# THE EFFECT OF RESOLVINS ON DERMAL WOUND HEALING

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**A thesis submitted to the**

**Graduate School—New Brunswick**

**Rutgers, The State University of New Jersey**

**in conjunction with**

**The Graduate School of Biomedical Sciences**

**the University of Medicine and Dentistry of New Jersey**

**in partial fulfillment of the requirements for the**

**Joint Degree of Master of Science**

**Graduate Program in Biomedical Engineering**

**Written under the direction of**

**Dr. Francois Berthiaume**

**and approved by**

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**New Brunswick, New Jersey**

**May, 2012**

# ABSTRACT OF THESIS

**THE EFFECT OF RESOLVINS**
**ON**
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A classical skin wound healing model can be divided into four distinct phases – instantaneous response, inflammatory, proliferation and the final remodeling phase. The balance between stimulatory and inhibitory mediators in each of these phases is critical in achieving optimal and efficient wound closure. It is well understood that inefficient or untimely resolution of inflammatory responses can result in profound delay in wound closure and increased tissue scarring as in the case with chronic wounds. This thesis studies the impact of exogenous administration of different types of pro inflammation resolution compounds such as Resolvin E1 (RvE1), Resolvin D1 (RvD1) and Resolvin D2 (RvD2) on dermal wound healing mechanism. *In vitro* migration assay studies performed using neutrophil cells confirmed the pro-resolution behavior of Resolvin compounds. All Resolvin compounds significantly blocked chemoattractant (fMLP) stimulated neutrophil migration. RvE1 demonstrated significantly better potency to block fMLP simulated neutrophil migration as compared to RvD1 and RvD2. This behavior was dose dependent and more pronounced at a concentration of 2000nM. Dorsal full thickness wound studies on wild type mice were performed using skin

substitute (Integra Dermal Regenerative® Skin or Alloderm® Regenerative Tissue Matrix) along with administration of 2000nM Resolvin at the wound site. Results from the study revealed that complete wound closure time significantly reduced from 28.6±1.52 days (control) to 19.4±1.52 days with RvE1 treatment, 22.8±1.79 days with RvD2 treatment and 24.4±2.19 days with RvD1 treatment ($n=5$, $p<0.05$). Histology of wounds treated with RvE1 at Day 30 revealed a well-structured and repaired site with organized layers of dense collagen and a completely re-epithelized surface as compared to the saline treated wounds (control). These results demonstrate that the addition of Resolvins to a wound site significantly accelerates the wound healing and re-epithelialization process.

# ACKNOWLEDGEMENTS

The list is long…

There are several people I would like to thank who have supported me through this endeavor. Firstly, I would like to start off with thanking Dr. Francois Berthiaume for his guidance and continuous support. He gave me the courage to think outside the box and to not be afraid of trying and failing. I would like to take his opportunity to thank everyone in Dr. Berthiaume's and Dr. Yarmush's laboratory, especially Melissa Przyborowski for training me on the surgery protocols and for always being willing to help when I needed it the most. A special thank you to Dr. Cai and Dr. Silver for willing to be a part of my thesis committee and for guiding me through this project. Shout out to Dr. Lagunoff and his team at UMDNJ for processing my histology slides at such short notice and taking time to share their expertise on wound healing with me.

Also, I would like to thank everyone in my team at Integra LifeSciences for being extremely supportive and understanding while I chased my educational pursuits.

Last but not the least; I would like to thank all my friends and my family for their faith in me.

Without any of your support, this work would never have been completed!

# DEDICATIONS

**To my Mom and Dad – my first teachers!**

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# CHAPTER 1

# INTRODUCTION

## 1.1 MOTIVATION AND SIGNIFICANCE

Wound care has evolved significantly in the past century from magical spells, incantations and potions to the use of sophisticated wound dressings, hemostats, hyperbaric oxygen chambers and the very recent regenerative dermal substitutes. Despite the emergence of new therapies and the evolution of wound care management, there still persists a need for more enhanced and efficient wound healing treatments. Currently, several therapies involving the administration of growth factors and stem cells to wound sites are being investigated to accelerate the wound healing process. The need for such therapies are more pronounced in cases involving chronic or non-healing wounds that are typical in patients having burns, diabetes mellitus or other immuno-suppressive predispositions. To put this into a qualitative perspective, according to *MedMarket Diligence Report*, in 2009 more than 100 million surgical procedures requiring some level of wound care management were performed globally [1]. High proportion of these wounds, stem from traumatic injuries, surgical injuries, burns and chronic conditions such as diabetes mellitus and vasculitis. To add to this, the prevalence of wounds caused due to diabetic ulcers are expected to grow at a rate of approximately 9% per annum with wounds caused due to pressure ulcers, venous ulcers and burns trailing close behind [1].

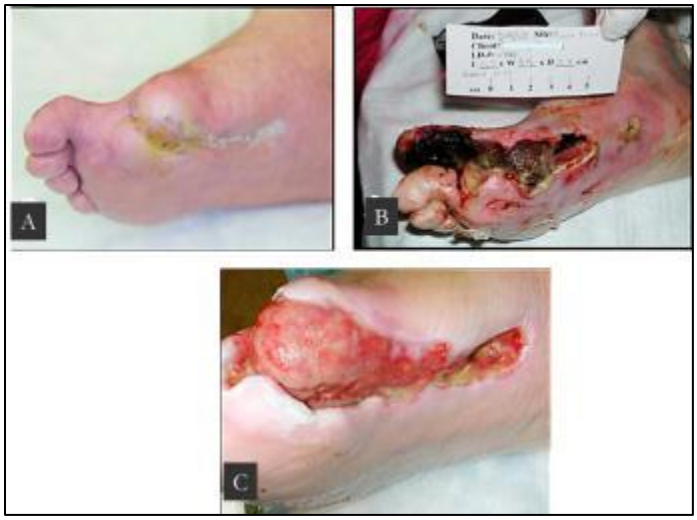



**Figure 1: Progression of Chronic Wounds: Diabetic Ulcers (http://www.hypertextbookshop.com)**

As of May 2008, according to the *Kalorama Information Journal*, the total number of chronic wounds adds up to approximately 6 million in the United States and 37 million globally. Among the various types of chronic wounds present, the most common and serious are diabetic ulcers due to rising incidence and prevalence of diabetes around the world [1]. If left untreated then the wounds can lead to tissue necrosis eventually requiring amputation. The National Health Interview Survey (NHIS) in 2008 estimated that the total prevalence of diabetes in U.S. is approximately 24 million people (7.0% of the population as a whole) [2]. Of these, an estimated 15% will develop foot ulcers during their lifetimes. More than 15% of foot ulcers result in amputation of the foot or limb [3] and foot ulcers precede approximately 85% of non-traumatic lower-limb amputations in people with diabetes [4,5]. This is only a US based statistic and on a global level this number is even more staggering. A recent statistic from the National Diabetes Information Clearinghouse sums the alarming state of the wound care market and

compelling need for better wound healing treatments. It states that diabetes makes it 46 times more likely to have an amputation [6]. Within one year after a diabetic foot amputation, 26.7% will have another amputation [6]. Three years after the first diabetic amputation, 48.3% will have another amputation. Within 5 years of a diabetes related amputation, 60.7% will have another amputation [6]. To add to that, 50% of all diabetics with an amputation are dead 3 years after the amputation and 65% of all of those with a diabetic amputation are dead 5 years from first amputation [6].

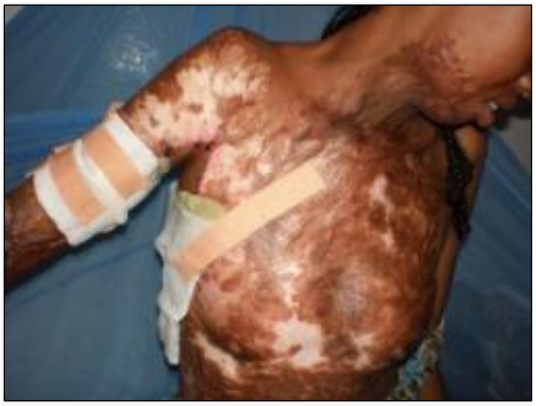


**Figure 2: Scarring from Burn Wound**
**http://woundcareassociates.com/wounds.php**

Burn wounds can be classified into minor burns, medically treated burns and hospitalized cases. Approximately 3.3 million minor burns are estimated to be treated in outpatient clinics [1]. These burns are typically treated using hydrogels and advanced wound care products. Medically treated burn wounds usually get more informed care to remove heat from the tissue, maintain hydration, and prevent infection. Approximately 6.3 million medical treated burns are estimated every year [1]. Whereas, burn wounds that require hospitalization are not as common but require more advanced and expensive care. These victims require

significant care, debridement, dermal regenerative substitutes and intensive reconstructive surgeries.

A significant attribute of all types of wounds is the occurrence of pathological infections. Infections usually lead to more extensive wound care time, the use of more expensive products and drugs, expensive antibacterial therapies, and increased morbidity and rehabilitation time.

Clearly, these statistics demonstrating the compelling socioeconomic impact of the current state of wound management emphasize the need for treatments that can potentially accelerate healing for better wound care. The old passive paradigm for wound management is now being looked at as being impractical. The emerging approach is more active and includes therapies that look at intervening and improving natural healing using such modalities as hyperbaric oxygen, electrical stimulation, skin surface negative pressure, exogenous growth factors and bioengineered skin substitutes.

From a business aspect, wound care market is considered the fastest growing medical sector as per the *Episom Business Intelligence Report* [7]. The future of wound care market is primarily driven by demographic demand and the continued pressure to rein in the high costs of chronic wounds. *Episom Business Intelligence Inc.* reported that as of May 2011 the global wound care market was estimated to be worth a staggering $7.2 billion and the US market was estimated to be around $89 million [7]. Another report from *Visiongain Inc.,* reported that the global advanced wound care market is expected to be worth $14.6 billion by 2021 [8].

## 1.2 PROJECT SCOPE

The objective of this project is to develop and test a therapy that can potentially accelerate and improve the wound healing process. The long-term goal is to develop the next generation of dermal skin substitutes that will speed up skin wound healing, reduce scarring and improve the appearance of healed wounds. Specifically, the scope of the project involves investigating and understanding the impact of adding pro-resolution molecules like Resolvin to a wound site. The hypothesis is that addition of Resolvin molecules during the early stages of wounding may accelerate the onset of the inflammation resolution phase and therefore drive subsequent stages of wound healing process. This strategy will be tested *in vivo* in a normal mice full thickness dorsal wound model. Specifically, this research will involve analyzing the impact of different types of resolvin molecules (derived from Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA)) in wound healing.

This project will be divided into two sections. The first section will focus on *in vitro* experiments performed to prove the pro-resolution behavior and the second section will focus on *in vivo* experiments performed to confirm the efficacy of therapy in an animal wound model.

# CHAPTER 2

# BACKGROUND

## 2.1 WOUND PHYSIOLOGY

### 2.1.1 SKIN WOUNDS: DEFINITION AND CLASSIFICATION

Wounds are defined as a "disruption of the normal anatomic structure and function", as per the Wound Healing Society [9]. A wound is typically classified as either being acute or chronic. Acute wounds are characterized by reparative healing processes that occur in an organized timely manner rather than regenerative processes. While in chronic wounds, such as venous stasis ulcers, pressure sores and diabetic ulcers, the reparative healing process occurs at a reduced rate that results in incomplete wound closure. Wound healing can be classified into three categories –

1] *Primary Intention Healing*

Such healing is typical in cases when the wound edges are brought adequately closer. These wounds heal spontaneously and result in scar formation oriented along the Langer's lines (collagen fiber alignment within the dermis) [9]. Most surgical wounds heal by primary intention healing. Wound closure is typically achieved with sutures, staples or adhesive tape. Healing post lacerations, well reduced bone fractures and flap surgeries are few examples of such healing mechanisms.

2] *Secondary Intention Healing*

Such healing is typical in cases when the wounds are large and coupled with extensive tissue loss following massive trauma such as third degree burns. Such wounds heal by the natural reparative process and are typically allowed to granulate

[9]. These phases are typically more pronounced than primary intention healing and usually result in increased scar formation.

3] *Tertiary Intention Healing*

Such healing is typical in cases when there is delayed primary closure. This occurs in cases when wounds are infected or contain foreign debris and cannot be closed until the complications are resolved. Healing by tertiary intention is also aided by surgical skin graft reconstruction in large open skin wounds. Failure to heal within a month, in these cases, can lead into a chronic state [9].

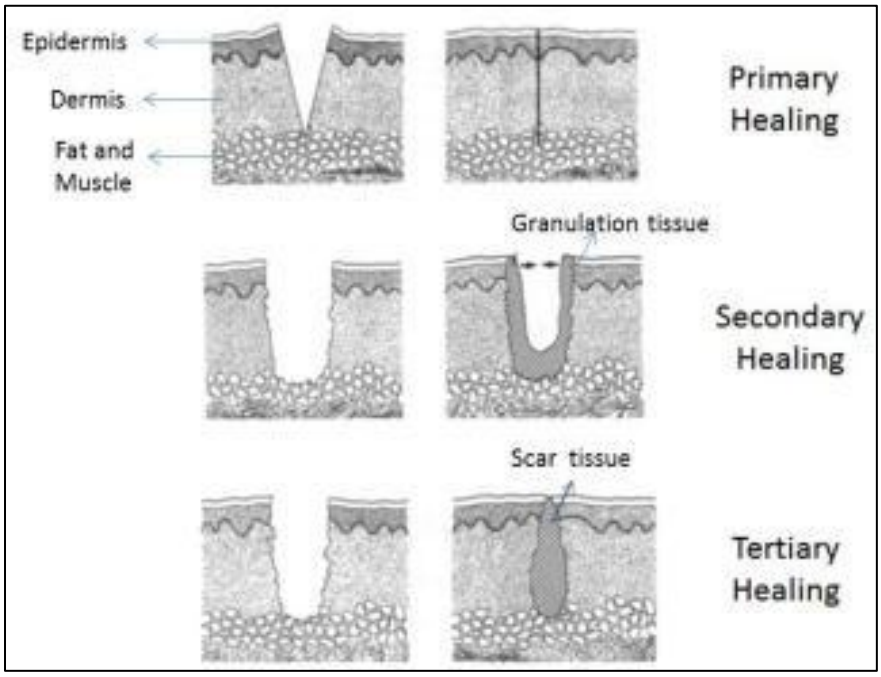



**Figure 3: Wound Healing Classification**
**Figure from Schwartz *et.al.* [10]**

Primary or tertiary wound healing is preferred to secondary healing since secondary intention involves a more severe wound contraction and scar formation. Additionally, full thickness wounds, like ones discussed in this thesis, do not heal spontaneously and require use of skin grafts.

### 2.1.2 SKIN WOUNDS: CLASSICAL HEALING MODEL

Wound healing is a very complex and intricate process. The immediate goal in wound repair is to achieve tissue integrity and homeostasis [11]. Full thickness wounds, such as the ones discussed in the thesis in later sections, damage many structures and layers such as epidermal keratinocyte layer, epidermal appendages (sweat glands, sebaceous glands and hair follicles), basement membrane and the dermis layer which comprises of fibroblasts, extracellular matrix (ECM), nerves and blood vessels [12].

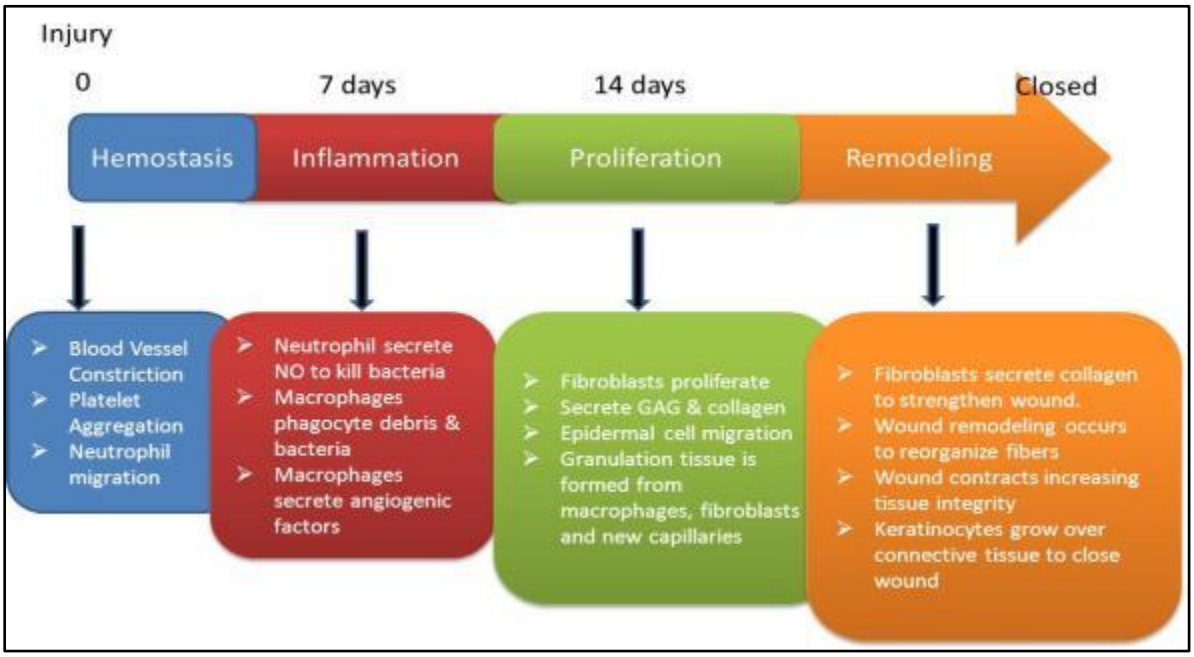



**Figure 4: Different Phases of Wound Healing**

As shown in **Figure 4**, the classical model of wound healing comprises of four fundamentally distinct stages – **instantaneous response phase, inflammatory phase, proliferative phase and remodeling phase.**

**a) Instantaneous Response Phase**

This immediate response to tissue injury is primarily triggered by ruptured cells and blood vessels present at the edges of the wound site. These cells transmit several 'stress' signals instantaneously after the tissue injury. Damaged local blood vessels respond by promoting platelet aggregation to eventually form a fibrin clot in order to achieve hemostasis and stop blood loss [12].

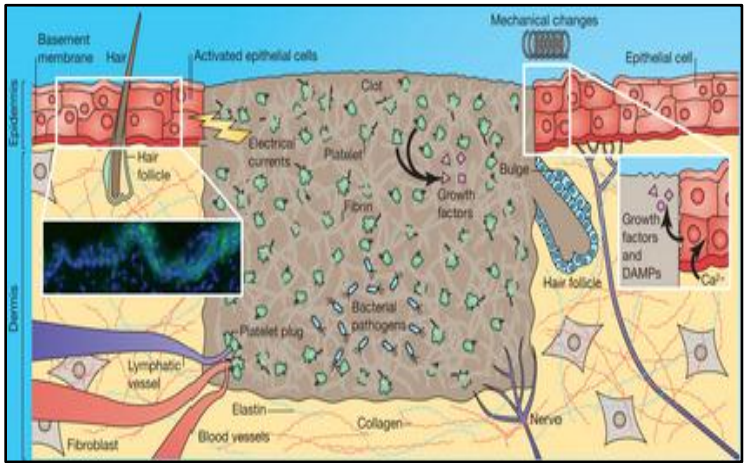



**Figure 5: Events during the Instantaneous Response Phase**
**Figure from Shaw T *et.al.* [12]**

This plug also provides structural support and integrity to the wound site while new collagen is being deposited [11]. Additionally, activated platelets are also an important source of growth factors (CXCL4: fibroblast growth factor, TGF-β: transforming growth factor, vascular endothelial growth factor etc.) that play an important role in the subsequent phases of wound healing [12].

