was applied to each tissue in a voltage treatment group for 30 minutes per day throughout the 5-day period.

4.4 - Treatment with Depolarization Solution: Depolarization solution formulations were applied to each tissue via addition to the growth media on day 1 of wounding and replaced on day 3.

4.4.1 - Depolarization Solution Formulation. A membrane potential altering solution was devised by Dr. Mike Levin in order to increase depolarization of cells in the wound model. The aim of the solution was to increase positive ion concentration in the cell with respect to the surrounding environment mimicking this effect observed in regenerative

species. Table 4.4 shows the components of the applied solution, their concentration, and function.

Component	Concentration	Function
Na^+	120 mM	Ensure higher [Na^+] outside cells than inside
Cl^-	Aim for < 50 mM	Maintain low concentration of negative ions in solution
K^+	120 mM	Ensure higher [K^+] outside cells than inside
Monensin	2.5 µM	Sodium and Potassium ionophore to increase cell permeability to positive ions and enhance depolarization

Table 4.4. Components of depolarization solution to be added to growth media.

This formulation was designed for treatment of amphibian models. This study provided the first results of treatment in mammalian skin wound model. Results could provide suggestions for optimization in this human engineered skin model.

4.4.2 – Application of Depolarization Solution. In order to apply the depolarization solution to the wounded tissues, components were combined directly with the cornification media used to feed the tissues. The cornification media, shown in Table 9.1 of the Appendix, was formed primarily of EPLILIFE-CF media ordered from Invitrogen. The distributor could not release ion concentrations of this formulation so these concentrations were assumed to be similar to those of DMEM and ion concentrations were adjusted to the desired concentrations list in Table 2. Potassium concentration was adjusted by adding potassium gluconate ($C_6H_{11}KO_7$) so as not to affect any other ion concentrations. This adjusted media was added to experimental groups treated with

depolarization solution on Day 1 when tissues were wounded and replaced on Day 3 when each experimental group received fresh media.

5. RESULTS

5.1 - Transepithelial Potential Data (TEP):

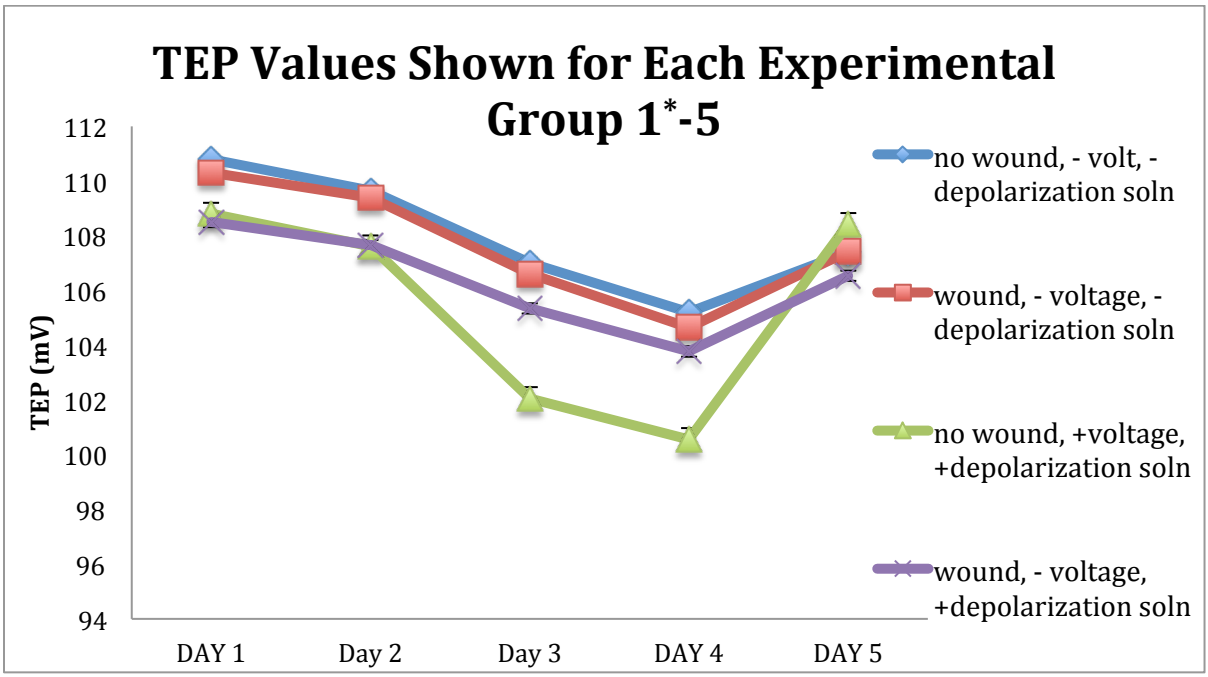

Figure 5.1. Shows average TEP values each day for 5 days after wounding. Error bars show standard deviation of each group (n=3). * Day 1 values were extrapolated from days 2-5 following trends observed in the literature [Dubé *et al.* 2010]. Experimental group names describe wound status and treatment with or without voltage (volt) or depolarization solution (dep).

Transepithelial potential measurements represent averages from one minute of collection at a sampling rate of 100 Hz for three tissues per experimental group for each day of data collection. Trends show a steady decrease in TEP from days 1 through 4 then a sharp increase from days 4-5 in all groups. Both samples treated with depolarization solution show statistically lower ($P<0.05$) TEP values than those measured in only cornification media each day, except day 5 when the group receiving depolarization solution but no voltage showed a significantly higher TEP value.

