

The Role of Inflammation in Wound Healing and Periodontal Disease

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THESIS

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TABLE OF CONTENTS

CHAPTER		PAGE
I.	INTRODUCTION	
A.	Background	1
B.	Significance	3
C.	Specific Aims	3
D.	Hypothesis	3
II.	CONCEPTUAL FRAMEWORK AND REVIEW OF LITERATURE	
A.	Wound healing overview	4
	1. Hemostasis/Coagulation	6
	2. Inflammatory phase	10
	3. Proliferative phase	18
	4. Remodeling phase	29
B.	Mucosal vs. Dermal healing	31
C.	Factors affecting wound healing and periodontal disease	33
	1. Diabetes and systemic issues	34
	2. Wound healing in smokers	35
	3. Wound healing in Stress	36
	4. Hormonal influence in wound healing	37
	5. Age effects on periodontitis and wound healing	38
D.	Periodontitis – An inflammatory disease	38
	1. Prevalence of periodontal disease	40
	2. Pathogenesis of periodontal disease	41
	3. Local and systemic inflammatory considerations	41
	4. Periodontitis connected with systemic disease and inflammation.....	43
E.	Summation	46
F.	PCR testing	47

III.	METHODOLOGY	
A.	Participants	51
B.	Wounding protocol	52
C.	Wound size assessment	54
D.	Detailed clinical protocol	54
E.	Detailed blood work protocol	59
F.	Detailed PCR protocol	61
G.	Statistical Analysis	62
H.	IRB Approval	63
IV.	RESULTS	
A.	Differences in periodontitis vs. healthy controls	63
B.	Wound healing rate	65
C.	Local inflammation	67
D.	Systemic inflammation	70
E.	Correlations between local and systemic inflammation	72
V.	DISCUSSION	
A.	Discussion	73
B.	Limitations of the Study	82
C.	Future Research	83
VI.	CONCLUSION	84
	CITED LITERATURE	86
	APPENDIX	95
	VITA	98

LIST OF TABLES

<u>TABLE</u>	<u>PAGE</u>
I. PHASES OF WOUND HEALING.....	5
II. GROWTH FACTORS, CYTOKINES, CHEMOKINES AND AND IMMUNOGLOBULINS ASSOCIATED WITH WOUND HEALING.....	15
III. PROLIFERATIVE PHASE.....	28
IV. SKIN VS. ORAL MUCOSA.....	32
V. PERIODONTAL DISEASE PROGRESSION.....	41
VI. PCR PRIMERS AND PROBES USED.....	49
VII. AVERAGE PROBING DEPTH, BLEEDING ON PROBING AND CLINICAL ATTACHMENT LOSS (WITH STANDARD DEVIATION)..	62
VIII. AVERAGE PROBING DEPTH, BLEEDING ON PROBING AND CLINICAL ATTACHMENT LOSS (WITH STANDARD ERROR).....	62

LIST OF FIGURES

<u>FIGURE</u>	<u>PAGE</u>
1. Timeline of the 3 phases of healing in mucosal tissues.....	4
2. Secondary hemostasis-Intrinsic and extrinsic coagulation pathways leading to the common pathway and resulting in the formation of a fibrin clot.....	9
3. Steps of blood preparation and RNA storage.....	58
4. Wound Healing Rate.....	63
5. Denotes local inflammatory cytokines that were measured through real time PCR	65
6. Denotes local inflammatory chemokines, adhesion molecules and growth factors that were measured through real time PCR.....	66
7. Cytokine analysis at baseline (non-stimulated blood).....	68
8. Cytokine analysis in stimulated blood.....	69

LIST OF ABBREVIATIONS

AA	Actinobacillus actinomycetemocomitans
AL	Attachment loss
A.M.	Andrew Monestero
BOP	Bleeding on probing
BMP	Bone morphogenic proteins
C5a	Complement component 5a
CAL	Clinical attachment loss
CD4+	Cluster of differentiation 4 molecule
CD8+	Cluster of differentiation 8 molecule
CRP	C-reactive protein
CRC	Clinical research center
CTGF	Connective tissue growth factor
ECM	Extracellular matrix
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
FMLP	Formyl-Methionyl-Leucyl-Phenylalanine
GMCSF	Granulocyte-macrophage colony-stimulating factor
H	Hours
ICAM-1	Intercellular adhesion molecule-1
IGF	Insulin-like growth factor
IL1	Interleukin 1
IL1- α	Interleukin 1 alpha (IL-1 α)
IL1- β	Interleukin 1 beta (IL-1 β)
IL2	Interleukin 2
IL4	Interleukin 4
IL6	Interleukin 6
IL8	Interleukin 8
IL10	Interleukin 10
IFN- γ	Interferon gamma
IRB	Institutional Review Board
KC	Cytokine CXCL1
KGF	Keratinocyte growth factor
LPS	Lipopolysaccharide
M1	Pro-inflammatory macrophage
M2	Anti-inflammatory macrophage
M-CSF	Macrophage colony stimulating factor
MCP-1	Monocyte chemo attractant protein-1 (CCL2)
MIP-1 α	Macrophage inflammatory protein-1 (CCL3, CCL4)
M.M.	Marc Manos
NK cell	Natural killer cell
PCR	Polymerase Chain Reaction
PD	Probing Depth
PDGF	Platelet derived growth factors
PGE2	Prostaglandin – E2

LIST OF ABBREVIATIONS

P.G.	Praveen Gajendrareddy
PTLBW	Pre-term and/or low birth weight
SPSS	Statistical Package for the Social Sciences
RANKL	Receptor activator of nuclear factor-KB ligand
TF	Tissue Factor
TGF- β	Transforming growth factor-beta
TNF- α	Tumor Necrosis Factor - alpha
VEGF	Vascular endothelial growth factors

SUMMARY

Objectives: In separate past studies, higher inflammatory responses have been associated with the pathogenesis of periodontal disease (periodontitis) and delayed wound healing in oral mucosa. The relationship between these factors is yet to be investigated. Using a standardized model of wound healing, this study objectively examined differences in oral mucosal healing rates and local tissue inflammation in response to wounding between individuals with periodontitis and healthy controls.

Materials and Methods: Subjects included 20 systemically healthy, non-smoking, adult male participants (age range 24-45 years); 10 individuals classified as having periodontitis and 10 healthy age-matched controls. Three excisional wounds were placed on the hard palate under local anesthesia, and were standardized for size, location and depth. Oral mucosal healing rates were determined using daily video graphs. Tissue was biopsied at 0h, 6h and 24h post-wounding and analyzed for local tissue inflammation through real-time PCR.

Results: Preliminary results indicate that wound healing in systemically healthy young men with periodontitis is the same as healing in men without periodontitis in an unaffected area of the mouth. Local inflammation at baseline (0 hours) and after wounding (6 hours and 24 hours) was also found to be the same (no statistically significant differences) in healthy young men with periodontitis and healthy young men without periodontitis in an unaffected area of the mouth.

Conclusion: These results suggest that moderate periodontal disease and its associated inflammation, even when untreated, does not affect healing rates in unaffected oral tissues. Despite higher levels of inflammation in the periodontally diseased tissues, baseline inflammation and inflammatory responses in the unaffected site (in local biopsied tissue) appeared similar between healthy controls and periodontitis subjects.

Clinical significance: These results may have clinical implications in young healthy males in the management of surgical procedures and implant placement into unaffected tissue sites. Tissue repair seems to occur normally in unaffected tissue despite these patients having untreated periodontal disease. Additional studies are required to explore additional factors such as saliva and systemic inflammatory status that have led to our findings.

I. INTRODUCTION

A. BACKGROUND

In today's society, we are surrounded with the notion of immediacy. The advent of the Internet and other technological advancements has provided people instantaneous results, which has sent our society into overdrive. Communication, attaining knowledge, decision-making and execution can all be completed rapidly via the click of a button. This is extremely evident in the business world where billions of dollars are made by focusing on efficiency and stream lining processes.

The expectation for speed has required the medical world to evolve as well. Clinicians often adhere to production-focused business models in order to succeed. Patients anticipate instant outcomes, which have become commonplace in many other aspects of their lives, placing pressure on the medical field to deliver. However, physiology is not instantaneous and will not speed up to appease an impatient society. Fortunately, researchers have made advancements, which they claim, can lend Mother Nature a hand. Unaltered wound healing requires time, but research in the field has isolated factors, which can promote more effective and efficient wound closure and healing.

Rapid wound healing reduces the likelihood of infections caused by foreign pathogens invading the underlying connective tissues of the body. The faster the body is able to repair itself the less post operative complications, pain and discomfort patients have to experience. This leads to a more satisfied and productive patient population.

