Hydrogels as Therapeutic Delivery

Vehicles for Wound Healing

BY

FADI S. MASOUD
B.Sc., Dalhousie University, Halifax, Nova Scotia, Canada, 1997
D.M.D., Semmelweis University, Budapest, Hungary, 2002
D.D.S., University of Southern California, Ostrow School of Dentistry,
Los Angeles, 2005

THESIS
Submitted as fulfillment of the requirements
for the degree of Masters of Science in Oral Sciences
in the Graduate College of the
University of Illinois at Chicago, 2012

Chicago, Illinois

Defense Committee:

Luisa DiPietro, Chair, Periodontics
Anne George, Advisor, Oral Biology
Praveen Gajandrareddy, Advisor, Periodontics

I dedicate this thesis to my wife for her love, patience, and encouragement; to my son for making all this work worthwhile and fun; to my parents and brothers for their continuous support through all the past years. Your endless love and infinite support made this possible.

ACKNOWLEDGEMENTS

I would firstly like to thank my thesis committee, Dr. Luisa A. DiPietro, Dr. Anne George, and Dr. Praveen Gajendrareddy for their great support, guidance and consideration. Your combined efforts provided me with the tools to successfully accomplish my research objectives. I would especially like to express thanks to Dr. DiPietro for her incredible generosity with her time, advice, guidance, patience, and mentorship throughout this process. It truly made this experience pleasant and meaningful. Special appreciation extended to Dr. Lin Chen and Dr. Shujuan Guo for their kindness in teaching me all the laboratory skills and directing me to achieving the best results. Also particular gratitude to Mr. Huang Chun-Chieh and Dr. Sriram Ravindran for their exceptional efforts in providing me with the research gels. Thanks to Julia Tulley, Wendy Cerny, and the other members of the Wound Healing and Regeneration Center for their assistance and support. I would also like to thank Dr. Keiko Watanabe for her efforts and continuous encouragement. Lastly, I would like to express my deepest gratitude for everyone's support and for making this experience an unforgettable one at UIC.

FSM

Table of Contents

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AGE	Advanced Glycation End-products
ANOVA	Analysis of Variance
bFGF	basic Fibroblast Growth Factor
BMP	Bone Morphogenetic Protein
EGF	Epidermal Growth Factor
FGF	Fibroblast Growth Factor
IL-1	Interleukin-1
MMP	Matrix Metalloproteinase
PBS	Phosphated Buffer Solution
PDGF	Platelet Derived Growth Factor
RGD	Arginine-Glycine-Aspartate
ROS	Reactive Oxygen Species
TGF-β	Transforming Growth Factor- β
TIMP	Tissue Inhibitor of Metalloproteases
TNF-α	Tumor Necrosis Factor- α
VEGF	Vascular Endothelial Growth Factor

SUMMARY

Wound healing is a complex and diverse process that aims to repair and restore anatomical continuity and function after injury. When wounds do not heal in a proper and timely sequence, or if the healing process does not reestablish structural integrity, then the wound is considered chronic or delayed. Periodontal disease exhibits a great deal of characteristics observed with chronic wound healing. A long lasting inflammatory process that produces continuous injury to vital structures with minimal or no resolution. Both local and systemic factors play a role in delaying wound healing. Diabetes is one of the major systemic causes of delayed wound healing. Exploiting scaffolds as mediums to facilitate wound healing can be a modality to reestablish normal tissue structure, or engineer the tissue back to normal. This study utilizes an animal model to look at the potential of enhancing wound healing using hydrogel scaffolds. Recent studies have shown potential of hydrogels, based on the naturally occurring leucine zipper peptide, to be an effective therapeutic delivery mechanism. Leucine zipper gels provide a structure that allows both, incorporation of bioactive motifs along with soluble mediators to improve healing.

The aim of this study is to determine the effect of a peptide self-assembling leucine zipper gel, as compared to other established hydrogels, on wound healing in diabetic mice. Four hydrogel scaffolds were prepared for utilization in this study including, pluronic F-127 gel, chitosan gel, leucine zipper gel-control, and leucine zipper-RGD gel. Pluronic gel preparation was at time of application and the remaining three were previously prepared (Dr. Ann George's laboratory). Four groups of mice with 6 mice in each group, (n=6) were evaluated for wound closure. An additional four groups with 2 mice in each group (n=2)

were used for histological sampling. Two 8mm diameter full thickness circular excisional wounds were prepared with biopsy punch instruments on the dorsal surface of each mouse. One wound was treated with approximately 100µl of a hydrogel and the other untreated wound served as a control. The wounds were evaluated, examined for any abnormalities, and photographed on day 0, 4, 6, 8, 12, 14, and 16. The surface area of each wound was measured using computer software (Axiovision, Carl Zeiss Microimaging GmbH, Germany). On day 16 the mice were sacrificed. The groups designated for histological examination were sacrificed on day 8 and 16. Wound samples from experimental and control mice were paraffin embedded, sectioned, and stained with hematoxilin and eosin and Masson's trichrome.

The wound closure data was analyzed using 2-way ANOVA. Wound closure showed statistically significant difference between gels and controls or between the gels. More importantly both leucine zipper gels showed no difference when compared to each other. This was further supported by histological findings demonstrating similar findings between control and experimental sites. The leucine zipper gels did not show any difference from their corresponding controls or the other hydrogels.

These results indicate that the peptide self assembling leucine zipper gel has no adverse affect on wound healing in diabetic mice when compared to other well established scaffold hydrogels. Therefore there is great potential for the leucine zipper hydrogel to help in improving delayed wound healing by incorporating various active motifs. This presents a noteworthy possibility of applying therapeutic hydrogels, based on the self-assembling leucine zipper peptide, as part of periodontal therapy. This hydrogel can act as

a carrier for any number of factors that could help in resolution of the periodontal lesion and even enhance regeneration and healing.

I. Introduction

The natural process of wound healing is a very precise and complex set of independent yet overlapping phases. The four stages of hemostasis, inflammation, proliferation and remodeling strive to reform damaged tissue structure with the utmost efficiency. However, elements that change this process can be introduced at any part of these four phases. Local and systemic factors have been shown to alter the proper sequence, timing, duration, and extent of wound healing (Guo and DiPietro 2010). Failure to progress through the normal stages of healing leads into a state of pathologic inflammation, thus producing a chronic wound (Menke et al. 2007). Poor anatomical and functional outcomes are the consequence of a healing process that is delayed, incomplete, or uncoordinated (Lazarus et al. 1994). Despite the various etiologies of non-healing wounds, most ulcerations are caused by ischemia secondary to diabetes mellitus, venous stasis, and pressure (Nwomeh et al. 1998).

The most recent World Health Organization data reported 346 million people worldwide have diabetes with 80% of diabetes deaths occurring in low and middle income countries. It is projected that these deaths will double between 2005 and 2030 (WorldHealthOrganization August 2011). In the United States diabetes affects 25.8 million people (8.3% of population) of all ages, with 7.0 million undiagnosed (NationalDiabetesInformationClearinghouse(NDIC) 2011). The diabetic patient population is prone to develop chronic non-healing wounds in the form of diabetic foot. Diabetic foot ulceration is a very serious complication preceding 84% of all diabetes related amputations (Brem and Tomic-Canic 2007). Non-healing wounds result in enormous health care

expenditures, reaching approximately more than $3 billion per year, along with the tremendous financial and psychological burden on the patients (Menke et al. 2007).

The problem of delayed wound healing has been the topic of vast amounts of research. There is a wide spectrum of treatment modalities, in the literature, striving to improve the healing process in chronic wounds. Application of tissue engineering principles to facilitate the reestablishment of tissue integrity is one of these many applications (Langer and Vacanti 1993). Various studies have examined the application of hydrogel scaffolds as wound dressings and carriers of different components that could facilitate healing.

The novel approach of this study involves examining the effect of self assembling peptide hydrogels, based the on the leucine zipper motif, in a diabetic mouse wound. We hypothesize the leucine zipper hydrogels will improve wound closure and show improved healing compared to the untreated wounds.

II. Background

A. Overview of wound healing

A wound is generally defined as a disruption of normal anatomical structure and function. Healing is a complex and multifaceted process which results in the restoration of anatomical continuity and functions (Diegelmann and Evans 2004). It is a dynamic, interactive process involving soluble mediators, blood cells, extracellular matrix, and parenchymal cells (Singer and Clark 1999).

The literature has produced a wealth of information about cutaneous wound healing. The cutaneous wound has been the most common paradigm utilized for exploring the different factors involved in wound healing. Assessment of cutaneous wounds involves description of wound appearance, location of the periwound skin and cutaneous appendages, color, position, capillary refill, venous filling, bruits and pulse status, varicosities, presence of bleeding, erythema, edema, induration, fibrin, necrosis of the wound, surrounding gangrene, exudates, odor, lymphaginitis, joint abnormalities, historic origin of the wound, and description of both spontaneous and induced pain (Lazarus et al. 1994). Delays in the normal healing process are characterized by a period of chronicity and frequent relapse, have a significant impact on socioeconomic well being of the population, and result in enormous health care expenditures (Nwomeh et al. 1998). With respect to the oral cavity, healing of both oral mucosal and dermal wounds proceeds through the same stages with the oral mucosa showing rapidity and lack of scar formation (Szpaderska et al. 2003).

Classically the model of wound healing has been divided into three sequential phases: (1) inflammatory, (2) proliferative and (3) remodeling or resolution. Some refer to hemostasis as a separate phase preceding the inflammatory phase. Therefore, four continuous, overlapping, and precisely programmed phases make up the intricate processes of wound healing. These phases progress in a specific and harmonious manner. Any interruptions, aberrancies, or prolongation in the sequence can lead to delayed wound healing or a non-healing chronic wound (Guo and DiPietro 2010).

Authors have further clarified optimal wound healing in human adults to involve (Gosain and DiPietro 2004; Guo and DiPietro 2010):

1) rapid hemostasis

2) appropriate inflammation

3) mesenchymal cell differentiation, proliferation, and migration to the wound site

4) suitable angiogenesis

5) prompt re-epithelialization (re-growth of epithelial tissue over the wound surface)

6) proper synthesis, cross-linking, and alignment of collagen to provide strength to the healing tissue

Acute wounds heal in an orderly progression, maturing through artificially defined phases of coagulation, inflammation, matrix synthesis and deposition, angiogenesis, fibroplasia, epithelialization, contraction, and remodeling (Stadelmann et al. 1998).

B. Normal Wound Healing

1. Hemostasis

All skin wounds are basically a disruption in the continuity of the natural skin barrier. In uninjured healthy skin, the superficial epidermis and deeper dermis maintain a protective barrier from the external environment and any external insults. Generally, this type of disturbance produces outflow of blood from injured vessels. Initially the vascular response to trauma involves a transient 5- to 10-minute period of intense vasoconstriction that assists in hemostasis (Stadelmann et al. 1998). The process of hemostasis consists of 2 major processes: development of a fibrin clot and coagulation (Li et al. 2007). Initial formation of clot serves as a temporary shield protecting the denuded wound tissues and provides a provisional matrix over and through which cells can migrate during the repair process (Martin 1997). As the blood spills into the site of injury, the platelets come into contact with the exposed collagen and other elements of the extracellular matrix and this triggers of clotting factors and cytokines such as platelet derived growth factor (PDGF) and transforming growth factor-β (TGF-β) (Diegelmann and Evans 2004). Activated platelets undergo aggregation and adhesion while releasing mediators (e.g., serotonin, adenosine diphosphate, and thromboxane A2) and adhesive proteins (e.g., fibrinogen, fibronectin, thrombospondin, and von Willebrand factor VIII)(Li et al. 2007). Formation of a platelet plug, further platelet aggregation and secretion is increased by these mediators and locally generated thrombin (Li et al. 2007). A clot forms comprised of platelets embedded in a mesh of cross-linked fibrin fibers derived by thrombin cleavage of fibrinogen, together with smaller amounts of plasma fibronectin, vitronectin, and thrombospondin (Martin 1997). The deposition of fibronectin creates a type of scaffolding structure on which fibroblasts

can migrate into the wound. Fibronectin is produced primarily in the first 24 to 48 hours after injury (Stadelmann et al. 1998). Coagulation plays the other critical role in hemostasis via intrinsic and extrinsic pathways triggered by platelet aggregation and damaged tissue. In addition the fibrin clot serves as a reservoir of cytokines and growth factors released from the degranulating platelets. This initiates the chemotactic signals to draw circulating inflammatory cells to the wound site, initiate reepithelialization and connective tissue contraction, and stimulate wound angiogenic response (Martin 1997).

Approximately 20 minutes after the injury, active vasodilation takes place at a later stage producing an increase in vascular permeability and facilitating the way for cellular infiltrates to the wound site (Stadelmann et al. 1998).

2. Inflammation

This inflammatory response is composed of two major components: (1) a vasomotor-vasopermeability response resulting in regional vasodilation and increased capillary permeability, and (2) a leukocyte infiltrate that occurs in response to specific chemotactic factors generated in the wound (Stadelmann et al. 1998). PDGF, released by platelets, initiates the chemotaxis of neutrophils, macrophages, smooth muscle cells, and fibroblasts (Diegelmann and Evans 2004). TGF-β on the other hand attracts macrophages and stimulates them to secrete additional cytokines, such as fibroblast growth factor (FGF), PDGF, tumor necrosis factor-α (TNF-α), and interleukin -1 (IL-1) (Diegelmann and Evans 2004). Hence there is an influx of a variety of cells including polymorphonuclear leukocytes (PMN) and mononuclear leukocytes, which mature into wound macrophages, and later into lymphocytes (Stadelmann et al. 1998).

Neutrophilic leukocytes are first responders to the wound site, arriving within minutes of the injury. They function as the first line of defense in clearing contaminating bacteria, foreign material, non-functional host cells, damaged matrix components, and serve as a source of pro-inflammatory cytokines (Li et al. 2007). Mast cells play a role in releasing granules filed with enzymes, histamines, and other active amines which are mediators responsible for the characteristic signs of inflammation around the wound site (Noli and Miolo 2001). This increases permeability of the surrounding vessels and so facilitates passage of cellular components into the wound site (Noli and Miolo 2001).

Neutrophil infiltration ends after a few days; senescent and apoptotic neutrophils are removed by tissue macrophages via phagocytosis. In turn these macrophages accumulate at the wound site and recruit blood borne monocytes. The macrophages phagocytose any remaining contaminant microbes and cellular debris, and release growth factors and cytokines, e.g. PDGF and TGF- β, that intensify the preceding platelet and neutrophil signals (Martin 1997). Macrophage play a key role in regulating both wound closure and dermal healing and their depletion delays re-epithelialization, reduces collagen deposition, and impairs angiogenesis (Mirza et al. 2009).

3. Proliferation

Major events during this phase are the creation of a permeability barrier (i.e., reepithelialization), the establishment of appropriate blood supply (i.e., angiogenesis), and restoration of the injured dermal tissue (i.e., fibroplasia)(Li et al. 2007). The stage of cellular proliferation, characterized by cellular propagation and migration of different cell types, occurs 2-10 days after injury (Gurtner et al. 2008). Keratinocytes migration over the

injured dermis is the first event to take place (Gurtner et al. 2008). However, fibroblasts start to appear within 2 to 3 days, becoming the dominant cell in the healing wound after the inflammatory phase subsides (Stadelmann et al. 1998).

Reepithelialization involves several processes including the migration of adjacent epidermal keratinocytes into the wound, proliferation of keratinocytes to provide the advancing and migrating epithelial cells, the differentiation of the neoepithelium into a stratified epidermis, and the restoration of an intact basement membrane zone that connects the epidermis and the underlying dermis (Li et al. 2007). Migration is an early event in wound reepithelialization aiming to restore an intact epidermal cover of the injured site (Hell and Lawrence 1979). Kertinocyte migration starts approximately 12 hours after wounding with cellular flattening and elongation development of pseudopodlike projections of lamellipodia, loss of cell-cell and cell-matrix contacts, retraction of intracellular tonofilaments, and formation of actin filaments at the edge of their cytoplasm (Li et al. 2007). The early provisional matrix formed by fibrin, fibronectin, and type V collagen enables keratinocytes to migrate and dissect under eschar and debris that may be covering the wound (Li et al. 2007). However, in order for epidermal cells to migrate between the collagenous dermis and the fibrin eschar, activation of plasmin by plasminogen activator produced by these epidermal cells and collagenase production are required (Bugge et al. 1996; Pilcher et al. 1997). Plasminogen activator activates collagenase which facilitates the degradation of collagen and extracellular-matrix proteins (Singer and Clark 1999). Keratinocytes also go through a process of cellular proliferation, maturation and finally restoration of the barrier function of the epithelium (Gurtner et al. 2008). Keratinocytes reattach themselves to the underlying substratum, reconstitute the

basement membrane, and then resume the process of terminal differentiation to generate a stratified epidermis (Li et al. 2007).

Approximately four days after injury, granulation tissue, rich with new capillaries, invades the wound space. This is accompanied by the movement of macrophages, fibroblast, and blood vessels into the wound space (Singer and Clark 1999). Growth factors, notably PDGF and TGF-β1, along with extracellular-matrix molecules, stimulate fibroblasts of the tissue around the wound to proliferate, express appropriate integrin receptors, and migrate into the wound space (Gray et al. 1993; Singer and Clark 1999; Xu and Clark 1996). TGF-β released by platelets, macrophages, and T-lymphocytes is considered to be a master control signal that regulates a host of fibroblast functions (Roberts and Sporn 1993). It increases transcription of the genes for collagen, proteoglycans, and fibronectin leading to an increase in their production (Roberts and Sporn 1993). At the same time it decreases secretion of proteases responsible for the breakdown of the extracellular matrix and stimulates tissue inhibitor of metallo-proteases (TIMPs) (Hall et al. 2003).

Fibronectin and hyaluronate form the greater part of early extracellular matrix that develops. This matrix serves as a scaffold for fibroblasts to migrate and adhere. Fibroblasts produce glycosaminoglycans and collagen which are important components of wound repair. As the fibroblasts multiply, collagen is produced and its levels continue to rise for approximately 3 weeks (Stadelmann et al. 1998). Early in wound healing, after 48 to 72 hours, there is a predominance of collagen type III produced by fibroblasts present in granulation tissue (Li et al. 2007). In hypertrophic and immature scars type I and III collagen are present at a ratio of 2:1 (Stadelmann et al. 1998). This changes to a 4:1 ratio,

an increase in type I collagen replacing type III as the site remodels and the scar matures in a return to normal skin structure (Stadelmann et al. 1998). Angiogenesis takes place along with this process forming new blood vessels and capillaries. New vasculature associated with fibroblasts and macrophages replace the fibrin matrix with granulation tissue and this forms a new substrate for keratinocyte migration which will take place at a later stage (Gurtner et al. 2008).

Formation of new vasculature, a process termed angiogenenesis or neovascularization, is another essential component in the restoration of the injured site. Local factors in the wound microenvironment such as low pH, reduced oxygen tension and increased lactate initiate the release of factors needed to stimulate a new blood supply (Diegelmann and Evans 2004). This is a complex process that relies on the extracellular matrix in the wound bed as well as migration and mitogenic stimulation of endothelial cells (Singer and Clark 1999). It has been found that several players have angiogenic activity, including vascular endothelial growth factor (VEGF), TGF-β, angiogenin, angiotropin, and angiopoietin 1 (Li et al. 2007; Singer and Clark 1999). VEGF and bFGF are considered to be the two most important positive regulators of angiogenesis (Gurtner et al. 2008). Multiple cell types such as keratinocytes, fibroblasts, epidermal cells, and endothelial cells, are able to produce these factors (Diegelmann and Evans 2004; Li et al. 2007; Singer and Clark 1999). VEGF is a potent cellular mitogen for endothelial cells and induces cellular migration and development (Li et al. 2007). During the first three days of wound repair, bFGF is probably the primary pro-angiogenic stimulus (Nissen et al. 1996). During the later formation of granulation tissue on days 4 through 7, VEGF is the predominant pro-angiogenic factor (Nissen et al. 1998).

Fibroblasts become the predominant cellular component as proliferation continues, and this cell type is responsible for producing the new matrix needed to restore structure and function to the injured tissue (Diegelmann and Evans 2004). Fibroblasts rebuild connective tissue structures in the injured site. Collagen synthesis by fibroblasts involves the secretion of collagen into the extracellular wound environment (Stadelmann et al. 1998). Once in the extracellular space, collagen then polymerizes into collagen fibers and covalently cross-links to increase tissue tensile strength. In adult mammals, the process of restoration of collagen in skin is incapable of completely re-establishing normal architecture, and injured sites only regain about 80-90% of normal tensile strength (Diegelmann and Evans 2004).

Wound contraction starts soon after injury and peaks at 2 weeks varying with depth of the wound (Li et al. 2007). Myofibroblasts are fibroblast with large bundles of actin containing microfilaments along the cytoplasmic face of the plasma membrane and by cell-cell and cell-matrix linkages (Singer and Clark 1999); this cell type appears in the wound and is thought to be important to wound contraction. These cells align and the wound contracts in direction of skin tension lines (Li et al. 2007). Extracellular fibronectin may function as a connection between the myofibroblast and collagen fibers (Li et al. 2007). The process of contraction can produce a significant reduction of wound surface area, in turn accelerating the closure process.