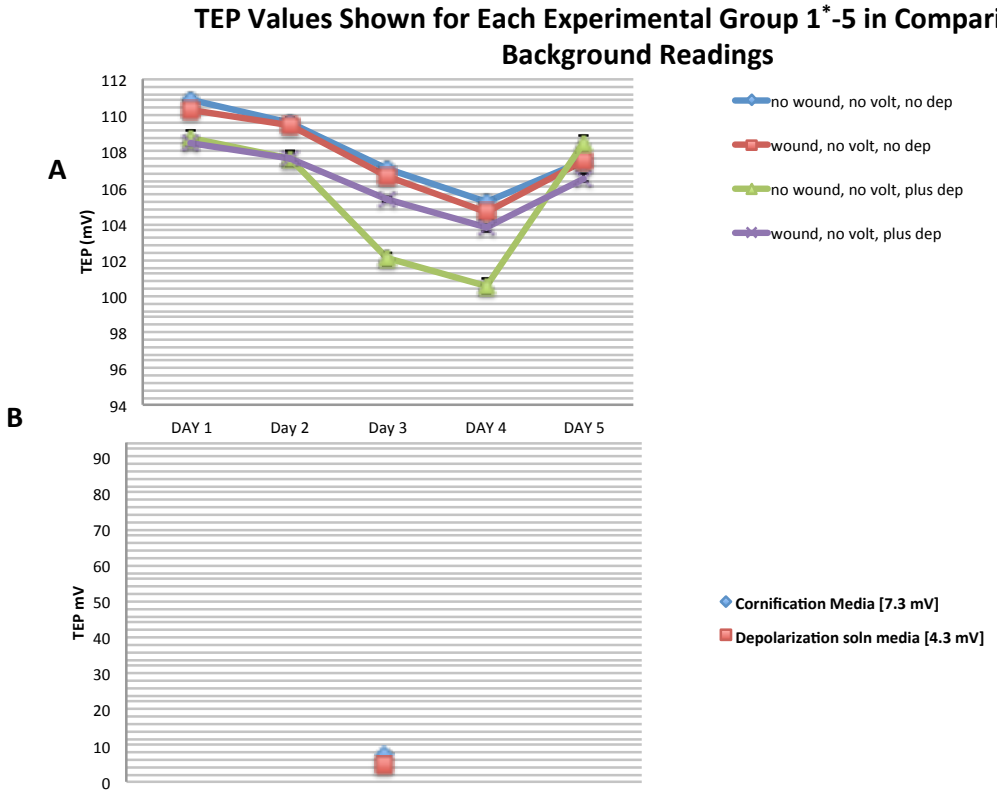

Figure 5.2. Shows background potential difference (**B**) with respect to recorded vales for experimental groups (**A**). Background values were recorded between electrodes in the culture media in the absence of tissues in order to gather a background reading for TEP measurements. Error bars show standard deviation for each group (n=3).

In order to assure that the potential difference recorded across electrodes represented the potential difference across the tissues and not inherent values across electrodes or results of resistance and potentials created in the media formulations, well plates with media in the absence of tissues were used to measure TEP values. These background readings served as blanks used to be sure that the noise collected in the absence of the tissues was at a minimum. As seen in Figure 5.2, the values recorded in the absence of the tissues

were on the scale of tens of mV while TEP values recorded across the tissues were found to be over 100 mV.

5.2 - Histological Analysis:

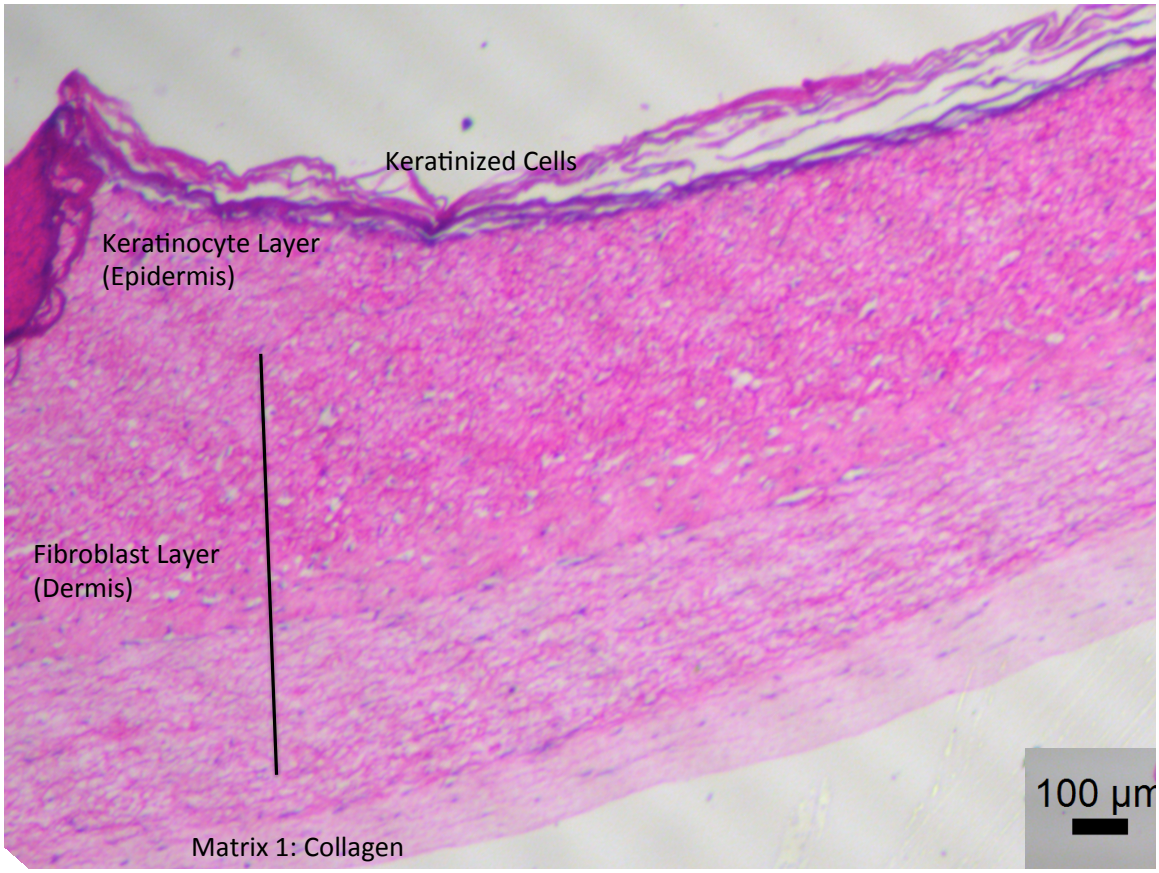

Figure 5.3. Shows the different layers in the construction of the tissue engineered skin equivalent.

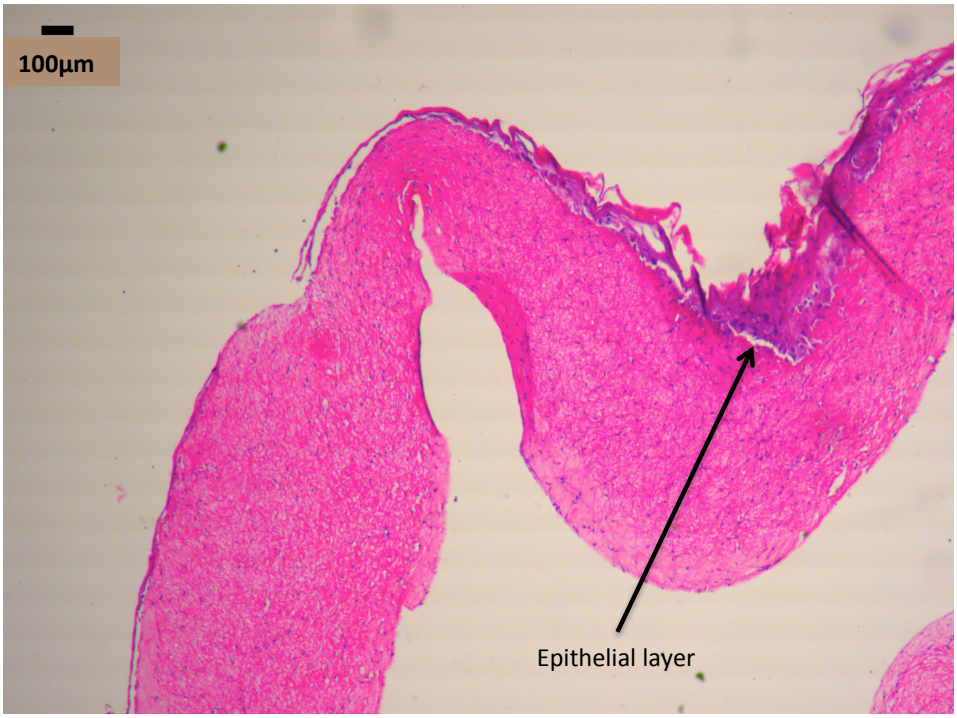

Figure 5.4. Skin sample with successful epithelialization in the marked area.

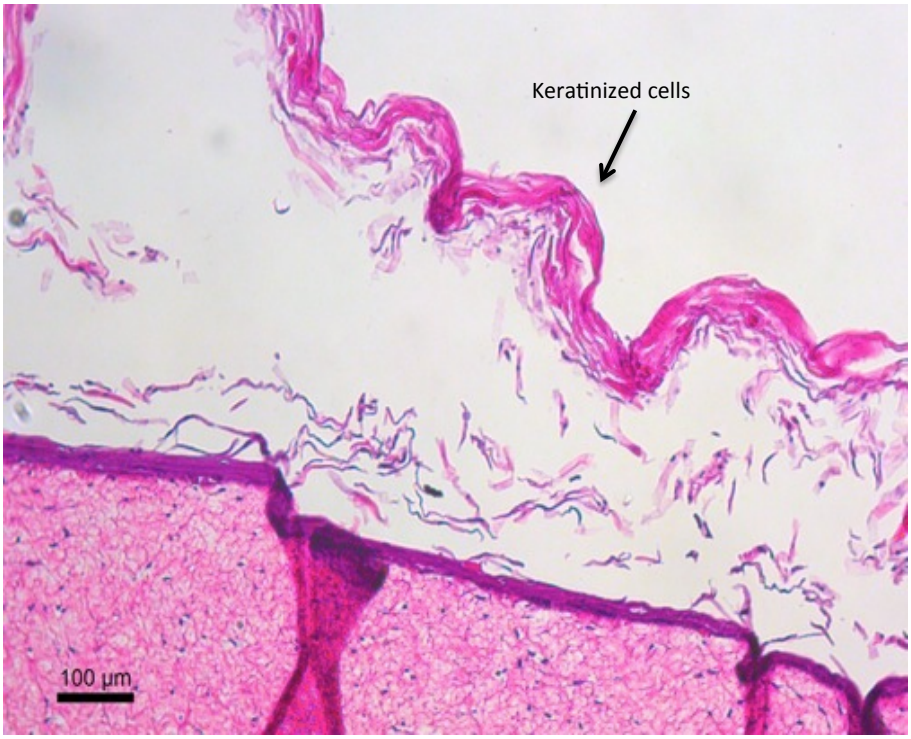

Figure 5.5. Extensive keratinization in epidermis of tissue. The presence of keratinized cells lifting off from the top layer of skin demonstrates good cell differentiation.