**b) Inflammatory Phase**

During initial stages of this phase, the priority of inflammatory responses is to counteract microbial wound infections and this takes precedence over wound closure [13]. From a macroscopic level, an acute inflammatory response is associated with

signs of local redness, swelling, heat and pain; and from a microscopic level it is linked with edema and accumulation of leukocytes, specifically polymorphonuclear neutrophils (PMNs), monocytes, macrophages and mast cells [14].

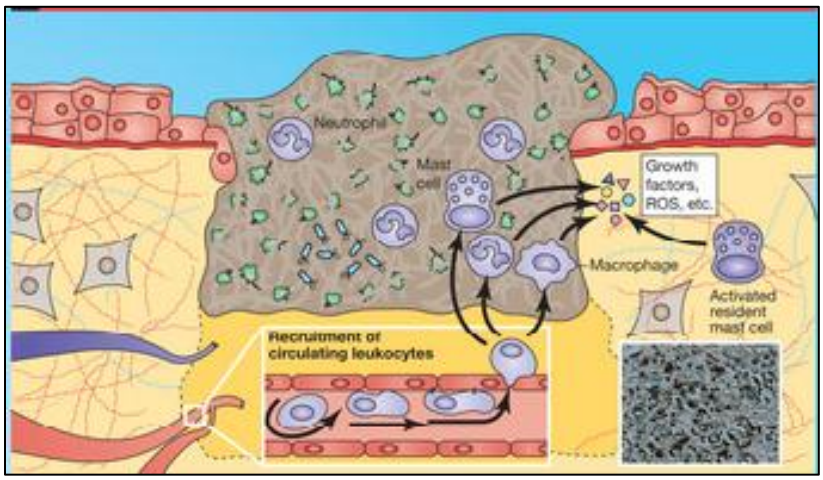



**Figure 6: Inflammatory Phase Events**
**Figure from Shaw T *et.al.* [12]**

Inflammatory response begins instantaneously with the infiltration of leukocytes (primarily PMNs) from damaged blood vessels to the wound site. PMNs are the most abundant cells in the wound site during the first two days post initial wounding [11]. Fibronectin and other platelet released growth factors are known to attract the PMNs into the wound site. These neutrophils engulf cellular debris and bacteria. Neutrophils destroy bacterial cells by releasing nitric oxide and reactive oxygen species (ROS) in a process known as 'respiratory burst' [12]. Simultaneous with clot formation, blood from ruptured blood vessels comes in contact with local tissue collagen and triggers blood platelets to release several inflammatory factors. Breached blood vessel cell membranes secrete inflammatory factors like thromboxanes and prostaglandins that cause the vessel to constrict to prevent further blood loss [11]. Vasodilation of blood vessels occurs within minutes of this initial vasoconstriction. Vasodilation further increases in influx of inflammatory cells like

leukocytes into the wound site. Pro-inflammatory factors like serotonin, bradykinin, protagladins, prostacyclins, thromboxane and histamine are released into the local wound site [15]. These pro-inflammatory factors also serve as growth factors and increase cell proliferation and migration.

After two days post injury, monocytes from the bloodstream start infiltrating the wound site and eventually mature into macrophages. The main goal of macrophages is to phagocytize bacteria, damaged cells and tissue debris. Macrophages are also known to release several cytokines and growth factors that attract cells that play a key role in initiating proliferation phase in wound healing. Macrophages are stimulated by low oxygen levels in a wound site and produce factors that induce and accelerate angiogenesis [16]. Additionally, macrophages are also involved in stimulating cells that reepithelialize the wound, create the granulation tissue bed and lay down neo-extracellular matrix in the wound site [17, 18].

Inflammatory cells in the process of cleaning up cell debris also stress the surrounding tissue by generating reactive oxygen species (ROS) and nitric oxide (NO). In order to protect themselves, surrounding local cells are known to induce cytoprotective and detoxifying programs [12]. Therefore, uncurbed inflammation is considered a hallmark of chronic or ‘non-healing’ wounds [11].

Successful wound repair requires effective and timely resolution of the inflammatory responses, i.e., down regulation of pro-inflammatory molecules (such as IL-10 or TGF-β1) and up regulation of anti-inflammatory molecules (IL-1 receptor antagonist or soluble TNF receptor) [11]. Complete resolution of an acute

inflammatory response and the return of the local tissues to homeostasis are necessary for activation of subsequent steps of wound healing process.

**c) Proliferative Phase**

The initiation of proliferative phase is marked by the infiltration of fibroblasts into the wound site. This occurs within two or three days after the initial tissue injury. Angiogenesis or neovascularization occurs concurrently with fibroblast proliferation. Angiogenesis is imperative for complete wound healing since fibroblasts and epithelial cells require oxygen and nutrients for proper functioning. Angiogenesis occurs when endothelial stem cells, from uninjured blood vessels, develop pseudopodia and penetrate through extracellular matrix components into the wound site to develop new blood vessels [16]. Endothelial cells are attracted to the wound area by fibronectin present on the fibrin scab and chemotactically by angiogenic factors released by other cells (macrophages and platelets) [19].

Fibroblasts function in laying down fibrous tissue or fibroplasia at the wound site. By the end of the first week, fibroblasts are the predominate cells in the wound site. Fibroblasts are recruited from uninjured neighboring cutaneous tissues and migrate across the wound using the fibrin cross-linking fibers [19]. Fibroblasts function in laying down the granulation tissue. This tissue serves as rudimentary tissue and contains blood vessels, fibroblasts, inflammatory cells, endothelial cells, myofibroblasts and new provisional extracellular matrix. Fibroblasts also secrete cytokines that attract epithelial cells to the wound site.

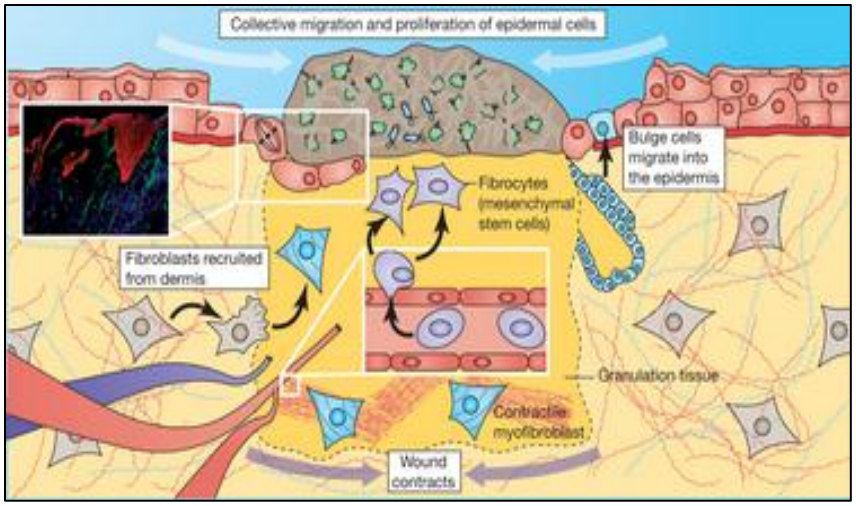



**Figure 7: Events during Proliferative Phase**
**Figure from Shaw T *et.al.* [12]**

One of the primary functions of fibroblasts cells is collagen deposition in the wound site [19]. Rapid production of Type III collagen and fibronectin occurs within the first 3 days since tissue injury [20]. Later during the later maturation phase, Type III is replaced with Type I collagen.

Epithelial cells (keratinocytes) migrate across the granulation tissue bed and function in reepthelializing the open wound site. Keratinocytes migrate from the edges of the wound site and move towards the center of wound (also known as the epithelial cell tongue motion). Keratinocytes are known to develop extending cellular processes like lamellipodia that assist in propelling them across the wound bed [21]. It is known that faster migration of keratinocytes can result in reduced tissue scarring [21]. Simultaneously, increased proliferation of keratinocytes at wound edges occurs to provide sufficient keratinocytes for complete coverage of wound site. Once migration is complete, keratinocytes secrete proteins that form new basal membrane. Basal cells differentiate to regenerate strata found in reepthelialized skin.

Wound contraction plays a critical role in defining the dynamics of wound closure. In full thickness wounds, contraction is known to peak between 5-15 days post initial wounding and continues even after the wound is completely reepthelialized [20]. Myofibroblasts play a key role in wound contraction [22]. Myofibroblasts contain the same kind of actin filaments present in smooth muscle cells [23]. Myofibroblasts form connections to the ECM at the wound edges and pull them contracting the wound size [24]. The end of the proliferative stage is marked by the breakdown of provisional ECM which leads to a decrease of hyaluronic acid and increase in chondroitin sulfate which gradually triggers the fibroblasts to stop migrating and proliferating [25].

**d) Remodeling/Maturation Phase**

The goal in this phase is to restore normal architecture and functionality of tissue. This is achieved by balancing several degradation and synthesis signals [12]. The initiation of the maturation phase can vary from 3 weeks to a year or longer depending upon the size of the wound [26].

During maturation phase, Collagen type III is gradually degraded and converted to Collagen type I. As the Collagen type I deposition increases the strength of the wound increases to 50% of that of normal tissue to ultimately 80% as strong as normal tissue [17]. The newly formed blood vessels are developed and form functional vascular networks [12]. The ECM is reorganized and remodeled during this phase [12].

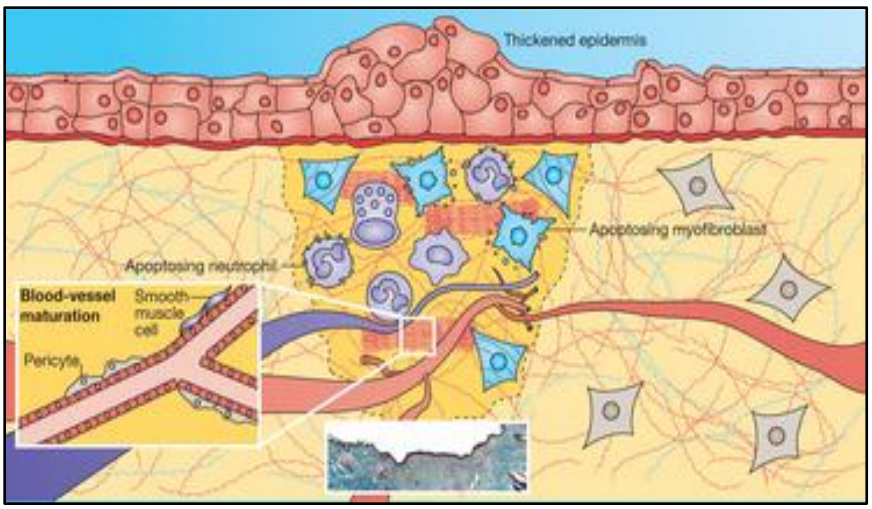



**Figure 8: Events during Remodeling Phase**
**Figure from Shaw T *et.al.* [12]**

### 2.1.3 REGENERATION VERSUS REPAIR

Repair refers to an instant physiologic remodeling of a tissue after injury in order to restore normal tissue functionality without necessarily replacing damaged tissue. Regeneration refers to substitution of damaged tissue with new tissue that is identical in both morphology and functionality. It has been reported that basic conditions required for tissue regeneration are usually conflicting from those that promote tissue repair [27]. Dermal substitutes force wound healing to follow a more regenerative or less scarring pathway by reducing wound contraction.

In case of superficial injuries and burns, the wound healing model follows a regenerative paradigm. In such cases, the dermis layer is relatively intact and sufficient epidermal cells remain that can proliferate and regenerate complete intact epidermal layer with normal architecture and functionality. In such cases regeneration occurs spontaneously within four weeks or less [28]. In cases of deep acute wounds, the epidermis layer is completely destroyed and the dermis is also considerably damaged. Thus, the healing process in this case is more reparative than regenerative.

Wound repair involves the synthesis of scar tissue, to fasten wound closure process, without restoration of the normal structure and function of the tissue [28].

## 2.2 CURRENT THERAPIES AND THEIR DISADVANTAGES

### 2.2.1. Dermal Substitutes

Skin substitutes can be divided into three main classes, namely, biological, biosynthetic and purely synthetic substitutes. The biological skin substitutes allow for excellent re-epithelialization due to the presence of a basement membrane. Synthetic skin substitutes aid in reduced scarring and providing a better aesthetic result [29]. Autogenic skin grafts are also predominantly used. Few examples of biological skin substitutes are Matriderm® (MedSkin Solutions, Germany) which is bovine dermal matrix and Alloderm® (LifeCell Corporation, New Jersey) which is human dermal matrix. Few examples of synthetic skin substitutes are Oasis® (Healthpoint Ltd., Texas), Integra® Dermal Regeneration Template (Integra LifeSciences, New Jersey) and Biobrane® (Bertek Pharmaceuticals, Texas).

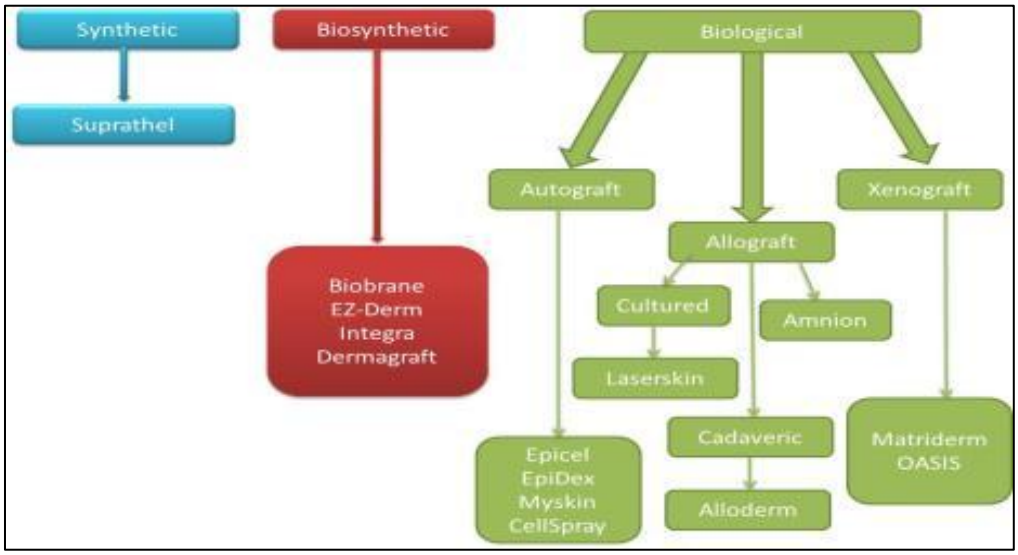



**Figure 9: Different Types of Skin Substitutes**

Within two weeks of graft placement in a wound bed, these substrates are sufficiently vascularized to support an epidermal skin graft which goes on to regenerate the epidermis layer. Though current dermal substitutes have offered a big revolution in management of wounds, one of its biggest drawbacks is the fact that they lack inherent antimicrobial activity and are therefore at high risk for infections especially due to slow vascularization rate [29, 30]. Another drawback is the shearing of skin substituents in the wound bed due to inadequate angiogenesis and deposition of non-degraded fibers in the wound bed due to poor biodegradation [31].

### 2.2.2. Stem Cell Therapy

Published studies have demonstrated that bone marrow derived stem cells and vascular progenitor cells released into the bloodstream during tissue injury spontaneously migrates towards the wound site and contributes to wound healing process [32]. Several published studies have reported that mesencyhmal and hemopoietic stem cells that were directly injected into injuries have demonstrated enhanced wound site regeneration, complete functionality restoration and with reduced scarring [33, 34].

### 2.2.3. Surgical Debridement

This is the surgical removal of a patient's dead, damaged, or infected tissue to improve the healing potential of the remaining healthy tissue. Such a surgery is often extensive and requires additional follow ups to ensure complete removal of gangrene tissue especially in the case of chronic wounds. Often, this leads to additional follow

up surgeries to removal more tissues. This treatment is usually followed with the use of skin grafts or substitutes.

### 2.2.4. Hyperbaric Oxygen Treatment

This therapy relies on supplying direct oxygen to damaged tissue. Oxygenation of damaged tissue has shown to provide healing effects. This technology taps potential of transporting oxygen via the plasma since the hemoglobin transportation pathway is saturated quickly at a low oxygen concentration. However the patient will be required to be placed in a sealed high pressure oxygenated chamber. This chamber is hooked to oxygen supply tank therefore decreasing the patient mobility and increasing discomfort during therapy. Another major drawback of this technique is that other regions of the body will also be exposed to unnecessary high pressured oxygen which may cause vasoconstriction, toxicity and tissue damage [35].


**Figure 10: Hyperbaric Oxygen Chamber**
**(*www.bronsonhealth.com*)**

### 2.2.5. Amputation

Amputation is the final and last course of action against tissue necrosis, especially in the case of peripheral chronic wounds. This is done to prevent spread of gangrene to healthy tissue. Though unwanted, this is the last resort to stop progression of advanced tissue necrosis. Such surgeries are often accompanied with severe infections and additional surgeries. Life expectancy of patient after amputation is also known to significantly reduce.

# CHAPTER 3

# PROPOSED APPROACH

## 3.1 PHYSIOLOGICAL IMPORTANCE OF RESOLUTION PHASE

Eming S. *et.al.,* consider inflammatory responses as an 'inevitable consequence' of tissue injury [11]. In a skin repair model, infiltrating leukocytes are considered to be the primary cells involved in triggering the inflammatory response. These cells not only help in battling invading pathogens but also assist in overall tissue remodeling and reconstruction. Therefore, excessive or reduced influx of leukocytes into the injured site may have significant impact on downstream cellular migration, cell proliferation, differentiation of wound site tissues and ultimately will affect the quality of the healing response [11]. Additionally, successful wound repair also requires effective and timely resolution of the inflammatory responses, i.e., down regulation of pro-inflammatory molecules (such as IL-10 or TGF-β1) and up regulation of anti-inflammatory molecules (IL-1 receptor antagonist or soluble TNF receptor) [11].

Counter regulation of inflammation is achieved by several systemic physiological mechanisms. These involve pathways that activate increase in the circulating levels of glucocorticoids, acute phase response and anti-inflammatory cholingeric efferent neural pathways in order to control and resolve the inflammatory response [36]. A class of endogenous produced lipid molecules from ω-3 polyunsaturated fatty acids, called resolvins and lipoxins are known to counter regulate inflammation and activate resolution [36]. Without these controls

mechanism, tissues would be overwhelmed by persistent inflammatory cell infiltrates, edema and tissue damage [36].

Recently, experimental models have demonstrated the importance of the inflammation resolution phase in wound healing. Excessive and chronic inflammation has been shown to be associated with impaired wound healing. It has been well documented that resolution of the initial acute inflammatory phase is critical in the progress of wound repair. In recent studies, Nrf-2, target of the keratinocyte growth factor-1 was identified as novel transcription factor regulating the inflammatory response during repair. The wound healing response in Nrf-2 knocked out mice was characterized by a prolonged inflammatory response following wound closure [37]. Several other researches have shown an increase in wound site re-epithelialization when inflammatory responses were appropriately reduced [38, 39].

Neutrophils are known to be dominant effectors during the early stages of inflammatory response. However, at times neutrophil granule contents are inadvertently spread into the extracellular matrix which leads to local tissue damage and results in amplification of acute inflammatory signals [40]. Further, due to increased activation and recruitment of neutrophils, endogenous chemical mediators such as eicosanoids (such as prostaglandin E2 and leukotriene B4) are released that could further cause more tissue damage [40]. It has been understood that untimely onset of the inflammation resolution phase in these clinical conditions may be responsible for the impairment in the quality of wound healing.

The progression from an acute to chronic inflammation as in arthritis, periodontal disease and cardiovascular disease, to name a few, is widely viewed as an

excess of pro-inflammatory mediators [41]. It is well known that chronic wounds fail to achieve complete wound closure and instead remain in a state of chronic inflammation. Unbalanced proteolytic activity during the inflammatory phase due to the lack of reduced resolution activity is primarily responsible for chronic conditions [11]. It is understood that tissue hypoxia, necrotic tissue remains and bacterial components are capable of sustaining a continued influx of neutrophils and macrophages, keeping the local tissue in a high stress inflamed state [11].