Over the last few decades Scientists have been supplementing nature's processes and facilitating faster wound healing. A number of growth factors and biologics on the market today have been touted to reduce healing time. They aim at stimulating wound closure as well as facilitate both soft tissue and bone growth. Some of these

factors and biologics include platelet derived growth factors (PDGF)¹, vascular endothelial growth factors (VEGF)², bone morphogenic proteins (BMP)³, transforming growth factor-beta (TGF- β)⁴, insulin-like growth factor (IGF)^{1,5}, epidermal growth factor (EGF)^{2,6} and fibroblast growth factor (FGF)^{3,7}. Although evidence can be found for and against the use of these supplements, in the end it is the immune system that enables the human body to repair and regenerate new tissues^{4,8}.

In the presence of injury to both mucosal and dermal systems a cascade of events is initiated to quickly and effectively minimize damage and repair wounded sites. This cascade of events involves a number of phases including hemostasis, inflammation, proliferation and remodeling. These stages all have critical functions that enable the body to heal adequately without excessive damage to the surrounding tissue. Each of these phases of wound healing must happen in a precise and regulated manner.

If these phases are interrupted or prolonged deleterious effects may occur, including but not limited to inadequate or non-healing wounds and or excessive scarring or keloid formation. It has been shown that the inflammatory phase of wound healing plays a key role in these less than ideal results.

Within this paper an overview of wound healing will be presented the detailed phases and steps will be reviewed. The inflammatory phase will be dissected and correlated to both effective mucosal and dermal wound healing.

Inflammation will also be discussed as a key etiologic factor within periodontal disease. Periodontal disease involves inflammatory changes at both local and systemic levels⁹. Change in the periodontal status results in changes to circulating inflammatory mediators. Previously published studies have shown a reduction in inflammatory markers such as IL-1 β , IL-6 and CRP following the treatment of periodontitis¹⁰. These links between healing, inflammation and periodontitis have

been hypothesized in the past ¹¹ but not tested with a standardized human mucosal wound model.

B. SIGNIFICANCE

A number of previous studies have demonstrated that increased inflammation in the supporting structures of teeth causes periodontal pathogenesis^{12,11}. A number of additional studies have shown that increased levels of inflammation systemically can cause impaired wound healing^{13,14,15}. In previous studies this group has used a standardized wound model to demonstrate the effects of gender, aging, stress, depression, and inflammation on mucosal healing. To date no studies have directly compared the effects of periodontal inflammation to that of wound healing in the oral mucosa. Within this study we will use this previously proven model to assess local inflammation in response to injury, wound closure and the relationships to systemic inflammation and periodontitis.

C. SPECIFIC AIMS

Within this study and review we aim to profile the differences in inflammation and mucosal healing among individuals with and without periodontal disease.

To accomplish this goal, we propose two specific aims:

1. To measure differences in wound healing rates in participants with untreated moderate periodontal disease and participants without periodontal disease.
2. To measure differences in local inflammatory markers: TNF- α , IL-1 β , IL-1 α , ICAM, IL-6, IL-8, MCP-1, MIP-1 α , and VEGF.

D. HYPOTHESIS

We hypothesize that the presence of untreated periodontal disease will relate to an increase in localized inflammatory response resulting in impaired mucosal wound healing. We believe the greater the periodontal disease severity the more exacerbated these effects will be.

II. CONCEPTUAL FRAMEWORK AND REVIEW OF LITERATURE

A. WOUND HEALING OVERVIEW

Wound healing, both dermal and mucosal, is often characterized by either three or four overlapping phases, depending on the literature cited: (1) hemostasis (2) inflammatory phase, (3) proliferative phase and (4) remodeling phase. In some studies, hemostasis is encompassed within the initial stages of inflammation, resulting in only 3 phases of wound healing as shown in Figure 1.

Figure 1.

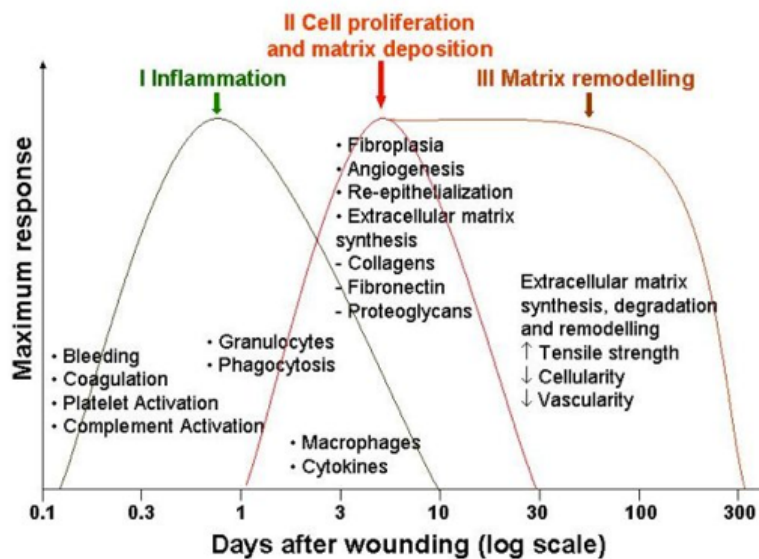


Figure 1. Timeline of the 3 phases of healing in mucosal tissues

www.worldwidewounds.com

To achieve optimal wound healing in adult humans, a number of events within these phases must occur in a timely and orderly manner. These events include: “(1) rapid hemostasis; (2) appropriate inflammation; (3) mesenchymal cell differentiation, proliferation, and migration to the wound site; (4) suitable angiogenesis; (5) prompt re-epithelialization; and (6) proper synthesis, cross-linking, and alignment of collagen to provide strength to the healing tissue. Interruptions, aberrancies, or prolongation in any of these events can lead to delayed wound healing or a non-

healing chronic wound¹⁶.” Table I. (below) outlines the main events in each of the stages of wound healing.

TABLE I.
PHASES OF WOUND HEALING

Phase	Events
Hemostasis Immediately	<ul style="list-style-type: none"> • Vascular constriction • Thrombus formation achieved through platelet aggregation and fibrin clot • Release of pro-inflammatory cytokines and growth factors (TGF- β, PDGF, FGF, EGF) • Fibrinolysis
Inflammation Immediately 48-72 hours IL 1 alpha – releases signaling right away	<ul style="list-style-type: none"> • Neutrophil infiltration (clear invading microbes and cellular debris) • Monocyte infiltration and differentiation to macrophages (clear apoptotic cells and release cytokines – helping to resolve inflammation) • Lymphocyte infiltration (T lymphocytes)
Proliferation (overlaps inflammatory phase) Starts day 1 ends 3-7 days	<ul style="list-style-type: none"> • Re-epithelialization – fibroblasts and endothelial cells are most prominent • Angiogenesis • Collagen synthesis • ECM formation
Remodeling Weeks to months (skin for several years)	<ul style="list-style-type: none"> • Collagen remodeling • Vascular maturation and regression

Table I. Overview of the main events that occur within the phases of wound healing and general timeline of events

B. HEMOSTASIS/COAGULATION

Hemostasis, the first step in wound healing, is the physiological process in which blood loss is limited during injury while preserving tissue perfusion and stimulating local repair. Hemostasis and coagulation pathways provide an intricate balance between clot formation and clot dissolution. If this balance is disrupted, the body will find itself in a state of either hyper coagulation and thrombosis or hypo coagulation and hemorrhage.

Coagulation occurs initially with platelet aggregation and fibrin formation. Once the platelets have accumulated at the site of injury fibrin forms and intricate web throughout the platelet plug stabilizing and sealing off the site of injury in the vessel lumen. In hemostasis, this coagulation process continues with the conversion of circulating hemostatic proteins called prothrombin to thrombin that in turn converts circulating fibrinogen (a circulating glycoprotein) to fibrin (a fibrous non-globular protein) thus forming an initial clot. This fibrin clot adheres and reinforces the initial platelet plug¹⁷.

Hemostasis can be divided into 3 phases: primary, secondary and tertiary hemostasis^{18,19}.

Primary hemostasis, which begins immediately after endothelial damage, includes both a vascular phase and a platelet phase of coagulation. The combination of these two events leads to limitations in the amount of blood loss.

The vascular phase relates to the contractile nature of the vascular smooth muscle. The injured vessel's diameter is quickly decreased upon insult. This is accomplished through smooth muscle spasm in the vessel walls resulting in the retraction of severed vessels and subsequent vasoconstriction of arteries and veins. The body uses this mechanism to slow blood flow in the area of injury. The accumulation of blood outside the area of the injured vessel creates a hematoma that increases extravascular pressure also slowing down bleeding in the area by collapsing adjacent vasculature including capillaries and veins. Blood flow is then diverted around the site of the injury through accessory vasculature ¹⁹.

The platelet phase of hemostasis includes adhesion and aggregation of these thrombocytes to the endothelial surface at the site of injury forming a soft platelet plug. The soft platelet plug temporarily seals the break in the vessel wall. These platelets then release chemical mediators such as thromboxane A₂, which have prothrombotic properties. These mediators stimulate the activation of new platelets and increase the aggregation of existing platelets, forming an initial soft platelet plug. These chemical mediators also lead to the initiation of secondary hemostasis.