In analysis of wound models, the level of migration of epithelial cells into the wound bed, signifying the initiation of the wound closure process, was assessed across different experimental groups as a determinant of wound healing. Figure 5.6 represents an un-healing wound, with no cell migration into the wound bed, while Figure 5.7 shows successful cell migration into the wound site to initiate closure. Table 5.1 displays the distribution of observed cell migration into wound beds across experimental treatment groups.

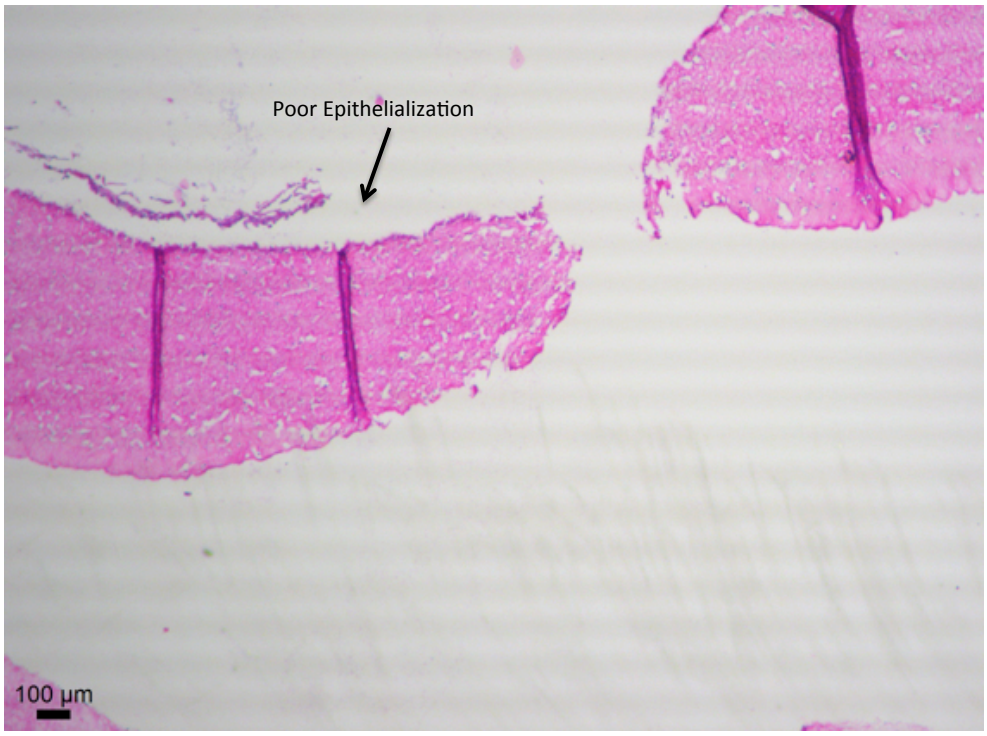

Figure 5.6. Shows wounded tissue treated with depolarization solution but no applied voltage (experimental group: +wound, -voltage, +depolarization solution). Skin shows suboptimal epithelial layer and no movement of cells into the wound site to initiate closure.

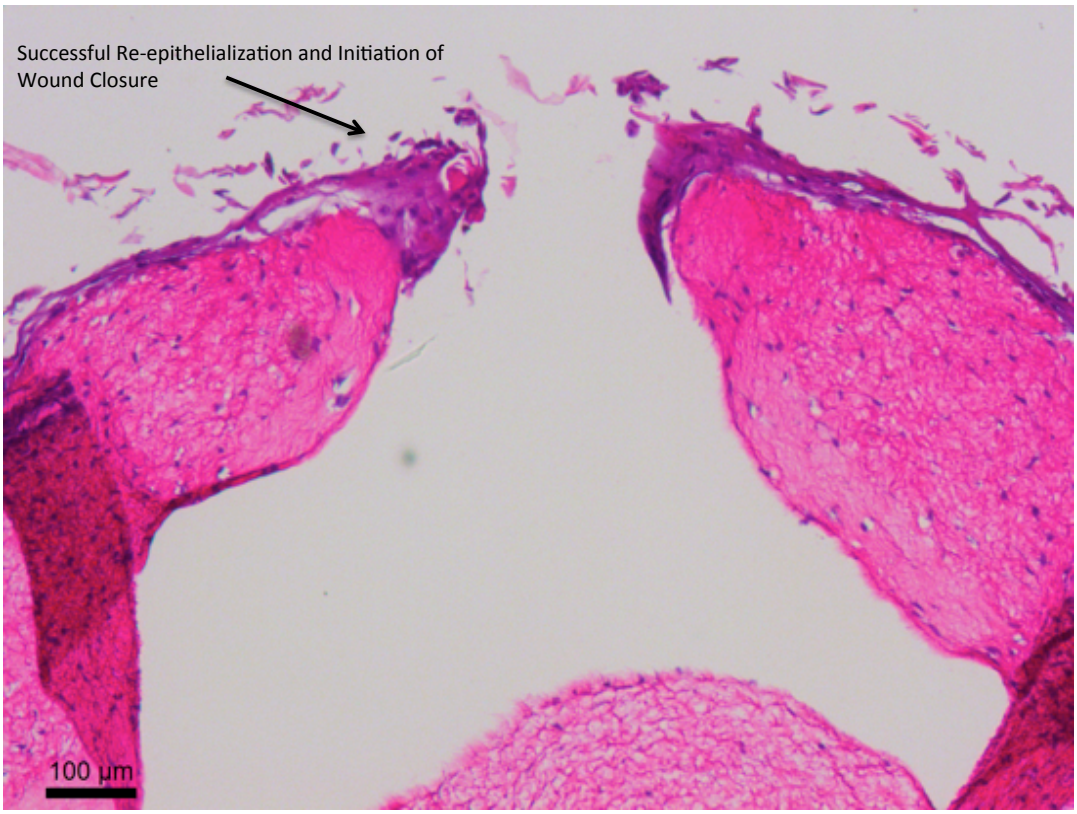

Figure 5.7. Shows a wounded tissue treated with applied electric field throughout treatment period but no depolarization solution (experimental group : +wound, +voltage, -depolarization solution). Movement of epithelial cells into the wound site has begun to signify a healthy healing of the tissue and initiation of wound closure.