Several studies have demonstrated that inflammation is directly linked to the extent of scar formation [11]. As an example, fetal wounds are known to heal without scarring. Even though there are several differences between the wound healing mechanism in fetal and adult tissues, the hallmark of fetal wound repair is the lack of inflammation. Additionally adult-like scar formation was observed when inflammation was provoked in fetal wounds [29]. As a second example, studies in mice with reduced systemic estrogen levels demonstrated impaired healing response [11]. This was linked to the fact that estrogen is known to dampen the inflammatory response and curbing inflammatory responses was impacted due to reduced levels of estrogen [30].

Therefore, complete resolution of an acute inflammatory response and the return of the local tissues to homeostasis are necessary for wound healing. Pro-resolution mediators like Resolvin and Lipoxins are different from traditional anti-inflammatory mediators. These mediators help in the restoration of tissue homeostasis and also promote clearance of inflammatory cells without associated immunosuppression. During the resolution phase, members of the versatile Resolvin

family have been shown to halt neutrophil infiltration, trigger macrophage phagocytosis of apoptotic neutrophils, accelerate the removal of phagocytes from the injured site, reduce pain by exhibiting analgesic properties and stimulate the development of molecules involved in antimicrobial defense [13].

## 3.2 RESOLVINS: SYNTHESIS, MECHANISM AND PREVIOUS WORK

Resolvins are a class of lipid molecules that are endogenously produced from the omega-3 polyunsaturated fatty acids (ω-3 PUFA) like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (also found in fish oils). A significant percentage of fatty acid composition in the nervous system, retina, sperm cells etc., consists of phospholipids composed of EPA and DHA. It is well known that ω-3 PUFAs can counteract the onset of several inflammatory diseases [36]. These lipid mediators were identified as potent endogenous regulators of excessive inflammatory responses that act via several cellular targets to stimulate resolution and preserve immune vigilance [36]. These include down-regulation of cell adhesion molecules on endothelial cells and leukocytes, reduced chemotaxis and transendothelial migration, reduced activation of neutrophils, inhibition of pro-inflammatory mediators and stimulation of phagocytosis of apoptotic neutrophils and macrophages [36, 43]. Resolvins and Lipoxins are known to act as endogenous anti-inflammatory mediators at low concentrations (pM or nM) and at specific G-protein couple membrane-spanning receptors [36]. For example, nanomolar concentrations of resolvins are known to dramatically reduce dermal inflammation, peritonitis, dendritic cell migration and interleukin production [43].

The term ‘resolvins’ or ‘resolution-phase interaction products’ was coined by Professor Charles Serhan and colleagues because these compounds were first encountered in resolving inflammatory exudates [43]. Compounds derived from eicosapentaenoic acid (EPA) are designated as resolvins of the E series, while those formed from the precursor docosahexaenoic acid (DHA) are denoted as either resolvins of the D series.

## [1] Resolvin E series

Endogenous Synthesis:

In the absence of aspirin (normal healing condition), cytochrome P450 monoxygenase converts EPA to 18R–hydroperoxy EPE intermediate. This intermediate is acted upon by 5-lipoxygenase, present in PMN molecules to form 5S, 12R, 18R trihydroxy EPA or Resolvin E1 (RvE1).

In the presence of aspirin, the cyclooxygenase enzyme (COX-2) in microvascular endothelial cells is acetylated. The aspirin activated COX-2 enzyme oxygenates EPA to form an 18R–hydroperoxy EPE intermediate. The intermediate is reduced to form a 5S-hydroperoxy 18R hydroxy EPE intermediate by the action of 5-lipoxygenase in PMN molecules [43]. The 5S-hydroperoxy 18R hydroxyl EPE intermediate undergoes enzymatic epoxide hydrolysis to produce 5S, 12R, 18R trihydroxy EPA or Resolvin E1 (RvE1).

Aspirin plays an important role in the synthesis of Resolvin molecules, it acetylates COX-2 enzyme and facilitates the synthesis [43]. Thus, at local sites of inflammation, aspirin treatment enhances the conversion of the ω-3 PUFAs like EPA

and DHA to 18R-oxygenated products, i.e. resolvins of the E and D series, which carry potent anti-inflammatory signals [43].

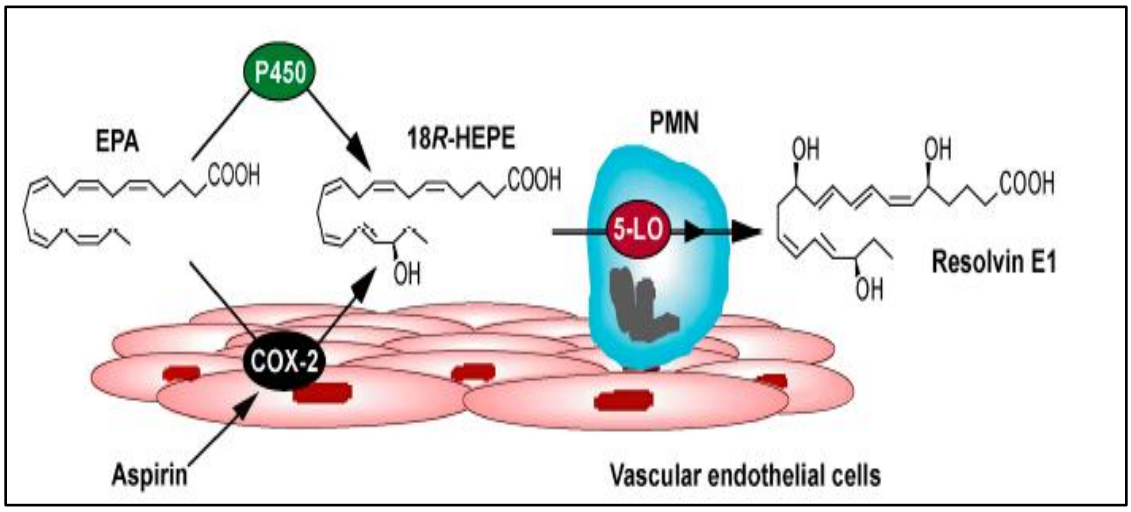



**Figure 11: Synthesis of RvE1 molecule via P450 cytochrome and COX-2 pathways Figure from Arita *et.al.* [44]**

Mechanism of Action:

RvE1 is considered the first ω-3 PUFA derived lipid mediator with receptor mediated anti-inflammatory and pro-resolving actions. The high potency of RvE1 can be attributed to its specific stereochemistry and double bond nature that promotes in selective recognition by G-protein coupled receptor (GPCR) CMKLR1 (also known as ChemR23) [44]. The significance of RvE1and CMKLR1 interaction were revealed in dendritic cells that are known for stimulating down regulation of pro-inflammatory cytokine IL-12 production [44] and transepithelial neutrophil migration [45]. RvE1 also activates this CMKLR1 receptor expressed in monocytes cells. On activation, this receptor up regulates mitogen activated protein kinase (MAPK) activity that controls monocyte proliferation, migration and apoptosis. Another study showed that CMKLR1 knockout mice are unable to resolve zymosan-induced peritonitis

exhibiting the importance of this receptor in inflammation resolution [57]. RvE1 is also interacts with Leukotriene $B_4$ receptor BLT1 expressed in neutrophils. Activation by RvE1 results in dampening pro-inflammatory mediators specifically by attenuating neutrophil migration [46, 59].

Previous Studies:

Several in-vivo and in-vitro experimental studies have demonstrated the potent anti-inflammatory behavior of RvE1. For instance, administration of RvE1 reduced ischemia in kidney injury [36]. RvE1 also demonstrated reduction of inflammation in mice white adipose tissues. Topical application of RvE1 to inflamed gingival tissue stimulates restoration and reduces inflammation in a rabbit model induced with periodontitis [36]. RvE1 stimulates resolution of allergic airway inflammation by down regulation IL-17, cytokine that helps in sustaining allergen induced airway inflammation, in a mice model [47]. Additionally, a clinical study showed that synthetic RvE1 reduced ocular inflammation in patients with dry eye condition (Clinicaltrails.gov identifier: NCT00799552). RvE1 are also known to reduce pro-inflammatory markers in human pancreatic islets [44]. Administration of small volumes of RvE1 (100ng/mouse) is known to reduce leukocyte infiltration by 50-70% [44]. As a summary, presence of RvE1, at nanomolar concentrations, is known to reduce dermal inflammation, peritonitis, dendritic cell migration and IL12 production, attenuates IL8 mediated neutrophil migration and intracellular ROS generation [44, 48].

Resolvyx Pharmaceuticals, Inc., a leading therapeutics company, published results of a study demonstrating that RvE1 effectively suppresses IL-23 and IL-17, two key inflammatory mediators of chronic inflammatory disease, in a preclinical model of asthma. The IL-23/IL-17 pathway has been linked to chronic inflammation, tissue remodeling, pathological neovascularization and bone loss. [49].

## [2] Resolvin D series

Endogenous Synthesis

In the absence of aspirin (normal healing process), COX-2 enzyme in human microvascular endothelial cells converts DHA to 17S-hydroxy-DHA intermediates. Enzymatic epoxidation generates a 7S, (8)-epoxide intermediates which are acted upon by 5-lipoxygenase to yield 7S, 8R, 17S-trihydroxy-DHA or Resolvin D1 (RvD1) and 7S, 16R, 17S trihydroxy-DHA or Resolvin D2 (RvD2).

In the presence of aspirin, aspirin acetylates COX-2 enzyme present in microvascular endothelial cells. Acetylated COX-2 enzyme oxygenates DHA at carbon 17 to form 17R-hydroperoxy DHA intermediate compounds [43]. These intermediates are then reduced to 17R-hydroxy-docosa hexaenoic acid (17R-HpDHA) which is further reduced by 5-lipooxygenase to produce a number of trihydroxylated isomers called 7S, 8R, 17R-trihydroxy-DHA or AT-RvD1 and 7S, 16R, 17R-trihydroxy-DHA or AT-RvD2. [36, 50, 60]. The key difference between Resolvin D series and their aspirin triggered counterparts is the stereochemistry switch of hydroxy group at carbon 17.

RvD1 and RvD2 are enzymatically deactivated by eicosanoid oxidoreductase to novel 8-oxo- and 17-oxo-RvD1. Oxidation of these molecules results in dramatic decrease in bioactivity. At the same time, AT-RvD1 and AT-RvD2 resists rapid deactivation. These may demonstrate the vitality of aspirin and ω-3 fish oils in humans [60].

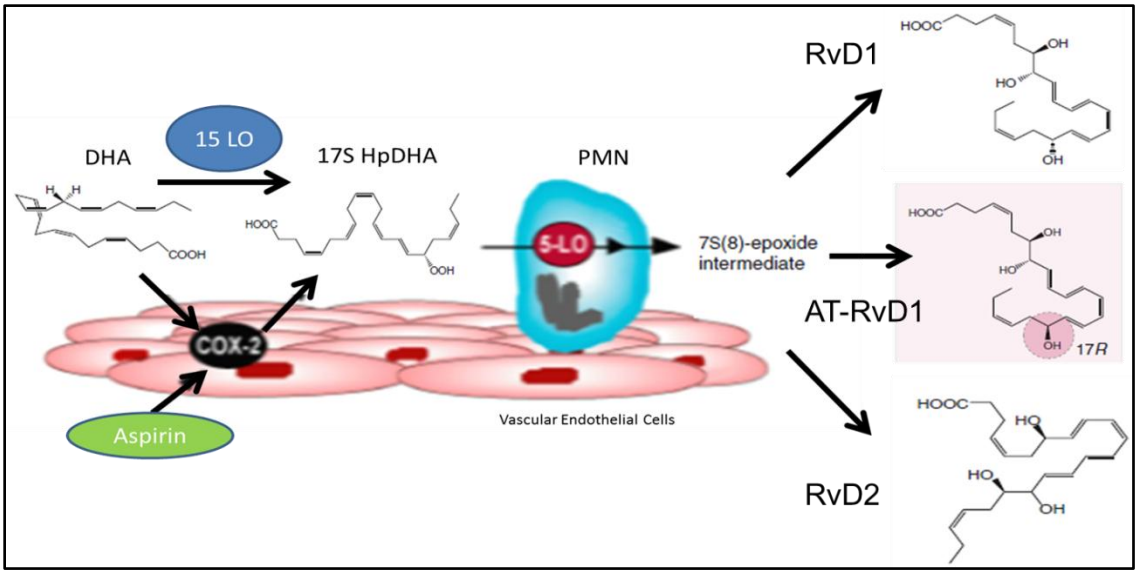



**Figure 12: Synthesis of RvD1, AT-RvD1 and RvD2**
**Figure is modified from Arita *et.al*. [44]**

Mechanism:

Similar to Resolvin E1, the stereospecificity and double bond nature of Resolvin D series molecules makes them ideal substrates for receptors. Two G-coupled receptors, GPR32 and ALX/FPR2 expressed in monocytes were identified to bind RvD1 [36]. On activation, these receptors enhance phagocytic and clearance activity of macrophages [57]. Additionally, RvD2 is also known to be a potent inhibitor of Transient receptor potential subtype V1 (TRPV1) and A1 (TRPA1) which explains its analgesic properties [51]. RvD2 decreases leukocyte–endothelial interactions by

endothelial-dependent nitric oxide production, and by direct modulation of leukocyte adhesion receptor expression.

Previous Studies:

D resolvins and AT-D resolvins have been experimentally shown to display potent pro-resolution activity such as clearing neutrophils from inflamed tissue sites, slowing down neutrophil migration and inhibiting neutrophil infiltration [36]. Resolvin D1 (RvD1) demonstrated potential to reduce inflammatory response. This action shows ability to control oxidative tissue injury forced by neutrophils present in an inflamed tissue [36]. Resolvin D1 and D2 are also known to block the production of TNF-induced transcripts that are responsible for the production of pro-inflammatory cytokine interleukin-1β [40]. Resolvin D2 (RvD2) have been shown to impede neutrophil-endothelial cell interactions and neutrophil trafficking by production of nitric oxide [36, 52]. RvD2 also promotes activation of microbial phagocytosis by macrophages. In a murine sepsis model, RvD2 administration reduced pro-inflammatory cytokine levels, namely IL-6, IL-1β, IL-23, IL-17 and IL-10, and tumour necrosis factor TNFα levels while enhancing bacterial clearance [52]. Additionally, AT-D resolvins and D-resolvins, at nanomolar concentrations, have demonstrated ameliorating effects in induced colitis model and these effects have been associated with dampening PMN infiltration [52].

## 3.3 HYPOTHESIS

The inflammation resolution phase can at times be the rate limiting step in the wound healing process. As discussed in the previous section, Resolvins have been shown to treat a broad range of acute and chronic diseases caused due to failure of resolving inflammatory responses and restoring immune homeostasis.

Previously, Sarkar *et.al.*, demonstrated that administration of Stromal Derived Factor-1 (SDF-1) decreased early stage inflammatory response and simultaneously delayed contraction and promoted re-epithelialization [61]. This study demonstrated that decreasing the inflammatory phase could have profound impact on the overall wound healing process. Similarly, the proposed approach to accelerate skin wound closure involves administrating Resolvins molecules to the wound site during the first week post tissue injury. The hypothesis is that Resolvin molecules, due to their pro-resolution characteristics, can assist in expediting the inflammatory resolution phase and accelerate the wound healing process by promoting wound closure. Wound closure will be determined macroscopically by evaluating the extent of reepithelialization and wound contraction. Additionally, immunochemical analysis will be performed to determine if there is any difference in collagen deposition, re-epithelialization progress and tissue reconstruction compared to the vehicle or control. Three commercially available Resolvin molecules - RvE1, RvD1 and RvD2 will be investigated in this study.

# CHAPTER 4

# MATERIALS AND METHODS

## 4.1 EXPERIMENTAL DESIGN

The basis of this study is to investigate the effect of exogenous administration of RvD1, RvD2 and RvE1 on the rate of dermal wound closure in wild type mice. *In vitro* studies were performed to characterize and quantify the pro-resolution behavior of each Resolvin molecule in order to determine optimal efficacy dosage parameters for *in vivo* studies.

The experimental study was divided into two sections. The first section focuses on *in vitro* experiments, i.e. developing and optimizing a neutrophil migration assay to demonstrate pro resolution activity of Resolvin molecules. The second section focuses *in vivo* experiments performed using a full thickness dermal wound model and based on results obtained from *in vitro* experiments.

## 4.2 *IN VITRO* STUDIES

### 4.2.1 MATERIALS

1) Corning HTS Transwell® 24 well plate

2) Resolvin D1 (Cayman Laboratories, Catalog No: 10012554)

3) Resolvin D2 (Cayman Laboratories, Catalog No: 10007279)

4) Resolvin E1 (Cayman Laboratories, Catalog No: 10007848)

5) Culture medium

   a. Roswell Park Memorial Institute (RPMI) – 500ml supplemented with 8ml L-glutamine and 3g HEPES (Fisher Scientific) and 10% (50ml) fetal bovine serum (FBS, Invitrogen) and 1% (5ml) Penicillin Streptomycin.

   b. Iscove's Modified Dulbecco's Medium (Invitrogen) – 500ml supplemented with 20% (100ml) fetal bovine serum (FBS, Invitrogen) and 1% (5ml) Penicillin Streptomycin.

6) Hematocytometer and Trypan Blue for cell counting

7) Transwell Inserts with 3µm or 8µm pore size.

### 4.2.2 METHODS

**Step 1: Differentiation from HL60 to Neutrophils [62]**

1. Human Promyelocytic Leukemia (HL-60) cells were maintained in IMDM. Cells were passaged once a week when the density would approach 1-2 million cells/ml.
2. Neutrophil-like cells were derived from HL-60 cells through differentiation with DMSO.
3. Cells were differentiated in RPMI culture medium containing 1.3% DMSO. Cells were left to differentiate for 5 days.
4. After 5 days, differentiated cells are found attached to the bottom of the flask. The cells are trypsinized, washed, counted and resuspended to obtain a concentration of at least 100,000cells/100ul of culture media.

**Step 2: Preparation of Resolvin and Control Solutions**

Calculation:

Supplied concentration = 25µg/250µl ethanol

Molecular Weight of Resolvin $\simeq$ 376.49g/mol

No of moles of solute present= 25µg / 376.49g/mol = 0.066 µmol

Hence, concentration in ethanol = (0.066 µmol/250µl) $\simeq$ 266µM

Therefore, for 500nM, 10µl of Resolvin was added to 5ml of RPMI media (vehicle for in vitro studies) or saline (vehicle for in vivo study). For 2000nM, 40µl was added to 5ml of RPMI media or saline.

**Step 3: Exposing cells to Resolvins or Control**

1. 5ml of differentiated HL-60 cells were exposed to either 5ml of Resolvin or 5ml of RPMI media (control solution), at specific concentrations, for a period of 15 minutes at 37ºC.

**Step 4: Performing Migration Assay**

1. A 24 well plate was used for the migration study.
2. 600µl of $10^{-7}$M fMLP (N-formyl-methionine-leucine-phenylalanine), a strong neutrophil chemoattractant, was added to the well plates.
3. 3µm or 8µm filter was then inserted into each well.
4. 300µl of differentiated HL-60 (dHL60) cells, with a concentration of 100,000cells/100ul (treated and control cells) was added to the top chamber above the filter.
5. The entire plate was placed in a 37ºC & 5%$CO_2$ incubator for a period of 2 hours.