Secondary hemostasis is defined as the formation of fibrin through the coagulation cascade. Davie and Ratnoff in 1964 best described the involvement of circulating coagulation factors using the waterfall cascade model²⁰. The waterfall cascade model is comprised of two initial pathways of coagulation, the extrinsic and the intrinsic pathways.

The extrinsic pathway, commonly initiated by trauma, requires the addition of tissue factor (TF) to the common circulating factors in the blood to initiate transformation of factor VII to factor VIIa.

The intrinsic pathway, as so named, uses only clotting components that are found within the blood. This pathway is typically initiated by endothelial damage to the vessels. This stimulus leads to the conversion of factor XII to XIIa, factor XI to XIa, factor IX to IXa in combination with factor VIIIa.

Both extrinsic and intrinsic coagulation pathways lead to the common coagulation pathway. Within the common coagulation pathway, factor X converts to factor Xa, which combines with its cofactor Va resulting in the formation of the prothrominase complex. This complex catalyzes the conversion of circulating prothrombin to thrombin. Thrombin is the master regulator of the coagulation cascade. Thrombin cleaves activation peptides from fibrinogen to form fibrin. Thereby, resulting in the formation of the initial loose fibrin clot, which in conjunction with platelet function work to seal the vessel wall. Figure 2. Depicts the common coagulation cascade

outlining both the extrinsic and intrinsic pathways and the factors that are stimulated to produce the fibrin clot.

Figure 2.

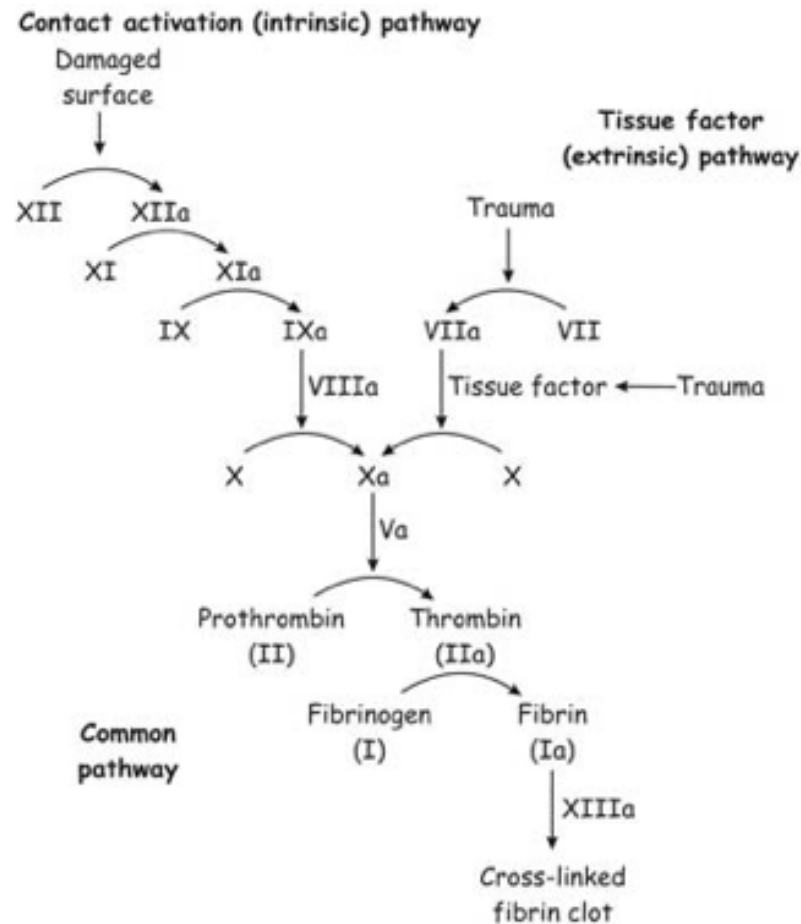


Figure 2. Secondary hemostasis-Intrinsic and extrinsic coagulation pathways leading to the common pathway and resulting in the formation of a fibrin clot

The third and final step of hemostasis involves the fibrinolytic system. It is initiated to disrupt secondary clotting elsewhere in the body as the clot at the site of injury is being formed. Fibrinolysis is an imperative step in the hemostatic process as it results in the dissolution of clots once they have served their purpose in hemostasis. This process works to alleviate intravascular coagulation at distant unrelated sites

throughout the body. Thereby limiting the coagulation process to the site of injury and eliminating the subsequent development of disseminated intravascular coagulation (DIC) and atherosclerotic vascular disease.

C. INFLAMMATORY PHASE

The inflammatory phase is a protective attempt to remove injurious stimuli and plays a key role in the progression of wound healing. Interruption or prolonged inflammation can result in deleterious effects including, but not limited to, inadequate or non-healing wounds or excessive scarring or keloid formation. Disorders of the inflammatory process or chronic inflammation can lead to a host of diseases. Inflammation must occur in a precise and regulated manner to assist in wound healing.

Immediately after an injury has occurred, innate immune cells initiate an inflammatory response at the site of injury. These cells include a first line of defense, which consists typically of a neutrophil influx, and a subsequent secondary defense including more specific leukocytes (NK cell, Basophils, Eosinophils), mast cells, dendritic cells, fixed macrophages and some specialized T cells. The purpose of these cells is to phagocytize invading foreign bacteria and pathogens and to produce and release pro-inflammatory mediators to stimulate further vascular response within the first hour of insult.

The early inflammatory phase is marked by rapid neutrophil infiltration. These cells are drawn to the site of injury by chemotactic factors such as Interleukin-8 (IL-8), Complement component 5a (C5a), Formyl-Methionyl-Leucyl-Phenylalanine (fMLP), and Leukotriene B4 released from platelets and other inflammatory mediators.

Cell surface receptors allow neutrophils to recognize concentration gradients of chemotactic factors, which direct migration through blood vessels and interstitial tissue to the site of insult. The neutrophils begin to accumulate within minutes and build up a defense to rid the area of debris and bacterial infiltrate. Neutrophils are the front line of defense for invading pathogens via phagocytosis. Neutrophils also recruit and activate many other mediators, which amplify the initial phase of the acute inflammatory process. These pro-inflammatory mediators known as leukotrienes and prostaglandins are created from arachidonic acid, an essential polyunsaturated fatty acid. This fatty acid enters the cyclooxygenase pathway to form prostaglandin and thromboxane or the lipoxygenase pathway to form leukotriene. Prostaglandins have a variety of physiological effects including the production of fever, pain and vasodilation and act on an array of cells. Leukotriene mediates leukocyte adhesion and activation and also acts as a chemo attractant for neutrophils¹⁵.

The acute inflammatory response is a self-limited protective mechanism.

Neutrophils have been found to possess the ability to change phenotypes with time.

Not only do neutrophils act as the body's first line of defense but they also generate

protective inflammatory mediators that in turn promote the resolution of the inflammation that they started. There is an active tapering of the disease returning tissues to a homeostatic health state from the inflammatory battle²¹. The enzymatically generated protective mediators include lipotoxins derived from arachidonic acid as well as resolvins, protectins and maresins. These findings allow us to infer that the resolution of acute inflammation is a clearly orchestrated active endeavor not a passive clearance of acute inflammatory exudate.

Macrophages are another commonly derived inflammatory cell and are activated from local surrounding tissues and distant sites, as circulating monocytes, to take on one of two forms. The first macrophage form, M1 phenotype, is a pro-inflammatory cell; it produces an array of pro-inflammatory cytokines, including: Tumor necrosis factor- α (TNF- α) and a number of Interleukins including IL-1 α , IL-1 β , IL-6 and IL-8. A number of studies have found an up-regulation of IL-1, IL-6 and TNF- α , during the inflammatory phase of wound healing. These cytokines are imperative in the recruitment and activation of immune cells, primarily circulating leukocytes, used to further rid the wound site of bacterial pathogens that could cause infection. The second macrophage form (M2 phenotype) is anti-inflammatory in nature and supports proliferation by producing growth factors. Other key inflammatory cells include mast cells, which are commonly found throughout the body. At the time of injury, these cells degranulate helping the body maintain tissue integrity and function. The last cells to arrive to site of injury, typically about 7 days post insult, include B-lymphocytes and T lymphocytes. These cells are more commonly

involved later in the proliferative phase or early in the remodeling phase of wound healing.

T lymphocyte function as it relates to wound healing is still unclear, however it has been shown that reduced levels of T lymphocytes can impair wound-healing outcomes. “CD4+ helper T cells have been suggested to accelerate wound healing while CD8+ cytotoxic suppressor T cells have been shown to inhibit wound healing”²².