4. Remodeling

Remodeling includes deposition of the matrix and its subsequent changes over time (Li et al. 2007). This stage of wound repair begins 2–3 weeks after injury and could last for

a year or more, during which all of the processes activated after injury slow down and cease (Gurtner et al. 2008). In actuality, remodeling occurs throughout the entire wound repair process. For example, the fibrin clot formed in the early inflammatory phase is replaced by granulation tissue that is rich in type III collagen and blood vessels during the proliferative phase (Li et al. 2007). Subsequently it is replaced by a collagenous scar predominantly of type I collagen with much less mature blood vessels (Li et al. 2007). Most of the endothelial cells, macrophages and myofibroblasts undergo apoptosis or exit from the wound, leaving a mass that contains few cells and consists mostly of collagen and other extracellular-matrix proteins (Gurtner et al. 2008). Type III collagen first appears after 48 hours and reaches its maximal levels between 5 to 7 days (Li et al. 2007). In the next 6–12 months, the acellular matrix is actively remodeled from a mainly type III collagen to one predominantly composed of type I collagen (Gurtner et al. 2008). Collagen degradation takes place via several proteolytic enzymes, matrix metalloproteinases (MMP's), which are secreted by macrophages, epidermal cells, endothelial cells, and fibroblasts (Gurtner et al. 2008; Singer and Clark 1999; Stadelmann et al. 1998). Total amount of collagen increases early in repair, reaching a maximum between 2 and 3 weeks after injury and within one year or longer, the dermis gradually returns to a stable phenotype that is similar to its preinjury state consisting largely of type I collagen (Li et al. 2007). Early in the healing process collagen fibrils are arranged in a haphazard manner, but with time they are reorganized to a structure that maximizes the sites strength (Baum and Arpey 2005). Although there is a reorganization of the extracellular matrix structure, the wound site can only reach 70-80% of its preinjury tensile strength (Baum and Arpey 2005; Li et al. 2007; Stadelmann et al. 1998).

C. Chronic wounds and delayed healing

Chronic wounds are defined as wounds that have failed to progress through normal stages of healing and hence entered a state of delayed, incomplete, and uncoordinated repair (Menke et al. 2007). These types of wounds do not progress through an orderly process and have lost ideal synchrony of events that lead to normal healing (Fig. 1) (Li et al. 2007). When wounds do not heal in proper and timely sequence, the result is an inability to reestablish normal structural integrity resulting in poor anatomical and functional outcomes (Menke et al. 2007; Stadelmann et al. 1998). These types of wounds are characterized by chronicity and frequent relapse and can be a major disability (Menke et al. 2007).

Excessive scar tissue formation is a form of a chronic wound differing from acute wounds by formation of an abundance of granulation tissue and exhibiting excessive fibrosis leading to scar contraction and loss of functions (Stadelmann et al. 1998). It appears that a failure of apoptosis, which plays a key role in vascular and fibroblastic cells elimination from normally healing granulation tissue, produces a high degree of cellularity that ultimately heals with an abundance of scar tissue (Desmouliere et al. 1995; Stadelmann et al. 1998).

Ulcerated skin wounds are probably the most common types of chronic wounds. They can be created or perpetuated by many factors, including vascular insufficiency, either venous or arterial, prolonged inflammation, pressure necrosis, physical agents, diabetes, infection, and cancer (Stadelmann et al. 1998). Approximately 70% of ulcers are

caused by ischemia, secondary to diabetes mellitus, venous stasis, or pressure (Menke et al. 2007).

Whereas normal wound healing involves an inflammatory process that is self-limiting, chronic non-healing wounds exhibit hyper-inflammation (Menke et al. 2007). Over-abundant neutrophil infiltration is responsible for the chronic inflammation characteristic of non-healing pressure ulcers (Diegelmann 2003; Diegelmann and Evans 2004). In normally healing wounds, neutrophils are almost nonexistent after the first 72 hours, while in chronic wounds they are present throughout the healing process (Menke et al. 2007). They seem to persist due to continued recruitment and activation in response to tissue trauma by pressure, bacterial overgrowth, leukocyte trapping, or ischemic reperfusion injury (Menke et al. 2007). Neutrophils release large amounts of enzymes such matrix metalloproteinase 8, a collagenase that has substrate preference for collagen type I (Diegelmann 2003; Nwomeh et al. 1999). Normally MMP's are inhibited by nonspecific protienase inhibitor α2-macroglobulin and specifically by TIMP-1(Menke et al. 2007; Nwomeh et al. 1999). Additionally, the disproportionate number of inflammatory cells create inflammatory cytokine predominance (Nwomeh et al. 1998). Neutrophils also release elastase, which is able to destroy growth factors, such as PDGF and TGF-β (Diegelmann and Evans 2004). The chronic wound's microenvironment appears to be one of massive destruction due to invasion of high neutrophil numbers and their destructive enzymes which have an overpowering effect over the already highly active connective tissue components (Diegelmann 2003). Hence, the overall result in chronic wounds is one in which there is a higher level of degradative versus protective enzymes in which the normal controlling feedback mechanism is no longer functional (Menke et al. 2007).

Cells in chronic wounds are phenotypically altered and unable to function properly. Fibroblasts demonstrate a phenotypic change which produces decreased migration and proliferation (Brem et al. 2007). Keratinocytes on the edge of chronic wounds are unable to migrate properly leading to a wound that cannot be closed (Li et al. 2007). Inability of these cells to respond to signaling factors, altered capacity to function, and the inadequate expression of proliferative factors e.g. TGF-β1 partake in creating these conditions (Li et al. 2007; Menke et al. 2007).

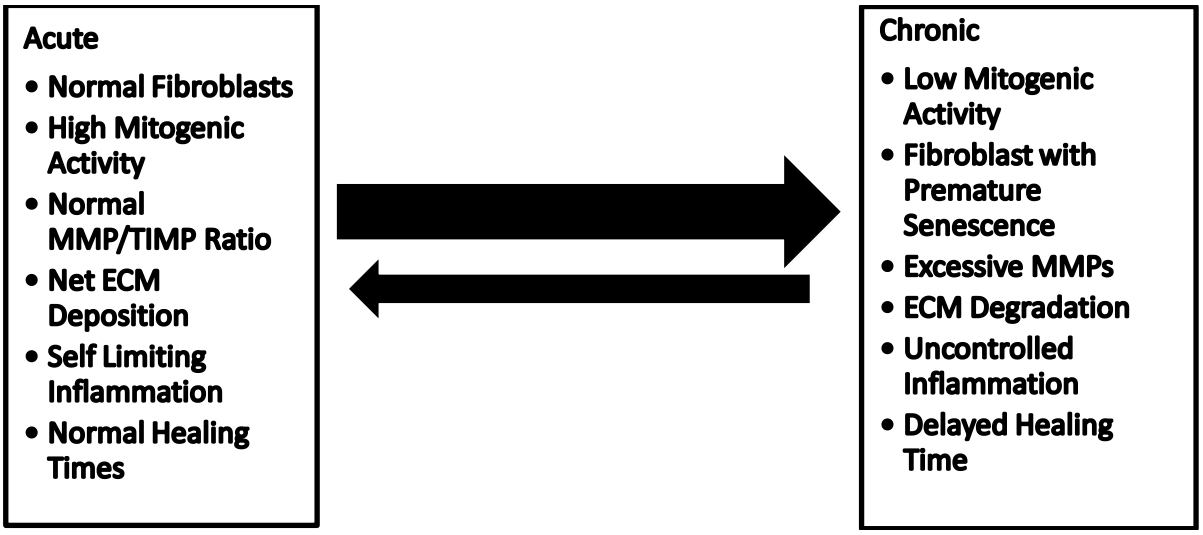

Figure 1. Disequilibrium of Chronic Wound Healing (*Reproduced from Menke et al. 2007*)

D. Effect of diabetes on wound healing

Factors that affect wound healing (Fig.2) can be divided into local factors directly influencing characteristics of the wound itself and systemic factors that pertain to overall health or disease state which in turn affects the ability to heal (Guo and DiPietro 2010).

Systemic factors, such as diabetes act through the local factors that ultimately affect wound healing (Guo and DiPietro 2010). Diabetes is one of the most well-known and widely spread systemic diseases that can radically influence the healing process. Fifteen percent of all persons with diabetes are anticipated to develop chronic non-healing diabetic foot ulcers which lead to lower leg amputation 84% of the time (Brem and Tomic-Canic 2007). Diabetic foot ulceration is associated with wound infection and osteomyelitis, which may lead to amputation (Blakytny and Jude 2006).

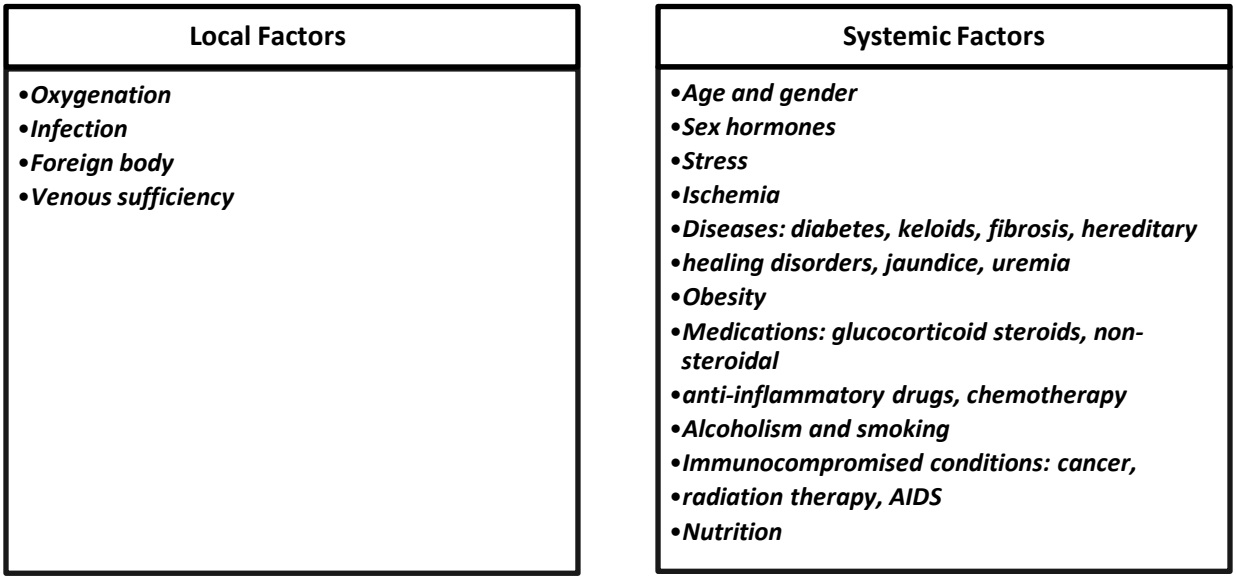

Figure 2. Factors Affection Wound Healing (*Reproduced from Guo and DiPietro 2010*)

Cellularly, wounds of diabetics exhibit an increase in inflammatory cells, and an absence of cellular growth and migration of the epidermis over the wound, along with narrowing or occluding of blood vessels along the wound edges (Ferguson et al. 1996). Diabetic patients have impaired leukocyte function and inadequate migration of neutrophils and macrophages to the wound (Delamaire et al. 1997).

Nitric oxide, a free radical produced by the enzyme nitric oxide synthase from L-arginine, is normally released from macrophages as part of their anti-microbial activity (Blakytny and Jude 2006). It has been shown that the production of this mediator is altered in diabetic patients with elevated nitric oxide synthesis detected in diabetic foot ulcers (Blakytny and Jude 2006). In diabetic patients, elevated levels of plasma nitric oxide have been associated with slow healing. It has also been suggested that altered nitric oxide production leads to a loss of macrophage activity against infection, and excessive amounts may be inhibitory to angiogenensis, decrease endothelial cell and lymphocyte proliferation, and may inhibit platelet and leukocyte activation (Blakytny and Jude 2006).

An overview in Figure 3 of the complex effect of diabetes on wound healing illustrates the most noteworthy contributory physiologic factors.

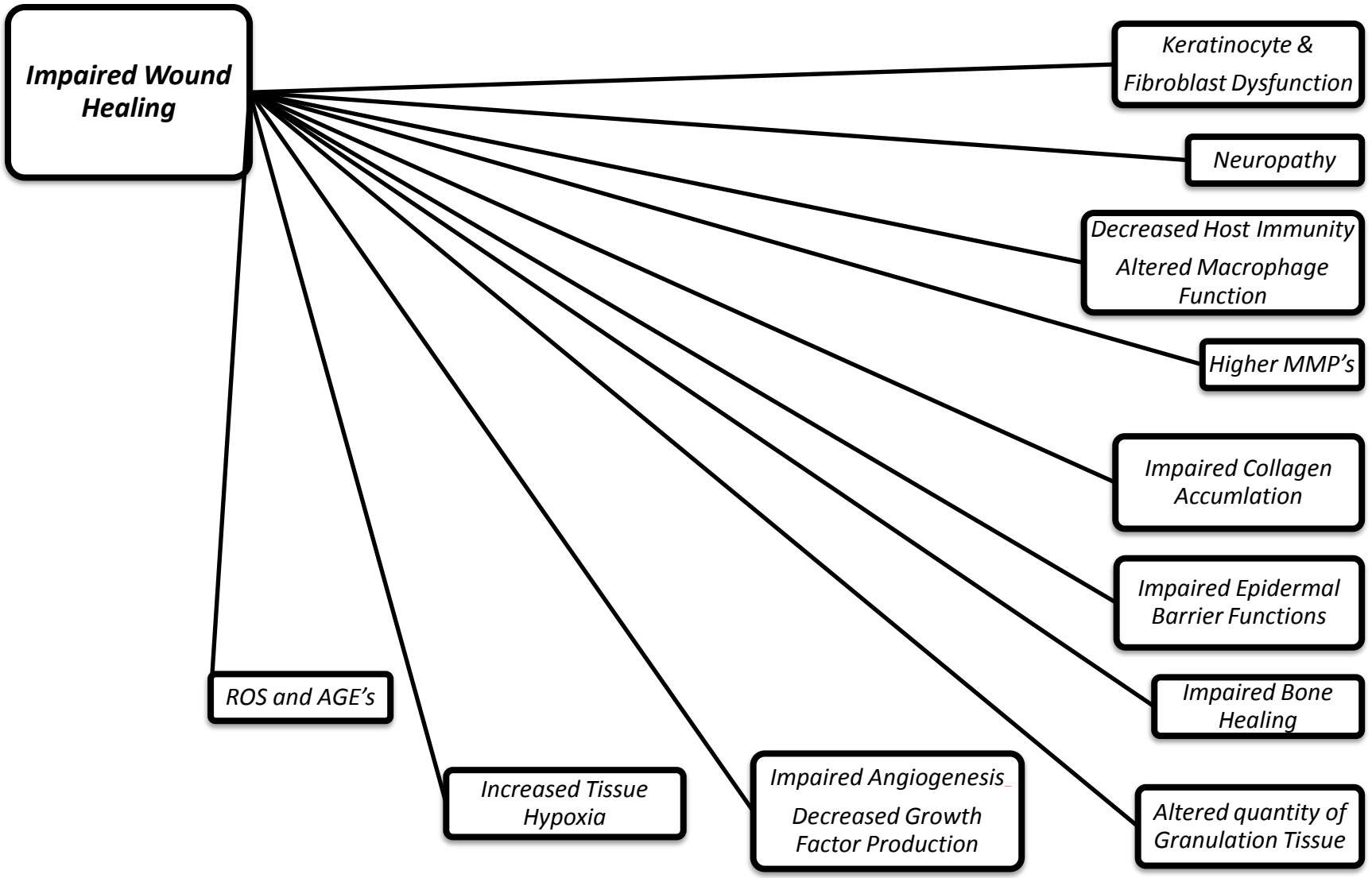

Figure 3. Physiological Effects of Diabetes on wound healing (*Adapted from Guo and DiPietro 2010 and Brem and Tomic-Clanic 2007*)
MMP's: matrix metalloproteases; ROS: reactive oxygen species; AGEs: advanced glycation end-products

E. Tissue Engineering

The term tissue engineering originated in 1987 at a bioengineering panel meeting held at the National Science Foundation. In early 1988 the first tissue engineering meeting was held at Lake Tahoe, California in which a working definition was formulated (Nerem 1991):

> Tissue engineering is the application of the principles and methods of engineering and the life sciences toward the fundamental understanding of structure-function relationships in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain, or improve functions.

Three general strategies can be followed for the creation of new tissue. First, isolated cells or cell substitutes may be used to supply the needed function. A second approach use, tissue inducing substances introduced into target areas. Finally, cells placed on or within matrices may be implanted and incorporated into the body. (Langer and Vacanti 1993) A combination of all of these approaches may also be used. Utilizing scaffolds to facilitate wound healing has been attempted as a modality to reestablish normal tissue structure, or engineer the tissue back to normal. As mentioned above, a scaffold based tissue engineering approach can include three elements (George and Ravindran 2010):

> 1) Biomaterials from which scaffolds are formulated. These materials provide a three dimensional template for tissue reconstitution. These can be either natural or synthetic materials that are designed to help in organization, growth, and cell differentiation. These biomaterial scaffolds should be able to provide both chemical and physical signals to facilitate this process.
>
> 2) A suitable cellular population present from an autologous, allogenic, xenogenic, or stem cell source. Isolated cells can be expanded in a cell culture

and then seeded onto the scaffold to guide their growth and tissues regeneration in three dimensions (Yang et al. 2001). Proliferation and differentiation of these cells is instrumental for success of the process.

3) The incorporation of biomolecules including angiogenic factors, growth factors, differentiation factors and signaling molecules.

1. Scaffolds

Biomaterials utilized as scaffold should have a formulation based on inertness and biodegradability. A truly inert framework or scaffold has limited interaction with the surrounding tissue environment while facilitating cellular growth and function (Yang et al. 2001). However, current research revolves around making these biomaterials more interactive with these processes. This can be based on their mode of application by acting as carriers introducing cells into damaged or diseased tissue, or by augmenting the function of endogenous progenitor cells (George and Ravindran 2010). Biodegradability entails that the material produces nontoxic products while leaving a desired living tissue (Yang et al. 2001). Scaffolds utilized for wound healing can be implemented as space filling agents, as delivery vehicles for bioactive molecules, or as three-dimensional structures that organize cells and present stimuli to direct formation of a desired tissue (Drury and Mooney 2003). Importantly, any scaffold designed to encapsulate cells must have that ability without damaging the cells, have no toxicity to the cells and the surrounding tissue, allow appropriate diffusion of nutrients and metabolites to and from the encapsulated cells and surrounding tissue, and requires sufficient mechanical integrity and strength to withstand manipulations associated with implantation and in vivo existence (Drury and

Mooney 2003). Thus, the success of a material as a scaffold depends on its critical physical, mass transport, and biological design variables. Physical properties include mechanical characteristics, formation mechanism and dynamics, degradation behavior. Mass transport properties are exemplified by diffusion capabilities. The biological variables necessary are indicated by a required nontoxicity. (Drury and Mooney 2003).

Traditionally, materials with the desired characteristics included natural polymers, synthetic polymers, ceramics, metals, and combinations of these materials. Metals and pure ceramics area not biodegradable and their processability are very limited. However, bioceramics such as hydroxyapatite, bioactive glasses, and calcium phosphate ceramics have been shown to exhibit bioactive, biocompatible behavior and have been used as filler material for bone defect repair and as artificial bone matrix. Bioceramics have been categorized into three categories: bioinert (alumina and zirconia), surface bioactive groups (sintered hydroxyapatite, bioglass, alumina-wollastonite glass ceramic), and bioresorbable (nonsintererd hydroxyapatite, a- or b- tricalcium phosphate, tetracalcium phosphate, octocalcium phosphate) (Yang et al. 2001). Polymers can be divided into both natural, such as collagen, glycosaminoglycan, starch, chitin, and chitosan, and synthetic such as poly (a-hydroxy) esters, polyanhydrides, polyorthoesters, and polyphosphazens. Variety in degradability among naturally occurring polymers has prompted the development of synthetic polymers that degrade via chemical hydrolysis and are insensitive to enzymatic processes. A class of synthetic polymers includes poly (a-hydroxy) esters and copolyesters of lactic acid and glycolic acid. In the United States, polyglycolic acid, or polyglycolide, polylactic acid, or polylactide, polydioxanone, and copolymers thereof are the only synthetic, degradable polymers with an extensive U.S. Food and Drug Administration

approval history. (Yang et al. 2001) These copolymers are hydrophobic and processed under severe conditions, which present a challenge in incorporating viable cells. Highly hydrated polymers or hydrogels presented an alternative scaffold made up of hydrophilic polymer chains from either synthetic or natural origins. (Drury and Mooney 2003)

Hydrogels are attractive biomaterials to use as scaffolds for tissue engineering (Peppas et al. 2006). Characteristics that make them perfect candidates as scaffolds for growing cells and tissues include water swellablity with water insolubility and cross-linked networks that exhibit high water contents and tissue-like elastic properties (Nicodemus and Bryant 2008). Fibroblasts, osteoblasts, vascular smooth muscle cells, and chondrocytes successfully immobilize and attach to hydrogel scaffolds (Peppas et al. 2006). The combination of high water content, biocompatibility, excellent mechanical properties and minimally invasive delivery capability makes hydrogels particularly attractive for tissue-engineering applications (Drury and Mooney 2003; Peppas et al. 2006). Hydrogels have been used as scaffolds for tissue engineering, and as immunoisolation barriers for microencapsulation technology in which allogeneic or xenogeneic cells are protected from the host's immune system through separation from the immune components via a semi-permeable membrane (Peppas et al. 2006). Combining both natural and artificial polymers can be utilized to provide proper scaffold degradation behavior after implantation.