Table 5.1 Shows distribution of observed cell migration into wound bed across experimental treatment groups.

Experimental Group	Number of Tissues with Observed Initiation of Wound Closure
+ Wound -Voltage -Depolarization Solution	1 out of 3
+Wound +Voltage -Depolarization Solution	3 out of 3
+Wound -Voltage +Depolarization Solution	0 out of 3
+Wound +Voltage +Depolarization Solution	0 out of 3

6. DISCUSSION

6.1 - TEP Data: The first objective of the study was to record TEP values in the designed tissue engineered skin model on the order of those recorded in mammals that follow relevant trends when wounded. Data collected was successful in completing this objective. Trends in TEP values collected reflect trends observed in *in vivo* values recorded in pigs (Figure 6.1). Values recorded were on the order of tens to several hundreds of mV, which reflect standard values found in *in vivo* in human studies. (Nuccitelli *et al.* 2008, McCaig *et al.* 2005). The magnitude of the values is of less importance to this study than the trend followed by the values since TEP values vary greatly depending on location within the body and model species. The measurement of a TEP in this *in vitro* skin model that follows previously observed trends in *in vivo* skin wounds suggests that this model could provide a successful system for the optimization of varying electrical properties as a wound treatment parameter.

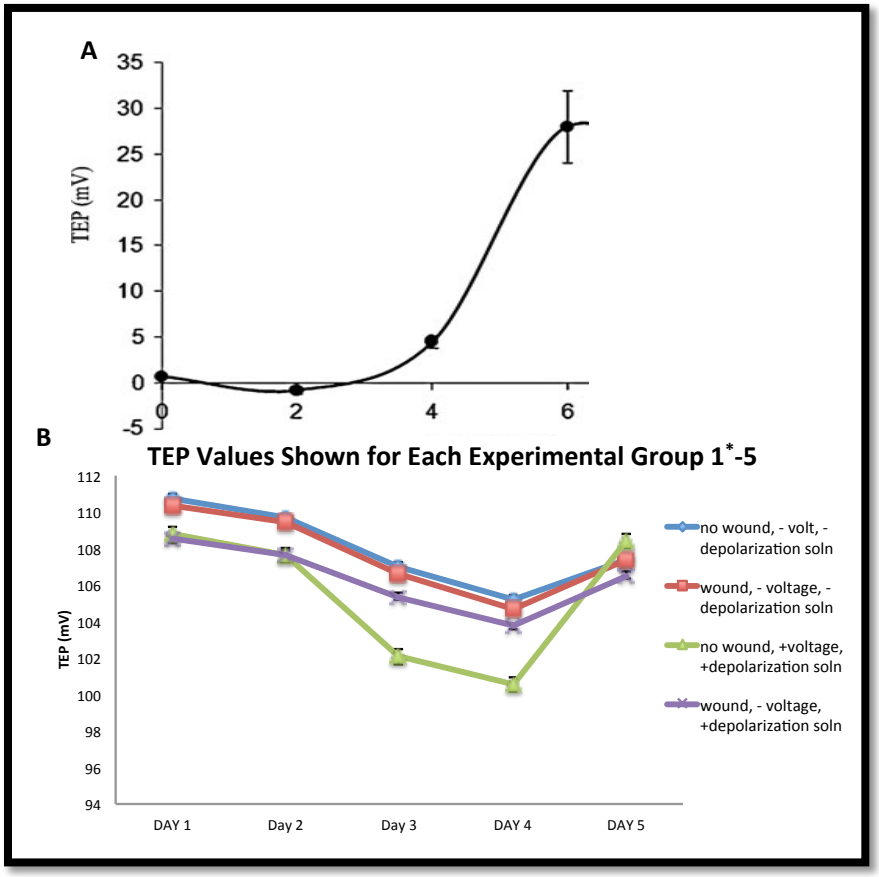

Figure 6.1. Shows comparison between *in vivo* TEP trends observed in back wounds on pigs and those recorded in this research in the *in vitro* skin model. Figure 22A shows TEP values recorded in pigs [Dubé *et al.* 2010] and Figure 22B shows *in* vitro TEP data collected in this study.

Analysis of the average values presented for different experimental groups shows that the tissues, both wounded and unwounded, that were cultured in cornification media showed significantly higher ($P<0.05$) TEP values each day (with the exception of day 5) than those cultured in depolarization solution. This suggests that the depolarization solution may have had some effect on transepithelial potential. No initial hypotheses were formed based on the effects that the depolarization solutions may have on the overall TEP values of the tissues. The solutions were designed to increase depolarization of individual cells but the overall effect this may have on the structure or magnitude of the potential across the tissue was unknown. Data collection should be repeated in order

to confirm the effect suggested by this data that the solutions might act to decrease overall TEP values of the tissues.