GROWTH FACTORS, CYTOKINES AND CHEMOKINES IN WOUND HEALING

“[Upon tissue injury, the] disruption in the epidermal barrier causes the underlying keratinocytes to release a [pre-stored] inflammatory mediator, interleukin-1(IL-1). This inflammatory mediator is the first signal that alerts the surrounding cells to damage. Concurrently, blood components are released into the wound site initiating the clotting cascade. Platelets degranulate releasing alpha granules, which secrete growth factors such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and transforming growth factor- β (TGF- β). PDGF [and IL-1 are proinflammatory cytokines that] are important in attracting neutrophils to the wound site to remove [harmful] bacteria¹⁶.” TGF- β works at the site of injury to convert monocytes into macrophages. Macrophages play a key role in granulation tissue development, site debridement and augmentation of the inflammatory response by releasing both growth factors PDGF, EGF and FGF (fibroblast growth factor) and pro-inflammatory cytokines IL-1 and IL-6. These biologically active

polypeptides act to alter a number of different processes within a target cell including growth, differentiation and metabolism. These actions occur within the body through innate cellular signaling pathways including endocrine, autocrine, paracrine, and juxtacrine communication. This behavioral effect on the cell is stimulated by the binding of these molecules to specific surface receptors on cells or extra cellular matrix proteins¹⁰.

The combination of these growth factors, pro and anti-inflammatory cytokines play a crucial role in the initial stages of wound healing. The body's innate immune cells including both macrophages and neutrophils are key manufacturers of pro-inflammatory cytokines. These cytokines include: Tumor necrosis factor α (TNF- α); a number of interleukins including Interlukin-1 β (IL-1 β), IL-4, IL-6, IL-8, IL-10 and Granulocyte macrophage colony stimulating factor (GMCSF). TNF- α , IL-1 and IL-6 are pro-inflammatory mediators that are upregulated during the inflammatory phase of wound healing. IL-10 and IL-4 are anti-inflammatory mediators²³. These cytokines work together to stimulate the recruitment and activation of immune cells to prevent infection at the injured site, as well as regulate the proliferation of epithelial cells and tissue fibroblasts enabling the body to remodel the injured tissues leading to more efficient wound closure.

Another molecule in the body that is imperative to wound healing is the Chemokine. A chemokine is a small heparin-binding protein that directs the circulating leukocytes to sites of injury or inflammation through chemotaxis the movement of a

cell across a chemical gradient. Through the stimulation and migration of inflammatory cells to sites of inflammation or injury, chemokines actively participate in the wound healing process¹⁰. In Table II. one can review some of the major growth factors, cytokines, chemokines and immunoglobulin involved in wound healing, what cells they originate from as well as their biologic and cellular effects on the body.

TABLE II.
GROWTH FACTORS, CYTOKINES, CHEMOKINES AND AND IMMUNOGLOBULINS
ASSOCIATED WITH WOUND HEALING:

Growth Factor	Wound Cell Origin	Cellular and Biologic Effects
TGF- β	Keratinocytes Platelets Macrophages/monocytes T lymphocytes Neutrophils	<ul style="list-style-type: none"> • Works with epidermal growth factor -binds to EGF receptor • Mitogenic and chemotactic for epidermal and endothelial cells • Stimulates angiogenesis • Stimulates wound matrix formation • Anti-inflammatory cytokine • Inhibits scar formation
GMCSF	Macrophage/monocytes Endothelial cells Fibroblasts	<ul style="list-style-type: none"> • Stimulates macrophage differentiation/proliferation
VEGF	Macrophages Fibroblasts Keratinocytes	<ul style="list-style-type: none"> • Granulation tissue formation • Mitogen for endothelial cells (not fibroblasts)
PDGF	Platelets Macrophages/monocytes Smooth muscle cells Endothelial cells	<ul style="list-style-type: none"> • Chemotaxis: fibroblasts, smooth muscle, monocytes, neutrophils • Mutagenesis: fibroblasts, smooth muscle cells

		<ul style="list-style-type: none"> • Stimulation of angiogenesis • Collagen synthesis
FGF	Fibroblasts Endothelial cells Smooth muscle cells Chondrocytes	<ul style="list-style-type: none"> • Angiogenesis • Granulation tissue formation • Reepithelialization • Matrix formation and remodeling <ul style="list-style-type: none"> -Through the recruitment of fibroblasts, keratinocytes, chondrocytes and myoblasts
KGF	Keratinocytes Fibroblasts	<ul style="list-style-type: none"> • Works with FGF • Stimulates keratinocytes
EGF	Platelets Macrophages Monocytes	<ul style="list-style-type: none"> • Stimulates proliferation and migration of all epithelial cell types
INFLAMMATORY CYTOKINES:		
Cytokine	Wound Cell Origin	Cellular and Biologic Effects
TNF-α	Macrophages	<ul style="list-style-type: none"> • PMN margination and cytotoxicity • \pm Collagen synthesis • Reepithelialization
IL-1	Macrophages Keratinocytes	<ul style="list-style-type: none"> • Fibroblast and keratinocyte chemotaxis • Collagen synthesis
IL-2	T Lymphocytes	<ul style="list-style-type: none"> • Increases fibroblast infiltration and metabolism
IL-6	Macrophages PMNs Fibroblasts	<ul style="list-style-type: none"> • Fibroblast proliferation • Acute-phase protein synthesis by the liver
IL-8	Macrophages	<ul style="list-style-type: none"> • Macrophage and PMN chemotaxis • Keratinocyte proliferation

IFN-γ	T Lymphocytes	<ul style="list-style-type: none"> • Macrophage and PMN activation • Retards collagen synthesis and cross-linking -stimulates collagenase activity
ANTI-INFLAMMATORY CYTOKINES:		
IL-4	T Lymphocytes Basophils Mast cells	<ul style="list-style-type: none"> • Inhibition of TNF, IL-1, IL-6 production; fibroblast proliferation, collagen synthesis
IL-10	T Lymphocytes Macrophages Keratinocytes	<ul style="list-style-type: none"> • Inhibition of TNF, IL-1, IL-6 production; inhibits macrophage and PMN activation
CHEMOKINES:		
Chemokine	Wound cell origin	Cellular and biologic effects
MIP-1α	Macrophages	Activate Neutrophils, Eosinophils and Basophils Induce synthesis of IL-1, IL-6 and TNF from fibroblasts and macrophages
MCP-1	Monocytes	Recruits monocytes, memory T-cells and dendritic cells to sites of inflammation
IMMUNOGLOBULINS (ANTI-BODIES):		
Immunoglobulin	Wound Cell Origin	Cellular and biologic effects
ICAM-1	Endothelial cells Leukocytes	Binds leukocytes to endothelial cells Transmigrates into tissues

Table II. Overview of the main growth factors, cytokines and immunoglobulins involved in wound healing, the cells they are derived from and their effects in the wound healing process¹⁶.

PROLIFERATIVE PHASE (Wound contraction, Re-epithelialization, Angiogenesis, Collagen synthesis, formation)

WOUND CONTRACTION AND RE-EPITHELIALIZATION

The proliferative phase overlaps the inflammatory phase in wound healing and is composed of a number of subcomponent stages, including wound contraction, re-epithelialization, angiogenesis, collagen synthesis and extra cellular matrix formation. Skin and oral mucosa are composed of stratified squamous epithelium. This serves as an important barrier from physical, chemical and microbial damage from the outside environment as well as regulating the integrity and function of the underlying connective tissue²⁴. For these reasons wound contraction and re-epithelialization after insult have to be vigorous processes to restore the barrier function.

Wound contraction is an essential component of wound healing. This process works to reduce the surface area of a wound by bringing the wound's edges closer together, thereby reducing the amount of re-epithelialization needed to facilitate closure. Studies have found that wound contraction can account for nearly half of wound closure²⁵. "Contraction is an active process mediated by differentiation of wound cells into myofibroblasts." This differentiation of the myofibroblast cells starts approximately one week after initial wounding when the granulation tissue has established itself²³. "[These newly formed myofibroblasts] are attached to a polymerized fibronectin network that serves as a bridge between cells and newly synthesized collagen fibrils." Integrins and other proteins directly interact with

these collagen fibrils to regulate deposition and crosslinking of these fibrils. Wound contraction is thus achieved when these fibrils are lined up perpendicular to the wound margins using their actin-rich cytoskeleton, to achieve proper constrictive force allowing for wound closure¹⁶.

Growth factors such as TGF- β , PDGF and FGF work to induce fibroblast contraction²⁶ while inflammatory mediators, such as interferon- γ (INF- γ), TNF- α , and IL-1 α can inhibit wound contraction²⁷. The combination of wound contraction and re-epithelialization allows the body to wall off the exposed underlying connective tissue from the outside environment.

“[Re-epithelialization is one of the most critical components in effective wound healing and closure. In normal healthy skin and mucosa,] epithelium is separated from the underlying connective tissue by a basement membrane. Keratinocytes of the basal layer attach to it via [hemi desmosomes]. The [more superficial] keratinocytes [connect] to neighboring cells by specialized cell-cell adhesions (desmosomes, adherens and gap junctions) [forming sheets of keratin]. During tissue homeostasis, the daughter cells of the proliferating basal cells that lose their contact with the basement membrane are slowly pushed to the [more superficial] layers of the epithelium. [The daughter cells then] undergo gradual terminal differentiation until they become anucleated and flattened, eventually shedding away from the epithelial surface²⁸.” This process is crucial to maintaining epithelial

integrity and is tightly regulated to promote barrier function during tissue homeostasis²⁹.