2. Types of Hydrogels

Both synthetic and naturally derived materials may be used to form hydrogels for tissue engineering scaffolds. Synthetic materials include polyethylene oxide, polyvinyl alcohol, polyacrylic acid, polypropylene furmarate-co-ethylene glycol, and polypeptides.

Representatives of naturally derived materials include agarose, alginate, chitosan, collagen, fibrin, gelatin, and hyaluronic acid. (Drury and Mooney 2003)

Similarity in mechanical and structural properties, to many naturally occurring tissues and the extracellular matrix, allowed successful applications of these hydrogels as scaffolds. The hydrogel structure depends on cross-links formed between polymer chains via chemical bonds and physical interactions. (Drury and Mooney 2003) Covalent cross-links can lead to stable hydrogels, while other types of cross-links could be used to reverse gelling properties of the hydrophilic polymers under desired conditions (Peppas et al. 2006). Many hydrogels that are under study possess covalent cross-linked networks that are stabilized permanently. This feature has led to efforts to make them degradable through either proteolytic cleavage or hydrolysis. The degradation rate for proteolytically degradable hydrogels can become a rate-limiting factor for tissue formation because it is dependent on cell-mediated protease activity which is difficult to regulate or accelerate. For hydrolyzable hydrogels, the degradation rate is difficult to control and universally occurring hydrolysis may result in continuous deterioration of the mechanical properties and expose the cells to undesired mechanical signals. (Liu et al. 2009)

Another feature that is important to the function of hydrogels is availability of specific protein domains. Protein domains of importance include a vast number of naturally occurring amino acid sequences that reliably fold into three-dimensional structures with distinct functions. Many of these domains have been exploited as biomedical engineering materials such as scaffolds for tissue engineering, active or reactive drug delivery systems, and in vivo and in vitro biosensors (Banta et al. 2010). One

approach to incorporating critical protein domains is the creation of self-assembling peptides.

Natural peptide and protein-based self-assembling systems are present throughout the different aspects of biology. Self-assembly is defined as spontaneous organization of molecules under thermodynamic equilibrium conditions into a structurally stable arrangement by the driving force of non-covalent interactions, including hydrogen bonds, ionic bonds, electrostatic interaction and van der Waals interaction. Based upon natural systems, self-assembling peptides can be designed so that their molecular and nanoscale structure are compatible with cell culture and enhance the differentiation of precursor cells. Peptide or protein based self-assembling gels provide the advantage of being a nanoscale biological material that can be integrated with other organic or inorganic components to form nanocomposites. These nanobiomaterials have biomimetic characteristics that present exceptional physico-chemical properties which have a fundamental task in activation of cellular interaction and initiation of tissue regeneration. (George and Ravindran 2010)

Self assembling peptides that can be created present the opportunity to apply biomimetic domains of importance in a variety of settings. A biomimetic material is able to mimic cellular and extracellular protein domains, with emphasis on presentation of signals in a controlled spatio-temporal manner. (George and Ravindran 2010) Self-assembling biomaterials therefore present a number of advantageous qualities as compared to other scaffolds such as synthetic polymers or tissue-derived biopolymers. This includes complete compositional definition, the ability to produce complex fibrillar or network structures, sensitivity to stimulus, and modularity. Such peptide-based self-assembling

forms have gained special attention due to their ease of synthesis, ability to incorporate native or non-native chemistry, specifiable biological activity, and the availability of design rules for producing predictably folded and oligomerized structures. (Jing et al. 2008)

A repeating motif of leucine residues is the most widely studied self assembling peptide. It allows dimerization of different polypeptides and was coined "leucine zipper" due to its ordered interdigitation that forms a zipper like structure which represented part of the scaffold that molds a protein to interact with its target sited on DNA (Landschulz et al. 1988). The leucine zipper can be considered one of the most straightforward elements for protein-protein interactions known (Burkhard et al. 2001). It consists of two polypeptide chains that, by wrapping around one another, form a rope-like helical bundle (Ryadnov et al. 2008). This coiled-coil motif, embodied in the leucine zipper, is a simple structural architecture that represents a highly versatile protein folding design (Burkhard et al. 2001). The coiled-coils design is basically heptad repeated units designated as *abcdefg,* where the *a* and *d* positions are occupied by hydrophobic residues such as leucine and the *e* and *g* positions are occupied by charged residues (Banta et al. 2010; George and Ravindran 2010; Petka et al. 1998; Stevens et al. 2005). The side chains of the nonpolar leucine residues lie in a plane along the length of a helix; the hydrophobic nature of the plane leads to helical folding and formation of multistranded coiled-coils (Banta et al. 2010; George and Ravindran 2010). Charged residues are frequently found at coiled-coil interfaces, thus playing a role in modulating the assembly and stability properties of coiled-coils (Burkhard et al. 2001). A consequence of changes in temperature, pH, ionic strength, or solvent is conformational changes of native and *de novo* coiled-coils (Wang et al. 1999). Coiled-coils can be involved in signal-transducing events, can act as a molecular recognition

system, can provide mechanical stability to cells, and are also involved in movement processes. (Burkhard et al. 2001) Coiled-coil perform various natural functions ranging from structural support (R-keratin, actin, etc.) to DNA binding (basic leucine zipper transcription factors), receptor oligomerization (mannose binding protein), and membrane fusion (HIV, influenza).

Petka was first to devise a scheme for creating a biomimetic self assembling hydrogel based upon this naturally occurring leucine zipper motif (Petka et al. 1998). Application of the leucine zipper motif in hydrogel formation stemmed from poorly understanding the molecular origins of gel formation, limitations for systematic engineering of gel properties, and limited ability to tune gel-solution transition conditions (Petka et al. 1998). A multidomain (triblock) protein chain was created with two short leucine zipper end blocks flanking a water soluble polyelectrolyte domain which under appropriate pH and temperature adopted the required helical confirmation (Petka et al. 1998). These hydrogels are reversible in response to high pH or high temperature where the leucine zipper domains are denatured (Banta et al. 2010; George and Ravindran 2010; Kennedy et al. 2005). The structure is potentially able to have multiple bioactive motifs incorporated into it while the leucine zipper self-assembling properties are unaffected (George and Ravindran 2010). This type of protein-protein recognition can result in the formation of reversible hydrogels with precise and independent control of length, composition, charge density of the polyelectrolyte domain, and relevant architectural features of the associated end block (Petka et al. 1998). It can be imagined that a variety of synthetic peptide materials with artificial allosteric sites introduced at critical core positions in the sequence such that self-assembly can be enhanced through incremental

changes in environmental stimuli (pH, metal ion, or small-molecule) within a well defined concentration range under physiologically appropriate conditions (Zimenkov et al. 2006). Achieving this control would be valuable in designing hydrogels of predetermined physical and biological properties (strength, porosity, and sensitivity to enzymatic degradation). Response to the proper environmental stimuli would make these hydrogel systems appealing for use in molecular and cellular encapsulation, controlled reagent delivery (e.g. drugs), and tissue engineering (Petka et al. 1998; Shen et al. 2007).

Incorporating specific motifs such cell attachment motifs, arginine-glycine-aspartic acid (RGD) into a scaffold could be a way of controlled grouping of different cell types, thus mimicking tissues (Fischer et al. 2007; Liu et al. 2009; Mi et al. 2006). A triblock polypeptide based on the leucine zipper motif formed a thermo reversible hydrogel which was shown to have antibody binding ability (Cao and Li 2008). The leucine zipper has also been shown to accommodate motifs from the hydroxyapatite nucleating domain and cell adhesive motifs of dentin matrix protein 1 (Gajjeraman et al. 2008). Ultimately, the goal is to create a synthetic biomaterial scaffold that influence cell adhesion, differentiation, and migration of specific cell types to create artificial tissues (Holmes 2002).

3. Hydrogels in Wound Healing

Hydrogels' structural similarity to the extracellular matrix found in tissues give them the capacity to mimic its many roles i.e. bring cells together and control tissue structure, regulate cellular functions, and allow diffusion of nutrients, metabolites, and growth factors (Lee and Mooney 2001). Various hydrogels have been employed as wound healing scaffolds.

a. Chitosan Gel

One of the most common applications is the chitosan hydrogel. On open wounds in a mouse model, it was shown to induce significant wound contraction and thereby accelerating wound closure and the healing process (Ishihara et al. 2001; Ishihara et al. 2002). Chitosan has been shown to stimulate collagen synthesis and incorporate into it FGF (Zhong et al. 2010). It has shown controlled release ability of growth factors such as FGF upon *in vivo* biodegradation of the hydrogel itself (Ishihara et al. 2003). Chitosan and chitosan embedded with FGF showed significantly accelerated wound closure in an animal model because it appeared that chitosan reduces the proteolytic environment of the wound and potentiates FGF activity leading to faster closure (Park et al. 2009). In wounds of diabetic mice, the application of chitosan containing FGF enhanced wound vascularization and granulation tissue formation and improved healing (Obara et al. 2005). Clinical data of a human study utilizing a mesh chitosan membrane as a wound dressing showed efficient adherence, hemostasis, healing, and re-epithelization of the wound (Azad et al. 2004). A comparison of native, lipase degraded, and an o-carboyxmethylated chitosan displayed various levels of antimicrobial activity (Kim et al. 2003). Despite its favorable characteristics chitosan has some disadvantages such as unsatisfactory mechanical properties, severe shrinkage, and deformation after drying (Zhong et al. 2010).

Chitosan is a natural polymer most widely used in wound healing due to its many advantages including biocompatibility, biodegradability, hemostatic activity, and antibacterial properties (Zhong et al. 2010). Chitosan is structurally similar to naturally occurring glycosaminoglycans and is degradable by enzymes in humans. It is derived from chitin, which is found in athropod exoskeletons. Chitosan is a linear polysaccharide of (1–

4)-linked d-glucosamine and N-acetyl-d-glucosamine residues from chitin (Drury and Mooney 2003).

b. Pluronic F-127 gel

Pluronic F-127's (Poloxamer 407, PF-127) use in wound healing has mainly been as a delivery vehicle for a variety of soluble mediators, including antibodies, cytokines, and growth factors (Dibiase and Rhodes 1996; Mori et al. 2008; Veyries et al. 2000). Viability of transferring epidermal growth factor (EGF) via a pluronic F-127 gel carrier has been established (Dibiase and Rhodes 1996). However, little data is published on the application of this formulation. There is limited data available on the potential of pluronic F-127 in enhancing wound healing. Formulation of its use as an artificial skin to treat burn wounds has also been reported (Schmolka 1972). This pluronic F-127 based artificial skin significantly enhanced the rate of burn wound healing and it was speculated that this was possible by stimulation of EGF (Nalbandian et al. 1987).

Pluronic F-127 is a thermoreversible hydrogel widely used in pharmaceuticals as a carrier for various routes of administration including oral, topical, intranasal, vaginal, rectal, ocular, and paranteral. Pluronic F-127 has a good solubilizing capacity, low toxicity and is, therefore, considered a good medium for drug delivery systems. It is more soluble in cold solutions than in room temperature or solutions as a result of increased solvation and hydrogen bonding at lower temperatures. The aqueous solutions have the ability of reverse thermal gelation, which means that it is liquid at low temperatures and gels when warmed or at room temperature. This process is reversible upon cooling. Pluronic F-127's ability to gel at higher temperatures allows it to transform from an applied cold solution to

solid gel form when heated by body temperature. Upon its application onto skin or injection into a body cavity, the gel acts as a solid artificial barrier and a sustained release depot. (Escobar-Chavez et al. 2006)

This study aims to present another form of hydrogel scaffold into the wound healing process. As mentioned earlier self-assembling peptide hydrogels have been evolving and present great potential in different applications. Presently, most of the published data is based around employment of synthetic self-assembling peptides on wound healing (Kao et al. 2009; Meng et al. 2009; Schneider et al. 2008). Using a leucine zipper based hydrogel in wound healing on the basis of biomimetic principles presents a novel approach in this field. Drawing on principles of natural process, we can formulate tools that can produce more specific and accurate results. The potential for this hydrogel to be tailor made as required for various functions is possibly its greatest advantage as a wound healing scaffold.

F. Remaining Questions about Hydrogels and the Healing Process

The field of hydrogels is rapidly expanding, with improved functionality occurring at a rapid rate. The advent of biomimetic hydrogels that can be created with many motifs suggests new strategies for the application of hydrogels in wound healing. While chitosan and pluronic gels have been described to be useful in the treatment of wounds, little is known about how gels based upon leucine zipper motifs might affect wound healing. In this study the leucine zipper hydrogel will be compared to both chitosan and pluronic F-127. These gels are well established in the literature as either a scaffold promoting wound healing, a carrier of different mediators, or both. We hypothesize that a peptide self assembling leucine zipper gel, in comparison to pluronic F-127 and chitosan gels, improves

wound healing in diabetic mice. It provides a favorable structure for the incorporation of bioactive motifs along with soluble mediators to improve healing.

III. Material and Methods

A. Animals

Wound closure studies were performed on genetically modified female diabetic mice that are homozygous for the diabetes spontaneous mutation (*Lepr^{db}*; Jackson Labs). These mice become identifiably obese around 3 to 4 weeks of age; elevations in plasma insulin and blood sugar begin at 10 to 14 days and at four to eight weeks, respectively. The affected mice are generally polyphagic, polydipsic, and polyuric. In addition to these symptoms, the mice have impaired wound healing (Michaels et al. 2007). Mice, at 6 weeks of age, were purchased from Jackson Laboratory and allowed to acclimate in the animal facility for 1 week prior to starting experiments, in accordance to UIC ACUC recommendations.

B. Hydrogel preparations

Four types of hydrogels were fabricated for this study.

1. Pluronic F-127 gel:

The gel was prepared by mixing 30mg Pluronic F-127 powder in 120µl chilled phosphate buffer solution (PBS) buffer solution to produce a 25% concentration. This gel mixture was prepared at the time of the experiment and was continuously chilled to maintain liquid form prior to application.

2. Chitosan gel:

Protocol for this hydrogel preparation was provided by Dr. Anne George's laboratory, UIC College of Dentistry (Ravindran et al. 2010). The hydrogel was a blend of

collagen and chitosan with 1mg/ml of type I collagen and 1mg/ml chitosan in a growth medium with 1 X Hank's balanced salt solution and 5% (w/v) NaHCO3. Since collagen and chitosan are monomers in 0.1M acetic acid, the pH of the mixture was raised by the addition of 1M NaOH solution (10 μl) to aid polymerization of the monomeric mixture. The mixture was incubated at 37°C and 5% CO2.

3. Leucine zipper gel with or without a bioactive motif:

Fabrication protocols for leucine zipper gels were provided by Dr. Anne George's laboratory, UIC College of Dentistry. A published leucine zipper peptide of six heptad repeat amino acid sequence was used (A1: SGELENE VAQLERE VRSLEDE AAELEQK VSRLKNE IEDLKAE) (Petka et al. 1998). This A1 sequence was modified with a cysteine substitution (A2: CSGELENE VAQLERE VRSLEDE AAELEQK VSRLKNE IEDLKAE) in order to obtain the directional leucine zipper domain binding as well as to facilitate covalent binding for stronger stability. Two A2 sequences were separated by a spacer of two sets of 5 repeats of flexible peptides (C1: 5(AGAGAGPEG)). An extra flexible tail containing cysteine (C2: AGAGAGPEGC) was also incorporated to the end of the protein structure. The combined A2-C1-C1-A2-C2 peptides formed the leucine zipper-blank protein construct. Functional motifs were incorporated into the two C1 blocks (A2-C1-X-C1-A2-C2, X=RGDS). The final construct was then cloned into a pQE9 vector (QIAGEN, Sample and Assay Technologies) and the protein was expressed in *E.coli* and purified according to the manufacturer's protocol. The average protein yield was about 80mg/L.

Hydrogels were formed after dissolving the prepared protein structure at a concentration of 7% w/v in 100mM, pH 7.4 phosphate buffer. The self assembled hydrogel forms within 3 hours when incubated at 37°C. The hydrogel is then stored at 4°C.

C. Surgical Wounding Procedure

The study protocol was approved by the University of Illinois, Chicago, Animal Care Committee. Mice were anesthetized with ketamine/xylazine (100/5 mg/kg) by intraperitoneal injection. The injected amount ranged from 250µl to 500µl, with some mice responding at the minimum dosage and others needing an additional application. Depth of anesthesia was monitored by the animal's reaction to a foot or tail pinch; additional anesthetic was added as needed depending on the mouse's response.

For the purposes of this experiment 4 groups of n=6 mice were set up to evaluate wound closure and 4 groups of n=2 for histological examination. The wound closure experiment was completed in two stages; performed on half the group n=3 in each phase. Prior to commencing the wounding procedure, blood glucose readings for each mouse were determined using a standard glucometer. A small puncture wound was made in the tail area to draw blood for the glucometer strip. Following that, the dorsal surface of each mouse was shaven and disinfected with alcohol swabs. Two 8mm diameter full thickness circular excisional wounds were prepared on the dorsal skin with biopsy punch instruments. Excisional wounds were performed by folding the dorsal skin and pressing the biopsy punch through both sides; wounds were placed at least 10 mm apart.

Gel application was performed with a micropipette for the pluronic gel and a thin metal spatula for the remaining hydrogels. One wound was treated with approximately 100µl of one hydrogel and the other wound was left untreated as a control. Each hydrogel was applied in a fashion that established a uniform thickness covering the wound and extending 1-2mm beyond the edges. Following gel application, the wounds were covered with a tegaderm dressing.

D. Treatment Protocol

For the purpose of evaluating the effect of different hydrogels on healing, 4 groups of mice were set up to assess wound closure (n=6) and for histological examination (n=2);

The surgical procedure was completed in three different time frames as shown below:

TABLE I
PHASES OF WOUND TREATMENT PROTOCOL

GROUPS	Phase I	Phase II	Phase III
Group A: Pluronic gel	N=3	N=3	N=2
Group B: Chitosan gel	N=3	N=3	N=2
Group C: Leucine Zipper-Blank	N=3	N=3	N=2
Group D: Leucine Zipper-RGD			N=3, N=3 & N=2

The first two phases involved evaluation of wound closure with application of the pluronic, chitosan, and leucine zipper-blank group; each phase involved treating half of the group (n=3). This was done in order to evaluate initial outcomes and have the ability to make any technical adjustments (e.g. anesthetic dosages and wounding technique). Due to the availability of the leucine zipper-RGD at a later time, the surgical procedure for that group was performed separately (Phase 3); this group was also divided in half so that 3 animals were treated and evaluated separately. However, the treatments were completed within

the same time frame. At the start of the first phase blood glucose levels were determined and recorded for each mouse at various time points (Table II). This included one group planned for the second phase although this group had to be eliminated from the study due to wound complications. Following that blood glucose levels were only recorded on first day for the second and third phase groups. The third phase also involved four groups with n=2 for the purpose of histological sampling wounds from each treatment group at two time points. Mice were sacrificed on day 16 post wounding in accordance with the University of Illinois, Animal Care Committee protocols using a carbon dioxide chamber and cervical dislocation.

E. Wound observation and measurement procedures

The tegaderm was removed from all mice on the fourth day post op. Wound closure progression was recorded by photographic record. A Canon Powershot A650 IS (Cannon U.S.A. Inc.) camera was fixed on a stand at a reproducible position. The camera was set for macro photography with no flash setting to document wound closure; all wounds were photographed on day 0, 4, 6, 8, 12, 14, and 16 with a cm scale ruler aligned next to the wound. Ruler alignment next to wound served as reference for magnification adjustment. Wounds were measured on each of the designated days using Axiovision (Carl-Ziess Inc.). This software has the capability to digitize the wound surface area and produce a measurement based on the photo. Measurements were tabulated for each control and experimental wound in each group for each day using Microsoft Excel (Microsoft Corp.). A mean and standard deviation figure for each day was calculated and tabulated in GraphPad

Prism (Graphpad Software Inc.) for analysis. The data was translated into graphs and analyzed using two-way ANOVA with Bonferroni correction.

F. Histological sampling

One mouse from each group designated for histological examination was sacrificed on day 8 and 16. Samples of experimental and control wounds were collected from each mouse. Samples were paraffin embedded sectioned and stained using hematoxylin and eosin and Masson's trichrome stain.