Further analysis of the TEP trends show that all tissues both wounded and unwounded follow the same TEP trend and that the wounded tissues did not show increased or decreased potential differences compared to unwounded tissues. It was expected that TEP value for unwounded tissue would remain constant and an initial decrease followed by a larger increase in TEP values would be observed for wounded tissues. As seen in Figure 6.1, this trend of an initial decrease, followed by a more exaggerated increase is seen in all tissues whether wounded or unwounded. The suggested explanation for these results is that the electrodes placed in the tissues to measure TEP wounded the tissues enough to elicit the observed TEP wound response in all tissues. Efforts to insert the platinum electrodes just below or above the tissue and maintain their position often resulted in insertion either across the surface of the tissue or entirely through it. In future studies, a system for electrode placement should be devised that will maintain electrodes on the surface of the tissues without penetrating them. This could potentially be achieved with the design of a mechanical insert for the well plate that will hold the platinum wires in place.

As noted in Figure 5.1 of the results section, day 1 TEP values shown were extrapolated from the data based on previously published trends found in *in vivo* wound models (Dubé *et al.* 2010). The recorded day 1 values were discarded since the amplification gain was incorrectly set and TEP ranges fed to DAQ card were not amplified to a scale optimal for DAQ card collection. The resulting day 1 values were recorded on the scale of millivolts and the DAQ system was unable to precisely record

values this small. Thus, even applying the correct gain factor to these recorded values could not assure that values were accurate and the day 1 data was discarded.

6.2 - Histological Analysis: Histology slides were initially analyzed for the presence of components of healthy skin tissue. Figure 5.3 shows the different layers of tissue including dermis, epidermis, and keratinized epidermal cells. The presence of all tissues layers suggests successful tissue formation and cell differentiation. Figure 5.4 highlights the epidermal layer in one tissue sample. A thick layer of the darker stained keratinocytes signifies a healthy, growing tissue. Some tissues produced better epidermis layers than others and as can be seen in Figure 5.4, the thick epidermis layer does not coat the entire tissue, leaving some areas without an epidermis layer. Figure 5.5 shows a thick layer of keratinized cells signifying good cell differentiation. However, keratinization will occur only is areas which have formed a successful epidermis layer. Two main reasons for suboptimal epithelialization in some tissues are suggested. One is that rat-tail collagen was used in the replacement of bovine collagen in order to cut down on costs of tissue production. In some previous trials of tissue growth, rat-tail collagen was found to reduce the quality of the final tissues. Another potential reason could be that tissues were allowed to grow for too long in the cornification media and some of the epidermis tissue began to die. Previous trials with these tissues had suggested the longest time grown in cornification media to achieve optimal tissues was around two weeks (Garlick *et al* 2009). In this study, tissues were wounded after 8 days of being in cornification media and data was collected for 5 days after wounding. Tissues were taken down after 15 days in cornification media. Based on the resulting tissues in this study it would be suggested

for future studies to wound the tissues around 3-4 days after transfer to cornification media.

Next, wounded tissues were analyzed for wound closure and for effects of different treatments on wound closure success. A large variation in wound closure success was observed as is represented in the comparison of Figures 5.6 and 5.7. The initial sign of wound closure is the migration of the epidermis cells into the wounds site, as can be observed in Figure 5.7. Some wounded tissues, such as that observed in Figure 5.6 showed no real initiation of the wound closure process. The beginning of the wound closure process would require a healthy and actively growing tissue and as some tissues did not present any healthy epidermis layer, they also did not show any signs of wound closure.

The experimental design of this study proposed the analysis of wound closure rate as a determinant for wound healing. However, since degree of wound closure proved very difficult to measure, this determinant of healing was not analyzed and instead observation of cell migration into the wound site was used to mark the initiation of a wound healing process as a determinant of healing. Histological analysis of all sample groups shows a distribution across experimental treatment groups of tissues with noticeable migration of epithelial cells into the wound site for the group treated with applied electric field but no depolarization solution. These trends suggest an improved response toward initiation of wound closure in tissues treated with applied external electric field in comparison to control tissues with no applied electric field and an inhibition of initiation of wound closure in tissues treated with depolarization solution as compared to control tissues without treatment with depolarization solution. However,

further repeats of this study should be done in order to conclude any reliable difference among groups. Based on the results from these initial trials, this study suggests further optimization of the depolarization solution for future trials. This depolarization formulation was based concentrations found to be successful in amphibian models and limited success of the formulation in this study suggests the necessity of reformulation and further characterization of the effects of the solution on mammalian cell lines.

6.3 - Sample Size and Time of Study: Experimental groups were all designed with a sample size of three tissues, with the exception of the group that received treatment with both applied electric field and depolarization solutions, which only had two tissues. Initial designs for the study included six tissues per experimental group and two rounds of data collection. However, multiple rounds of tissue contamination cut replicates down to one collection with the sample size in half. Contamination was a large issue in this study due to both the long culture time and intricacy of seeding technique as well as the removal of all antibiotic/antimitotic factors in the last stage of culture. The cornification media formulation used in the protocol developed by the Garlick Lab contained no antibiotic factors as they had been shown to decrease quality of resulting tissues. In two separate rounds of tissue culture, bacterial contamination was observed after the switch to cornification media. Finally, in the last round of culture, antibiotics were re-included in the cornification media before insertion of electrodes to prevent contamination throughout data collection. It is suggested that any future studies include antibiotics in media formulation throughout data collection. Due to these contamination issues, small sample sizes only suggest the trends observed in the results section and the experiments should be repeated in order to provide more conclusive results.

As shown in the results section as well as Figure 6.1, data was only collected for a time period of 5 days after wounding. This time constraint was set by the amount of time after transfer to cornification media that tissue remains viable and the fear of contamination with prolonged data collection. It is the hope that future studies will be able to collect data for a longer time period in order to observe a more complete healing of the wound. It has been determined that optimal tissue growth is observed at a maximum of 2 weeks after transfer to cornification media so in order to maximize culture time after wounding, future studies should induce wounding 3-4 days after transfer to cornification media allowing for up to 10 days of data collection within this optimal window of growth time. In order to reduce concerns of contamination during data collection, antibiotics should be included in the cornification media.