“[Upon wounding, the basement membrane of both skin and mucosa] is disrupted and keratinocytes become exposed to wound-induced electrical fields. Pro-migratory matrix molecules, including Type I collagen in the connective tissue, fibronectin fibrils in the clot, and pro-migratory growth factors [are] secreted by the wound cells. Keratinocytes and other wound cells [synthesize proteinases that] contribute to collagen denaturation, growth factor release and activation and to modulation of the molecular structure of the fibrin-fibronectin clot¹⁶.”

Keratinocyte activation is paramount in the restoration of the epithelial barrier after wounding. In mucosa Epithelial cells begin to migrate within 12 to 24 hours over the wound, peaking at 24 to 36 hours. They progress at a rate of 0.5 mm /day. Wounds are generally keratinized by 2 weeks³⁰. The epithelial keratinocytes become activated after wounding through a number of pathways stemming from the exposed area including: (1) activation of growth factors and cytokines released from inflammatory cells adjacent to the clot, (2) pro-migratory matrix molecules within the wound site and (3) wound generated electrical fields³¹.

Upon activation, through wounding, hemidesmosomal attachments are dissolved between the keratinocytes and the basement membrane. A number of desmosomal cell connections are required for idyllic re-epithelialization however lateral

desmosomal connections between the wound edge keratinocytes are drastically decreased³².

“[A number of] cytokines, including keratinocyte growth factor (KGF), epidermal growth factor (EGF) and insulin-like growth factor (IGF) [also] promote keratinocyte migration [and] are able to cause alterations in the organization of the cytoskeleton and cell shape^{33,34}.” These morphological changes to the keratinocytes appear only hours after wound infliction and seem to be prerequisites for the migration of these keratinocytes.

Histological studies have shown that during the first 24 hours of wounding, keratinocytes start their migration from the outer edges of the wound and slowly move along the provisional matrix, initiating re-epithelialization of the wound defect. Once this migration is well underway, typically around 48-72 hours after injury, the proliferation of basal keratinocytes adjacent to the migrating epithelial front begins, seeding more migratory, non-proliferating cells to the wound^{35,36}. It is unclear how significant a role these cells play, but some studies have stated that even bone marrow derived cells may act as transit-amplifying keratinocytes to assist wound re-epithelialization^{37,38}.

There have been a number of suggested models of keratinocyte migration during re-epithelialization. Stenn and Malhotra proposed a model in 1992, in which the basal keratinocytes actively pull along the supra-basal cell layers facilitating the migration

of cells across the wound matrix. In another proposed mechanism the epithelial cells actively move together as a sheet sliding and crawling along the cells at the wound margin^{39,40}. This proposed epithelial cell migration pattern has been observed in vivo in corneal wounds. A leap frog model has also been purposed which states “the cells at the wound margin do not in fact migrate, but the supra-basal keratinocytes leap over the basal keratinocytes and attach to the wound matrix forming a new leading front⁴¹.” This leads to a more intra-epithelial migration. No matter what the method of epithelial cell migration the process of mucosal re-epithelialization has been shown to be an imperative step proliferative phase of wound repair and it cannot be established without an adequate blood supply. This brings us to another process in the proliferative phase of wound healing.

ANGIOGENESIS

Wound healing would not be possible without blood and systemic circulation. Blood and the vessels that transport it are critical in delivering the necessary cytokines, chemokines, growth factors and other mediators that allow for effective wound healing, without angiogenesis at the site of injury none of this would be possible. Angiogenesis has been defined as the physiological process in which novel blood vessels form from their pre-existing counterparts. The process of angiogenesis has many similarities to that of re-epithelialization. Endothelial cells become activated by a number of cell mediated factors and start to migrate within

the first 24 hours of wounding into the wound provisional matrix in both skin and oral mucosa.

One stark difference that has been found between the oral mucosa in relation to skin is the concentration of these vascular capillaries and the relative blood flow within them appears significantly higher intra-orally⁴². Indicators such as deeper pink hue of the gingiva, increased rates of epithelial migration and overall speedier wound healing in the oral cavity are all indirect effect of this increased vascularity. Using Doppler flowmetry in rhesus monkeys Canady found this to be the case. He found that blood flow was significantly higher in oral cavity in comparison to facial skin. The results of his study can most likely be attributed to the ever-abundant capillary supply found in the oral mucosa. Similar studies have found up to a two fold increase in capillary density during wound healing to that of uninjured normal tissue¹⁵.

The formation of new blood vessels is inherently associated with the formation of granulation tissue in wound healing. A new capillary network is established after injury to the tissue by the angiogenic process. This angiogenic process is stimulated by a number of angiogenic factors, such as vascular endothelial growth factors (VEGFs) that are released from platelets, inflammatory cells and resident fibroblasts during injury. A major inducer of VEGF expression in the wound site is hypoxia in the tissue. After wounding, injured tissues must attain a normal level of

oxygenation, once this normoxia is attained VEGF production diminishes, signaling the end of the angiogenic process.

It has been shown that wounds that tend to suffer from scar formation in skin have more robust angiogenesis than palatal mucosal wounds that heal without scars ⁴³.

During tissue maturation, the regression of some vessels aids in more seamless healing. This regression of vessels is often linked to reduction or lack of angiogenic stimuli as well as inhibition of angiogenesis. Thrombospondin-1 is a key factor that aids in the inhibition of angiogenesis. A delicate balance must be formed between angiogenesis and inhibition of new blood vessel formation for an ideal healing outcome.

COLLAGEN SYNTHESIS AND ECM FORMATION

The coordinated expression of extracellular matrix molecules, proteinases and integrins are paramount in wound healing. “[Proteinases work to] cleave matrix molecules to reveal functionally active domains within these molecules and release matrix-bound growth [factors]²⁴.” Wound matrix molecules contact with the integrins of keratinocytes to facilitate pro-migratory cell stimulation and subsequent wound closure. This contact promotes the binding of growth factors and mitogenic cytokines to their cell surface receptors aiding in the re-approximation of the wound edges.

In order for this re-epithelialization and wound closure to occur, an underlying connective tissue layer must simultaneously be working to proliferate and repair itself. “Connective tissue wound healing is a critical process that restores the blood supply, integrity, mechanical strength and function of the injured tissue{Larjava:2012wt}.” The extracellular matrix (ECM) in the oral mucosal has a number of stark differences than that of the connective tissue in skin, including type of fibroblasts derived in each. These fibroblasts have not only phenotypical differences but varying functional traits as well. Due to their ‘replicatively younger’ phenotype, oral mucosal fibroblasts exhibit a preferential healing response in vivo^{44,45}. Oral mucosa can be differentiated into a more robust keratinized masticatory mucosa, composed of denser connective tissue firmly affixed to underlying bone and a thinner non-keratinized lining mucosa, containing freely movable loose connective tissue.

Extracellular matrix (ECM) serves as a template that guides subsequent tissue maturation. Fibroblasts use this matrix as an initial scaffold; these cells are the major producer of the collagen matrix. Type I, Type III and Type V collagen are the main components of soft connective tissue in both skin and the oral mucosa. Type III collagen is most abundant in early granulation tissue formation. Type I collagen accumulation speeds up within the first few days, attaining its peak formation between 7-14 days. By two weeks 75% of the immature granulation tissue is composed of these Type I collagen fibers^{46,47}. Type III collagen makes up the majority of the remaining granulation tissue at approximately 22%. This level

remains relatively constant from one week up until approximately 3 months post wounding. Type V collagen accounts for only a negligible proportion of the overall collagen in the wounds. It accounts for approximately 3% of the total collagen at its peak around 21 days post wounding and this amount gradually declines for the next 3 months.

In normal uninjured tissue, fibroblasts remain metabolically active but in a relatively dormant state as they are not receiving outside signals from their environment causing them to proliferate. While in this quiescent state, these cells remain securely attached and maintain tissue structure by slowly synthesizing, degrading and organizing extracellular matrix^{48,49}.

“[In skin and in the PDL,] fibroblasts form an interconnected network through cell-cell adhesions which allows them to exchange information directly via gap [junctions]⁵⁰. Quiescent fibroblasts are also responsive to paracrine signaling molecules released from other cells present in the connective tissue or in the [epithelium]^{51,52}.” Through these mechanisms, fibroblasts are able to feel changes in the tissues through minute variance in mechanical tension and help to modulate the ECM to meet changing functional demands⁵³. This can be exemplified intra-orally through changes in the periodontium; specifically, the PDL widening or shortening in response to occlusal forces or orthodontic tooth movement.

Dermal and mucosal wounding induce a number of changes in the signaling environment of fibroblasts. These resultant changes work to activate cells from their otherwise homeostatic state. In wounding, cell-to-cell contacts between fibroblasts are disrupted. This mechanical tension breaks the direct cell-to-cell signaling network and promotes the release of ECM molecules or their components that fibroblasts do not normally encounter^{54,55}. “[In addition to the increased function of the fibroblasts, other] cells in the wound area, including inflammatory, epithelial, vascular, adipose and neuronal cells, are activated to secrete or release paracrine factors that modulate fibroblast [functions]¹⁰.”