IV. Results

The goal of this study was to determine the effect a self-assembling leucine zipper gel, as compared to other established hydrogels, on wound healing in diabetic mice. Leucine zipper gels provide a structure that allows both the incorporation of bioactive motifs along with soluble mediators to improve healing. Two leucine zipper gel variants were developed for application in this study. One gel did not contain any bioactive motifs and the other had RGD incorporated into it. Our study showed that both leucine zipper gels had no adverse effects on wound closure when compared to the controls. In addition, when compared to chitosan and pluronic F-127 hydrogels, they exhibited no difference on wound closure. Histological samples were collected on day 8 and day 16 from the control and experimental sites and stained using Hematoxylin and Eosin and Masson's Trichrome stain. Results from studies are summarized below.

A. Blood Glucose Levels

Determination of the blood glucose levels was done as a level of verification of the mice's overall status prior to commencing the study (Table II). According to the vendor (Jackson Laboratories) the mice should exhibit non-fasting blood glucose levels of higher than 250mg/dl. Our initial readings did not show such high readings. Upon inquiry, the vendor informed us that fluctuations of glucose levels have been noted and as the mice age the blood glucose levels will increase. For this reason we performed one experiment in which we delayed the procedure to allow all mice to reach blood glucose levels higher than 250mg/dl. This group of mice (identified as mice 4, 5, and 6 on Table II) was monitored,

and at 13 weeks of age most, but not all, these mice had levels higher than 250mg/dl. This group of 13 week old mice was then subjected to the wounding procedure. On day 6, it was found that the wounds of all of these mice were not healing properly, and instead the paired wounds had fused, producing one contiguous large wound. . The experiment was terminated at this point and the mice were euthanized. Having determined that older mice were unable to tolerate the experimental procedure, all remaining experiments were performed on mice at week 7 of age (Mice 1, 2, 3 and 7, 8, 9 on Table II). All data regarding the effect of hydrogels on healing is therefore derived from mice aged 7 weeks, a population with somewhat variable blood glucose levels.

TABLE II

BLOOD GLUCOSE LEVELS

	Week 6	Week 7	Week 9	Week 10	Week 13	
Group A						
Mouse 1	502	502	No Readings Obtained			
Mouse 2	191	258				
Mouse 3	158	301				
Mouse 4	204	159	150	340	406	terminated
Mouse 5	161	191	90	115	376	
Mouse 6	301	158	173	151	>500	
Mouse 7	>500					
Mouse 8*	>500					
Mouse 9*	>500					
Group B						
Mouse 1	375	375	No Readings Obtained			
Mouse 2*	240	404				
Mouse 3	100	320				
Mouse 4	320	120	192	209	378	terminated
Mouse 5	281	251	109	118	>500	
Mouse 6	238	188	171	181	>500	
Mouse 7	345					
Mouse 8	>500					
Mouse 9	>500					
Group C						
Mouse 1	120	243	No Readings Obtained			
Mouse 2	258	315				
Mouse 3	319	269				
Mouse 4	203	118	143	204	184	terminated
Mouse 5	189	141	174	128	168	
Mouse 6	258	319	124	143	191	
Mouse 7*	>500					
Mouse 8	470					
Mouse 9*	>500					
Group D						
Mouse 1	161					
Mouse 2	169					
Mouse 3	133					
Mouse 4	278					
Mouse 5	153					
Mouse 6	265					

(*) These mice were eliminated from the experiment due to death or fusion of the wounds, Group A: Mouse 5 died on day 4, Mouse 6 died on day 10; Group B: Mouse 2 wound fusion on day 6; Group C: Mouse 4 died on day 8, Mouse 6 died on day 4

B. Wound closure

The first part of this study focused on following the progress of wound closure over a period of 16 days (Fig 1). Wounds were photographed at each time point (day 0, 4, 6, 8, 10, 12, 14, 16) and measurements of the wounds' surface areas were completed on each mouse for the experimental and control sites throughout this period of time (Table 1). Multiple photographs were taken when needed to verify measurement and to avoid inaccuracies in measurement due to wound position in the photographic record. Mean surface areas were calculated for each group at each time point. In group B (chitosan gel) and C (leucine zipper-blank gel) two mice were eliminated from the experiment due to fusion of both wounds. Two mice died in group A (pluronic PF-127 gel) and one in group C (leucine zipper-blank gel).

Wound closure progression for both leucine zipper gels was similar when compared to each other (Fig 2). When a comparison of each leucine zipper gel was made to their controls, equivalent results were observed (Fig 3, 4). The two other chitosan and pluronic F-127 gels produced analogous kinetics of closure (Fig 5, 6). It was observed that for each group there was a decrease in wound size until closure at approximately similar rates for both control and experimental groups. This appeared to hold true when separately comparing the experimental sites and control groups to each other (Fig.7, 8).

These results were analyzed using two-way ANOVA with Bonferroni correction. No statistically significant difference was found between the experimental and controls, between the four gels, or between the two leucine zipper gels.

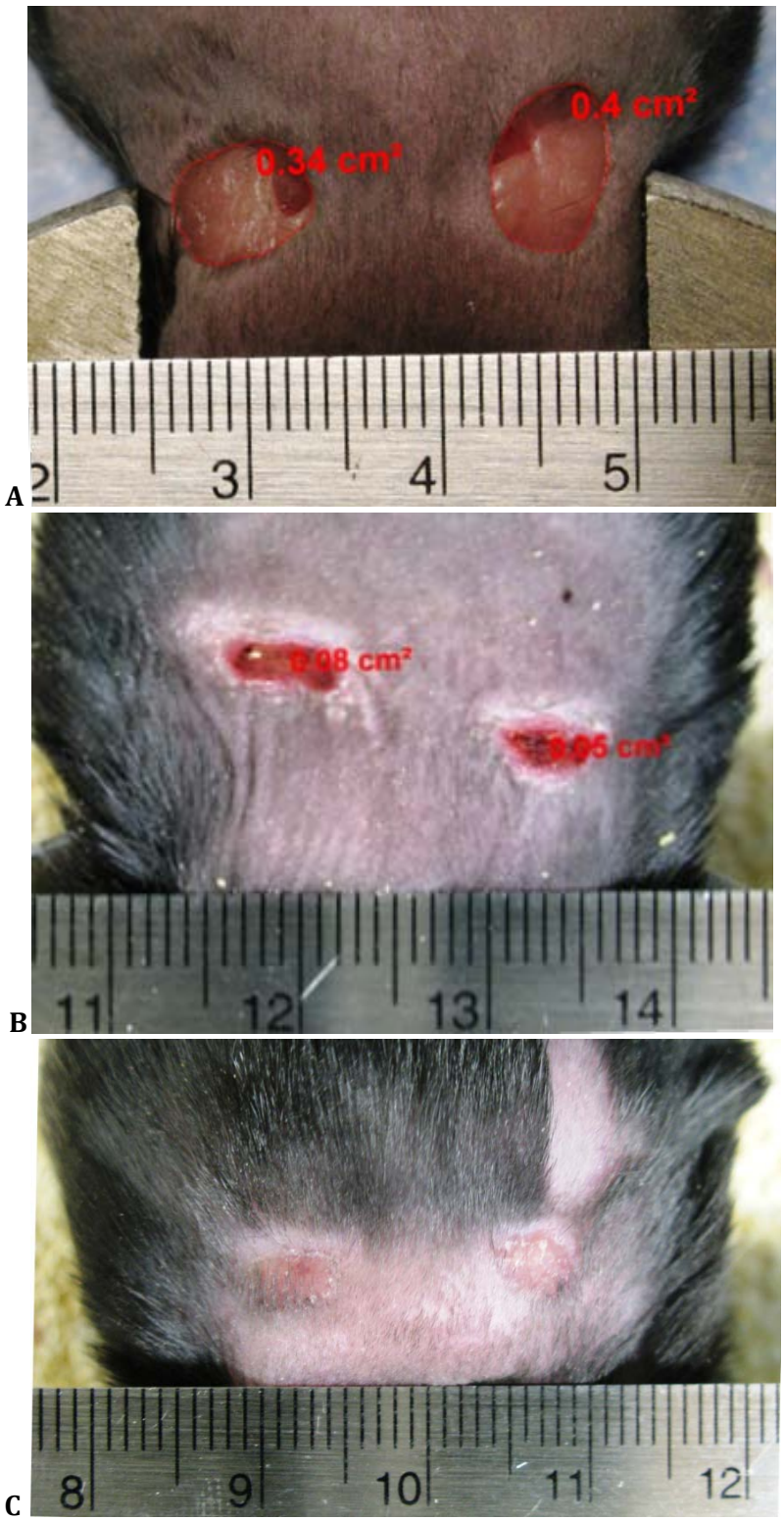

Figure 4. Photographic documentation and measurements of wounds. Examples of progression from first day of wounding until epithelial closure. (A) Day 0, (B) Day 8, (C) Day 16

TABLE III

WOUND CLOSURE MEASUREMENTS

(Surface areas in (cm^2) Means and Standard Deviations Included)

(Group A Pluronic F-17, Group B: Chitosan, Group C: Leucine zipper-Blank, Group D: Leucine zipper-RGD)

fused: both control and experimental wounds fused, dead: mouse died during observation period

	Time	Day 0		Day 4		Day 6		Day 8		Day 10		Day 12		Day 14		Day 16	
Group A		Control	Experiment	Control	Experiment	Control	Experiment	Control	Experiment	Control	Experiment	Control	Experiment	Control	Experiment	Control	Experiment
	Mouse 1	0.475	0.475	0.2905	0.2905	0.13	0.19	0.08	0.1	0.04	0.04	0	0	0	0	0	0
	Mouse 2	0.53	0.45	0.452	0.2905	0.32	0.21	0.27	0.24	0.263333333	0.24	0.265	0	0.26	0	0.28	0
	Mouse 3	0.435	0.42	0.355	0.452	0.1	0.17	0.09	0.08	0	0.04	0	0	0	0	0	0
	Mouse 4	0.41	0.41	0.19	0.15	0.23	0.19	0.08	0.053333333	0.05	0.04	0.065	0.01	0.065	0	0.06	0
	Mouse 5	0.44	0.53	dead	dead	dead	dead	dead	dead	dead	dead	dead	dead	dead	dead	dead	dead
	Mouse 6	0.52	0.72	0.43	0.38	0.47	0.46	0.575	0.71	dead	dead	dead	dead	dead	dead	dead	dead
	AVG	0.468333333	0.500833333	0.3435	0.3126	0.25	0.244	0.219	0.236666667	0.088333333	0.09	0.0825	0.0025	0.08125	0	0.085	0
	SD	0.048648398	0.115689959	0.106955598	0.113347585	0.150499169	0.121573023	0.214837148	0.274266699	0.118649812	0.1	0.125465799	0.005	0.123043014	0	0.133041347	0
Group B																	
	Mouse 1	0.53	0.52	0.452	0.323	0.276666667	0.126666667	0.19	0.14	0.11	0.075	0.08	0.02	0	0	0	0
	Mouse 2	0.7	0.58	0.5165	0.5485	fused	fused	fused	fused	fused	fused	fused	fused	fused	fused	fused	fused
	Mouse 3	0.63	0.53	0.516	0.387	0.36	0.34	0.18	0.2	0.06	0.09	0.05	0.05	0	0	0	0
	Mouse 4	0.32	0.41	0.3	0.31	0.095	0.07	0.08	0.06	0.035	0.03	0.01	0.01	0	0	0	0
	Mouse 5	0.28	0.34	0.24	0.315	0.146666667	0.183333333	0.1	0.06	0.07	0.06	0.03	0.01	0.02	0.02	0	0
	Mouse 6	0.46	0.5	0.4	0.37	0.18	0.19	0.18	0.12	0.085	0.05	0.05	0.02	0.01	0.01	0.01	0.01
	AVG	0.486666667	0.48	0.404083333	0.375583333	0.211666667	0.182	0.146	0.116	0.072	0.061	0.044	0.022	0.006	0.006	0.002	0.002
	SD	0.166813269	0.088317609	0.114221021	0.090330735	0.106144556	0.100791314	0.051768716	0.058991525	0.027973201	0.023021729	0.02607681	0.016431677	0.008944272	0.008944272	0.004472136	0.004472136
Group C																	
	Mouse 1	0.3	0.36	0.452	0.581	0.185	0.275	0.12	0.41	0.07	0.095	0.01	0.01	0	0	0	0
	Mouse 2	0.28	0.33	0.355	0.355	0.26	0.19	0.3	0.195	0.04	0.06	0.01	0.01	0	0	0	0
	Mouse 3	0.47	0.37	0.452	0.355	0.29	0.23	0.31	0.16	0.02	0	0	0	0	0	0	0
	Mouse 4	0.42	0.56	0.39	0.51	0.23	0.275	fused	fused	fused	fused	fused	fused	fused	fused	fused	fused
	Mouse 5	0.36	0.61	0.36	0.41	0.175	0.26	0.09	0.12	0.12	0.11	0.01	0.03	0.01	0.005	0	0
	Mouse 6	0.44	0.52	dead	dead	dead	dead	dead	dead	dead	dead	dead	dead	dead	dead	dead	dead
	AVG	0.378333333	0.458333333	0.4018	0.4422	0.228	0.246	0.205	0.22125	0.0625	0.06625	0.0075	0.0125	0.0025	0.00125	0	0
	SD	0.077567175	0.119233664	0.047740968	0.100133411	0.048810859	0.036297383	0.1161895	0.129510296	0.043493295	0.048883365	0.005	0.012583057	0.005	0.0025	0	0
Group D																	
	Mouse 1	0.54	0.546666667	0.455	0.33	0.223333333	0.28	0.215	0.2	0.025	0.04	0.02	0.035	0	0	0	0
	Mouse 2	0.346666667	0.42	0.51	0.37	0.24	0.26	0.22	0.15	0.04	0.03	0.01	0.01	0	0	0	0
	Mouse 3	0.48	0.505	0.55	0.355	0.275	0.22	0.19	0.17	0.065	0.03	0.01	0.02	0	0.003333333	0	0
	Mouse 4	0.5	0.61	0.47	0.56	0.18	0.31	0.17	0.16	0.055	0.095	0	0.01	0	0	0	0
	Mouse 5	0.365	0.565	0.375	0.375	0.16	0.21	0.083333333	0.096666667	0.04	0.02	0	0.015	0.01	0	0	0
	Mouse 6	0.475	0.475	0.22	0.315	0.14	0.14	0.1	0.135	0.01	0.096666667	0	0.06	0	0.01	0	0
	AVG	0.451111111	0.520277778	0.43	0.384166667	0.203055556	0.236666667	0.163055556	0.151944444	0.039166667	0.051944444	0.006666667	0.025	0.001666667	0.002222222	0	0
	SD	0.07748596	0.067949464	0.118448301	0.089186135	0.051601213	0.060221812	0.058389655	0.034775737	0.019853631	0.034583501	0.008164966	0.019493589	0.004082483	0.004036867	0	0

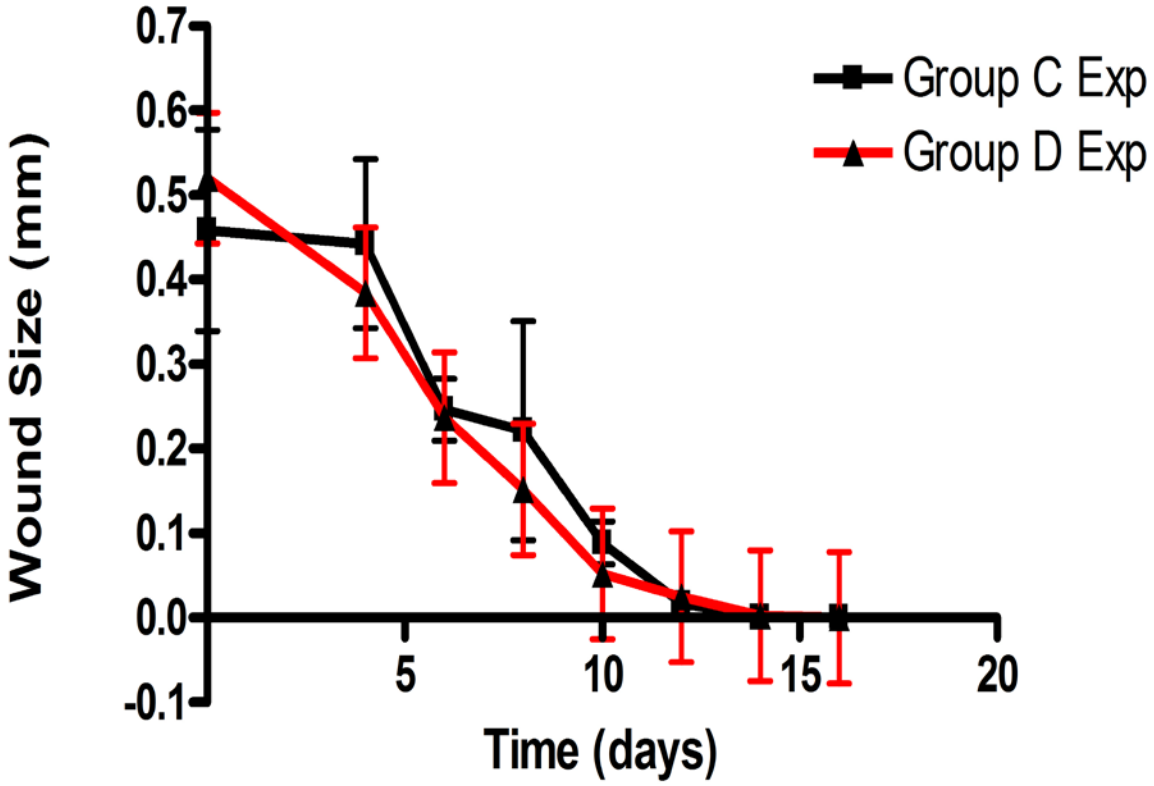

Figure 5. Comparison of wound closure for both leucine zipper gels
Group C is Leucine zipper-blank and Group D is leucine zipper-RGD. Lines represent wound closure during the observation time period. The data points represent mean values for each group (Grp. C, n=6; Grp. D, n=6 days 0-X, n=4 days X-16) and bars represent standard deviation values. Data, analyzed with two-way ANOVA, post-Bonferroni, showed no statistical significance between groups, $P < 0.05$.

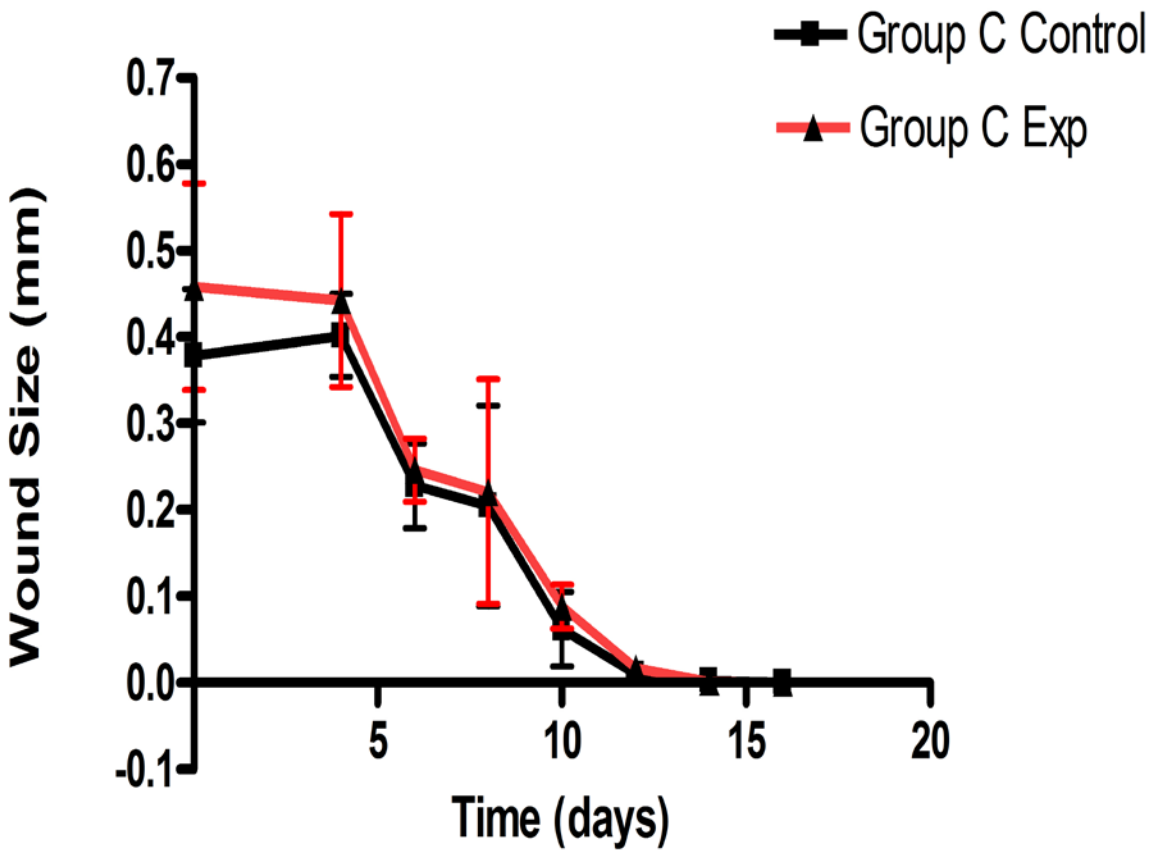

Figure 6. Effect of Leucine Zipper-Blank Gel on Wound Closure
Group C is the Leucine zipper-blank. Lines represent wound closure during the observation time period. Data points represent mean values (n=6 days 0-X, n=4 days X-16) and bars represent standard deviation values. Data, analyzed with two-way ANOVA, post-Bonferroni, showed no statistical significance between groups, $P < 0.05$.