6. Post the migration duration, the filters were removed and well plates were trypsinized to detach cells.
7. Cells along with the fMLP solution in the bottom of the well plate were pipetted out, centrifuged and resuspended

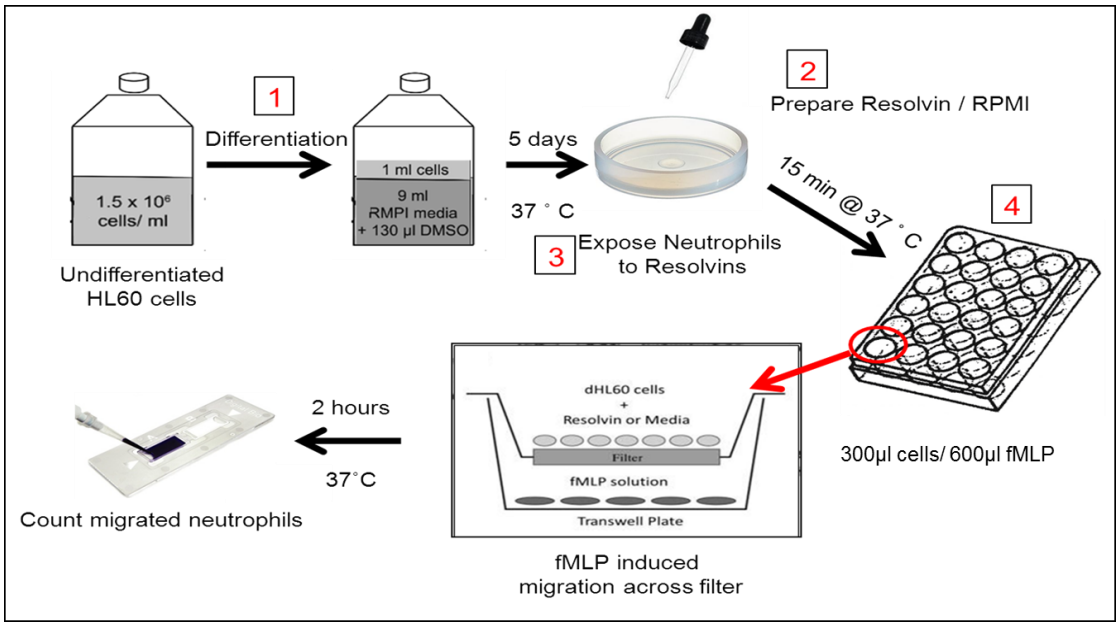



**Figure 13: Design of Neutrophil Migration Assay**

8. The cells were finally counted using a hematocytometer to determine the number of neutrophils that migrated through the filters in the presence or absence of Resolvin molecules.

## 4.3 *IN VIVO* STUDIES

### 4.3.1 RATIONALE FOR DESIGN OF EXPERIMENT

This study used wild type C57BL/6 mice. Though, there are significant anatomical and physiological differences between human and mouse skin, the mouse model is

used since it is convenient, low cost and a very common model for studying dermal wound healing kinetics [53].

Alloderm® and Integra® skin grafts were used as skin substitutes. The primary reason to use skin substitutes is because mice wounds are known to close primarily due to wound contraction rather than re-epithelialization due to the presence of panniculus carnosus muscle in the subcutaneous tissue. Humans lack this muscle and thus heal less through contraction and more through scar tissue deposition and re-epithelialization [53]. Therefore, skin substitutes were used in order to reduce wound contraction to develop a model closer to human wound healing. Additionally the use of skin substitutes also provides a base for consistent and uniform application of Resolvin molecules. Additionally, Tegaderm® wound dressing was used to cover the wound site at all times to prevent the wound from drying up and to create a physical barrier to prevent the mice from tampering with the wound site. Resolvin compounds were solubilized in saline. Resolvin molecules or saline solutions were administered directly into the skin substrate placed over the wound site every alternate day for the first 8 days. Since the hypothesis was based on Resolvin molecules attenuating the inflammatory phase, the treatment was given only for the first 8 days.

To test the hypothesis, four groups of experiments were performed:

a) Skin substitute + Wound Dressing+ Saline (at 0, 2, 4, 6, 8 days),

b) Skin substitute + Wound Dressing+ RvD1 (at 0, 2, 4, 6, 8 days),

c) Skin substitute + Wound Dressing+ RvD2 (at 0, 2, 4, 6, 8 days) and

d) Skin substitute + Wound Dressing+ RvE1 (at 0, 2, 4, 6, 8 days).

### 4.3.2 SAMPLE SIZE RATIONALE

It is considered that a difference in wound healing kinetics is meaningful if it is 50% different from the control group. Furthermore, the minimum level of statistical significance is set to 95%. **Therefore, in order for the experiment to have a power of 80%, and assuming a standard deviation of 25%, a sample size of at least 4 animals per experimental group is required.**

### 4.3.3 MATERIALS

1) 1 week old wild type mice (C57BL/6) were purchased from Charles River Laboratories (Wilmington, MA) and maintained in accordance with Rutgers Animal Welfare Facility guidelines.
2) Integra Dermal Regenerative® Template (Integra LifeSciences, NJ)
3) Alloderm® Regenerative Tissue Matrix (LifeCell, NJ)
4) Resolvin E1, D1 and D2 Compounds (Cayman Chemicals)
5) Tegaderm™ (3M™ Company)
6) Equipment for Surgery
    a. Heating Pad
    b. Mouse Induction Chamber and Nose Clamp
    c. Oxygen Tank
    d. Isoflurane
    e. Clippers
    f. Nair Hair Removal Cream
    g. Artificial tears ointment (Butler Schein #007312)
    h. Silk Braided Section 6.0 Sutures

i. Sterilized Scissors and Tweezers

j. Syringes (U-100 BD Ultra-Fine™ VWR item # BD328438)

7) Chemicals for Surgery

a. Betadine Purdue Surgical Scrub (VWR; #19-027132)

b. 70% Ethanol

c. Buprenorphine Hydrochloride 0.3mg/ml (Butler Schein; CAS#53152-21-9)

## 4.3.4 METHODS

### 4.3.4.1 SURGICAL PROCESS AND WOUND MODEL

All procedures on animals were approved by the Rutgers Committee on Research Animal Care (Rutgers Animal Protocol No: 09-033). Mice were housed four animals per cage before surgery in standard microisolator polycarbonate caging. Animal rooms were maintained at 64-79ºF with 30-70% humidity on a 12 hour light/dark cycle. Commercial rodent ration (LabDiet 5P00 Proloab RMH3000) was provided along with acidified water. One day before the surgery, the mice were anesthetized with Isoflurane and their dorsum regions were shaved, treated with Nair® (Church & Dwight Co., NJ) and cleaned. The mice were then placed individual in cages with alpha dry bedding.

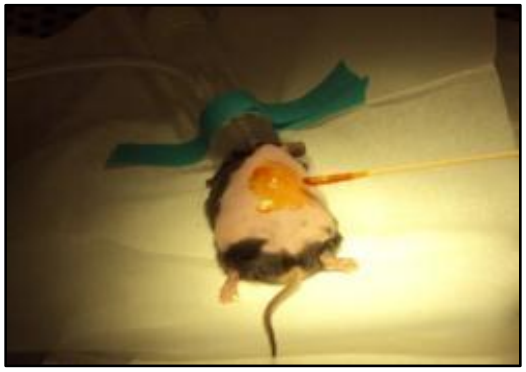


**Figure 14: Application of Betadine® on shaved dorsal mice sections**

On the day of the surgery, mice were again anesthetized with Isoflurane and the shaved dorsum region was treated with three-fold alternating application of Betadine scrub and 70% alcohol to disinfect surgery site. The skin was marked with a standardized $1cm^2$ template. A full thickness wound was created on the dorsal area of the mouse by excising a 1cm x1cm square of skin (epidermis, dermis and panniculus carnosus) using sterile scissors and tweezers. An equivalent sized skin substitute (Integra® or Alloderm®) was placed to conceal the exposed wound site. The edges of the substitute were tugged within the wound site edges. Tegaderm® wound dressing was placed over the wound site and sutured in place.

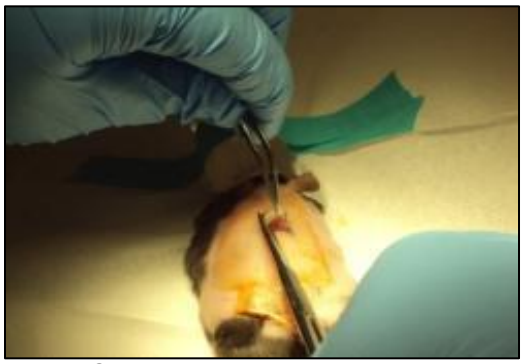


**Figure 15: $1cm^2$ wound creation on mice dorsal section**

Next, either the 100µl of 2000nM Resolvin D1 or D2 or E1 (treatment types) or saline (control) was injected into the skin substitute matrix. Also on Day 0, mice were subcutaneously injected with analgesic (buprenorphine) at a dosage of 0.05mg/kg (250µl) under the scruff region. The mice were placed back into their cages.

Similarly, on Days 2, 4, 6 and 8 the wound site were again injected with 100µl of 2000nM Resolvin D1 or D2 or E1 (treatment types) or saline (control). The wound site was monitored for wound closure every 2 or 3 days. Rodent food, water and bedding were replaced as required. Tegaderm® dressing was replaced often since the

animals frequently ripped the dressing off. Pictures of the wound site were taken using a high resolution camera. On or after Day 35, once the wounds were healed the mice were euthanized with $CO_2$ gas.

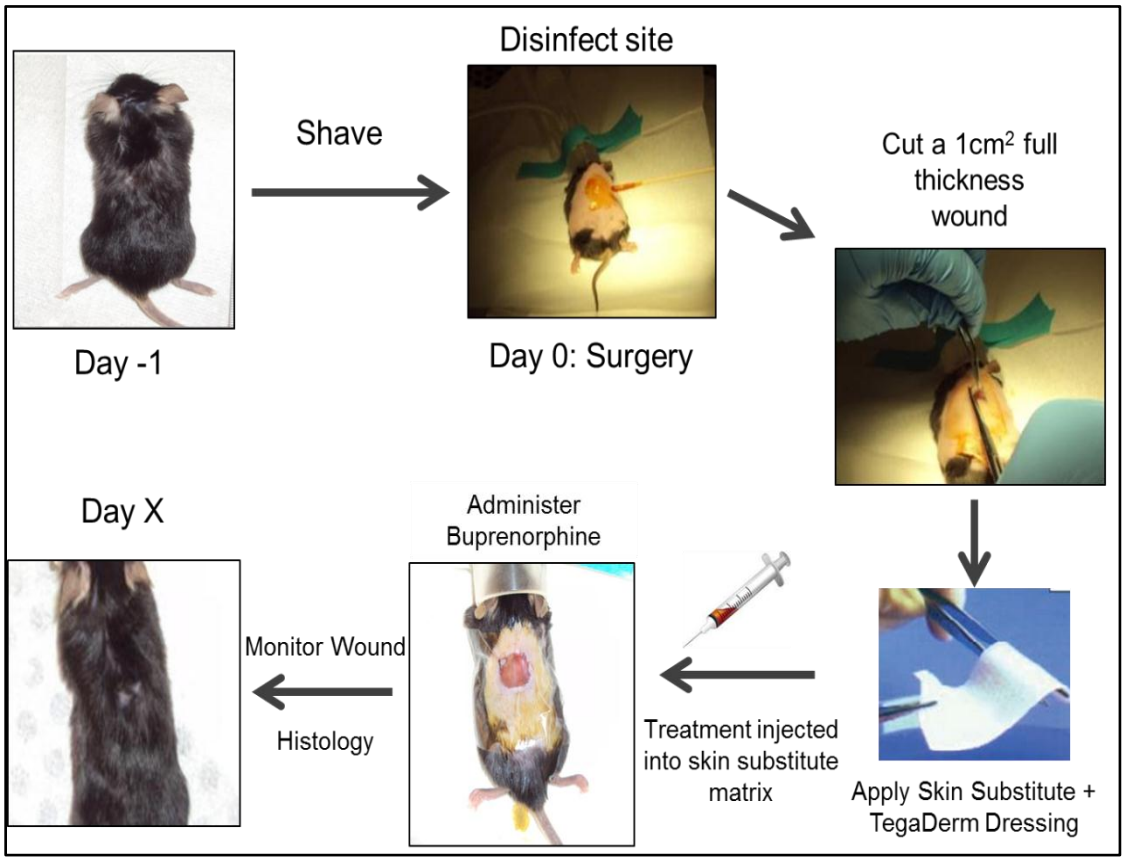



**Figure 16: Design of *In Vivo* study**

#### 4.3.4.2 MACROSCOPIC WOUND CLOSURE ANALYSIS

Pictures of the site were taken regularly for macroscopically examining the rate of wound closure. Pictures were taken using a Sony 10.1MP camera (optical lens f=5.8-23.2mm). For quantification of the wound area, the raw digital files were imported into NIH Image J software v1.40g (Image J, NIH, MD) for processing. A planimetry tool was used to calculate the scar area. Macroscopically, the wound edges were defined by dermal edges of exposed tissue or scab. Wound closure rate, in each case,

was analyzed on the basis of the normalized wound area. Normalized wound area parameter on a particular day was defined as the ratio of wound area on that day over the initial wound area.

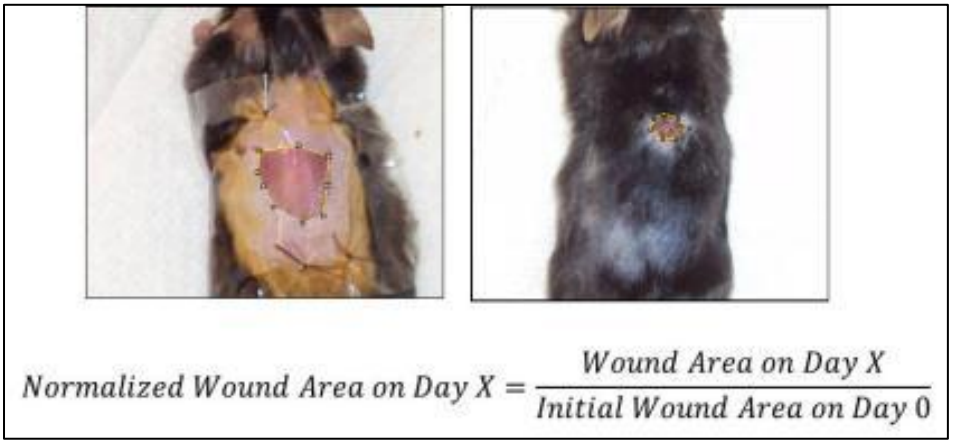



**Figure 17: Normalized Wound Area Determination using Image J software**

#### 4.3.4.3 HISTOLOGICAL ANALYSIS

Central wound cross sections were fixed in 10% formalin and embedded in paraffin, sectioned in 5µm sections and stained following standard Hematoxylin and Eosin (H&E) and Masson's Trichrome protocols. The histological images were obtained using an Eclipse 50I microscope (Nikon Instruments Inc.) with a 4X (Lens# CFI Plan N.A. 0.1) and 20X (Lens# CFI Plan Fluor N.A. 0.50) magnification. Tissues were sectioned along a transverse plane right through the center of the wound site to obtain different skin and fascia layers in one section.

Lateral wound margins in the histological sections were determined by the presence of appendages (hair follicles, sweat glands) and organized epidermis and dermis layers. H&E stain was performed to characterize the architecture of the wound site, collagen deposition and inflammation state during different stages of healing. The stain colors nuclei of cells blue and colors other, eosinophilic structures in

various shades of red, pink and orange. Masson's Trichome staining was selected since it is good technique for visualizing collagen fibers, basement membranes, fibrin and hyaline.

## 4.4 STATISTICAL ANALYSIS

Results are expressed as mean ± standard deviation. Independent Student's t-test (two tailed) analysis was used to evaluate significant difference between treatment groups. Also, One Way Analysis of Variance (ANOVA) using the Tukey approach for multiple comparisons was also used. A p-value $< 0.05$ was considered significant.

# CHAPTER 5

# RESULTS

## 5.1 *IN VITRO* STUDY

### 5.1.1 MIGRATION ASSAY OPTIMIZATION STUDY

This study was performed to optimize neutrophil migration assay by selecting appropriate parameters for cell/fMLP volume ratio, filter pore size and migration duration.

**Optimization Study # 1**

Determination of ratio of cell volume over chemoattractant solution for optimal diffusion dynamics

Result:

Chemoattractant fMLP was added to the bottom chamber of the well and cells were pipetted into the chamber above the filter. Two different types of volume ratios were tested - 200µl cells/600µl fMLP and 300µl cells/600µl fMLP. The cells, in this control case, were not exposed to Resolvin. Determining optimal ratio is critical since this can impact the neutrophil migration diffusional gradient. If the ratio is not balanced, then an upward diffusional gradient can prevent or reduce cell migration through the filter decreasing overall cell counts. An n=3 was tested for each group. The time of migration was set to 2 hours and 8µm filters were used. The results (**Figure 18**) demonstrated that either 200µl cells/600µl fMLP and 300µl cells/ 600µl fMLP can be used. The results using these ratios with controls were not significantly

different. But for the purpose of this study, ratio of **300µl cells/ 600µl fMLP** was used for all remaining studies.

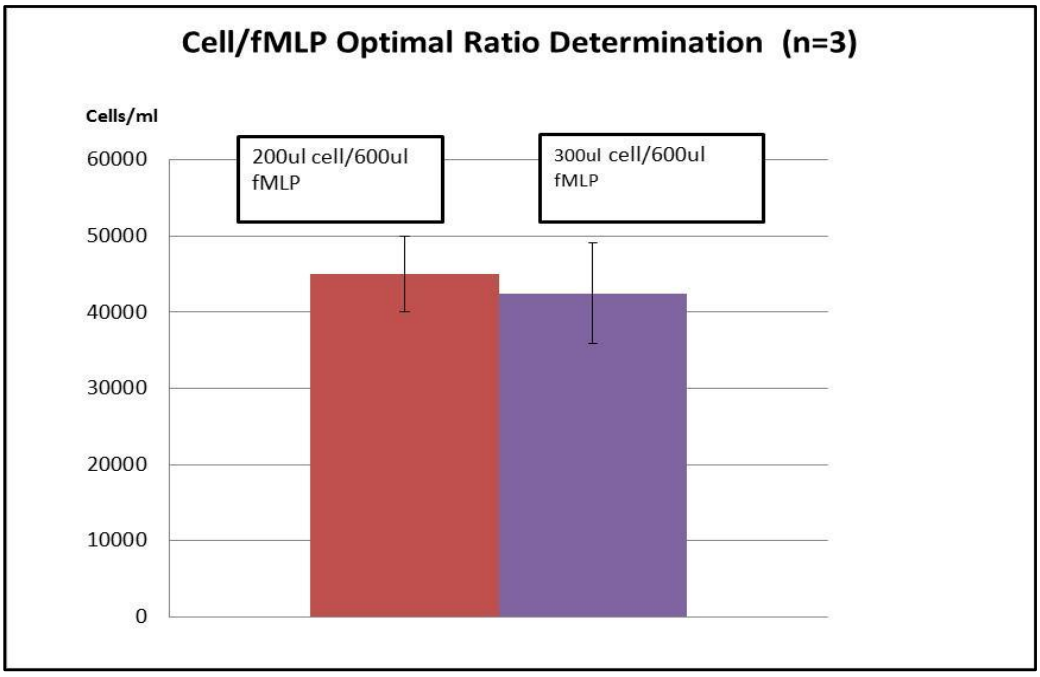



**Figure 18: Cell / fMLP Ratio Optimization Study**

**Optimization Study # 2**

Determination of Filter Pore Size for optimal neutrophil migration

Result:

Two different types of Transwell Inserts with pore size of 8µm and 3µm were tested. The final goal is to use control conditions that do not inhibit cell migration and provide sufficient cell count for analysis purposes. An n=3 sample size was tested for each group. The time of migration was set to 2 hours and ratio of 200µl cells/ 600µl fMLP was used. There is a significant increase ($p<0.05$) in the number of migrated cells when the filter type is changed from 3µm to 8µm (**Figure 19**). Therefore, in

order to work with greater cell counts and to reduce any variability, **8 micron filter** was used for all remaining studies.