7. CONCLUSION

In the conclusion of this study it can be stated that a successful tissue engineered human skin equivalent was cultured and the designed TEP electrode measurement system reported electrical properties similar to those observed in *in* vivo studies, thus providing an appropriate model for the testing of desired wound treatments and achieving the first specific aim of the experimental design. Future replicates of this study would be desirable in order to be sure results could be repeated.

The second objective of this study was to treat the tissues model developed in specific aim 1 with external electric fields and depolarization solutions and assess their effect on improved wound healing. Electrode and solution design allowed for successful treatment in these two parameters but further data collection would be required to assess their effect. Initial data analysis suggests differentiation between experimental groups but a larger sample size and more repeats of data are required to make sound conclusions on the treatment effects.

The main success of this research was the design of the electrode and DAQ system to apply electric fields to the tissue models and record TEP values. It is the hope that the successful experimental set-up devised in this study will be used to continue testing and optimization of treatment parameters.

8. FUTURE DIRECTIONS

As stated in the conclusion, the main goal for future directions of this research is the use of the devised model to repeat tests conducted in this study in order to prove repeatable results as well as continued variation and optimization in treatment parameters. Now that a successful tissue, electrode, and data acquisition system have been designed, future research can focus on the optimization of treatment parameters. Depolarization formulations and concentrations could be applied in varying concentration and time frame as well as added in conjunction with polarization solutions in future studies. Different strengths and polarities of electric fields could be tested to optimize induction of cell migration induce through applied voltage. Once these parameters have been optimized, the new designed treatment will hopefully exhibit similar results in animal and clinical trials and provide a new successful treatment for chronic wounds.

Another, smaller, focus for future research is the development of an electrode to measure a change in potential across the wound surface. Initial designs for electrode system included a gold in silk electrode to interface with the top of the epidermis of the skin and record potential across the tissue (Figure 8.1). However, insulation and flexibility of the electrode could not be optimized in time to include them in the DAQ system. Once functional electrodes have been fabricated, potential across the skin wound could be recorded providing further insight into the electrical properties of this tissue engineered wound model.

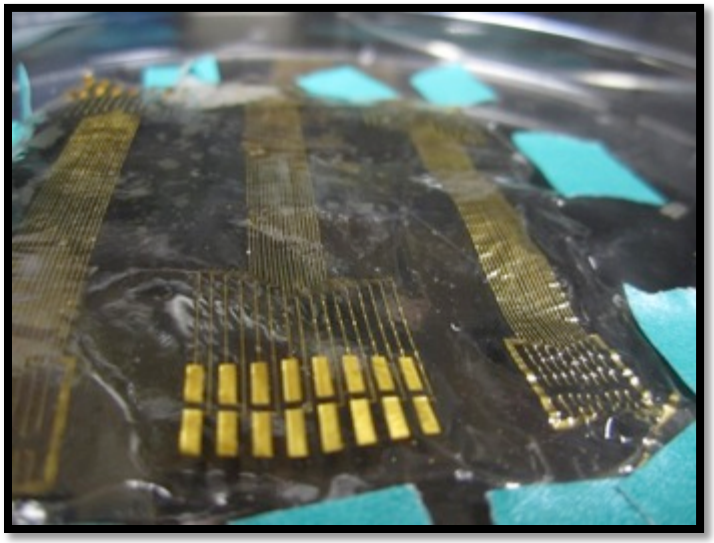

Figure 8.1. Initial attempts at silk electrode fabrication.

Designs for silk electrode interface into the DAQ system have already be completed (Figure 8.2) and upon successful optimization of the electrodes, the system should be able to record and supply multiple potentials across the skin surface at one time.