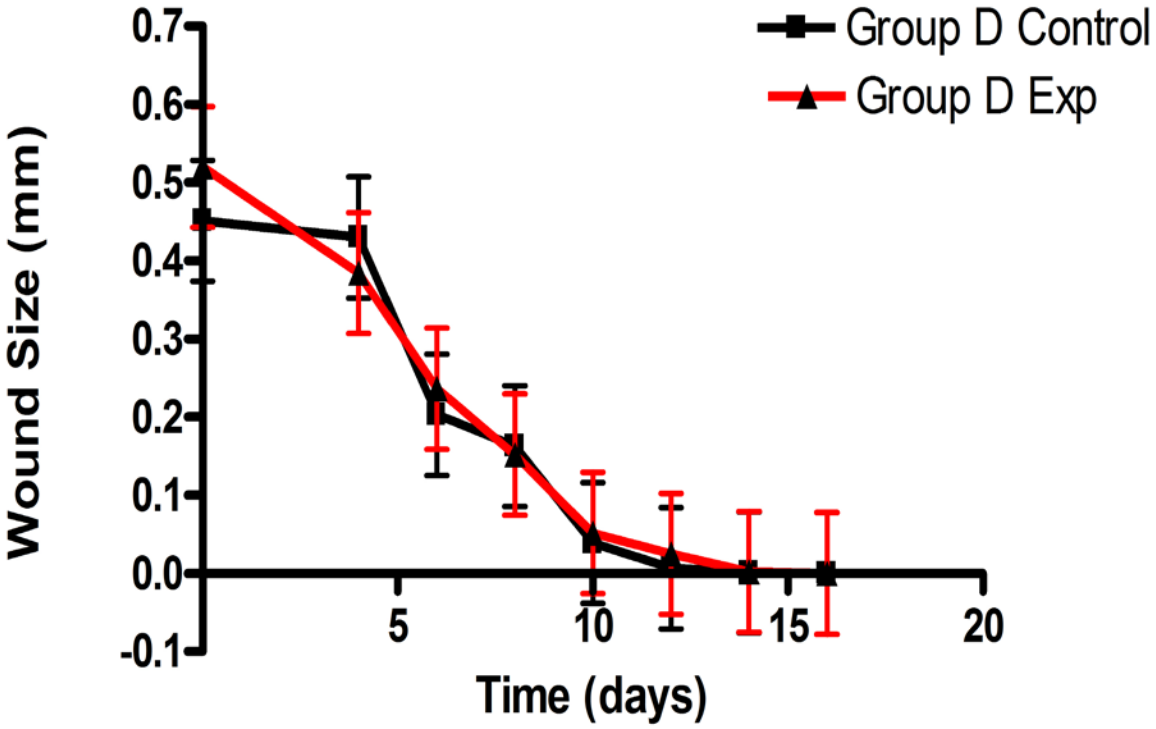

Figure 7. Effect of Leucine Zipper-RGD Gel on Wound Closure
Group D is Leucine zipper-RGD. Lines represent wound closure during the observation time periods. Data points represent mean values (n=6) and bars represent standard deviation values. Data, analyzed with two-way ANOVA, post-Bonferroni, showed no statistical significance between groups, $P < 0.05$

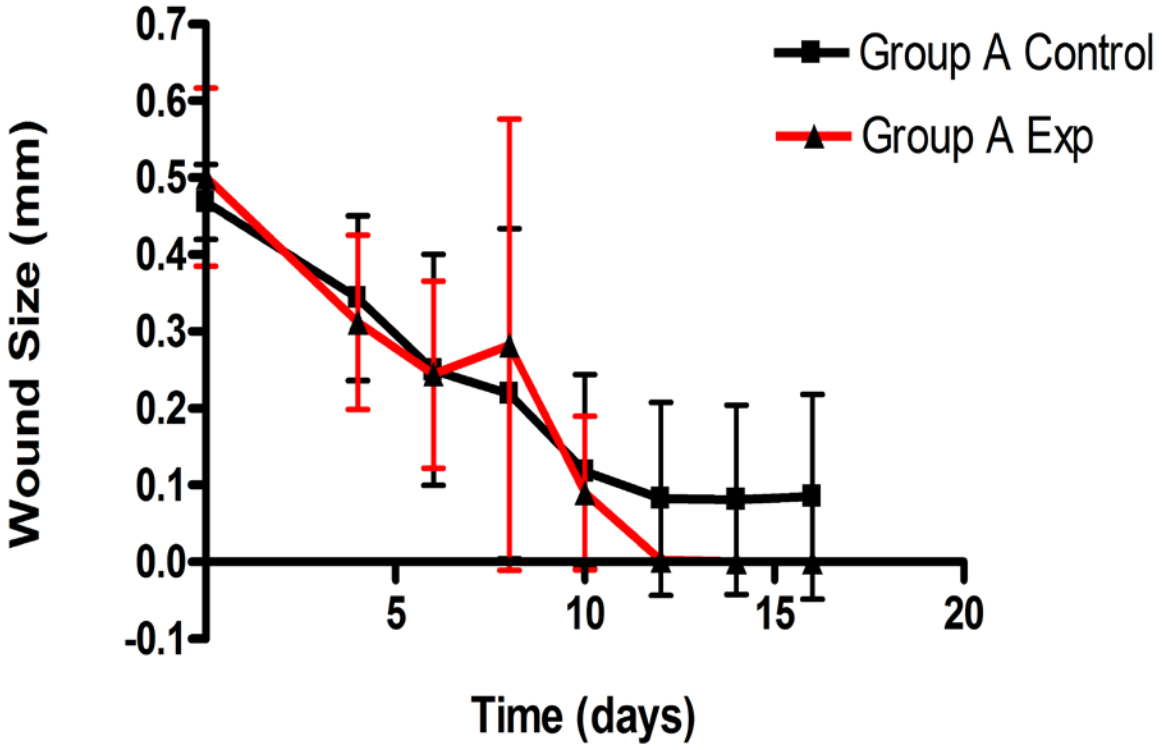

Figure 8. **Effect of Pluronic Gel F-127 on Wound Closure**
Group A is pluronic F-127. Lines represent wound closure during the observation time period. Data points represent mean values (n=6 days 0-X; n=4 days X-16) and bars represent standard deviation values. Data, analyzed with two-way ANOVA, post-Bonferroni, showed no statistical significance between groups, $P < 0.05$.

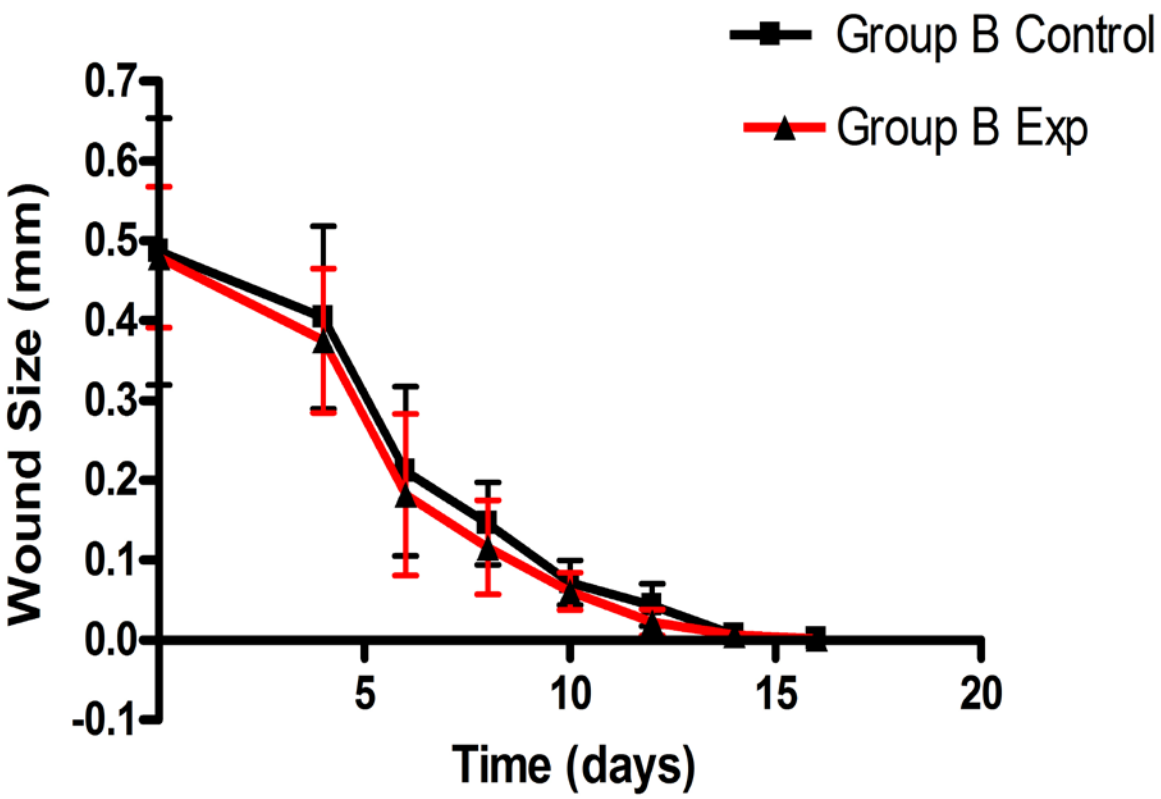

Figure 9. Effect of Chitosan Gel on Wound Closure
Group B is chitosan gel. Lines represent wound closure during the observation time period. Data points represent mean values (n=6 days 0-X, n=5 days X-16) and bars represent standard deviation values. Data, analyzed with two-way ANOVA, post-Bonferroni, showed no statistical significance between groups, $P < 0.05$

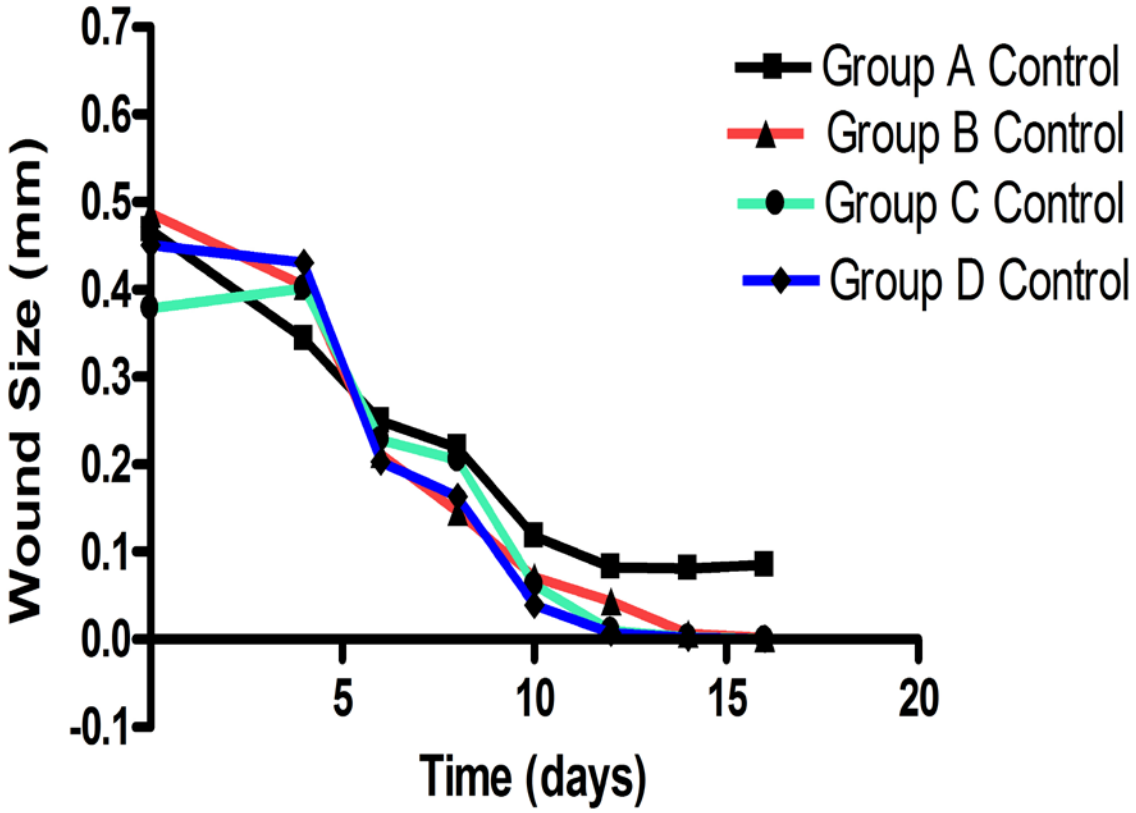

Figure 10. Comparison of wound closure in untreated control wounds
Untreated wounds of each group show similar wound closure progression. Group A is pluronic F-127, Group B is chitosan, Group C is leucine zipper-blank, and Group D is leucine zipper-RGD. Lines represent wound closure during the observation time period with recorded measurements at designated time points. The points represent mean values. Data, analyzed with two-way ANOVA, post-Bonferroni, showed no statistical significance between groups, $P < 0.05$

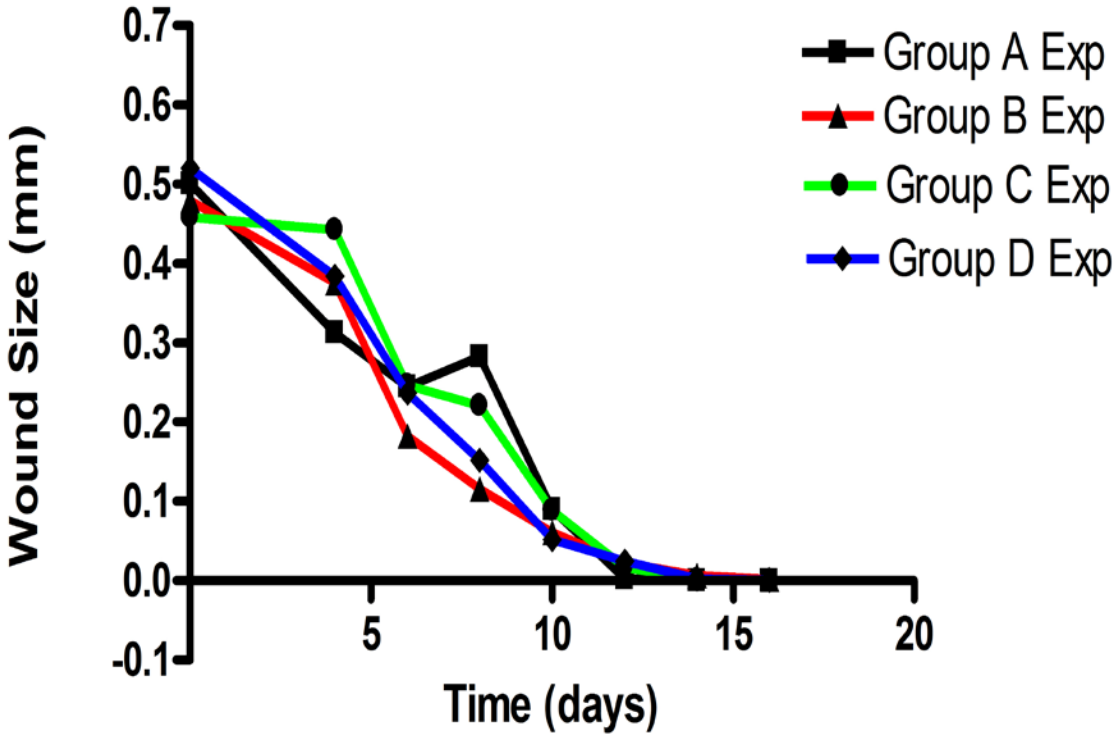

Figure 11. Comparison of wound closure of all treated experimental wounds
All treated wounds show wound closure progression that is similar in progression with each group showing bigger changes at different time points. Group A is pluronic F-127, Group B is chitosan, Group C is Leucine zipper-blank, and Group D is leucine zipper-RGD. Lines represent wound closure during the observation time period. The points represent mean values. Data, analyzed with two-way ANOVA, post-Bonferroni, showed no statistical significance between groups. $P < 0.05$

TABLE IV

NUMBER OF COMPLETE WOUND CLOSURES OUT OF TOTAL IN EACH GROUP

(Expressed in percentages)

Group A: Pluronic F-127, Group B: Chitosan, Group C: Leucine Zipper-blank, Group D: Leucine Zipper-RGD

Mice eliminated from experiment due to death or wound fusion

	Day 10		Day 12		Day 14		Day 16	
	Control	**Experiment**	**Control**	**Experiment**	**Control**	**Experiment**	**Control**	**Experiment**
Group A	0%	0% (0/4)	50% (2/4)	75% (3/4)	50% (2/4)	100%	50% (2/4)	100% (4/4)
	2 dead		2 dead		2 dead		2 dead	
Group B	0% (0/5)	0% (0/5)	0% (0/5)	(0/5)	60% (3/5)	60% (3/5)	80% (4/5)	80% (4/5)
	1 eliminated		1 eliminated		1 eliminated		1 eliminated	
Group C	0% (0/4)	25% (1/4)	25%(1/4)	25% (1/4)	75% (3/4)	75% (3/4)	100% (4/4)	100% (4/4)
	1 eliminated		1 eliminated		1 eliminated		1 eliminated	
Group D	0% (0/6)	0% (0/6)	50% (3/6)	0% (0/6)	83.3% (5/6)	66.7% (4/6)	100% (6/6)	100% (6/6)

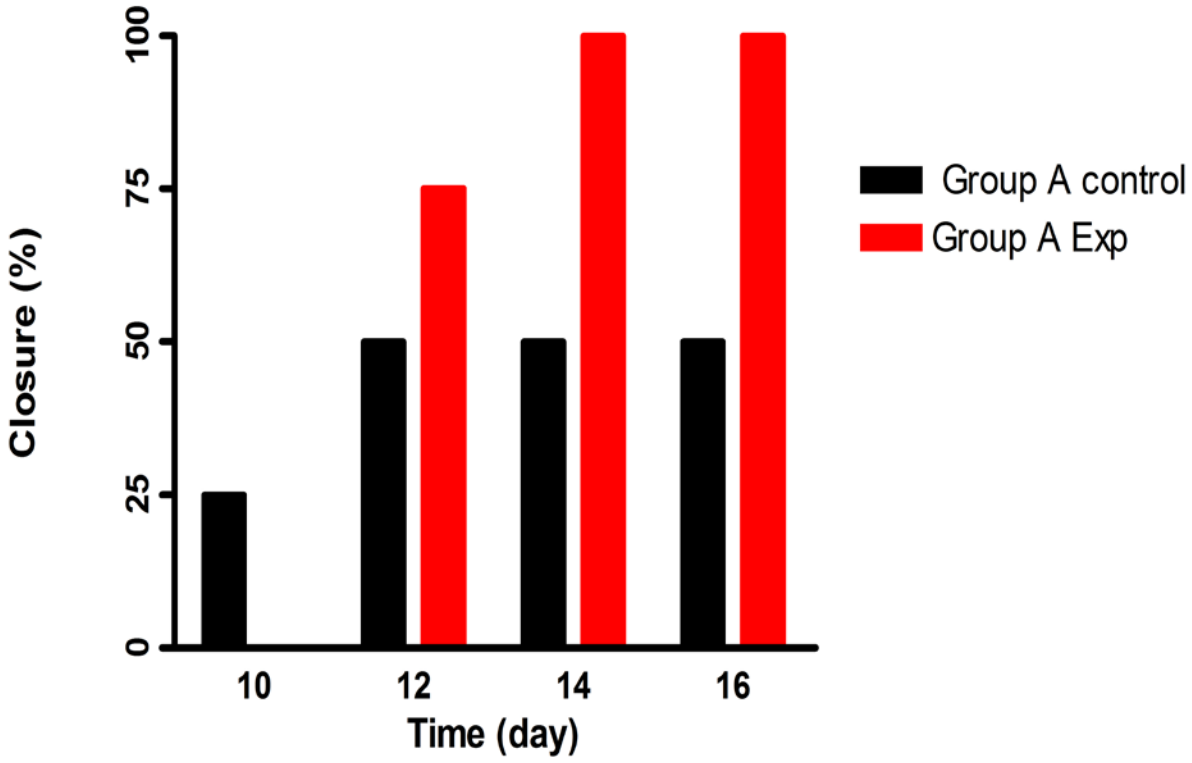

Figure 12. Wound closure percentage progression of Pluronic F-127 gel
Comparison of wound closure for both experimental and control groups showing higher percentage of closure in experimental groups. Wound closure first exhibited on day 10 and followed till day 16. Group A is Pluronic F-127 gel