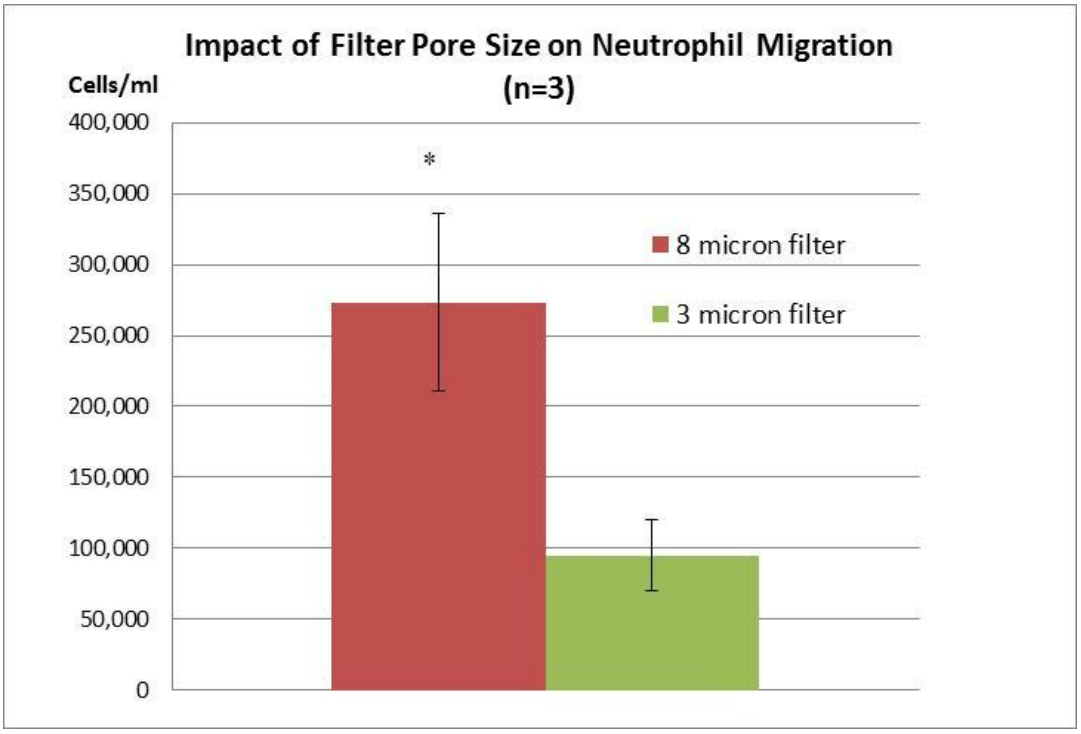



**Figure 19: Micron Filter Impact on Cell Migration**

**Optimization Study # 3**

Determination of optimal time for conducting migration study

Result:

Two different time conditions were tested, 1 and 2 hours. An n=3 sample size was tested for each group. 3µm filters and ratio of 200µl cells/ 600µl fMLP were used for this study.

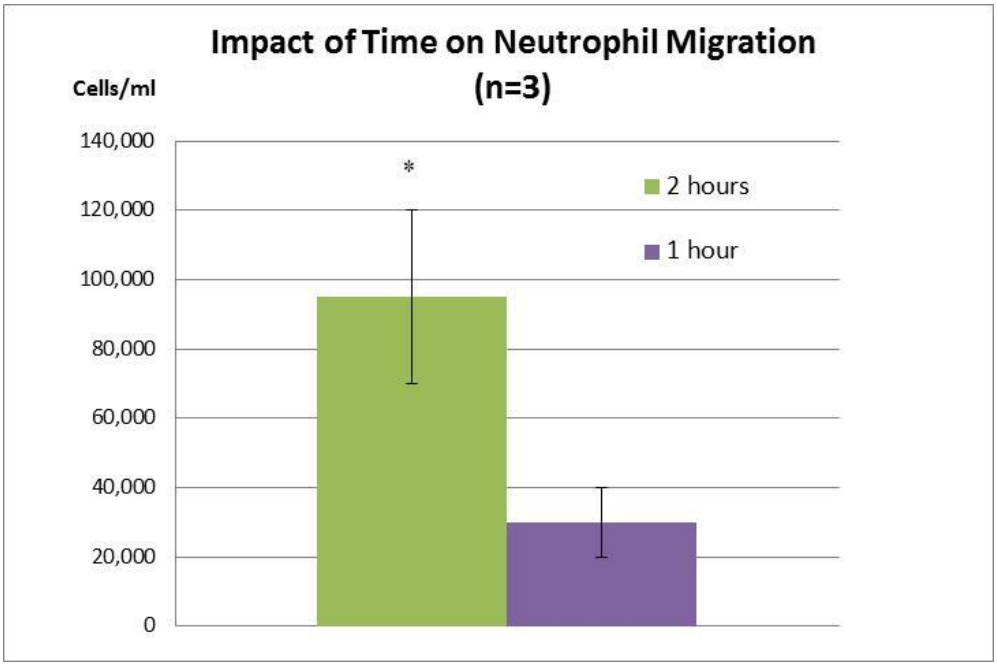



**Figure 20: Migration Time Impact on Cell Migration**

There is a significant increase ($p<0.05$) in the number of migrated cells when the time is changed from 1 hour to 2 hour (**Figure 20**). Therefore, in order to work with greater cell counts and to reduce any variability, **2 hour time** was used for all remaining studies.

**Optimal Neutrophil Migration Assay Parameters were:**

Cell/fMLP solution ratio = 300ul/600ul

Duration of Study = 2 hours

Filter Type = 8 microns

### 5.1.2 NEUTROPHIL MIGRATION ASSAY: IMPACT OF RESOLVINS

Cells were exposed to two different concentrations of Resolvin molecules - 500Nm and 2000Nm. Exposure to Resolvin molecules at 500nM and 2000nM concentration significantly blocked neutrophil cell migration ($p<0.05$) compared to the control condition (**Figure 21**). The control condition provided uninhibited migration of neutrophils directed towards the chemoattractant, $10^{-7}$M fMLP.

As expected, FMLP functioned as a chemoattractant resulting in the migration of neutrophils towards the lower chamber of the Transwell plate. However, incubation of neutrophil cells with Resolvin molecules for 15 minutes at 37ºC resulted in a dose and molecule type inhibition of neutrophil migration (**Figure 21**).

Literature suggested that the behavior of Resolvins to impede neutrophil migration is directly proportional to the Resolvin concentration [9, 48, 50]. Similar to data provided in published reports, RvE1 at 2000nM produces significant inhibition to neutrophil migration compared to RvE1 at 500nM, RvD1 at 500nM and RvD1 at 2000nM. RvD2 at 2000nM also produces significant inhibition to neutrophil migration compared to RvD1 at 500nM and 2000nM. Neutrophil migration is not impacted significantly when the concentration of RvD2 or RvD1 is increased from 500nM to 2000Nm. Therefore, for the *in vivo* studies, Resolvins at a concentration of 2000nM will be evaluated for enhanced wound closure ability.

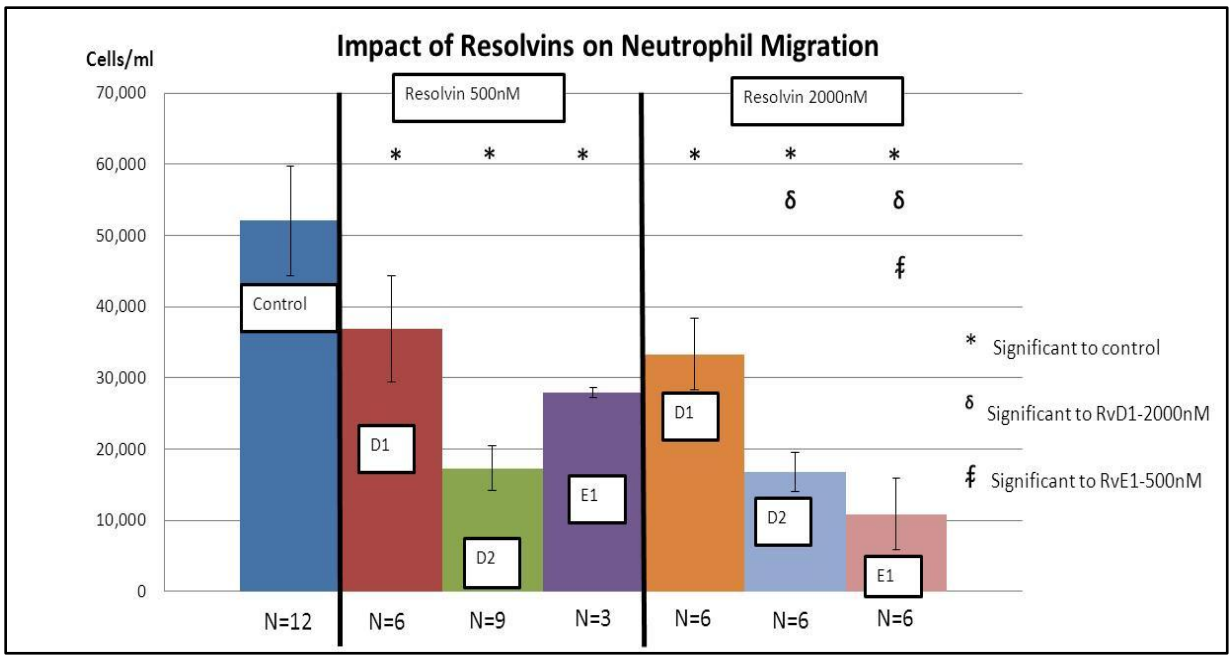



**Figure 21: Impact of Different Resolvin Molecules on Neutrophil Migration**

## 5.2 *IN VIVO* STUDY

In this study, wound healing closure was evaluated and compared between control (saline treatment), RvD1, RvD2 and RvE1 conditions. Four animals per test group were evaluated. Mice were treated with saline (control) or treatment (2000nM of RvD1, RvD2 or RvE1 in sterile saline) on Day 0, 2, 4, 6 and 8 post surgery day. Alloderm® was used skin substitute to reduce wound contraction and to aid in wound closure. Integra Regenerative® Dermal Substitute was also used in a small n=1 study to verify if changing the skin substitute impacted wound closure rate.

### 5.2.1 MACROSCOPIC WOUND CLOSURE ANALYSIS

The wounds were macroscopically evaluated post-surgery from Day 0 until complete wound closure (re-epithelialization). **By macroscopic observation, when the surface of the wound area exhibited a similar appearance as that of the**

**surrounding uninjured normal skin region with no inflammation, it was determined that complete wound closure (re-epithelialization) had occurred.** All wounds treated by saline demonstrated complete re-epithelialization by an average of 28.6 ± 1.52 days. All wounds treated by Resolvin drugs healed significantly faster as compared to the saline treated wounds ($p<0.05$) (**Figure 22**). RvE1 treated drugs closed the fastest. Complete wound closure was observed as early as Day 19.4 ± 1.52 post surgery. Wounds treated with RvD1 and RvD2 closed by an average of 24.4 ± 2.19 and 22.8 ± 1.79 days respectively. Wounds healed using Resolvin E1 treatment closed significantly faster than Resolvin D1 and D2 groups ($p<0.05$). **Figure 22** below plots the wound closure times for all four test groups (includes both AlloDerm and Integra skin substitutes).

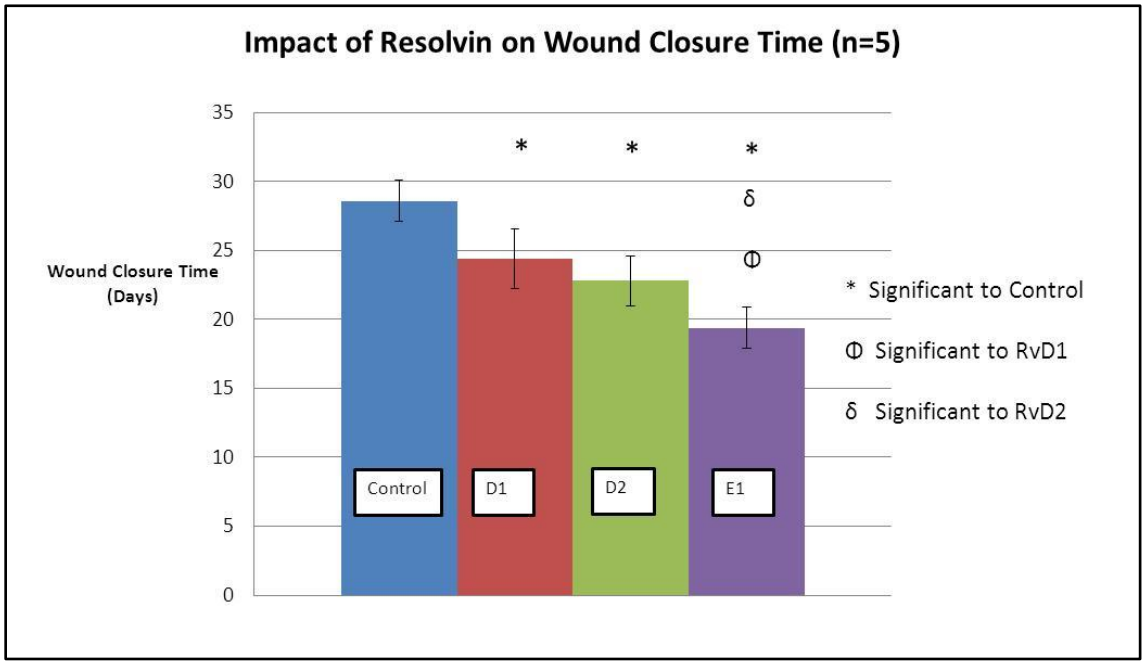
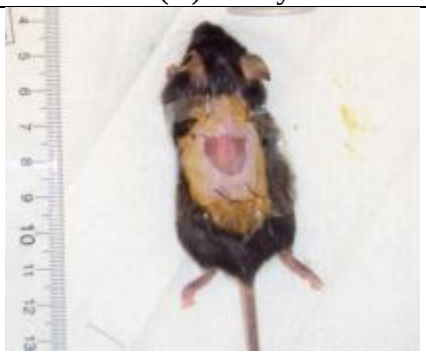
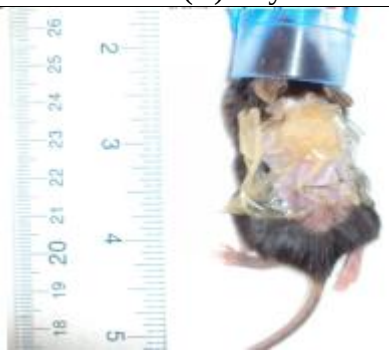
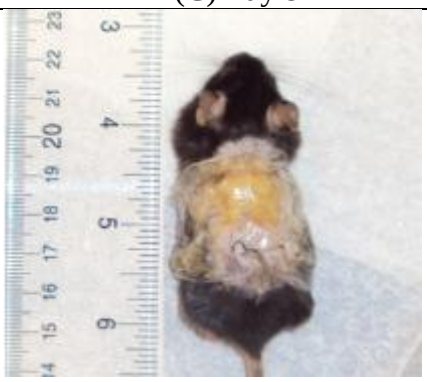
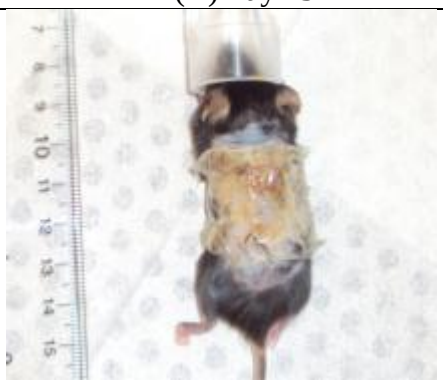
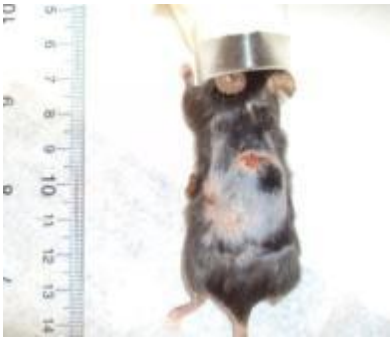
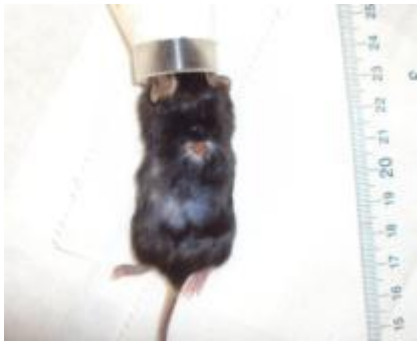



**Figure 22: Impact of Resolvins on Wound Closure Rate**

### 5.2.2 MACROSCOPIC WOUND CLOSURE ANALYSIS

**(A) Saline Treatment (Control)**

**Figure 23 (A-H)** macroscopically tracks the wound closure rate during the course of the healing process.

| (A) Day 0 | (B) Day 4 |
|---|---|
|  |  |
| (C) Day 8 | (D) Day 13 |
|  |  |
| (E) Day 16 | (F) Day 19 |
|  |  |

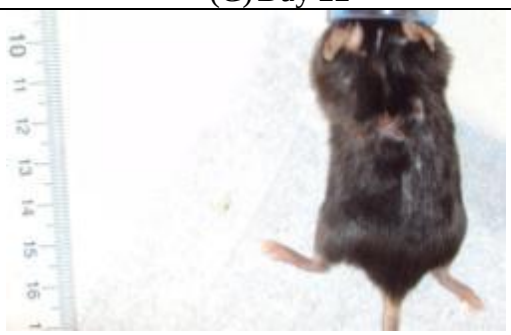
| (G) Day 22 | (H) Day 29 |
|---|---|
|  |  |

**Figure 23 (A-H): Wound Healing Process with Saline (Control) treated mice**

At Day 0 (surgery day; **Figure 23-A**), the full thickness wound has a surface area of approximately 1cm$^2$. During the first week **(Figures 23-B** and **23-C**), formation of the fibrin clot (for hemostasis) along signs of inflammation namely wound redness, excessive inflammatory exudates and swelling are prominent. By Day 13, the wound visually looks smaller and less inflamed. It is not until Day 29 (**Figure 23-H**) can the wound be considered completely re-epithelialized and closed.