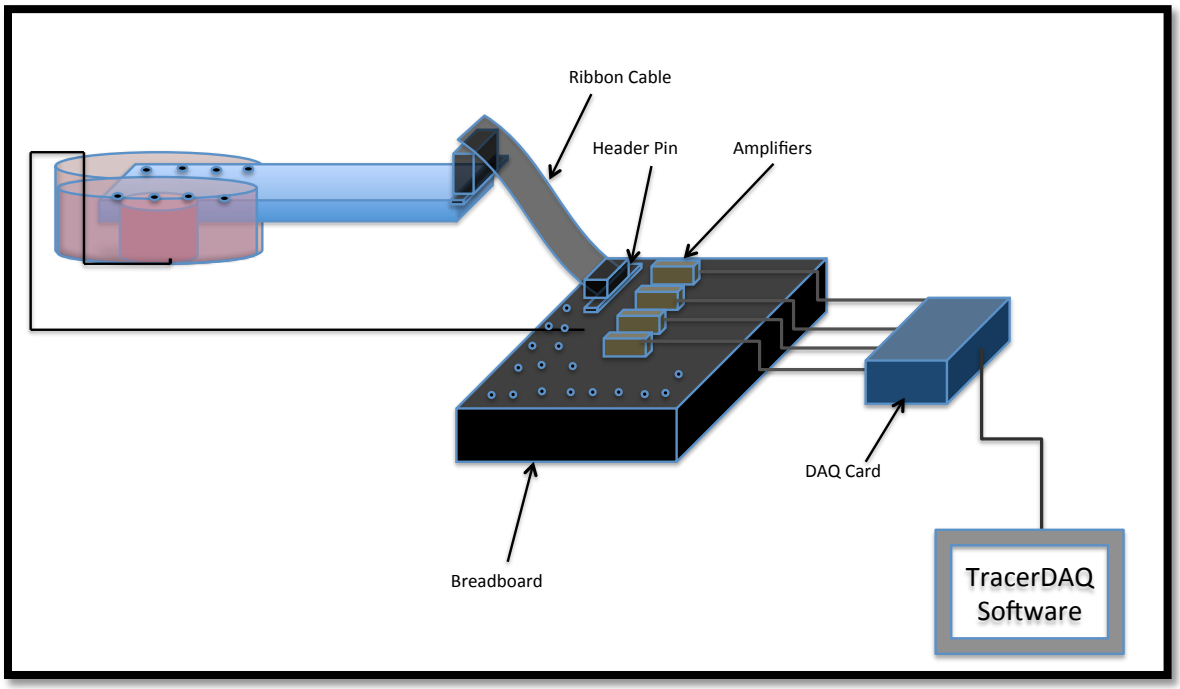

Figure 8.2. Interface of silk mat electrode and DAQ system to allow for the collection and application of multiple potential values at one time.

9. APPENDIX

Table 9.1. Shows media formulations used in the maintenance of the tissue engineered skin equivalent [T. DesRochers].

Material	**Source**	**[Stock]**	**Epi I (500mL)**	**Epi II (500 mL)**	**Cornification (500 mL)**
EPILIFE CF	Invitrogen # M-EPICF-500		361.5 mL	361.5 mL	236 mL
F-12	Invitrogen # 31765-035		120 mL	120 mL	237 mL
Adenine	Sigma # A2786-5G	18 mM	1 mL	1 ml	1 ml
Hydrocortisone	Sigma # H0888-1G	0.7 mM	1 ml	1 ml	1 ml
$CaCl_2$		0.5 M		1.8 mL	1.8 mL
T3	Sigma # T5516-1MG	10 nM	1 ml	1 ml	1 ml
ITS	Invitrogen # 41400-045		4 mL	4 mL	4 mL
Progesterone	Sigma # P8783-1G	2 nM	0.5 mL	0.5 mL	
Serum			0.5 mL (cFBS)	0.5 mL (FBS) Lot# ALG14153	10 mL (FBS) Lot# ALG14153

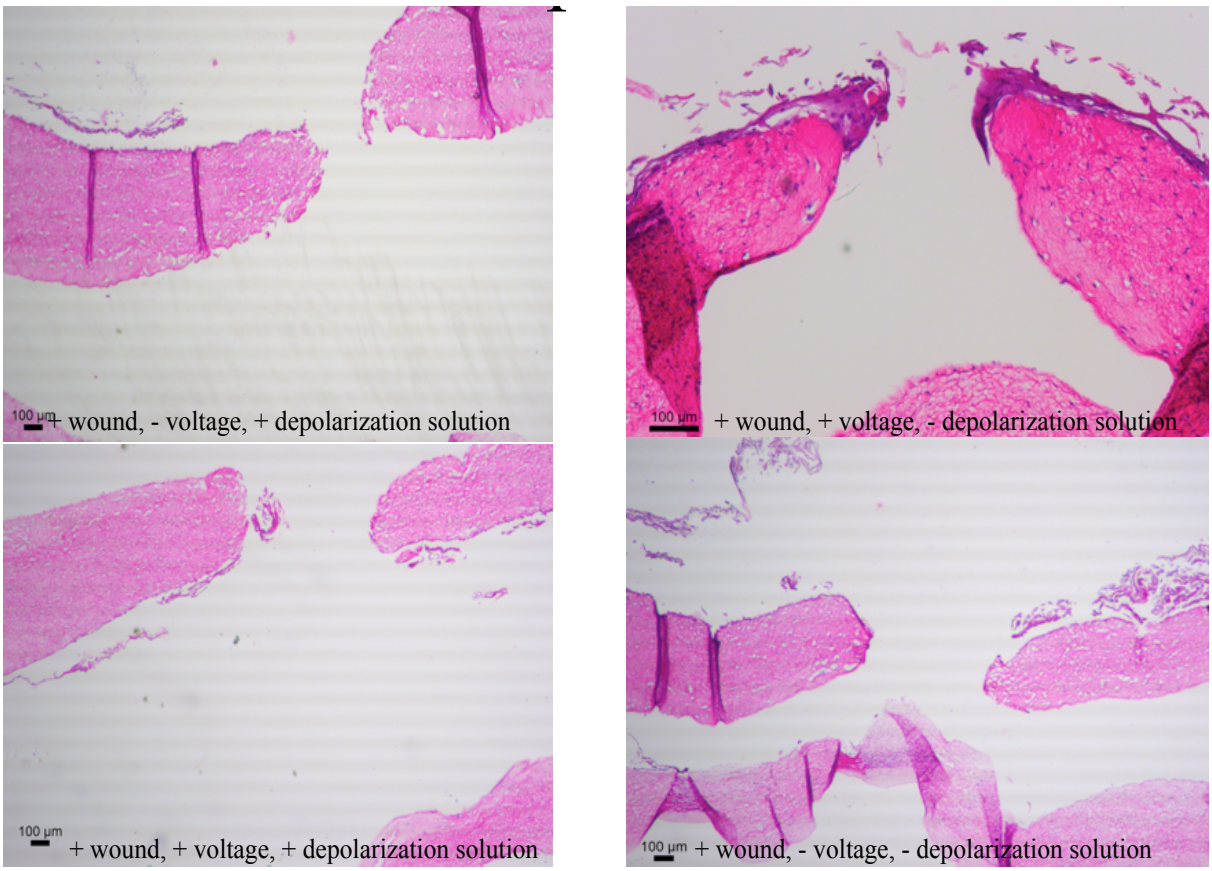

Figure 9.1. Sample histology across all experimental groups

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