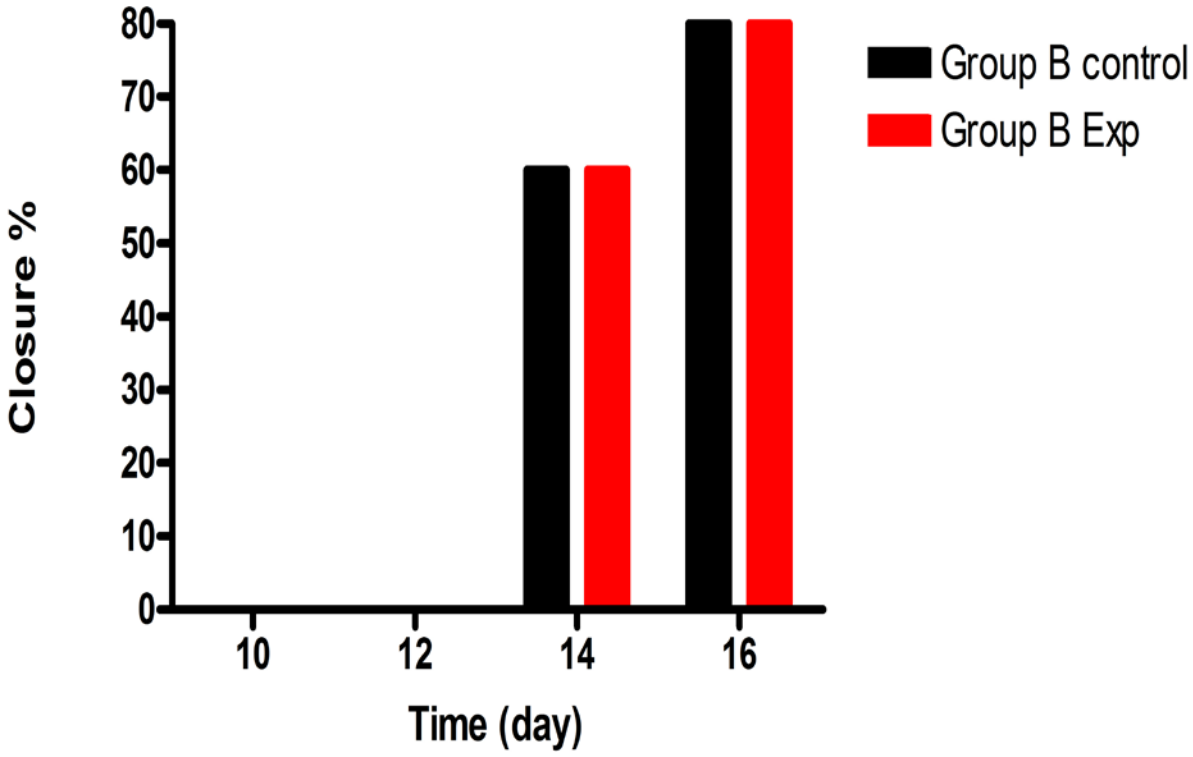

Figure 13. Wound closure percentage progression of Chitosan gel
Comparison of wound closure for both experimental and control groups showing identical percentage of closure for both groups. Wound closure first exhibited on day 10 and followed till day 16. Group B is Chitosan gel

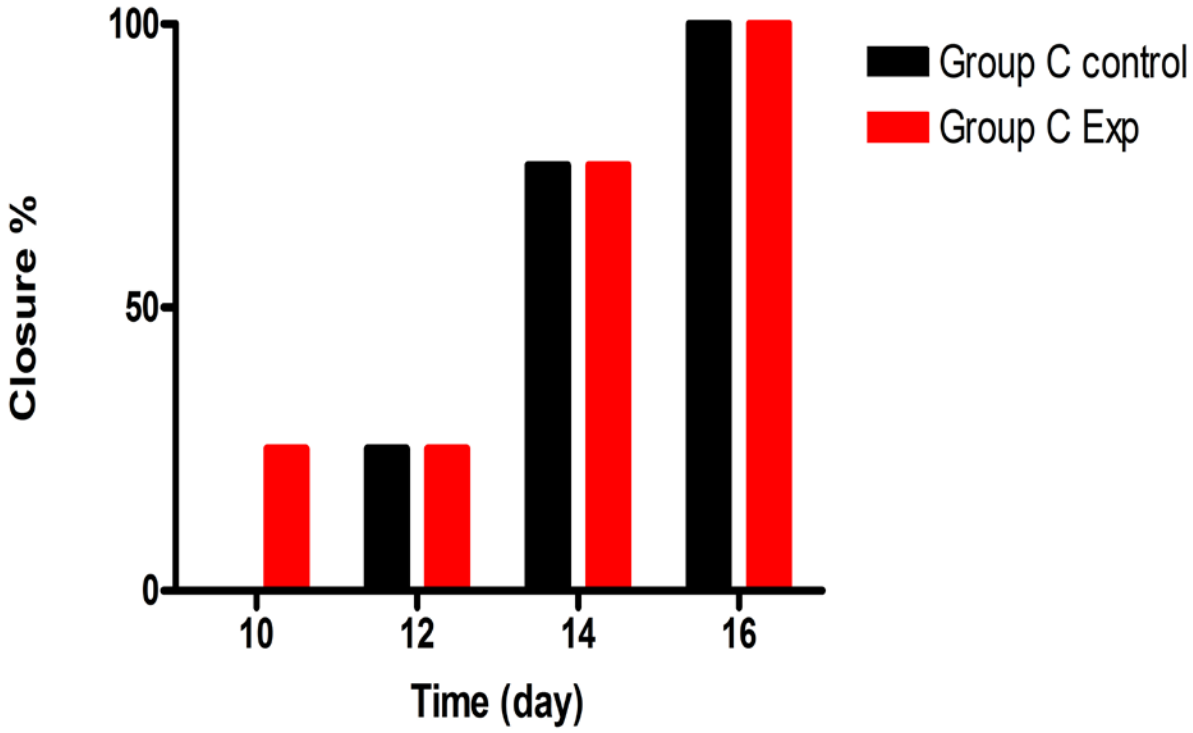

Figure 14. Wound closure percentage progression of Leucine Zipper-blank gel
Comparison of wound closure for both experimental and control groups showing earlier closure in experimental group followed by identical closure for both groups. Wound closure first exhibited on day 10 and followed till day 16. Group C is Leucine zipper-blank gel

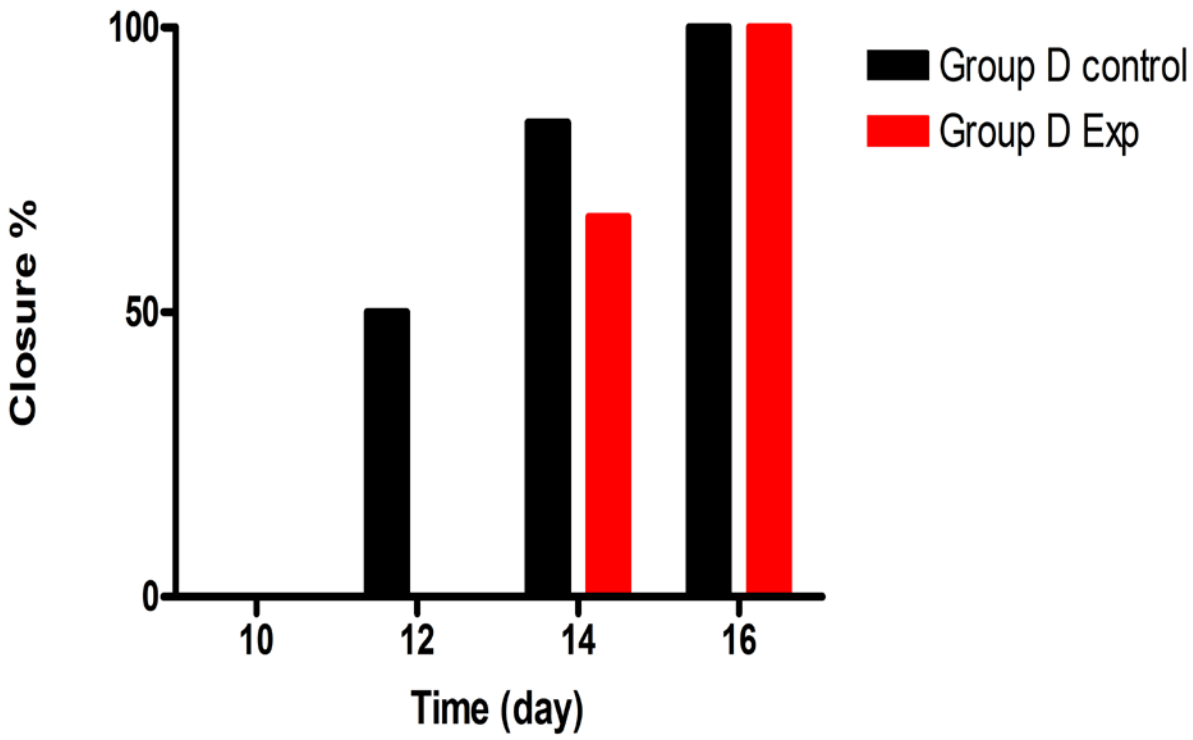

Figure 15. Wound closure percentage progression of Leucine Zipper- RGD
Comparison of wound closure for both experimental and control groups showing earlier closure in control group followed by increase in closure of experimental group and ending with identical results for both groups. Wound closure first exhibited on day 10 and followed till day 16. Group D is Leucine zipper-RGD gel

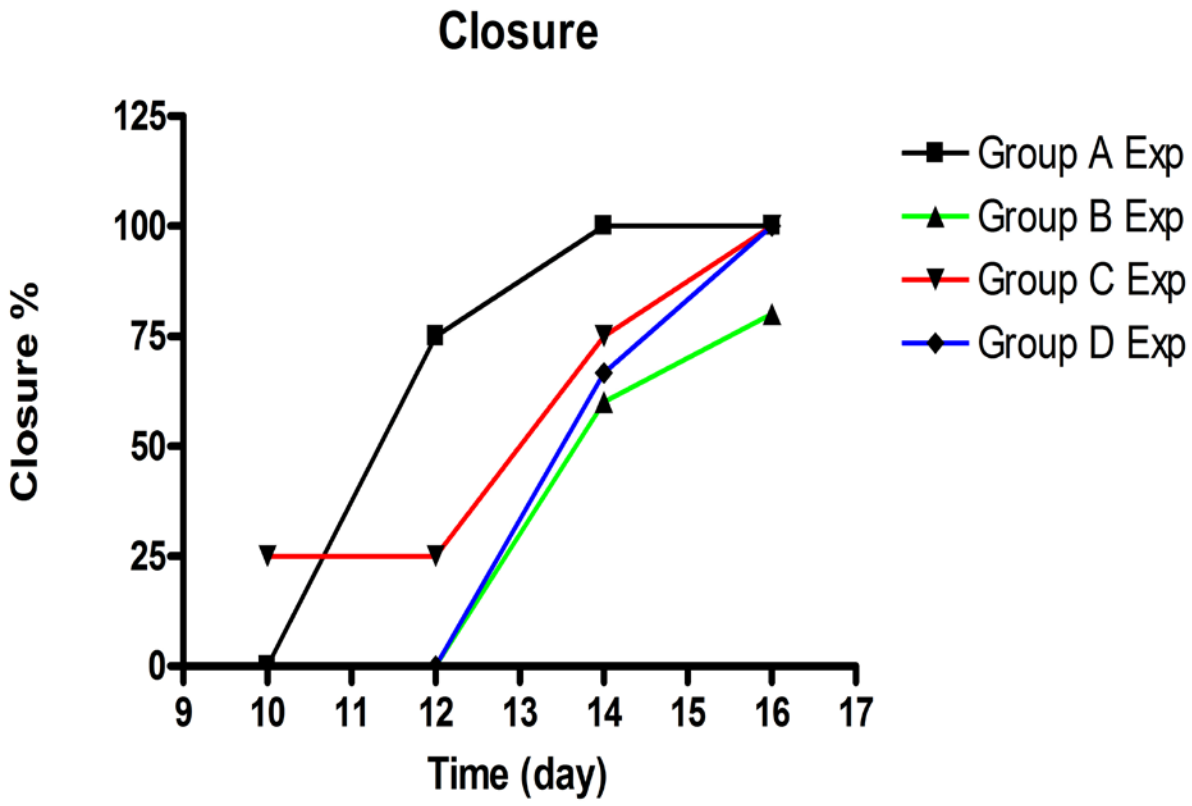

Figure 16. Progression of wound closure percentage for all experimental wounds
All treated wounds show wound closure progression that is starting at different time points with the highest percentage of closure exhibited on day 10 in the Leucine zipper-blank group. Group A is pluronic F-127, Group B is chitosan, Group C is Leucine zipper-blank, and Group D is leucine zipper-RGD. Lines represent wound closure during the observation time period. The points represent percentage closure

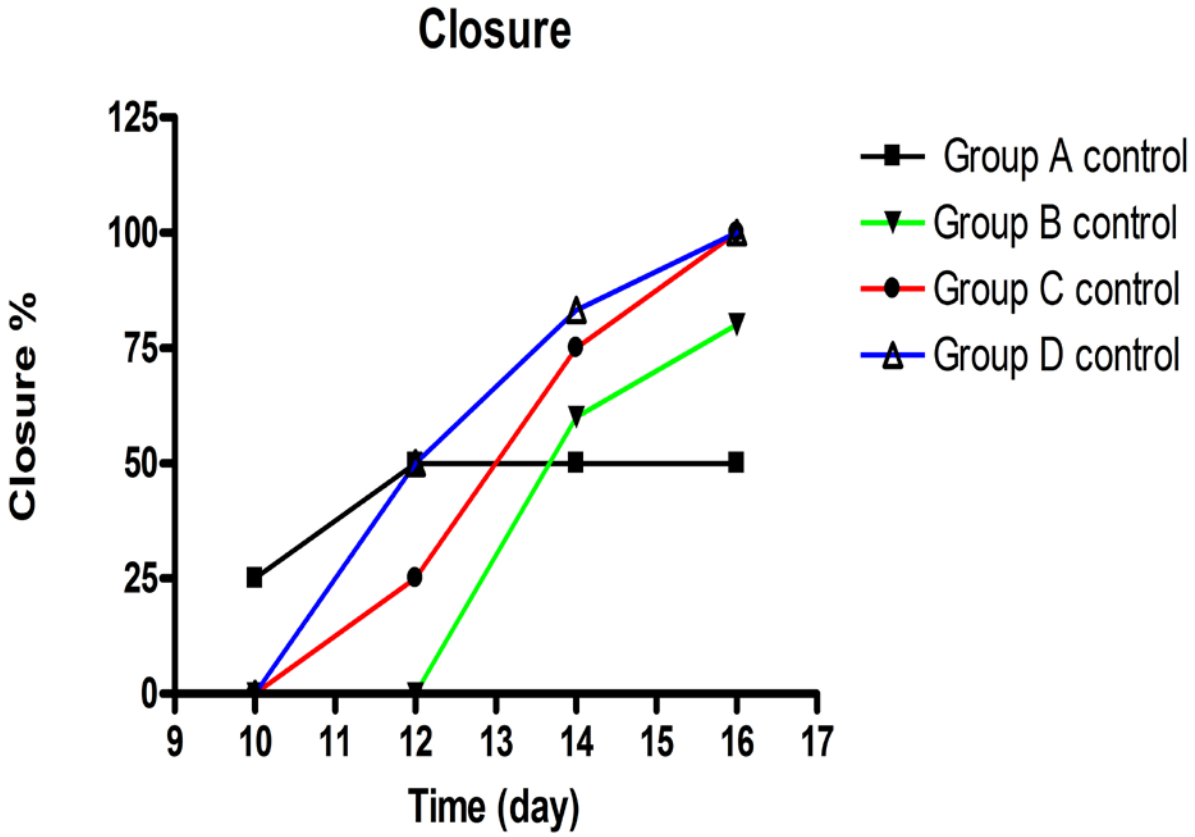

Figure 17. Progression of wound closure percentage for all control wounds
All treated wounds show wound closure progression that is starting at different time points with variability of percentage closure evident between the four groups. Group A is pluronic F-127, Group B is chitosan, Group C is Leucine zipper-blank, and Group D is leucine zipper-RGD. Lines represent wound closure during the observation time period. The points represent percentage closure

C. Histological Samples

Histological samples were collected and embedded in paraffin for hematoxylin and eosin and Masson's trichrome staining. The sections were evaluated for collagen density and architecture, overall cellularity, and epithelial closure and maturity. Examination of day 16 wounds revealed that no difference exists in the healing process between the leucine zipper gels and controls (Fig 11, 12). This was also characteristic of both the pluronic F-127 and chitosan gels (Fig 9, 10). Trichrome staining verified moderate collagen formation, which was clearly of a lesser density than adjacent normal tissue, present in the dermal beds of all samples. Most of the collagen fibers present in the wound bed were organized in a linear arrangement, indicative of lesser fiber maturity. Collagen fibers present in the wound bed displayed a looser arrangement than the adjacent normal tissue. Generally, the histological samples revealed no obvious difference in the levels or appearance of collagen

A moderate cellular population, composed mainly of a fibroblast and inflammatory cells mix, was observed. Additionally a modest number of blood vessels were present in the wound beds. All the samples displayed full closure but incomplete epithelial maturity. The overlying epithelium was generally hyperproliferative and keratinzation was apparent in all the samples.

On the whole, no noticeable differences in histologic architecture were detected among any of the groups.

Samples of day 8 wounds were also prepared and examined (data not shown). However, due to incomplete closure and immaturity of the dermal architecture exhibited by minute collagen presence at day 8, these samples were not highly informative.

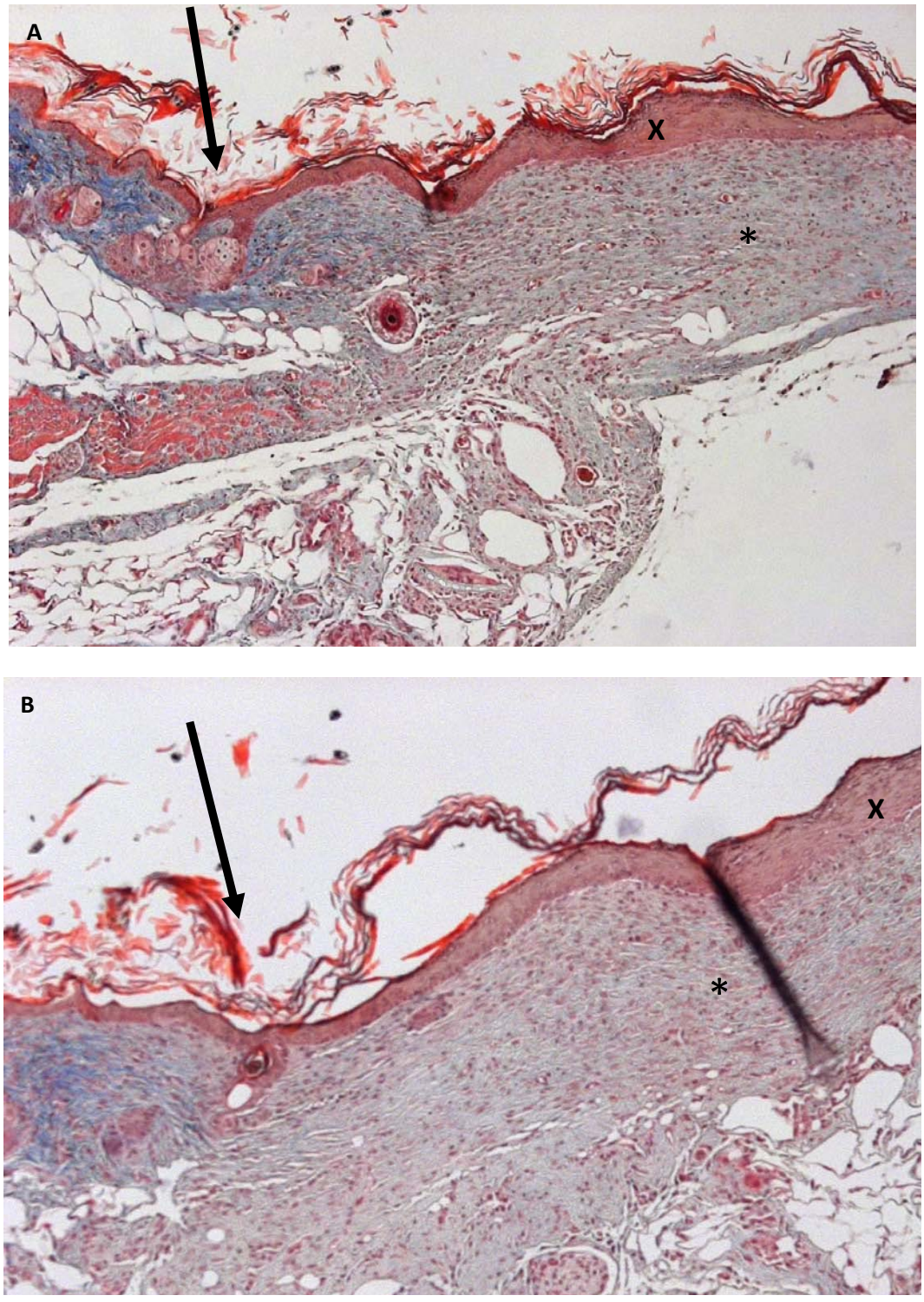

Figure 18. Group I Day 16
Representative sections from Group I, Pluronic F-127, gel stained with Masson's trichrome. (A) Untreated control group exhibits thickening of epithelial layers (X) with complete bridging and closure of wound bed. (B) Experimental group pluronic F-127 exhibits similar findings to control with characteristic thickening of epithelial layer (X) and complete closure of wound. Both sections showing evidence of immature collagen fibers (*) present in the wound bed**.** Solid Arrow: Wound edge