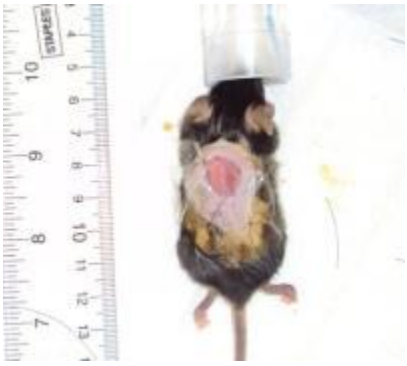
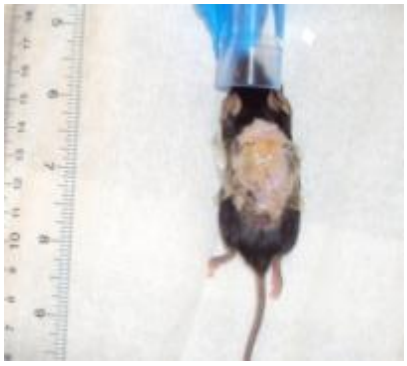
| Saline Treatment – Control | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Animal #** | **1** | | **2** | | **3** | | **4** | | **Average** | |
| **Day** | **Area (cm$^2$)** | **% Closure** | **Area (cm$^2$)** | **% Closure** | **Area (cm$^2$)** | **% Closure** | **Area (cm$^2$)** | **% Closure** | **Avg % Closure** | **Std. Dev** |
| Day 0 | 1.03 | 0.00 | 0.98 | 0.00 | 0.93 | 0.00 | 0.92 | 0.00 | 0.00 | 0.00 |
| Day 2 | 1.13 | -9.13 | 0.91 | 6.96 | 1.02 | -10.61 | 0.88 | 5.15 | -1.91 | 9.24 |
| Day 4 | 0.86 | 16.67 | 0.97 | 0.24 | 1.17 | -25.99 | 0.77 | 16.40 | 1.83 | 20.07 |
| Day 6 | 0.77 | 25.54 | 0.90 | 8.19 | 1.12 | -21.14 | 0.81 | 11.85 | 6.11 | 19.64 |
| Day 8 | 0.76 | 26.95 | 0.97 | 1.15 | 1.18 | -26.94 | 0.53 | 43.17 | 11.08 | 30.69 |
| Day 11 | 0.72 | 30.73 | 0.85 | 13.14 | 0.79 | 14.85 | 0.48 | 48.45 | 26.79 | 16.47 |
| Day 13 | 0.62 | 40.32 | 0.87 | 10.73 | 0.81 | 12.64 | 0.21 | 76.95 | 35.16 | 30.97 |
| Day 16 | 0.29 | 71.60 | 0.31 | 68.44 | 0.33 | 64.60 | 0.20 | 78.14 | 70.70 | 5.73 |
| Day 19 | 0.15 | 85.71 | 0.14 | 85.47 | 0.32 | 65.70 | 0.14 | 85.09 | 80.49 | 9.86 |
| Day 22 | 0.09 | 91.08 | 0.10 | 100.00 | 0.07 | 92.63 | 0.02 | 97.96 | 95.42 | 4.25 |
| Day 26 | 0.03 | 97.39 | 0.03 | 100.00 | 0.03 | 100.00 | CLOSED | 100.00 | 99.35 | 1.31 |
| Day 29 | CLOSED | 100.00 | CLOSED | 100.00 | CLOSED | 100.00 | | | 100.00 | 0.00 |

**Table 1: Wound Closure Rate in Saline Treated (Control) Mice**

As shown in **Table 1**, average % wound closure observed for control mice is between 11.08 % and 35.16% during Day 8 and 13. This time period is critical since it marks the attenuation of the inflammatory phase (resolution stage) and initiation of the proliferation stage [73]. Negative wound closure rates, as seen in some cases during the initial wound healing stage, is most likely an artifact due to difficulty in visually seeing the actual wound due to exudate presence and swelling at wound site. By Day 29, 100% wound closure is achieved. In one case, complete wound healing was achieved by Day 26.

**(B) RvD1 Treatment**

**Figure 24 (A-H)** macroscopically tracks the wound closure rate during the course of the healing process. Similar with control treated wounds, initial week of wound healing is marked with cardinal signs of inflammation such as swelling and exudate accumulation under the wound dressing. With RvD1 treated mice, wounds look visibly smaller by Day 13. By Day 22, the wound is significantly closed. But, it is not until Day 26 can the wounds be considered completely re-epithelialized.

| (A) Day 0 | (B) Day 4 |
| --- | --- |
|  |  |

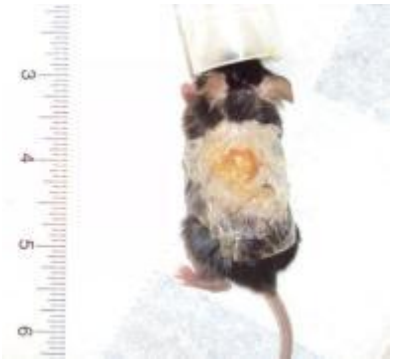
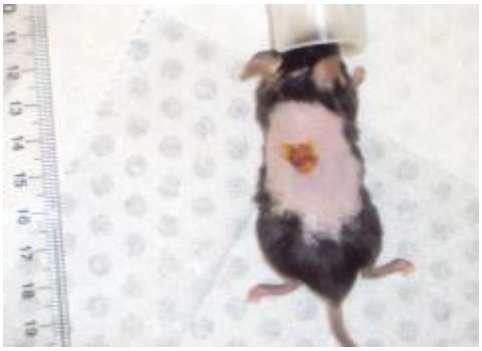
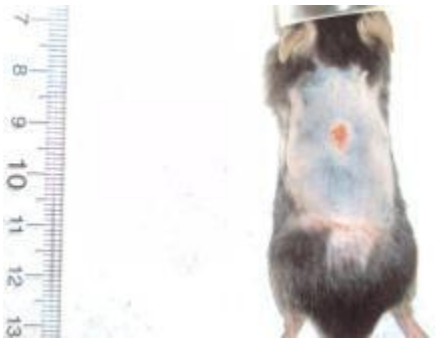
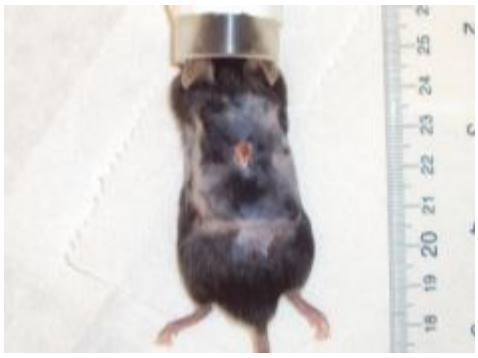
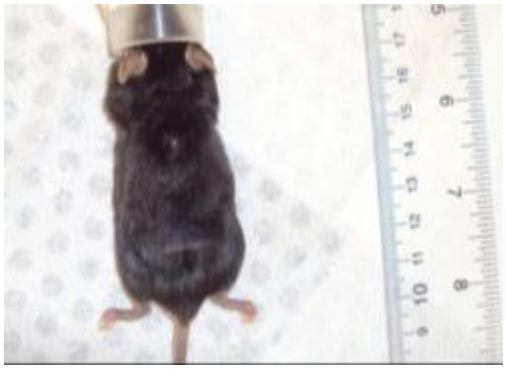
| (C) Day 8 | (D) Day 13 |
|---|---|
|  |  |
| (E) Day 16 | (F) Day 19 |
|  |  |
| (G) Day 22 | (H) Day 26 |
|  |  |

**Figure 24: (A-H): Wound Healing Process with RvD1 Treated Mice**

As demonstrated in **Table 2** below, average % wound closure observed for RvD1 treated mice is between 18.34 % and 50.22% during Day 8 and 13. This is higher than

the wound closure rate observed in control animals suggesting that wound closure was occurring at faster rate. 100% wound closure or complete re-epithelialization occurred only by Day 26 in most cases.

| RvD1 Treated Mice | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Animal # | 1 | | 2 | | 3 | | 4 | | Average | |
| Day | Area ($cm^2$) | % Closure | Area ($cm^2$) | % Closure | Area ($cm^2$) | % Closure | Area ($cm^2$) | % Closure | Avg % Closure | Std. Dev |
| Day 0 | 0.98 | 0.00 | 0.99 | 0.00 | 0.97 | 0.00 | 1.23 | 0.00 | 0.00 | 0.00 |
| Day 2 | 1.07 | -9.39 | 1.06 | -7.81 | 0.96 | 0.78 | 1.19 | 3.26 | -3.29 | 6.25 |
| Day 4 | 0.97 | 1.45 | 0.97 | 1.32 | 0.90 | 7.27 | 1.33 | -8.59 | 0.36 | 6.58 |
| Day 6 | 0.87 | 11.43 | 1.00 | -1.58 | 0.84 | 13.32 | 1.22 | 0.41 | 5.90 | 7.57 |
| Day 8 | 0.86 | 11.94 | 0.85 | 13.57 | 0.80 | 17.11 | 0.85 | 30.74 | 18.34 | 8.54 |
| Day 11 | 0.64 | 34.47 | 0.76 | 23.15 | 0.40 | 58.60 | 0.66 | 46.03 | 40.56 | 15.23 |
| Day 13 | 0.69 | 29.21 | 0.30 | 69.83 | 0.50 | 48.89 | 0.58 | 52.94 | 50.22 | 16.68 |
| Day 16 | 0.11 | 88.58 | 0.11 | 88.36 | 0.23 | 76.75 | 0.55 | 55.21 | 77.22 | 15.68 |
| Day 19 | 0.10 | 89.72 | 0.20 | 79.96 | 0.09 | 90.21 | 0.44 | 63.89 | 80.94 | 12.31 |
| Day 22 | 0.10 | 89.37 | CLOSED | 100.00 | 0.07 | 92.86 | 0.07 | 94.30 | 94.13 | 4.42 |
| Day 26 | CLOSED | 100.00 | | | CLOSED | 100.00 | CLOSED | 100.00 | 100.00 | 0.00 |

**Table 2: Wound Closure Rate for RvD1 treated mice**

**(C) RvD2 Treatment**

**Figure 25 (A-H)** macroscopically tracks the wound closure rate during the course of the healing process. Similar to control and RvD1 treated wounds, swelling and exudate accumulation is observed during the first week post injury. Exudate accumulation is known to subside by Day 13 and a more noticeable fibrin clot is visible. Wound closure is more evident post Day 13. But, was not until Day 22 that the wound could be considered 100% closed or completely re-epithelialized.

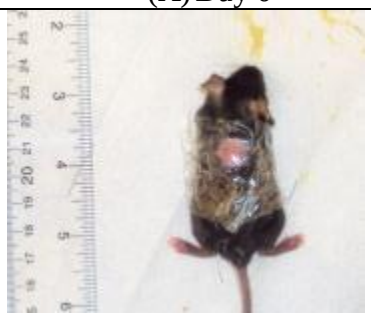
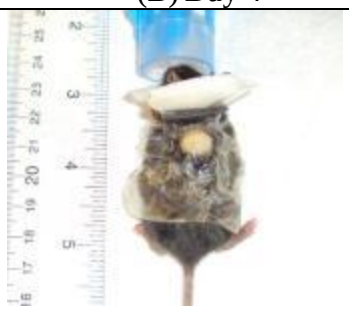
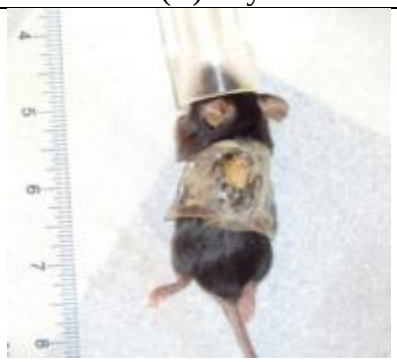
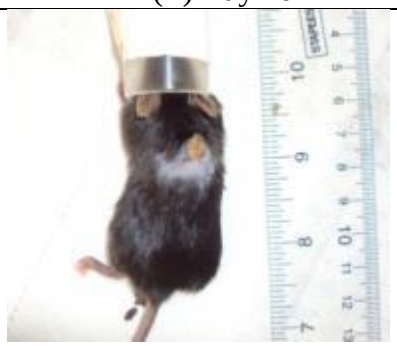
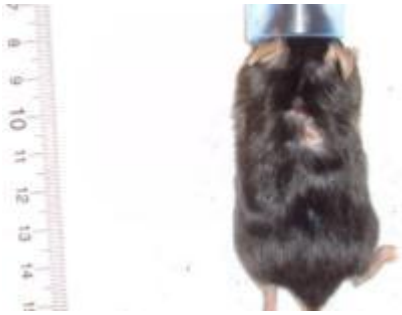
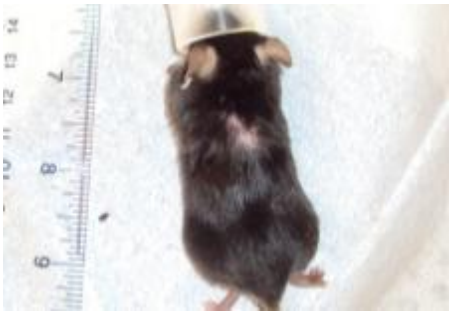
| (A) Day 0 | (B) Day 4 |
|---|---|
|  |  |
| (C) Day 8 | (D) Day 13 |
|  |  |
| (E) Day 16 | (F) Day 19 |
|  |  |
| (G) Day 22 | (H) Day 26 |
|  |  |

**Figure 25 (A-H): Wound Healing Process with RvD2 Treated Mice**

As shown in **Table 3** below, average % wound closure observed for RvD2 treated mice is between 30.05 % and 65.58% during Day 8 and 13. This is significantly higher than the wound closure rate observed in control animals suggesting that Resolvin treatment does indeed expedite the inflammation resolution and promote imitation of subsequent wound healing stages. 100% wound closure or complete re-epithelialization occurred only by Day 22 in most cases.

| RvD2 Treated Wounds | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Animal # | 1 | | 2 | | 3 | | 4 | | Average | |
| Day | Area ($cm^2$) | % Closure | Area ($cm^2$) | % Closure | Area ($cm^2$) | % Closure | Area ($cm^2$) | % Closure | Avg % Closure | Std. Dev |
| Day 0 | 1.03 | 0.00 | 1.05 | 0.00 | 1.15 | 0.00 | 1.13 | 0.00 | 0.00 | 0.00 |
| Day 2 | 0.90 | 12.97 | 1.03 | 1.55 | 1.13 | 1.57 | 1.12 | 1.37 | 4.37 | 5.74 |
| Day 4 | 0.77 | 25.19 | 0.91 | 12.67 | 0.77 | 33.61 | 0.77 | 31.84 | 25.83 | 9.49 |
| Day 6 | 0.72 | 29.76 | 0.88 | 15.53 | 0.78 | 32.45 | 0.99 | 12.94 | 22.67 | 9.86 |
| Day 8 | 0.75 | 27.38 | 0.74 | 29.30 | 0.73 | 37.07 | 0.83 | 26.44 | 30.05 | 4.83 |
| Day 11 | 0.65 | 37.06 | 0.31 | 70.50 | 0.29 | 74.44 | 0.62 | 44.90 | 56.73 | 18.53 |
| Day 13 | 0.58 | 43.72 | 0.26 | 74.84 | 0.34 | 70.10 | 0.30 | 73.66 | 65.58 | 14.71 |
| Day 16 | 0.44 | 57.75 | 0.13 | 88.04 | 0.10 | 90.97 | 0.27 | 75.95 | 78.18 | 15.09 |
| Day 19 | 0.15 | 85.75 | 0.06 | 94.36 | 0.03 | 97.80 | 0.04 | 96.53 | 93.61 | 5.43 |
| Day 22 | 0.05 | 95.46 | CLOSED | 100.00 | CLOSED | 100.00 | CLOSED | 100.00 | 100.00 | 2.27 |
| Day 26 | CLOSED | 100.00 | | | | | | | 100.00 | 0.00 |

**Table 3: Wound Closure Rate for RvD2 treated mice**

**(D) RvE1 Treatment**

**Figure 26 (A-F)** macroscopically tracks the wound closure rate during the course of the healing process. As seen with the other cases, initial week post wounding is marked with inflamed tissue signs. By Day 13, the exudate accumulation subsides significantly and wound size looks visibly reduced. With RvE1 treated wounds, by Day 16 the wound is almost 95% completely re-epithelialized. But by Day 19 the wounds can be considered completely re-epithelialized and closed.

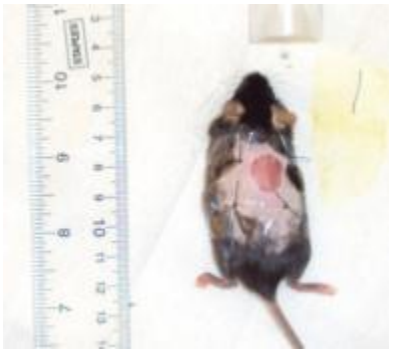
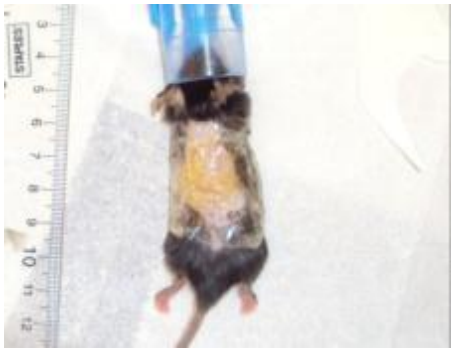
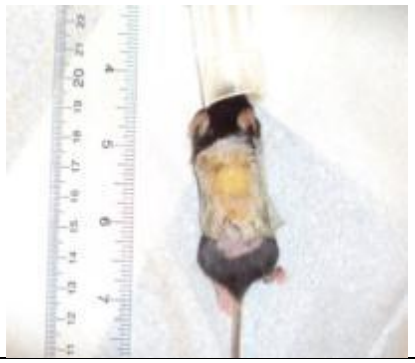
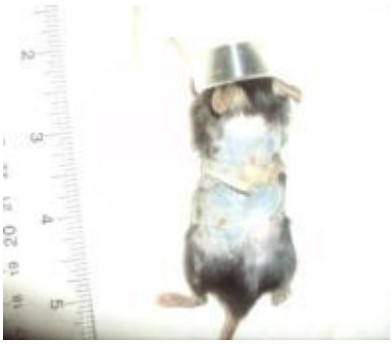
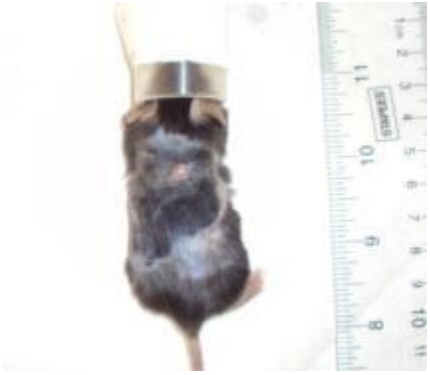
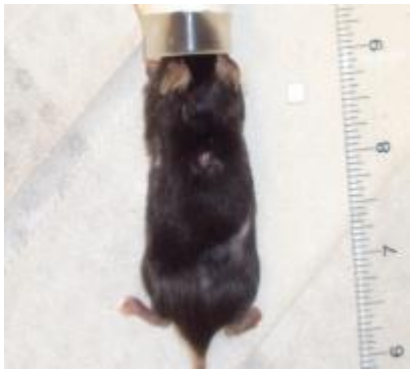
| (A) Day 0 | (B) Day 4 |
|---|---|
|  |  |
| (C) Day 8 | (D) Day 13 |
|  |  |
| (E) Day 16 | (F) Day 19 |
|  |  |

**Figure 26 (A-F): Wound Healing Process with RvE1 Treated Mice**

As shown in **Table 4,** % wound closure rate increases from 23.84% to 91.93% during Day 8 and Day 13. This is significant compared to the control treated animals, wherein the wounds had closed only by 30% by Day 13 and still showed signs of inflammation and exudate accumulation. This observation suggests that Resolvin E1

by means of promoting inflammation resolution also initiates subsequent wound healing stages and significantly accelerates overall wound closure times.

| RvE1 Treated Wounds | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Animal # | 1 | | 2 | | 3 | | 4 | | Average | |
| Day | Area ($cm^2$) | % Closure | Area ($cm^2$) | % Closure | Area ($cm^2$) | % Closure | Area ($cm^2$) | % Closure | Avg % Closure | Std. Dev |
| Day 0 | 1.01 | 0.00 | 1.00 | 0.00 | 1.05 | 0.00 | 0.95 | 0.00 | 0.00 | 0.00 |
| Day 2 | 0.95 | 6.45 | 0.91 | 9.71 | 0.97 | 7.48 | 1.08 | -14.05 | 2.40 | 11.05 |
| Day 4 | 0.89 | 11.66 | 0.82 | 18.60 | 0.88 | 15.80 | 1.02 | -6.90 | 9.79 | 11.48 |
| Day 6 | 0.86 | 14.56 | 0.78 | 21.98 | 0.83 | 20.87 | 0.85 | 10.93 | 17.08 | 5.24 |
| Day 8 | 0.88 | 13.19 | 0.74 | 26.01 | 0.58 | 44.36 | 0.84 | 11.79 | 23.84 | 15.10 |
| Day 11 | 0.40 | 60.69 | 0.38 | 61.69 | 0.17 | 83.67 | 0.57 | 39.55 | 61.40 | 18.02 |
| Day 13 | 0.07 | 92.64 | 0.04 | 95.63 | 0.04 | 96.59 | 0.16 | 82.86 | 91.93 | 6.28 |
| Day 16 | 0.05 | 95.10 | 0.05 | 94.72 | 0.05 | 95.16 | 0.04 | 95.32 | 95.07 | 0.25 |
| Day 19 | CLOSED | 100.00 | CLOSED | 100.00 | CLOSED | 100.00 | CLOSED | 100.00 | 100.00 | 0.00 |
| Day 22 | | | | | | | | | | |
| Day 26 | | | | | | | | | | |