Fibroblast regulation is an extremely specific and complex process. At this time this regulation process is still poorly understood. It has been hypothesized that the most likely mechanism of fibroblast action is based on a combination of concentration gradient of activating signals and cell type⁴⁸. These activating signals initiate fibroblast cell proliferation, prompt cell adhesion modulation to the ECM and provide direction to cells migrating over the injured sites blood clot. Once these cells have migrated over and into the blood clot, they begin proliferation and form granulation tissue. This new granulation tissue ECM initiates the repair process; which begins to form approximately 2-4 days after wounding. “[The tissue consists primarily of] components of the blood clot, fibroblasts and their progenitor cells that have [found their way] into the area, primitive connective tissue ECM produced by wound [fibroblasts], new and forming blood vessels and [inflammatory cells]^{55,56}.”

This primitive granulation tissue is hyper cellular and serves as a scaffold allowing for subsequent connective tissue healing. “Once fibroblasts have migrated into the granulation tissue, their primary role is to rapidly produce new connective tissue ECM to re-establish tissue strength and function. [This] fibroblast migration into the wound provisional matrix [is mediated by] an increasing but relatively low concentration gradient of [chemo attractants, namely; EGF, TGF-B, and PDGF⁴⁸. In addition], insulin-like growth factor-1 (IFG-1), FGF-2 and connective tissue growth factor (CTGF) [are also] present in the granulation [tissue]^{57,58}.”

Over time this primitive ECM slowly turns over from a poorly organized cell-rich granulation tissue into a mature connective tissue structure by remodeling. This typically occurs between 7-10 days post injury. For proper tissue remodeling to occur, both a reduction in the number of fibroblasts producing ECM and a down regulation of those remaining fibroblasts must occur. These remaining fibroblasts begin the process of ECM reorganization and remodeling with an end goal of complete regeneration. This goal of complete regeneration is accomplished when these tissues regain their original form and function.

Table III. Summarizes the major steps in both the proliferation and remodeling phases of wound healing.

TABLE III.
PROLIFERATIVE PHASE

Granulation tissue formation	Regulation of inflammation Proliferation Migration ECM deposition Re-epithelialization and angiogenesis
Wound contraction	Differentiation into myofibroblasts Wound contraction Reorganization of ECM
REMODELING PHASE	
Remodeling	Down regulate ECM deposition ECM degradation Collagen cross-linking ECM reorganization and maturation Down regulate cell proliferation Undergo apoptosis Assume senescence Assume quiescent state

Table III. Common event within the proliferation and remodeling phases ¹⁶.

REMODELING PHASE

The final phase of wound healing is the remodeling phase, in which tissue will obtain its final form. The timeline for this phase can range from a matter of weeks to months up to a number of years. This timing can be dependent on the size and type of wound and whether or not it is healing by primary or secondary intention. In a smaller wound, with little or no granulation tissue formation, wound contraction can occur in as little as 3-5 days. In larger wounds in which secondary intention healing is anticipated it may take more than 7-14 days for wound contraction to occur.

After wound contraction, ECM production and degradation returns to a more homeostatic state and the body transitions into a remodeling phase in which the transitional granulation tissue is converted into a more mature underlying connective tissue. At this time fibroblasts, macrophages, myofibroblasts and neovasculature are eliminating inflammatory tissue and aligning the freshly formed collagen fibrils into thicker, more perpendicular bundles along the wound edges.

As wounds mature, these early collagen fibrils are converted from type III collagen to type I collagen⁵⁵. Over time, these collagen fibers gradually form crosslinks leading to better stability of the tissues. This is a slow process; animal studies have demonstrated that wound tear strength three weeks after initial injury is only about 20% of its final strength. Tissue remodeling of an affected area can take months or even years to gain its original strength. Over this time, down regulation of cell proliferation and cellularity of the tissue is reduced. The abundance of pro-mitogenic chemokines, cytokines, and growth factors is greatly reduced in comparison to that of the proliferative phase. The reduction in inflammatory cells helps the tissue to mature and, if regulated ideally, regenerate new viable tissue without scar formation.

If complete regeneration does not occur in the remodeling phase scar formation can occur. Scars can range anywhere from a mild form, where collagen rich ECM is organized in a more parallel fashion, to the more versatile basket weave configuration present in normal skin and mucosa, leading to a weaker breaking

strength of 70-80% of normal skin. When there is an unbalanced deposition to degradation ratio of ECM an accumulation of ECM can form keloids or hypertrophic scars in the skin⁵⁹. Remodeling is a continual process that takes months to years to regain previous form and function. This timing is highly variable depending on wound size and severity. This also varies considerably between mucosal and dermal healing.

MUCOSAL VS. DERMAL HEALING

Mucosal and dermal wounds proceed through similar stages of wound healing, yet the healing of the oral mucosa can be distinguished from skin healing by the speed of site closure and minimal scarring seen in the mucosa. One key difference between dermal and mucosal healing is that oral mucosa resides in a moist saliva bath full of micro-flora, a unique molecular combination, which Loo 2010 and Reish 2009 believe may promote the wound healing process. Decreased inflammatory response is seen in wet wound healing and this may correlate to diminished scarring⁶⁰.

“Significantly lower levels of macrophage, neutrophil, and T-cell infiltration were observed in [mucosal] vs. dermal wounds. RT-PCR analysis of inflammatory cytokine production demonstrated that oral wounds contained significantly less IL-6 and [Cytokine CXCL1](KC) than did skin [wounds]⁶¹.” The lessened inflammatory response is due to reduction in cytokine production, thereby reducing the number of leukocytes and other immune cells recruited to the area of injury.

Reduced inflammatory phase in mucosal wound healing has been likened to that of fetal wound healing models. In fetal models, scarless healing occurs in the early stages of development. At this stage of development, inflammatory cells are absent. Therefore there are no neutrophils, T-cells, B-cells or macrophages to wreak havoc on a wounded site. Cytokines, such as IL-6 and IL-8 have also shown great attenuation in fetal wounds. The lack of these inflammatory cells may be key to identifying future improvements of wound healing with decreased healing times and scarring.

There is very little data and few studies that explore the mechanisms of re-epithelialization in oral mucosa specifically; therefore, the majority of our knowledge is derived from skin healing studies. These skin studies are assumed to be similar to oral mucosa because the process of wound healing is highly evolutionally and mechanically conserved. Below in Table IV you will see a number of differences between healing of adult skin and mucosa.

TABLE IV.
SKIN VS. ORAL MUCOSA

Adult skin (dermis)	Adult oral mucosa
Fibroblasts <i>in vitro</i>	
Slower migration	Faster migration
Slower collagen gel contraction	Faster collagen gel contraction
Lower expression of growth factors (HGF/SF and KGF)	Higher expression of growth factors (HGF/SF and KGF)
Lower expression of MMP-2 and MMP-3	Higher expression of MMP-2 and MMP-3
TGF-B promotes proliferation	TGF-B suppresses proliferation
Shorter proliferation lifespan	Longer proliferation lifespan
Quantitative and qualitative differences in production of hyaluronan and sulfated glycosaminoglycans	
Connective Tissue ECM	
Less tenascin-C	Abundantly tenascin-C
Less hyaluronan	More hyaluronan
Acute wound healing	
Dry, contaminated environment	Moist (saliva), contaminated environment
Strong and prolonged inflammation	More rapid, short and weaker inflammation
Slower re-epithelialization	Faster re-epithelialization
Strong angiogenesis response	Moderate angiogenesis response
High expression of VEGF	Lower expression of VEGF
TGF-B1/TGF-B3 isoform ratio higher	TGF-B1/TGF-B3 isoform ratio lower
Results in scar formation	Results in minimal scar formation

Table IV. Differences between skin and mucosa ¹⁶.

FACTORS AFFECTING WOUND HEALING AND PERIODONTAL DISEASE

A number of factors have been attributed in the literature to affect the impairment of wound healing and progression of periodontal ranging from systemic conditions such as diabetes to age, smoking, stress and hormonal imbalances. A brief review of pertinent literature surrounding these factors can be found below.

DIABETES AND SYSTEMIC ISSUES

Uncontrolled and mismanaged systemic conditions can greatly affect the effectiveness of the immune system and the body's ability to heal. That same uncontrolled or excessive inflammatory response as it relates to injury or infection can lead to debilitating chronic disorders. In uncontrolled diabetic patients, for instance, non-healing chronic ulcers can be commonly found on the lower extremities, which in a number of cases lead to subsequent amputation. In addition to these chronic conditions these patients also present with slow healing to acute injury and are at increased risk for complications such as infection and wound dehiscence. Why is this the case? There are a number of mechanisms that affect these altered healing outcomes a number of which stem from an altered inflammatory phase of healing.