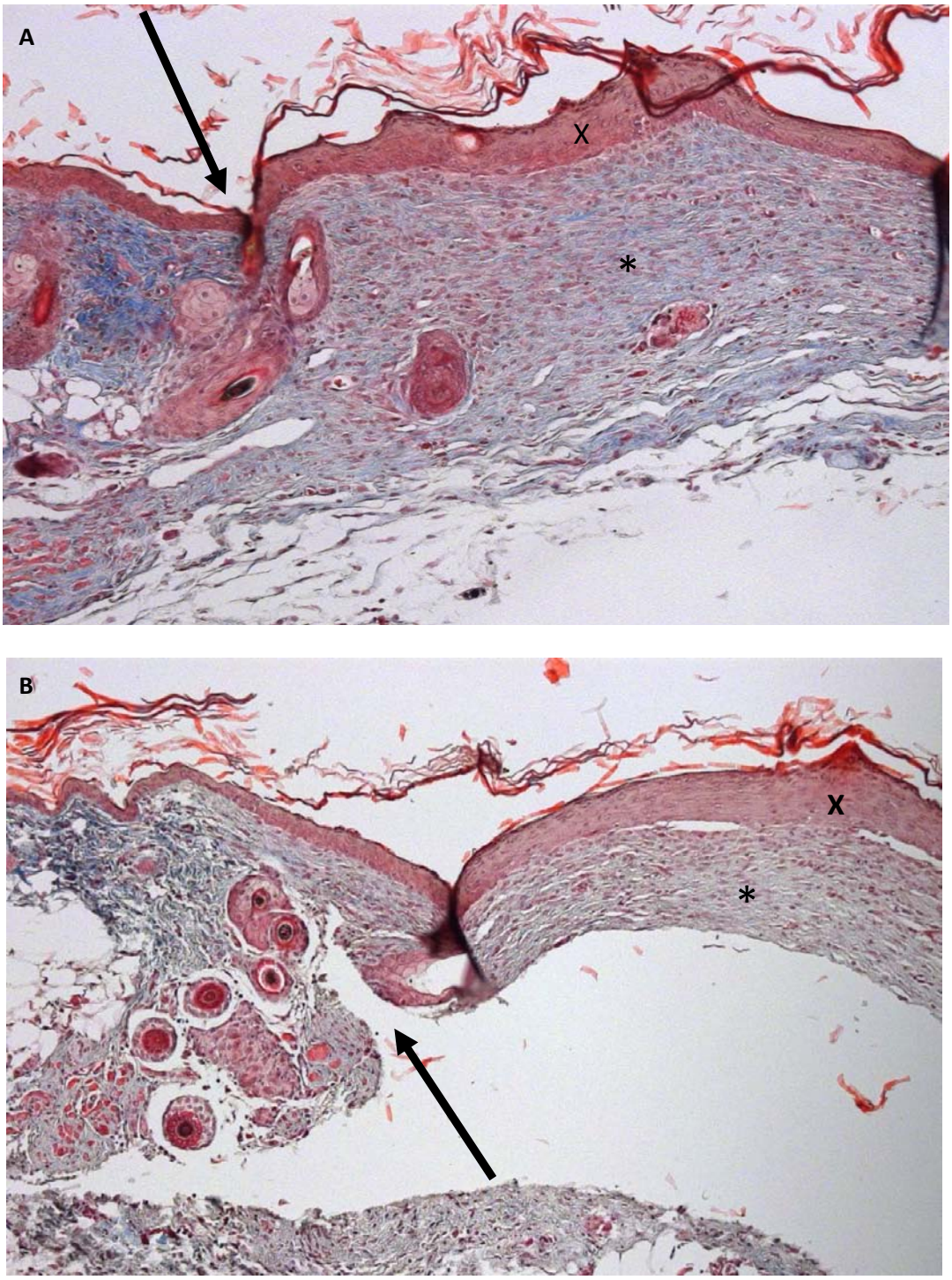

Figure 19. Group II Day 16

Representative sections from Group II, Chitosan gel, stained with Masson's trichrome. (A) Untreated control group exhibit characteristic thickening of epithelial layers (X) and complete closure of the wound bed. (B) Experimental group chitosan gel exhibits similar findings to control with thickening of epithelial layer (X) and complete closure of wound. Both sections showing presence of immature collagen fibers (*) present in the wound bed. Solid Arrow: Wound edge

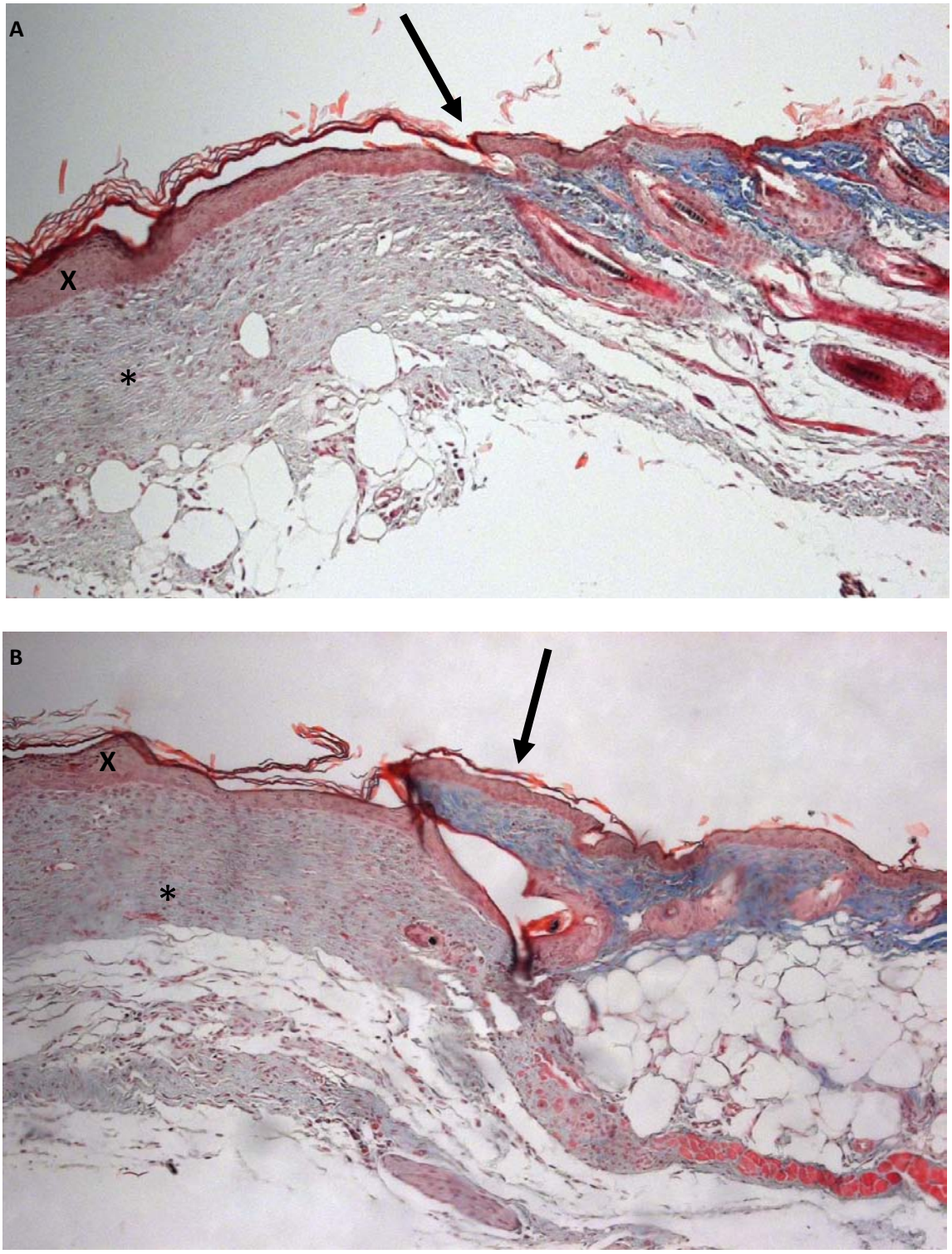

Figure 20. Group III Day 16

Representative sections from Group III, Leucine zipper-blank, stained with Masson's trichrome. (A) Untreated control group exhibits thickening of epithelial layers (X) and complete closure of the wound bed. (B) Experimental group leucine zipper-blank showing similar findings to control of thickened epithelial layer (X) and full closure of wound. Both sections have evidence of immature collagen fibers (*) formation in the wound bed. Solid Arrow: Wound edge

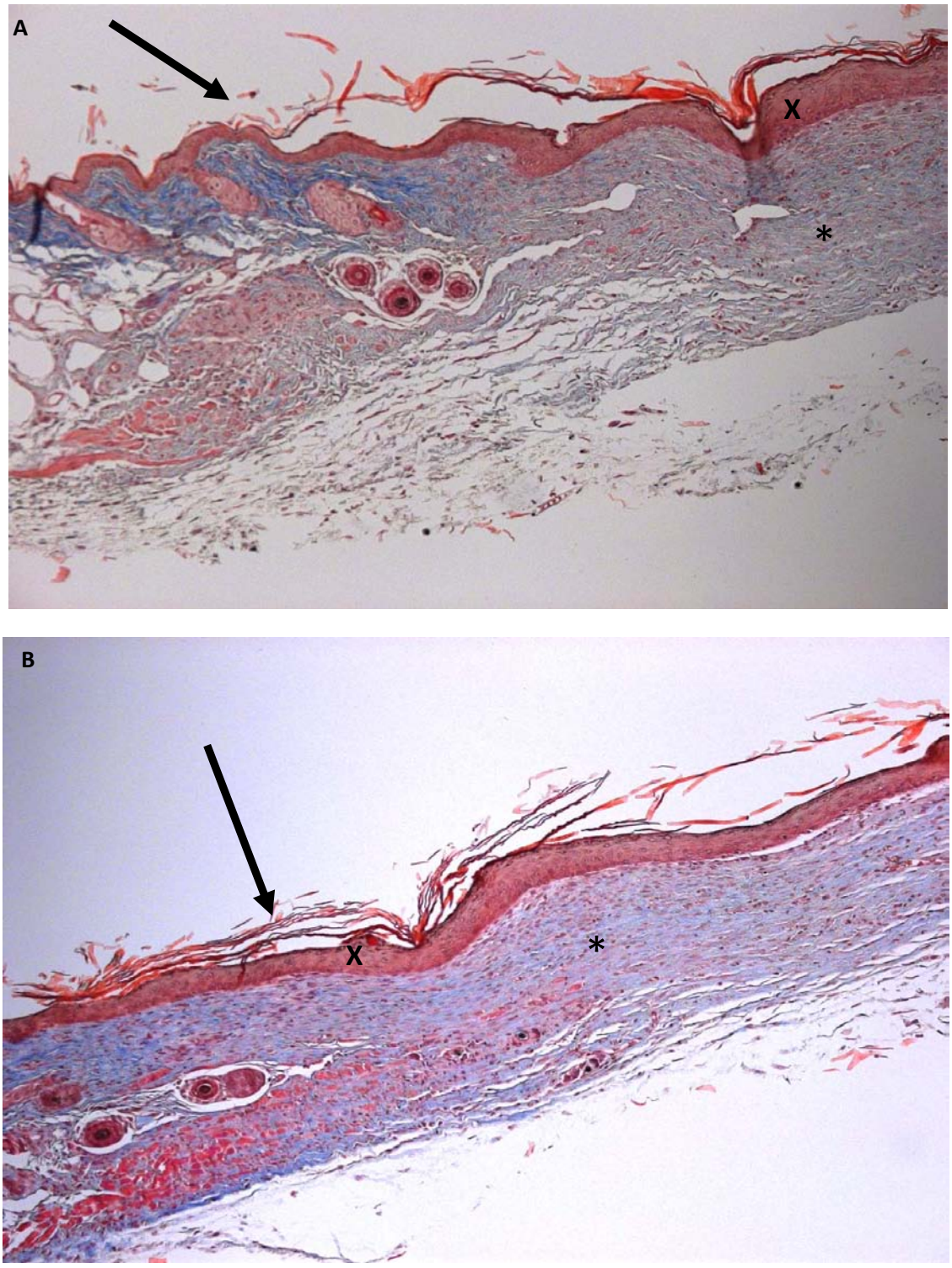

Figure 21. Group IV Day 16

Representative sections from Group IV, Leucine zipper-RGD, stained with Masson's trichrome. (A) Untreated control group displaying characteristic thickening of epithelial layers (X) and total closure of the wound bed. (B) Experimental group leucine zipper-RGD exhibits comparable findings to control with thickening of epithelial layer (X) and fully closed wound. Both sections illustrate presence of immature collagen fibers (*) present in the wound bed. Solid Arrow: Wound edge

V. Discussion

Wound healing is a clearly defined process that aims to achieve reconstitution of tissue structure and function; however it can be altered by various factors. Diabetes is known to be one of the major systemic diseases that has a profound effect in altering and retarding wound healing (Guo and DiPietro 2010). Delayed wound healing in diabetics is embodied mainly as the diabetic foot ulcer. Diabetic foot ulcers are estimated to occur in 15% of all patients with diabetes and precede 84% of all diabetes-related lower leg amputations (Brem and Tomic-Canic 2007). The Center for Disease Control estimated that more than 60% of non-traumatic lower-limb amputations occur in people with diabetes. In 2006, about 65,700 non-traumatic lower limb amputations were performed in people with diabetes.

A variety of treatment modalities have been explored to tackle the problem of chronic wounds and delayed healing. Currently strategies employing material scaffolds are being applied to engineer tissues and facilitate tissue healing (Drury and Mooney 2003). Scaffolds serve as a synthetic extra cellular matrix to organize cells into a three-dimensional architecture and to present stimuli, which direct the growth and formation of a desired tissue (Yang et al. 2001). The development of hydrogels provides an approach utilizing three dimensional polymer scaffolds combined with cellular components that can act as analogues to the natural extracellular matrices found in tissues (Lee and Mooney 2001).

This study employed a diabetic mouse model to compare the effect of four different hydrogels on wound healing. Application of all four gels on 8mm excisional wounds

provided an opportunity to observe the wound closure on a macro scale and follow changes over an adequate period of time. Within a period of 16 days all wounds were closed. The four gels applied showed no difference in rate of wound closure when compared to the controls and when compared to each other. Microscopically, a histological qualitative evaluation revealed that the four hydrogels showed no difference from the controls at selected time points. On day 8 the wound edges were identified with the characteristic widened epithelial and subepithelial layers. Epithelial closure was complete on day 16 with a maintained widened structure and evidence of immature connective tissue.

In the current set of experiments, we utilized genetically diabetic *db/db* mice as a model of impaired wound healing. Excisional wounds in these mice show a statistically significant delay in wound closure, decreased granulation tissue formation, decreased wound bed vascularity, and markedly diminished proliferation compared with controls and other diabetic mouse models (Michaels et al. 2007). Although the pathophysiology of hyperglycemia in *db/db* mice is different from that of human diabetes, the wound healing impairments present are analogous to those observed in human studies (Greenhalgh 2003). Since 90% of diabetic patients have type 2 diabetes, the *db/db* mouse strain, a model of type 2 diabetes, may be more representative of chronic wound healing in humans (Michaels et al. 2007).

Pluronic F-127 has been widely studied as a delivery conduit for various pharmacological agents and occasionally applied as a scaffold. Its administration in liquid state provides convenient application and intimate contact with tissue before forming a non-occlusive gel on warming and is able to act as a depot with increased contact time and

prolonged pharmacology action (Escobar-Chavez et al. 2006). Pluronic F-127 demonstrated favorable results as an accelerator of wound healing in third degree burn injuries created on pigs (Nalbandian et al. 1987). The authors concluded that the enhanced healing rate was due to an unknown mechanism, but suspected possible stimulation of EGF. However, pluronic F-127 hydrogel has shown favorable characteristics as a carrier of EGF (DiBiase and Rhodes 1991, 1996). It was noted that the exact flux of EGF required for therapeutic effect has yet to be determined and little data has been published on this subject since the studies documented previously (Escobar-Chavez et al. 2006). An intravenous application of pluronic F-127 produced a positive therapeutic effect on the inflammatory process in the burn area which may have helped to improve the healing (Paustian et al. 1993). An in vitro study, involving the oral environment, found that a very low dose of pluronic polyols (F-68 and F-127) can increase the attachment and early growth rate of human gingival fibroblasts which may lead to accelerated collagen synthesis and wound healing (Hokett et al. 2000). A recent review looked at the various applications of this gel, and included a handful of studies examining its effects on wound healing (Escobar-Chavez et al. 2006). This study supported the previous findings of favorable wound closure with the application of pluronic F-127 hydrogel both on a macro and microscopic level. However, it was not found that the gel accelerated wound closure or altered tissue structure in any way.

Chitosan is an extensively used hydrogel scaffold in wound healing studies. The results of this study did not show any positive or negative affect by the chitosan gel. There was no difference in wound closure between the chitosan and control site. Histologically, both sites showed similar characteristics. In two studies using genetically diabetic mice,

chitosan was shown to have no significant effect on accelerating wound closure in one and higher rate of closure in an another (Mizuno et al. 2003; Obara et al. 2005). However, in another application in an aged mouse model, chitosan accelerated wound closure in comparison to the controls (Park et al. 2009). These differences could be attributed to two different mouse models used or the chitosan preparations. Despite these difference, the application of chitosan and its derivatives as biomaterials for wound healing has been reported to possess characteristics favorable for promoting rapid dermal regeneration and accelerated wound healing (Shi et al. 2006). In order to exploit chitosan's favorable characteristics, it has been combined with a variety of materials such as growth factors, extracellular components, and antibacterial agents. Experiments incorporating bFGF with chitosan resulted in accelerated wound closure, induced wound contraction, granulation tissue, and capillary formation (Obara et al. 2005; Park et al. 2009). The versatility of chitosan provides great potential and a wide range of applications for its use in regenerative therapy. However, some disadvantages of chitosan such as unsatisfactory mechanical properties, severe shrinkage, and deformation after drying could discourage its application in tissue engineering (Zhong et al. 2010). Chitosan possesses a great deal of promise (Table 1), but as it was pointed out in a recent review, the majority of studies carried out are *in vitro* conditions and more *in vivo* studies need to be carried out (Shi et al. 2006).

TABLE V

APPLICATIONS AND BENEFITS OF CHITOSAN IN REGENERATIVE MEDICINE

(*Adapted from Shi 2006)*

Wound Healing
•Chemoattract macrophages and neutrophils to initiate the healing process •stimulate granulation tissue and re-epithelization •carry growth factors to accelerate the healing • limitation of scar formationand retraction •intrinsic antimicrobial activity and controlled releasing of exogenous antimicrobial agents to anti-infection
Tissue engineering
•Rapid degradation, non-toxic and biodegradable • easy to develop various forms •controlled release of cytokines, extracellular matrix and antibiotics •retain the normal cell morphology, promote the attachment, proliferation and viability of living tissues cells and even stem cells •modification with a variety of biomaterials • provide cell immunoisolation
Gene Therapy
•Non-toxic, biodegradable and biocompatible with high cationic charge potential •protect DNA from degradation by nucleases; • yield high transfection efficiency

The leucine zipper gels produced no significant difference in wound closure when compared to the controls or other gels. Histologically, both gels showed characteristics similar to the control sites on day 8 and day 16. Based on a review of the literature to date, the application of leucine zipper hydrogels in wound healing has not been evaluated previously. Nevertheless, wound healing studies employing other self assembling peptide hydrogels have been carried out (van der Veen et al. 2011). RADA16-I is a self assembling peptide hydrogel reported in those wound healing experimentations. It is a synthetic 16-amino acid sequence peptide consisting of arginine (R), aspartic acid (D), and alanine (A) with alternating hydrophobic and hydrophilic side chains (Yokoi et al. 2005). It possess the

RAD motif which is similar to the cell adhesion RGD motif and self polymerizes to form a scaffold of nanometric fibrous structures with β-sheet configuration (Kao et al. 2009).

RADA-16I hydrogel dressing for deep second degree burns in rats reduced edema, sped up the beginning and disappearance of eschar, and accelerated wound contraction when compared to chitosan and the control (Meng et al. 2009). An *in vitro* study using bioengineered human skin equivalents and the RADA-6I based hydrogel combined with EGF, found that those hydrogels accelerated the rate of wound coverage by 5 fold when compared to controls without scaffolds and by 3.5 fold when compared to the scaffold without the growth factor (Schneider et al. 2008). A more recent report exploring the use of a self assembling elastin-like peptide scaffold fused with keratinocyte growth factor on diabetic mice, showed a 2- and 3- fold enhancement in reepithelialization and granulation when compared to the controls (Koria et al. 2011).