**Table 4: Wound Closure Rate for RvE1 treated mice**

Additionally, another study was performed using Integra Regenerative® Dermal template (N=1) as skin substitute instead of Alloderm® to test if the wound healing dynamics observed with Alloderm® in the presence of Resolvin would change if another skin substitute is used. **Table 5** outlines the percentage wound closure during the course of healing process for each test type. Similar to the Alloderm®-Resolvin study, the resolvin treated mice wounds completely re-epithelialized by Day 22 while the control closed by Day 30. By Day 13, the Resolvin E1 treated wound was approximately 91% closed. Concurring with published reports [58], there was no significant difference in wound closure rates observed when either Integra Regenerative® Dermal skin or AlloDerm® Regenerative Matrix was used.

| Day | Saline - Control | | RvD1 | | RvD2 | | RvE1 | |
|---|---|---|---|---|---|---|---|---|
| | Area ($cm^2$) | % Dec | Area ($cm^2$) | % Dec | Area ($cm^2$) | % Dec | Area ($cm^2$) | % Dec |
| Day 0 | 1.30 | 0.00 | 1.12 | 0.00 | 0.95 | 0.00 | 0.89 | 0.00 |
| Day 2 | 1.27 | 2.21 | 1.16 | -3.10 | 0.84 | 12.14 | 0.81 | 8.70 |
| Day 4 | 1.07 | 18.05 | 0.98 | 13.12 | 0.80 | 15.98 | 0.79 | 10.95 |
| Day 6 | 0.91 | 29.89 | 0.86 | 23.29 | 0.78 | 17.98 | 0.76 | 14.75 |
| Day 8 | 0.87 | 32.97 | 0.84 | 25.03 | 0.77 | 19.44 | 0.73 | 17.68 |
| Day 11 | 0.72 | 44.94 | 0.65 | 42.11 | 0.22 | 77.28 | 0.19 | 78.56 |
| Day 13 | 0.33 | 74.44 | 0.21 | 81.14 | 0.05 | 95.21 | 0.08 | 90.99 |
| Day 16 | 0.15 | 88.30 | 0.07 | 93.60 | 0.08 | 92.05 | 0.03 | 96.56 |
| Day 19 | 0.12 | 91.03 | 0.02 | 98.00 | 0.07 | 92.58 | 0.01 | 99.07 |
| Day 22 | 0.04 | 96.83 | CLOSED | 100.00 | CLOSED | 100.00 | CLOSED | 100.00 |
| Day 27 | 0.04 | 97.23 | | | | | | |
| Day 30 | CLOSED | 100.00 | | | | | | |

**Table 5: Wound Closure Rates with Integra Regenerative ® Dermal Substitute (N=1)**

**DATA SUMMARY:**

As a summary, wound closure rates in all four groups (using both AlloDerm and Integra skin substitutes, total n=5 each) were plotted to analyze healing rates at different phases during wound healing. **Figure 27** reveals that there was a significant increase in wound closure between Day 8 and 13 for all Resolvin compounds compared to the control.

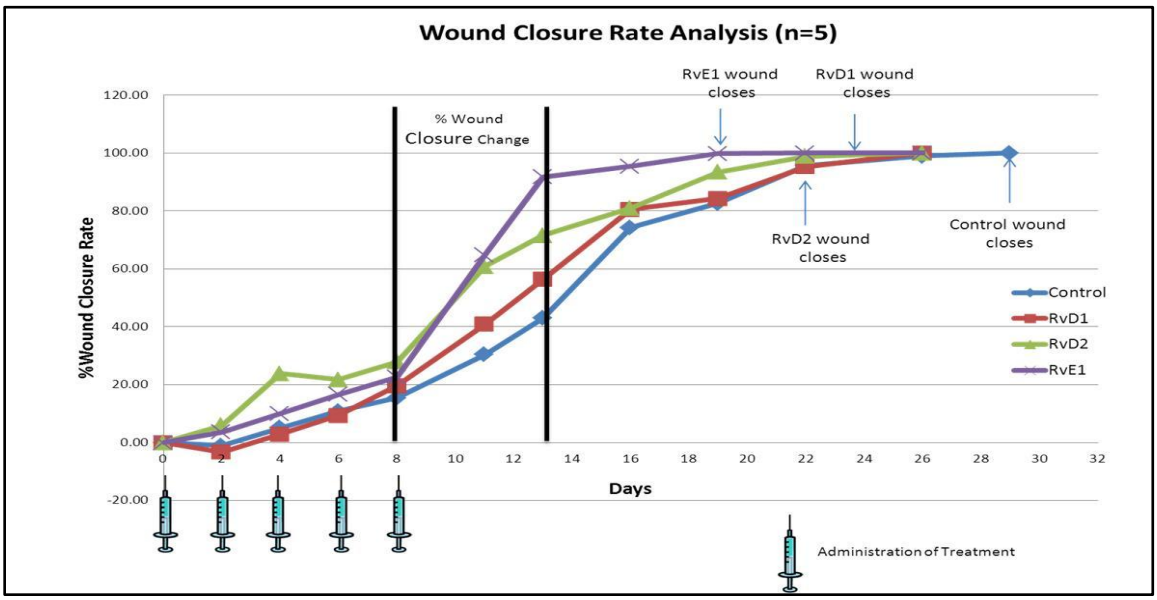



**Figure 27: % Wound Closure Rate Analysis in all Treatment Groups**

## 5.2.3 HISTOLOGICAL ANALYSIS

### 5.2.3.1. 5th Day Post Surgery

**(A) Saline Treated Wounds**

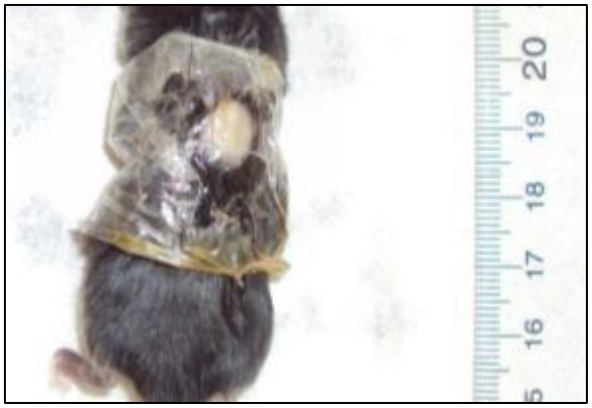



**Figure 28: Day 5 Gross Wound- Saline Treatment**

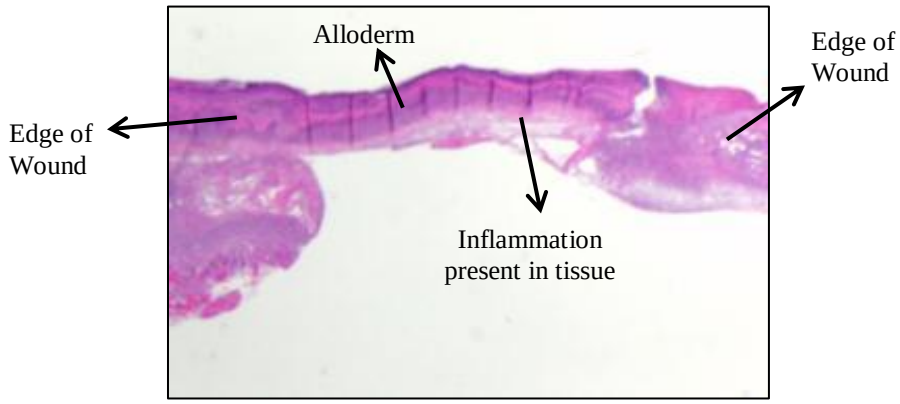



**Figure 29: H&E Stain for Day 5 Wound - Saline Treatment**

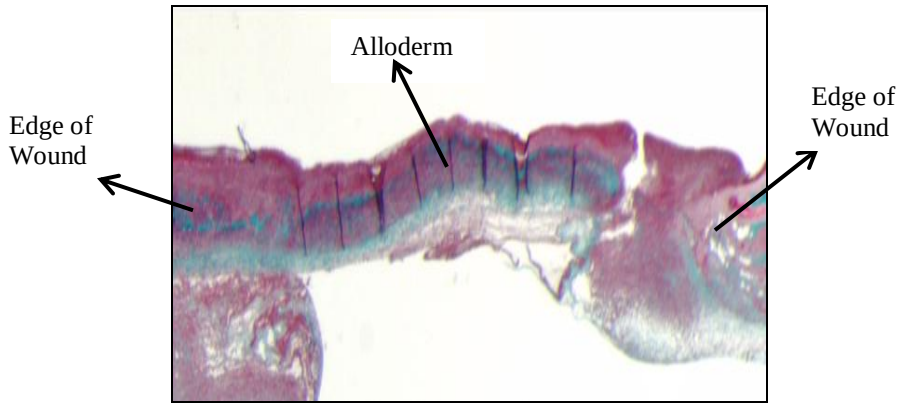



**Figure 30: Trichrome Stain for Day 5 Wound- Saline Treatment**

**B) RvE1 Treated Wounds**

**Figure 31: Day 5 Gross Wound - RvE1 Treatment**

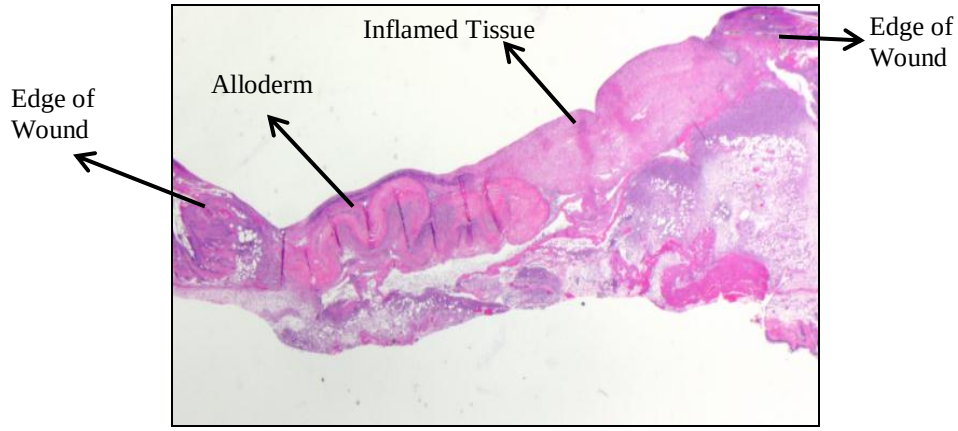



**Figure 32: H&E Stain for Day 5 Wound - RvE1 Treatment**

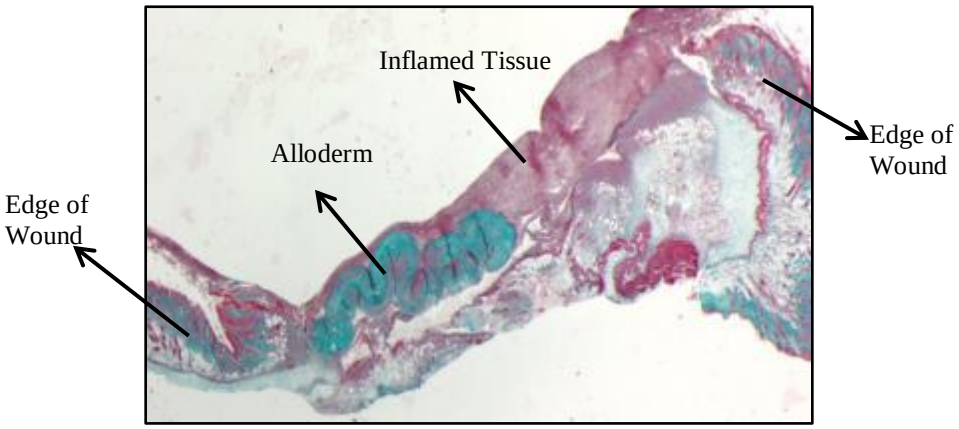



**Figure 33: Trichrome Stain for Day 5 Wound - RvE1 Treatment**

The edges of the wound site are called out in all the figures and have different characteristics as compared to the wound site (complete epithelial layer, organized collagen deposition and dermal appendages). The wound site is marked by the presence of AlloDerm® matrix. Wound sites were also identified by the lack of epithelial layer. At Day 5, the AlloDerm® matrix is relatively intact with no clear cellular infiltration and is identified by a characteristic bluish green color for collagen in the Trichrome stain. The wounds were filled with granulation tissue that was rich in fibroblasts, granulocytes, and an extracellular matrix without a clear organization.

#### 5.2.3.2. 10th Day Post Surgery

##### (A) Saline Treated Wounds

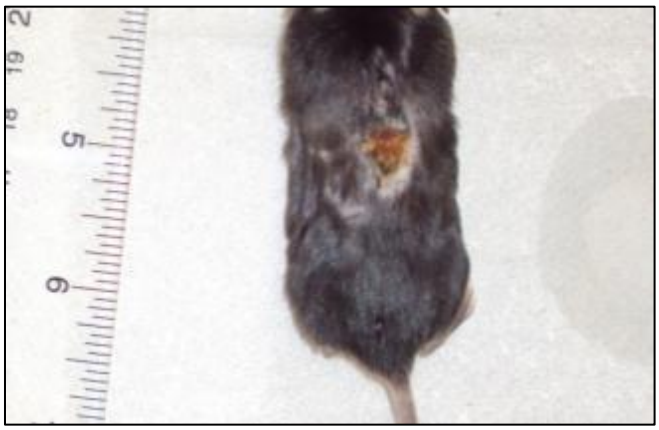


**Figure 34: Day 10 Gross Wound - Saline Treatment**

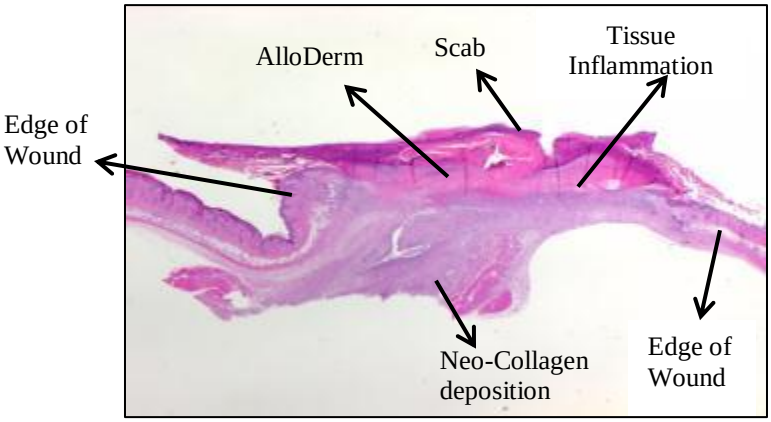



**Figure 35: H&E Stain for Day 10 Wound - Saline Treatment**

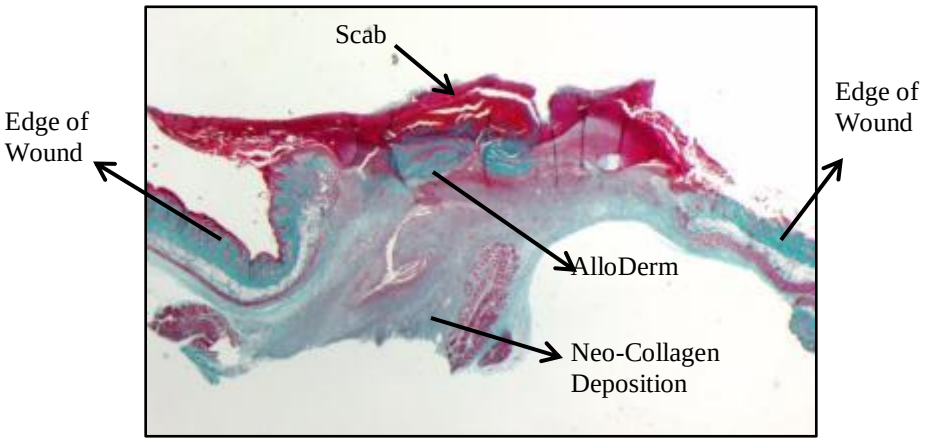



**Figure 36: Trichrome Stain for Day 10 Wound – Saline Treatment**

**(B) RvE1 Treated Wounds**

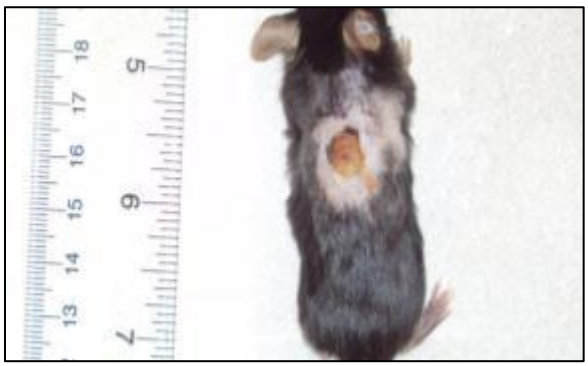


**Figure 37: Day 10 Gross Wound - RvE1 Treatment**

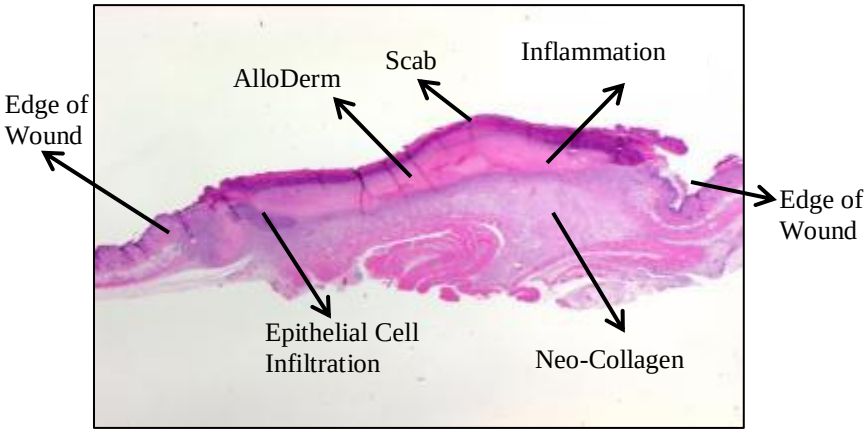



**Figure 38: H&E Stain for Day 10 Wound – RvE1 Treatment**

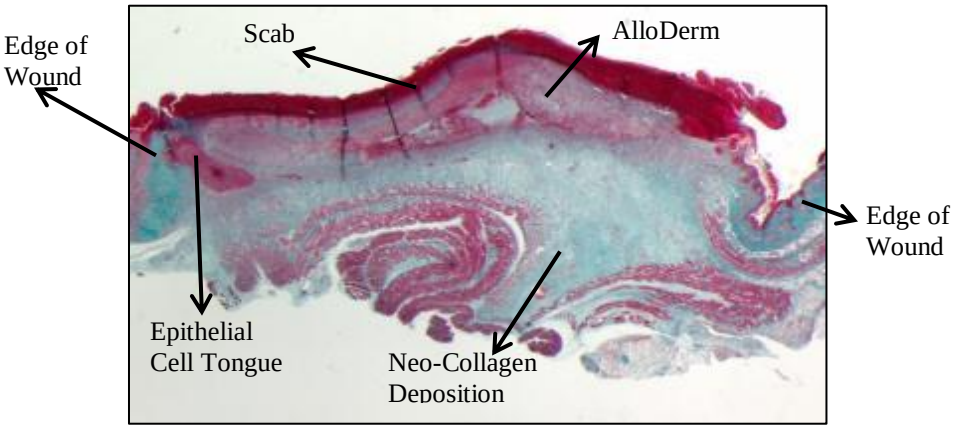



**Figure 39: Trichrome Stain for Day 10 Wound – RvE1 Treatment**

On Day 10, both wounds (saline and RvE1 treated wounds) stained bluish green for collagen in regions below the inflamed tissue. New collagen deposition is observed at this stage. The neo-collagen is differentiated from AlloDerm® due to the difference in color intensity. Neo-collagen appears lighter pink as compared to AlloDerm®. The wounds were still filled with granulation tissue that was rich in fibroblasts. The inflammatory infiltrate was still present, although it was reduced compared to the five days post-surgery sections. An infiltrating epithelial cell layer was observed in the RvE1 treated wounds at the wound margins.