This altered inflammatory response begins with an altered balance in the number of circulating proteases leading to an intensified inflammatory reaction and a decrease in vascularity due to an altered angiogenic response. Diabetic wounds contain an overabundance of neutrophils, the first line in inflammatory immune defense, sent out to clear the wound of bacterial infiltrates. However in this patient population these neutrophils have little effect on bacterial decontamination. This ineffective inflammatory response leads to more tissue damage and delayed wound repair⁶².

In addition to increased numbers of phagocytic inflammatory cells, diabetic patients also have an abundance of cytokines and chemokines. These inflammatory

mediators in addition to advanced glycation end products create an environment of excessive cellular apoptosis, leading to a highly dysfunctional repair process.

WOUND HEALING IN SMOKERS

An increased risk in the prevalence of periodontitis and the impediment of wound healing has been documented in cigarette smokers in a number of studies. Grossi found that; “smokers had an increased relative risk for periodontitis ranging from 2.05 to 7.28 for light and heavy smokers”, respectively. Smoking causes decreases in blood flow, peripheral PMN function as well as decreased levels of serum IgG⁶³. Smokers have also been found to have a higher prevalence of infection with periodontal pathogens than non-smokers. Significantly higher proportions of smokers harbored higher mean levels of *Actinobacillus actinomycetemcomitans* (A.A), a 3.1 fold increase, *Bacteroides Forsythus*, a 2.3 fold increase, and *Porphyromonas Gingivalis* than did non-smokers. A combination of an impairment in immune response and an increased prevalence of periodontal pathogens contributing to chronic subgingival inflammation and infection leads to and increased severity of periodontal disease in smokers⁶⁴.

In cases of inflammatory, traumatic or mechanical insult where blood flow to the gingival margin has been compromised the compounding superimposition of vasoconstrictive effects from smoking can result in greater post surgical gingival shrinkage. This leads to impaired wound healing in these patients. It has been shown that nicotine intake may effect: monocyte function and gingival fibroblast

proliferation (cell metabolism and function), fibroblast synthesis of fibronectin and type I collagen, decreased oxygen transport and metabolism, enzyme poisoning. Studies by Trombelli and Scabbia found that cigarette smokers respond less favorably than non-smokers to periodontal surgical therapy⁶⁵.

WOUND HEALING IN STRESS

“Currently, stress is classified as a risk indicator for periodontal disease. The role of psychological stress in wound healing has been addressed in a plethora of studies in the medical field. The impact of stress on periodontal wound healing may be influenced by factors that can be classified into the following two broad categories health-impairing behaviors, such as poor oral hygiene; and factors that have pathophysiological effects, such as altered cytokine profiles^{66,67}.”

These afore-mentioned behaviors that can greatly impair health in association with stress include: Poor oral hygiene, increased consumption of cigarettes, increased alcohol consumption, forgetfulness and difficulty concentrating, disturbed sleeping patterns and poor nutritional intake. A number of pathophysiological effects have also been noted to occur in stressed individuals leading to impairment of normal mucosal and skin wound healing, including higher glucocorticoid levels (cortisol) and higher catecholamine levels (epinephrine and norepinephrine), which may lead to any or all of the following: Hyperglycemia, which may impair neutrophil function and impair the initial phase of wound healing, reduced levels of growth hormone, which may down-regulate the tissue repair response, altered cytokine profiles,

which may affect recruitment of cells important to wound remodeling, such as macrophages and fibroblasts, reduced tissue matrix metalloproteinase levels, leading to impaired tissue turnover and reduced wound remodeling, decreased natural killer cell levels, reducing the host ability to mount an appropriate immune response to periodontal pathogens and an altered Th1/Th2 ratio, leading to an increased susceptibility to periodontal disease⁶⁸.

HORMONAL INFLUENCE IN WOUND HEALING

Previous studies performed by members of this group have analyzed the effects of sex hormones on mucosal wound healing. Other groups focusing primarily on dermal wound healing have reported an advantage in healing rates amongst the female population. In contrast, in 2009 the Engeland laboratory demonstrated that certain women (post-menopausal) heal mucosal wounds more slowly than men. Within this study, “blood collected at the time of wounding was used to assess circulating testosterone, progesterone and estradiol levels [as well as] in vitro cytokine production in response to LPS. No strong associations were observed between healing times and estradiol or progesterone levels. However, in younger subjects, lower testosterone levels related to faster wound closure. Conversely, in older women higher testosterone levels related to lower [overall] inflammatory [response] and faster healing times.” It is theorized that testosterone may impact re-epithelialization and angiogenesis responses within the proliferative phase of wound healing. This suggests that testosterone levels modulate human mucosal healing rates¹⁴.

AGE EFFECTS ON PERIODONTITIS AND WOUND HEALING

Holm-Pedersen & Loe (1971) studied wound healing in the gingiva of young (Mean age = 23 years) and old (Mean age = 70 years) human volunteers. A [2-mm] wide biopsy was performed in a standardized way and [healing] monitored for a period of 2 to 10 months. The authors reported that healing occurred somewhat faster in the young individuals. The most conspicuous difference between the 2 groups, however, was that while the free gingiva of young adults regenerated [to] pre-operative levels within 60 days, that of [old] individuals never did regenerate [completely]⁶⁹.

Lindhe & Nyman (1985) compared the effect of plaque control and surgical pocket elimination separately for one group of young (26-29 years) and one group of older (> 60 years) individuals, over a 5-year period. The authors stated that these 2 groups of individuals prior to treatment had "similar pocket depth and bone score values, reflecting a similar degree of periodontal breakdown. The [elimination] of plaque, [calculus] and diseased periodontal tissues resulted in the disappearance of the clinical symptoms of periodontitis irrespective of the age of the [patients]⁷⁰.

PERIODONTITIS - AN INFLAMMATORY DISEASE

Periodontitis, a disease characterized by host inflammation in response to the overabundance of pathogenic microorganisms, notably *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans*. Is commonly diagnosed by clinical

and radiographic evidence of destruction of the supporting structures of the tooth, the periodontium. “In susceptible individuals, dysregulation of inflammatory and immune pathways leads to chronic inflammation, tissue destruction and disease.{Cekici:2014ha}” Periodontal disease is one of the major causes of tooth loss in adult humans.

Traditionally, periodontal status has been divided into three broad categories: health, gingivitis, and periodontitis⁷¹. Using this criterion, a diagnosis of health implies that there is no loss of connective tissue attachment or clinically detectable gingival inflammation. Conversely, plaque-induced gingivitis is the presence of gingival inflammation without concurrent loss of connective tissue attachment, while plaque-induced periodontitis implies both gingival inflammation and concurrent loss of alveolar bone and supporting connective tissue.

Apical migrations of the junctional epithelium with its associated increased development of pocket epithelium are key features of periodontitis development. This apical migration of tissue is stimulated by a chronic inflammatory process adjacent to the alveolar process, leading to loss of this supporting structure as seen in both periodontitis and peri-implantitis. This is mainly due to local formation of bone resorbing osteoclasts, which degrade bone without any subsequent coupling of osteoblastic de novo bone formation. Stimulation of mononuclear osteoclast progenitors by receptor activator of nuclear factor- κ B ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) is crucial for osteoclastogenesis. This induces osteoclast progenitor cell differentiation and the fusion to mature,

multinucleated osteoclasts. M-CSF and RANKL are produced by osteoblasts/osteocytes and by synovial and periodontal fibroblasts and the expression is regulated by pro- and anti-inflammatory cytokines⁷². These cytokines and osteoblast progenitor cells play a central role in the pathogenesis of periodontal diseases.

Physiologic inflammation is a well-orchestrated network of cells, mediators and tissues. Early inflammatory phase defenses waged through innate immunity provide critical protection against invading periodontal pathogens. This, as well as the immunological functions of adaptive immune response, provides the necessary mechanisms to allow the host to efficiently protect oneself from these specific oral micro biota that attempt to invade the body and the systemic circulation. The response of innate and adaptive immunity in conjunction with RANKL and osteoclastic activity leads to the apical migration of the periodontal tissues and the resultant progression of periodontal disease.

PREVALENCE OF PERIODONTAL DISEASE

Recent studies have shown that this complex inflammatory disease affects nearly half of the US population. In 2012, data as part of the Centers for Disease Control's (CDC) National Health and Nutrition Examination Survey (NHANES) estimated that one out of every two Americans age 30 and over has periodontal disease.

"[Specifically], 47.2%, or 64.7million American adults, have mild, moderate, or severe periodontitis; in adult's age 65 and older, the prevalence rates increase to

70.1%⁷³.” “Gingivitis affects 50-90% of adults worldwide, depending on its precise definition⁷⁴.” This data illustrates that periodontal diseases are “one of the most prevalent non-communicable chronic diseases in [the] population” and highlights the importance of furthering our understanding of disease etiology to continually improve diagnostic and preventative measures.