In an investigation looking at periodontal ligament fibroblast, it was observed that the cells adhere to the RADA 16 scaffold differently from two other scaffolds containing cell adhesion motifs (Kumada and Zhang 2010). It has been suggested that cells adhere to the peptide scaffold via adhesion proteins which can be derived either from the serum of the added culture medium or produced by the cells (Sieminski et al. 2008). However, the most interesting conclusion made was that the interaction between integrins of the fibroblasts and cell adhesion motifs of the scaffold functionalized the fibroblasts to produce and secrete collagen I and III (Kumada and Zhang 2010).

Findings of this study, with respect to all three hydrogels, appear to be in line with results found in the literature. Application of the leucine zipper hydrogels did not produce any adverse effects on the wound or retard closure. These results suggest that hydrogels

composed of leucine zipper motifs have potential for development as a novel therapeutic agent. Although the hydrogels tested in our experiments did not improve healing, many more modifications are possible. An example of such modification, present in this study is the RGD motif. A wide array of modifications is possible at time of the hydrogel fabrication. This can include addition of other growth or connective tissue motifs within the protein construct, or the inclusion of soluble growth factors such as PDGF or BMP's, anti-inflammatory agents, and/or antibiotics at the time of fabrication.

Some caveats to the interpretation of the current studies are needed. First, the small sample population may mean that the results are under powered. One reason for the small number of subjects is that complications in the mice population and wound healing had an effect on the final number of wounds observed. Second, measurements of the wound surface area depend on exact determination of the wound edge. This could have been limited by hair growth as the study progressed and was only determined by one examiner. Therefore reproducibility of these observations with more examiners, a bigger mouse population and histological samples would certainly present greater validity to our conclusions.

The wide variation in the non-fasting blood glucose levels was observed in all groups. However, the complication that arose with eliminated groups could raise the concern of poor wound healing observed at an older age than 7 weeks. Even though the mice are genetically modified to be diabetic and their use as models for delayed wound healing has been well established, information about any correlation of the blood glucose levels with the overall health status and wound healing outcomes would be helpful.

Another important limitation to our studies is the relevance of studies performed in mice to the actual clinical situation. The murine model for human diabetes is not perfect, however given the similarities to human wound healing defects it is presently the best medium for testing therapeutic interventions which have the potential for clinical relevance (Michaels et al. 2007). Success of animal models has provided validity for progression to evaluate materials in clinical studies. Despite that success in human clinical studies has not always been possible. Undeniably, clinical studies are imperative to evaluate the complete effect of any proposed treatment in the human model.

Currently, there is limited information on self assembling peptide gel applications in wound healing and tissue regeneration. Most of the literature revolves around synthetic self assembling peptides such as RAD16. Recently, a novel *in vitro* application of a curcumin loaded β-hairpin (MAX8) hydrogel as an injectable agent for localized delivery showed promise (Altunbas et al. 2011). The leucine zipper coiled coil motif is one of the many naturally occurring and most widely studied motifs. The mutations required to engineer a responsive behavior have been mapped by the extensive literature that collectively described workings of the leucine zipper coiled coil in terms of aggregation number, strand orientation, strand specificity, and temperature stability (Banta et al. 2010). Our observations have shown that exploiting a self-assembling leucine zipper hydrogel, as a scaffold to facilitate wound healing, provides similar results as other hydrogel scaffolds. The positive observations made with other self assembling peptide motifs are encouraging, but following a biomimetic strategy could prove to be more effective in harmony with nature's guidelines. Further research and experimentation is

necessary in order to see full potential of the leucine zipper hydrogel application in the patient population.

CITED LITERATURE

Altunbas, A., et al. (2011), 'Encapsulation of curcumin in self-assembling peptide hydrogels as injectable drug delivery vehicles', *Biomaterials,* 32 (25), 5906-14.

Azad, A. K., et al. (2004), 'Chitosan membrane as a wound-healing dressing: characterization and clinical application', *J Biomed Mater Res B Appl Biomater,* 69 (2), 216-22.

Banta, S., Wheeldon, I. R., and Blenner, M. (2010), 'Protein engineering in the development of functional hydrogels', *Annual review of biomedical engineering,* 12, 167-86.

Baum, C. L. and Arpey, C. J. (2005), 'Normal cutaneous wound healing: clinical correlation with cellular and molecular events', *Dermatol Surg,* 31 (6), 674-86; discussion 86.

Blakytny, R. and Jude, E. (2006), 'The molecular biology of chronic wounds and delayed healing in diabetes', *Diabet Med,* 23 (6), 594-608.

Brem, H. and Tomic-Canic, M. (2007), 'Cellular and molecular basis of wound healing in diabetes', *J Clin Invest,* 117 (5), 1219-22.

Brem, H., et al. (2007), 'Molecular markers in patients with chronic wounds to guide surgical debridement', *Mol Med,* 13 (1-2), 30-9.

Bugge, T. H., et al. (1996), 'Loss of fibrinogen rescues mice from the pleiotropic effects of plasminogen deficiency', *Cell,* 87 (4), 709-19.

Burkhard, P., Stetefeld, J., and Strelkov, S. V. (2001), 'Coiled coils: a highly versatile protein folding motif', *Trends Cell Biol,* 11 (2), 82-8.

Cao, Y. and Li, H. (2008), 'Engineering tandem modular protein based reversible hydrogels', *Chem Commun (Camb),* (35), 4144-6.

Delamaire, M., et al. (1997), 'Impaired leucocyte functions in diabetic patients', *Diabet Med,* 14 (1), 29-34.

Desmouliere, A., et al. (1995), 'Apoptosis mediates the decrease in cellularity during the transition between granulation tissue and scar', *Am J Pathol,* 146 (1), 56-66.

DiBiase, M. D. and Rhodes, C. T. (1991), 'Investigations of epidermal growth factor in semisolid formulations', *Pharm Acta Helv,* 66 (5-6), 165-9.

DiBiase, M. D. and Rhodes, C. T. (1996), 'Formulation and Evaluation of Epidermal Growth Factor in Pluronic F-127 Gel', *Drug Development and Industrial Pharmacy,* 22 (8), 823-31.

Diegelmann, R. F. (2003), 'Excessive neutrophils characterize chronic pressure ulcers', *Wound Repair Regen,* 11 (6), 490-5.

Diegelmann, R. F. and Evans, M. C. (2004), 'Wound healing: an overview of acute, fibrotic and delayed healing', *Front Biosci,* 9, 283-9.

Drury, J. L. and Mooney, D. J. (2003), 'Hydrogels for tissue engineering: scaffold design variables and applications', *Biomaterials,* 24 (24), 4337-51.

Escobar-Chavez, J. J., et al. (2006), 'Applications of thermo-reversible pluronic F-127 gels in pharmaceutical formulations', *J Pharm Pharm Sci,* 9 (3), 339-58.

Ferguson, M. W., et al. (1996), 'The histology of diabetic foot ulcers', *Diabet Med,* 13 Suppl 1, S30-3.

Fischer, S. E., et al. (2007), 'Controlling cell adhesion to surfaces via associating bioactive triblock proteins', *Biomaterials,* 28 (22), 3325-37.

Gajjeraman, S., et al. (2008), 'Biological assemblies provide novel templates for the synthesis of hierarchical structures and facilitate cell adhesion', *Adv Funct Mater,* 18 (24), 3972-80.

George, A. and Ravindran, S. (2010), 'Protein Templates in Hard Tissue Engineering', *Nano Today,* 5 (4), 254-66.

Gosain, A. and DiPietro, L. A. (2004), 'Aging and wound healing', *World J Surg,* 28 (3), 321-6.

Gray, A. J., et al. (1993), 'A alpha and B beta chains of fibrinogen stimulate proliferation of human fibroblasts', *Journal of cell science,* 104 (Pt 2), 409-13.
Greenhalgh, D. G. (2003), 'Wound healing and diabetes mellitus', *Clin Plast Surg,* 30 (1), 37-45.
Guo, S. and DiPietro, L. A. (2010), 'Factors affecting wound healing', *J Dent Res,* 89 (3), 219-29.
Gurtner, G. C., et al. (2008), 'Wound repair and regeneration', *Nature,* 453 (7193), 314-21.
Hall, M. C., et al. (2003), 'The comparative role of activator protein 1 and Smad factors in the regulation of Timp-1 and MMP-1 gene expression by transforming growth factor-beta 1', *J Biol Chem,* 278 (12), 10304-13.
Hell, E. and Lawrence, J. C. (1979), 'The initiation of epidermal wound healing in cuts and burns', *British journal of experimental pathology,* 60 (2), 171-9.
Hokett, S. D., et al. (2000), 'Pluronic polyol effects on human gingival fibroblast attachment and growth', *J Periodontol,* 71 (5), 803-9.
Holmes, T. C. (2002), 'Novel peptide-based biomaterial scaffolds for tissue engineering', *Trends Biotechnol,* 20 (1), 16-21.
Ishihara, M., et al. (2001), 'Acceleration of wound contraction and healing with a photocrosslinkable chitosan hydrogel', *Wound Repair Regen,* 9 (6), 513-21.
Ishihara, M., et al. (2002), 'Photocrosslinkable chitosan as a dressing for wound occlusion and accelerator in healing process', *Biomaterials,* 23 (3), 833-40.
Ishihara, M., et al. (2003), 'Controlled release of fibroblast growth factors and heparin from photocrosslinked chitosan hydrogels and subsequent effect on in vivo vascularization', *J Biomed Mater Res A,* 64 (3), 551-9.
Jing, P., et al. (2008), 'Self-assembling peptide-polymer hydrogels designed from the coiled coil region of fibrin', *Biomacromolecules,* 9 (9), 2438-46.
Kao, B., Kadomatsu, K., and Hosaka, Y. (2009), 'Construction of synthetic dermis and skin based on a self-assembled peptide hydrogel scaffold', *Tissue Eng Part A,* 15 (9), 2385-96.
Kennedy, Scott B., et al. (2005), 'Controlled Structure in Artificial Protein Hydrogels', *Macromolecules,* 38 (17), 7470-75.
Kim, K. W., et al. (2003), 'Antimicrobial activity of native chitosan, degraded chitosan, and O-carboxymethylated chitosan', *J Food Prot,* 66 (8), 1495-8.
Koria, P., et al. (2011), 'Self-assembling elastin-like peptides growth factor chimeric nanoparticles for the treatment of chronic wounds', *Proc Natl Acad Sci U S A,* 108 (3), 1034-9.
Kumada, Y. and Zhang, S. (2010), 'Significant type I and type III collagen production from human periodontal ligament fibroblasts in 3D peptide scaffolds without extra growth factors', *PLoS One,* 5 (4), e10305.
Landschulz, W. H., Johnson, P. F., and McKnight, S. L. (1988), 'The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins', *Science,* 240 (4860), 1759-64.
Langer, R. and Vacanti, J. P. (1993), 'Tissue engineering', *Science,* 260 (5110), 920-6.
Lazarus, G. S., et al. (1994), 'Definitions and Guidelines for Assessment of Wounds and Evaluation of Healing', *Archives of Dermatology,* 130 (4), 489-93.
Lee, K. Y. and Mooney, D. J. (2001), 'Hydrogels for tissue engineering', *Chem Rev,* 101 (7), 1869-79.
Li, J., Chen, J., and Kirsner, R. (2007), 'Pathophysiology of acute wound healing', *Clin Dermatol,* 25 (1), 9-18.
Liu, B., Lewis, A. K., and Shen, W. (2009), 'Physical hydrogels photo-cross-linked from self-assembled macromers for potential use in tissue engineering', *Biomacromolecules,* 10 (12), 3182-7.
Martin, P. (1997), 'Wound healing--aiming for perfect skin regeneration', *Science,* 276 (5309), 75-81.
Meng, H., et al. (2009), 'The effect of a self-assembling peptide nanofiber scaffold (peptide) when used as a wound dressing for the treatment of deep second degree burns in rats', *J Biomed Mater Res B Appl Biomater,* 89 (2), 379-91.

Menke, N. B., et al. (2007), 'Impaired wound healing', *Clin Dermatol,* 25 (1), 19-25.
Mi, L., et al. (2006), 'Self-assembling protein hydrogels with modular integrin binding domains', *Biomacromolecules,* 7 (1), 38-47.
Michaels, J. th, et al. (2007), 'db/db mice exhibit severe wound-healing impairments compared with other murine diabetic strains in a silicone-splinted excisional wound model', *Wound Repair Regen,* 15 (5), 665-70.
Mirza, R., DiPietro, L. A., and Koh, T. J. (2009), 'Selective and specific macrophage ablation is detrimental to wound healing in mice', *Am J Pathol,* 175 (6), 2454-62.
Mizuno, K., et al. (2003), 'Effect of chitosan film containing basic fibroblast growth factor on wound healing in genetically diabetic mice', *J Biomed Mater Res A,* 64 (1), 177-81.
Mori, R., Shaw, T. J., and Martin, P. (2008), 'Molecular mechanisms linking wound inflammation and fibrosis: knockdown of osteopontin leads to rapid repair and reduced scarring', *J Exp Med,* 205 (1), 43-51.
Nalbandian, R. M., et al. (1987), 'Pluronic F-127 gel preparation as an artificial skin in the treatment of third-degree burns in pigs', *J Biomed Mater Res,* 21 (9), 1135-48.
NationalDiabetesInformationClearinghouse(NDIC) (2011), 'National Diabetes Statistics 2011 fact sheet', (http://diabetes.niddk.nih.gov/dm/pubs/statistics/index.aspx) U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES.
Nerem, R. M. (1991), 'Cellular engineering', *Ann Biomed Eng,* 19 (5), 529-45.
Nicodemus, G. D. and Bryant, S. J. (2008), 'Cell encapsulation in biodegradable hydrogels for tissue engineering applications', *Tissue Eng Part B Rev,* 14 (2), 149-65.
Nissen, N. N., et al. (1996), 'Basic fibroblast growth factor mediates angiogenic activity in early surgical wounds', *Surgery,* 119 (4), 457-65.
Nissen, N. N., et al. (1998), 'Vascular endothelial growth factor mediates angiogenic activity during the proliferative phase of wound healing', *Am J Pathol,* 152 (6), 1445-52.
Noli, C. and Miolo, A. (2001), 'The mast cell in wound healing', *Veterinary dermatology,* 12 (6), 303-13.
Nwomeh, B. C., Yager, D. R., and Cohen, I. K. (1998), 'Physiology of the chronic wound', *Clin Plast Surg,* 25 (3), 341-56.
Nwomeh, B. C., et al. (1999), 'MMP-8 is the predominant collagenase in healing wounds and nonhealing ulcers', *J Surg Res,* 81 (2), 189-95.
Obara, K., et al. (2005), 'Acceleration of wound healing in healing-impaired db/db mice with a photocrosslinkable chitosan hydrogel containing fibroblast growth factor-2', *Wound Repair Regen,* 13 (4), 390-7.
Park, C. J., et al. (2009), 'Accelerated wound closure of pressure ulcers in aged mice by chitosan scaffolds with and without bFGF', *Acta Biomater,* 5 (6), 1926-36.
Paustian, P. W., et al. (1993), 'Intravenous Pluronic F-127 in early burn wound treatment in rats', *Burns,* 19 (3), 187-91.
Peppas, N., et al. (2006), 'Hydrogels in Biology and Medicine: From Molecular Principles to Bionanotechnology', *Advanced Materials,* 18, 1345–60. .
Petka, W. A., et al. (1998), 'Reversible hydrogels from self-assembling artificial proteins', *Science,* 281 (5375), 389-92.
Pilcher, B. K., et al. (1997), 'The activity of collagenase-1 is required for keratinocyte migration on a type I collagen matrix', *J Cell Biol,* 137 (6), 1445-57.
Ravindran, S., Song, Y., and George, A. (2010), 'Development of three-dimensional biomimetic scaffold to study epithelial-mesenchymal interactions', *Tissue Eng Part A,* 16 (1), 327-42.
Roberts, A. B. and Sporn, M. B. (1993), 'Physiological actions and clinical applications of transforming growth factor-beta (TGF-beta)', *Growth Factors,* 8 (1), 1-9.

Ryadnov, M. G., Papapostolou, D., and Woolfson, D. N. (2008), 'The leucine zipper as a building block for self-assembled protein fibers', *Methods Mol Biol,* 474, 35-51.

Schmolka, I. R. (1972), 'Artificial skin. I. Preparation and properties of pluronic F-127 gels for treatment of burns', *J Biomed Mater Res,* 6 (6), 571-82.

Schneider, A., Garlick, J. A., and Egles, C. (2008), 'Self-assembling peptide nanofiber scaffolds accelerate wound healing', *PLoS One,* 3 (1), e1410.

Shen, Wei, Kornfield, Julia A., and Tirrell, David A. (2007), 'Dynamic Properties of Artificial Protein Hydrogels Assembled through Aggregation of Leucine Zipper Peptide Domains', *Macromolecules,* 40 (3), 689-92.

Shi, C., et al. (2006), 'Therapeutic potential of chitosan and its derivatives in regenerative medicine', *J Surg Res,* 133 (2), 185-92.

Sieminski, A. L., et al. (2008), 'Primary sequence of ionic self-assembling peptide gels affects endothelial cell adhesion and capillary morphogenesis', *J Biomed Mater Res A,* 87 (2), 494-504.

Singer, A. J. and Clark, R. A. (1999), 'Cutaneous wound healing', *N Engl J Med,* 341 (10), 738-46.

Stadelmann, W. K., Digenis, A. G., and Tobin, G. R. (1998), 'Physiology and healing dynamics of chronic cutaneous wounds', *Am J Surg,* 176 (2A Suppl), 26S-38S.

Stevens, M. M., et al. (2005), 'Molecular level investigations of the inter- and intramolecular interactions of pH-responsive artificial triblock proteins', *Biomacromolecules,* 6 (3), 1266-71.

Szpaderska, A. M., Zuckerman, J. D., and DiPietro, L. A. (2003), 'Differential injury responses in oral mucosal and cutaneous wounds', *J Dent Res,* 82 (8), 621-6.

van der Veen, V. C., et al. (2011), 'New dermal substitutes', *Wound Repair Regen,* 19 Suppl 1, s59-65.

Veyries, M. L., et al. (2000), 'Control of staphylococcal adhesion to polymethylmethacrylate and enhancement of susceptibility to antibiotics by poloxamer 407', *Antimicrob Agents Chemother,* 44 (4), 1093-6.

Wang, C., Stewart, R. J., and Kopecek, J. (1999), 'Hybrid hydrogels assembled from synthetic polymers and coiled-coil protein domains', *Nature,* 397 (6718), 417-20.

WorldHealthOrganization (August 2011), 'Diabetes Fact Sheet', (http://www.who.int/mediacentre/factsheets/fs312/en/index.html) World Health Organization.

Xu, J. and Clark, R. A. (1996), 'Extracellular matrix alters PDGF regulation of fibroblast integrins', *J Cell Biol,* 132 (1-2), 239-49.

Yang, S., et al. (2001), 'The design of scaffolds for use in tissue engineering. Part I. Traditional factors', *Tissue Eng,* 7 (6), 679-89.

Yokoi, H., Kinoshita, T., and Zhang, S. (2005), 'Dynamic reassembly of peptide RADA16 nanofiber scaffold', *Proc Natl Acad Sci U S A,* 102 (24), 8414-9.

Zhong, S. P., Zhang, Y. Z., and Lim, C. T. (2010), 'Tissue scaffolds for skin wound healing and dermal reconstruction', *Wiley Interdiscip Rev Nanomed Nanobiotechnol,* 2 (5), 510-25.

Zimenkov, Y., et al. (2006), 'Rational design of a reversible pH-responsive switch for peptide self-assembly', *J Am Chem Soc,* 128 (21), 6770-1.

VITA

NAME:	Fadi S. Masoud
EDUCATION:	Bachelor of Science, Biology, Dalhousie University, Halifax, Nova Scotia, Canada, 1997
	Doctorate of Dental Medicine, Semmelwies University, Budapest, Hungary, 2002
	Doctorate of Dental Surgery, University of Southern California, Ostrow School of Dentistry, Los Angeles, California, 2005
	Certificate of Periodontics, University of Illinois at Chicago, College of Dentistry, Chicago, Illinois, 2012
	Master of Science, Oral Sciences, University of Illinois at Chicago, Chicago, Illinois, 2012
TEACHING:	Department of Operative Dentistry, University of Southern California, Ostrow School of Dentistry, Los Angeles, California, 2004, 2005
	Department of Periodontics, University of Illinois at Chicago, College of Dentistry, Chicago, Illinois, 2009-2012
HONORS:	Dean's List, University of Southern California, Ostrow School of Dentistry, Los Angeles, California, 2004, 2005
PROFESSIONAL MEMBERSHIP:	American Academy of Periodontics Midwest Society of Periodontics Illinois Society of Periodontics American Dental Association California Dental Association (past member) Los Angeles Dental Society (past member)