### 5.2.3.3. 30th Day Post Surgery

#### (A) Saline Treated Wound

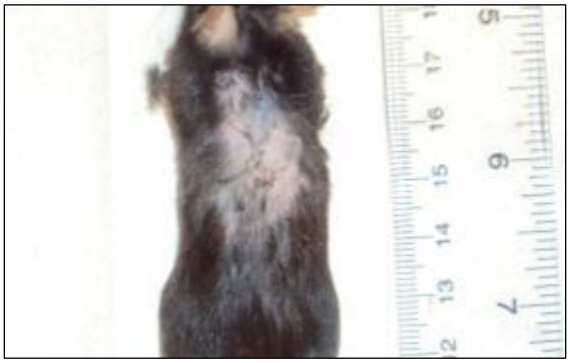


**Figure 40: 30th Day Wound – Control Treatment**

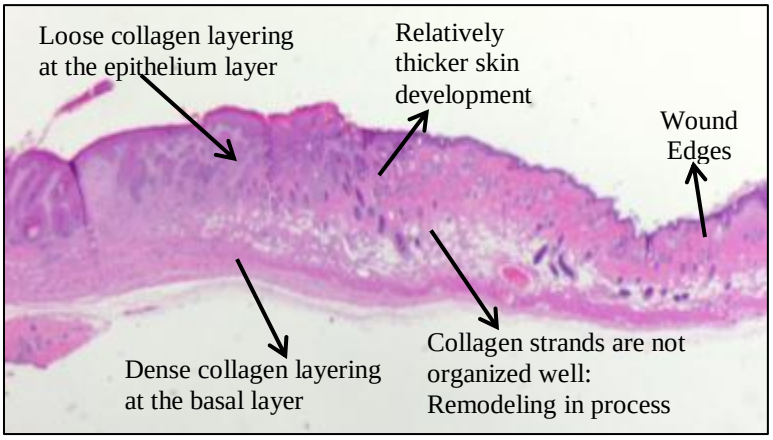



**Figure 41: H&E Stain for Day 30 Wound – Control Treatment**

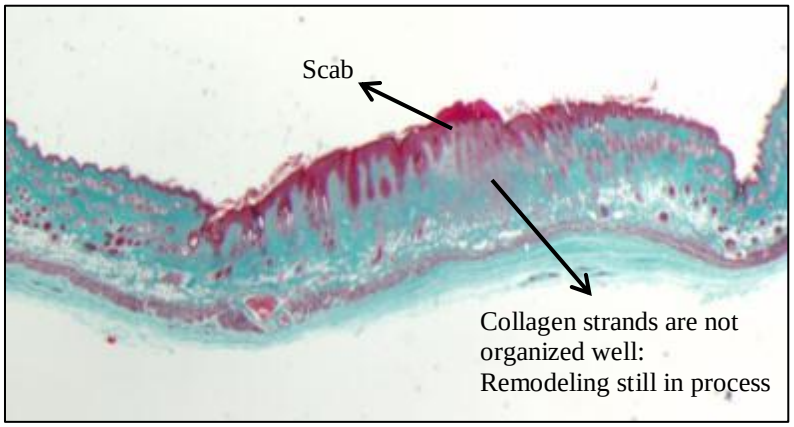



**Figure 42: Trichrome Stain for Day 30 Wound – Control Treatment**

**(B) RvE1 Treated Wound**

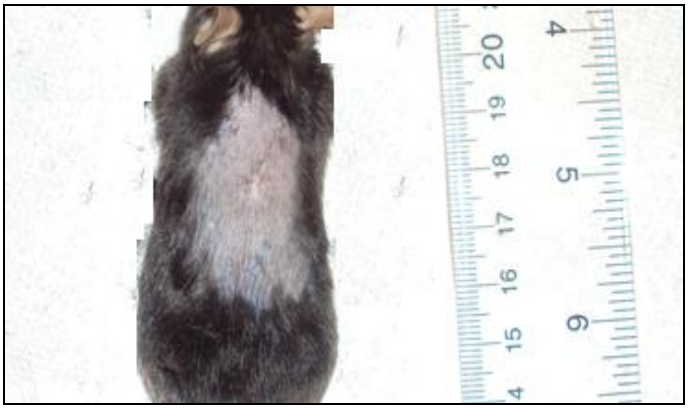


**Figure 43: 30th Day Wound – RvE1 Treatment**

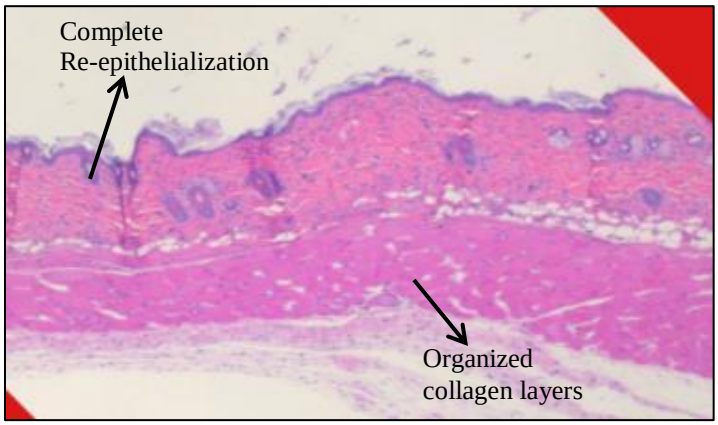



**Figure 44: H&E Stain for Day 30 Wound – RvE1 Treatment**

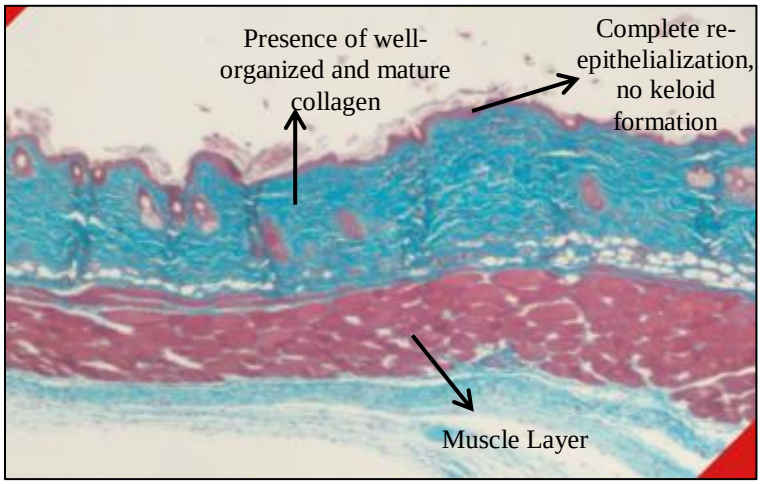



**Figure 45: Trichrome Stain for Day 30 Wound – RvE1 Treatment**

The most profound difference between saline treated versus RvE1 treated wounds were observed in the Day 30 histology slides. Saline treated wounds had not completely re-epithelialized by Day 30, the presence of scab demonstrated in complete wound closure. Whereas, RvE1 treated wounds had completely re-epithelized. Also, thicker epithelium substrate layer was seen in the case of saline treated wound possibly due to hyperplasia of epithelium cells. These wounds also demonstrated unorganized and random collagen deposition especially in regions closer to the epithelium layer.

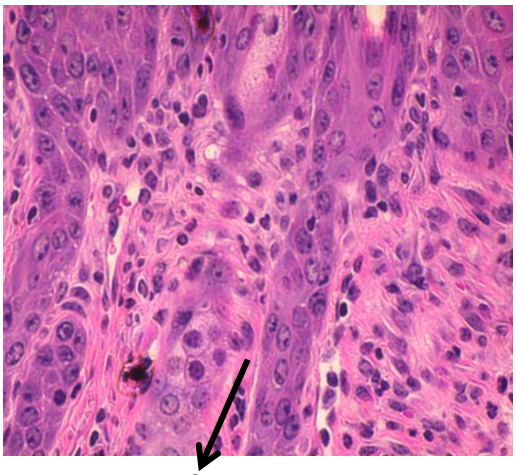



**Figure 46: Control Wound Day 30- 40X**

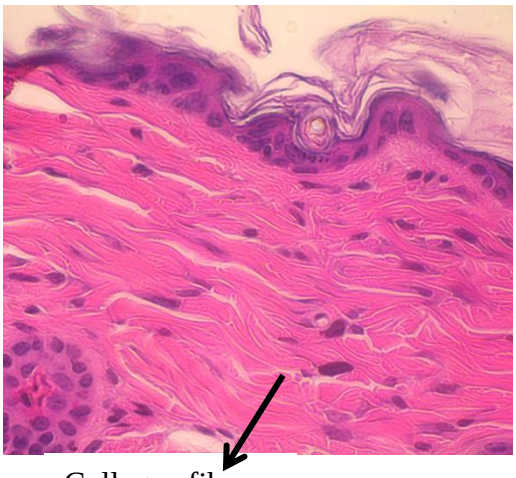



**Figure 47:RvE1 Wounds Day 30– 40X**

While, RvE1 treated wounds showed well organized, dense and mature collagen layers in the wound substrate. The collagen fibers identified were thicker and denser compared to the control treated wounds (**Figure 46** and **47**) Also, for RvE1 treated wounds, there were no significant differences between the remodeled wound site tissue and uninjured tissue. The only differentiating marker for wound site presence was the lack of hair follicles and other dermal appendages.

# CHAPTER 6

# DISCUSSIONS

## 6.1 DISCUSSION OF RESULTS

Although there have been several advances in the field of wound management the complex etiology of the healing process, especially in the case of chronic wounds, makes wound care extremely challenging. As mentioned earlier, the goal of this project is to develop a therapy that could accelerate the wound healing process and minimize overall wound closure time. The therapy tested in this project targets accelerating the wound healing process using pro-resolution molecules such as Resolvins. *In-vitro* neutrophil migration assay tests confirmed that Resolvin molecules, to varying degrees, significantly inhibit migration of neutrophil cells. This inhibition was dependent not only on the type of Resolvin molecule but also on the dosage administered. Cells were exposed to either media (control) or Resolvin molecules at 37ºC for a period of 15 minutes. Among the Resolvin molecules, Resolvin E1 molecule demonstrated significantly stronger potency to block fMLP induced neutrophil migration. This behavior was more pronounced at a dosage of 2000nM as compared to 500nM. The results of this study concur with previously published reports that demonstrate blockage of neutrophil migration by Resolvin molecules [48, 50]. In a study performed by Haas-Stapleton *et.al.*, he demonstrated the dose dependent behavior of RvE1 molecules inhibits IL8 induced neutrophil migration [48]. It was hypothesized that RvE1 that binds with Leukotriene $B_4$

receptor BLT1 expressed in neutrophils dampens the migration of neutrophils to the inflammation site while enhancing phagocytosis, ROS synthesis and fungicidal activity [48]. The variability in the degree of inhibition seen with RvD1, RvD2 and RvE1 can be attributed to the different inherent biochemical mechanism of action of the Resolvin molecules.

A reproducible $1.0cm^2$ dorsal full thickness excision wound model in wild type mice was developed to evaluate the dermal wound healing kinetics. Exogenous application of three different types of Resolvin compounds (RvD1, RvD2 and RvE1) in mice dermal wounds demonstrated significant decrease in wound closure times as compared to the untreated control group ($n=5$; $p<0.05$). Concurring with *in vitro* tests, RvE1 demonstrated the fastest wound closure time. Wound closure times observed on RvE1 treated wounds were significant compared to RvD1 and RvD2 treated wounds. Wounds treated with RvE1 closed approximately an average of 9 days before the saline treated wounds demonstrated complete re-epithelialization. Wounds, on an average, re-epithelialized approximately 92% by Day 13 for RvE1 treated wounds. At the same time, the untreated wounds had closed, on an average, by 35%. Similarly on an average, wounds treated by RvD1 and RvD2 closed by 41% and 66% respectively. The time between Day 8 and Day 13 is crucial since this time point marks the period of attenuation of the inflammatory stage and the amplification of the proliferative stage [55]. For Resolvin E1, maximum wound closure was observed during this time. As shown in Figure 27, the increase in percent wound closure in wounds treated with RvE1 was approximately 58% during Day 8 and Day 13 as compared to 24.08%, 31.88% and 35.53% with wounds treated with saline (control),

RvD1 and RvD2 respectively. This feeds back to the hypothesis that Resolvins molecules significantly increase wound closure and re-epithelialization rates in normal mice dermal wound model. The possible explanation for the increase in wound closure rates can relate back to Resolvins assisting in accelerating the resolution activities post inflammatory phase therefore resulting in earlier onset of subsequent wound healing stages and eventually resulting in faster re-epithelialization of wound site.

Additionally, data obtained from histology revealed that by Day 30, wounds treated with RvE1 exhibit a completely re-epithelialized wound site along with well-organized, dense layers of mature collagen fibers and completely repaired tissue architecture. In fact, the difference between the tissue architecture in the injured site was indistinguishable from the uninjured site. The only difference was the lack of dermal appendages present in the repaired tissue. At the same time, saline treated wounds (control) exhibited possible hyperplasia of epithelium cells verified by the presence of thicker epithelium substrate layer, incomplete re-epithelialization of wound surface and unorganized deposition of loose collagen fibers. Complete organization and dense layering of collagen fiber layers by Day 30 is testament to the fact that that the wounds treated with RvE1 underwent longer extent of remodeling or maturation as compared to the control wounds which still had not completely re-epithelialized by Day 30 [54]. These observations confirm that treatment with Resolvin, particularly RvE1 helps in accelerating the wound healing process.

**On a whole, the observed results confirm the hypothesis that Resolvin compounds accelerate the wound healing and re-epithelialization process in normal mouse dermal wound injury model.**

## 6.2 POTENTIAL NEXT STEPS

This therapy has shown potential in accelerating wound healing in normal wild type mice. Additional studies may need to be conducted to confirm the optimal dosage concentration, administered volume and frequency for maximizing wound healing efficacy. Further investigation into exploring the stability of Resolvin molecule *in vivo* under inflamed wound conditions may also need to be performed to better understand the working mechanism of Resolvin molecules. Also, more involved histological analysis may need to be performed to better interpret the impact of Resolvin compound administration on collagen deposition and neutrophil infiltration at the wound site. Since aspirin plays an important role in the rapid synthesis of these bioactive Resolvin molecules, it will also be interesting to see if topical application of aspirin will help in promoting faster inflammation resolution and accelerating wound closure.

The most obvious next step would be to test this therapy in a chronic wound model (diabetic mice). Chronic wounds, as established in Chapter 1 impacts several million people on a worldwide scale and is a billion dollar growing market. People suffering from chronic wounds are in dire need for treatments that can accelerate wound closure. Though only tested out in normal mice, this treatment shows promise in accelerating wound closure. Chronic inflammation is trademark of chronic wounds

and Resolvin molecules will help curb this inflammatory phase to initiate the subsequent steps of wound healing.

## 6.3 LIMITATIONS OF CURRENT STUDY

It is crucial to note the limitations of the current study. Wild type mice (Stain C57BL/6) were used for this study and these mice strains are known for rapid dorsal hair growth which can pose problems with maintaining the wound dressing. Ensuring that the Tegaderm wound dressing remains adhered on the wound is extremely important to reduce the occurrence of bacterial infections and to prevent wound site from drying up. Also, these mice are known for being extremely active which can create difficulties for maintaining the wound site without any external disturbances such as biting the dressing and suture off the wound site, rubbing the wound site against the cages etc. Thirdly, these mice strains are known to heal wounds by wound contraction. Almost 90% of healing is due to wound contraction as opposed to re-epithelialization [55]. Though the use of skin substitutes reduce the extent of wound contraction, this mouse strain may not be optimal for dermal wound model in order to mimic human dermal wounds. These external variations can impact the wound healing study. Instead of using wild type mice, diabetic (Lepr db gene mutation) mice can be used. These mice stains are known to demonstrate less contraction and greater degree of re-epithelialization [55]. Also, these mice strains are less active and typically bulky. Therefore, the occurrence of wound disturbance can also be minimized. However, these mice have an underlying diabetic condition and their innate healing mechanism can be impaired as a result of their disposition.

Additional difficulties in this study arose due to use of skin substitutes. Both Alloderm® and Integra Dermal Regenerative® template are extremely delicate collagen based grafts. Once hydrated in PBS, these grafts can be extremely difficult to handle and are often torn while handling during surgery. Another bigger concern with using these skin substitutes is the easy displacement and wrinkling of skin substitute during the initial days after surgery due to rapid movements and disturbances by the mice. This can potentially produce variability and impact the overall healing process.

## 6.4 SUMMARY AND CONCLUSION

In the recent years, the importance of the inflammation resolution phase has emerged significantly. Several clinical and laboratory studies have demonstrated the criticality of optimal functioning of this phase. In order to achieve efficient and optimal wound closure it is crucial that the inflammatory phase is efficiently resolved for subsequent stages to be initiated. Chronic conditions leading to exacerbated inflamed wound sites can impede complete wound closure and result in several complications that can lead to scarred tissues, ischemic tissue development, gangrene formation and even amputation. Treating abnormalities impeding the optimal functioning of this phase can be the solution to several chronic diseases such asthma, arthritis, periodontitis, cardiovascular diseases and even Alzheimer's disease [44]. Resolvin molecules are widely studied pro-resolution molecules that were first discovered by Charles Serhan in inflammatory exudates. During the resolution phase, members of the versatile Resolvin family have been shown to halt neutrophil infiltration; trigger macrophage phagocytosis of apoptotic neutrophils; accelerate the

removal of phagocytes from the injured site; reduce pain by exhibiting analgesic properties and stimulate the development of molecules involved in antimicrobial defense [56].

This project investigated the use of pro-resolution molecules like Resolvins to accelerate wound closure and to promote faster and more enhance wound healing. Three different types of Resolvin compounds were investigated in this study – Resolvin D1, Resolvin D2 and Resolvin E1. These compounds were exogenously applied in conjunction with a skin substitute to full thickness dermal wild type mice wounds. All wounds treated with Resolvin compounds demonstrated significantly accelerated wound closure. Resolvin E1 demonstrated the fastest wound closing. Wound closed at an average of 19.4 days as compared to untreated wounds which closed at an average of 28.6 days. Histological analysis revealed that wounds treated with Resolvin E1 exhibited a more 'completely' repaired tissue as compared to the untreated control wounds.

As mentioned earlier, there is need for a therapy that can accelerate wound healing, reduce scarring and efficiently restore normal tissue functionality. This therapy exhibits great potential to accelerate dermal wound closure and can be used as a tool to stimulate wound repair and regeneration in poorly healing tissues especially in chronic wound conditions.

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