PATHOGENESIS OF PERIODONTAL DISEASE

In the classic paper by Page and Schroeder in 1976 the pathogenesis of periodontal disease was explained in detail as a sequence of inflammatory stages. These stages, the initial, early and established lesions are sequential stages in gingivitis, where as the final advanced stage manifests clinically as periodontitis⁷⁵. These inflammatory stages are described in detail in Table V. This paper was pivotal in changing the thought process leading to the current ideas that the host response and hyperactive defense mechanisms are some of the primary factors leading to periodontal disease. This altered immune response can also explain individual differences in susceptibility to periodontitis in response to bacterial insult. Some individuals have a hyper acute response leading to localized or chronic aggressive attachment loss where other have a more attenuated response and never progress beyond a level of chronic inflammatory gingivitis.

LOCAL AND SYSTEMIC INFLAMMATORY CONSIDERATIONS

Periodontal disease involves inflammatory changes at both local and systemic levels⁹. Changes in the periodontal status results in changes to circulating

inflammatory mediators. For example, levels of inflammatory markers such as IL-1 β , IL-6 and CRP have been shown to decrease after treatment of periodontitis¹⁰.

TABLE V.
PERIODONTAL DISEASE PROGRESSION

Initial Lesions (Day 2-4)	<ul style="list-style-type: none"> o Vasculitis of vessels next to JE (alteration in most coronal part) o Loss of peri-vascular collagen o Exudation of fluid from gingival sulcus o Increased migration of leukocytes (PMNs) into the JE and gingival sulcus o Presence of extra-vascular serum proteins, especially fibrin
Early (Day 4-7)	<ul style="list-style-type: none"> o Lymphoid cells (lymphocytes) o Cytopathic alterations in resident fibroblasts o Further loss of the collagen fiber network supporting the marginal gingiva o Basal cell proliferation in the JE begins
Established (2-3 weeks)	<ul style="list-style-type: none"> o Continued manifestations of acute inflammation o Plasma cells without appreciable bone loss o Presence of extra-vascular immunoglobulins in the CT and JE o Continuing loss of CT substance noted in the early lesion o Proliferation, apical migration, and lateral extension of the JE. o Early pocket formation may or may not be present
Advanced Lesion	<ul style="list-style-type: none"> o Extension of the lesion into alveolar bone and PDL with significant bone loss o Continued loss of collagen next to the pocket epithelium with fibrosis at more distant sites o Cytopathically altered plasma cells with absence of altered fibroblasts o Formation of periodontal pockets with periods of wax and wane o Fibroblasts are reduced when inflammation is present.

Table V. Progression of periodontal disease as an inflammatory disease ⁷⁵.

PERIODONTITIS CONNECTED WITH SYSTEMIC DISEASE AND INFLAMMATION

Systemic disease plays a role in the pathogenesis of periodontal disease but determining the extent of that role is a difficult undertaking. There are a number of confounding factors that present significant obstacles in the design of necessary studies. Control groups for a number of these studies are not matched for age, gender, oral hygiene and socio-economic status. Due to the more chronic nature of this disease more multi-year longitudinal studies are needed including both participants with and without systemic disease.

That being said a number of associations have been found between periodontal disease and systemic disease and inflammation. One of the most notable being in leukocyte deficiency diseases, reduction in the number or function of Neutrophils (PMNs) can increase the progressive nature and severity of periodontal destruction. Medication induced gingival overgrowth in response to dental plaque has been associated with phenytoin, nifedipine, and cyclosporine. Hormonal variation has been shown to increase the severity of plaque-induced gingival inflammation. HIV and immunosuppressive drug therapy may predispose individuals to increased periodontal destruction. The histiocytoses diseases as well as a number of other genetic polymorphisms may present as necrotizing ulcerative periodontitis. These abnormal inflammatory and immune processes that are currently being assessed as possible contributing factors in periodontal disease¹³.

More common associations have been drawn and are frequently cited in regards to periodontitis and diabetes, low or pre-term birth weights and coronary artery disease. It has been well documented that the prevalence and severity of periodontitis are increased in poorly controlled diabetics. A degree of synergism has been noted between the two diseases based on the findings that decreasing glycemic control maybe exacerbated in patients with periodontitis.

While inflammation plays an obvious role in periodontal diseases, evidence in the medical literature also supports the role of inflammation as a major component in the pathogenesis of diabetes and diabetic complications.

Research suggests that, as an infectious process with a prominent inflammatory component, periodontal disease can adversely affect the metabolic control of diabetes. Conversely, treatment of periodontal disease and reduction of oral inflammation may have a positive effect on the diabetic condition, although evidence for this remains somewhat [equivocal]⁷⁶."

It has been shown in medical literature that infections have been strongly associated with adverse pregnancy outcomes like pre-term and/or low birth weight (PTLBW). There is substantial evidence on the direct association of genito-urinary infections and the incidence of PTLBW. Numerous cases of adverse pregnancy outcomes have also been recorded without maternal genito-urinary infection but high levels of the tumor necrosis factor alpha (TNF-alpha) and prostaglandin E2 (PGE2) in the amniotic fluid. These findings alluded to the presence of infection elsewhere in the body⁷⁷. A number of studies in the periodontal literature have shown a strong

positive relationship between periodontal disease and low birth weight. Women with active periodontal disease have been shown to be 8x as likely to have Pre term or Low birth rate than women with normal healthy dentition⁷⁸. Periodontal infections may mediate PTLBW through contamination of the fetoplacental unit by periodontal pathogens or through effects of inflammatory mediators on the fetoplacental unit. Speculation exists of a cross correlation between periodontitis and PTLBW, due to documented increases in systemic inflammation as well as the presence of periodontal pathogens including, *P. gingivalis*, *T. forsythia*, *F. nucleatum* and *C. rectus* in both amniotic fluid and subgingival plaque samples in patients who gave birth to PTLBW neonates. Although few studies exist on this subject matter, more research and studies are needed before definitive conclusions can be drawn.

At this point there is not enough evidence to establish a causal relationship between periodontal disease and cardiovascular disease and or stroke. A number of common risk factors have been associated with both diseases, including smoking, diabetes, behavioral factors, age and gender⁷⁹. Although conflicting evidence exists, data is becoming more available in regards to the atherosclerotic process and the theory that infections and chronic inflammatory conditions such as periodontitis may influence its progression. These chronic inflammatory processes may increase and promote thrombi formation and platelet plugs which in turn hinder blood flow and contribute to vascular pathogenesis¹³. C-reactive protein (CRP) has been cited in a number of recent studies as a risk factor for atherosclerosis. In the periodontal literature Kumar and Shojaee found elevated levels of salivary and gingival crevicular

CRP levels in periodontitis patients as compared to healthy controls^{80,81}. Kumar found that there was a two-fold increase in the CRP levels in subjects with either coronary artery disease or chronic periodontitis compared with those of healthy individuals. As we progress in our understanding of inflammation and its roles in systemic and local etiology we can see a number of complex correlations between periodontitis and diabetes, pre term or low birth rate, atherosclerosis as well as genetic and medication induced conditions.

SUMMATION

As stated throughout this review inflammation plays an influential role in both wound healing and periodontal disease. These links between healing, inflammation and periodontitis have been hypothesized in the past but not tested with a standardized human mucosal wound model. This group has previously used a standardized wound model to demonstrate the effects of gender, aging, stress, depression, and inflammation on mucosal healing. We will use this model to assess both local and systemic inflammation in response to injury, and healing through daily measurements of wound size and measurements of inflammatory mediators in the local tissue and blood.

The thought process on altered immune response and hyper-inflammatory phenotypes lead to our current research in which we set out to determine if differences exist in both inflammation and mucosal healing among individuals with and without periodontal disease. We hypothesized that the presence of untreated

periodontal disease would relate to increases in both systemic inflammation and localized inflammatory responses, resulting in impaired mucosal wound healing and that the greater the periodontal disease severity the more exacerbated these effects would be. This pilot study was completed measuring local healing through daily video

PCR TESTING

[Developed by Kary Mullins in 1982,] Polymerase Chain Reaction [or] PCR is a biochemical technology [used] to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. [These DNA sequences can be used for a variety of applications within the research lab, including:] DNA cloning for sequencing, DNA-based phylogeny, or functional analysis of [genes]⁸².

The primary method of action relies on thermal cycling. Through numerous cycles of repeated heating and cooling, DNA is denatured (melted) and enzymatically replicated. In the first step, the DNA double helix is physically separated at a high temperature. “In the second step, the temperature is lowered and the two strands become templates for DNA polymerase to selectively amplify the target DNA. DNA primers (short DNA fragments) containing sequences complementary to the target region [are used in conjunction with DNA polymerase to enable selective and repeated amplification. The DNA generated, as PCR progresses,] is used as [the] template for replication, [setting] in motion a chain reaction in which the DNA

template is exponentially [amplified]⁸³.”

“[The typical PCR protocol] consists of a series of 20-40 repeated temperature changes, called cycles, with each cycle commonly consisting of 2-3 discrete temperature steps, usually three. The cycling is often preceded by a single temperature step (called *hold*) at a high temperature (>90°C), and followed by one hold at the end for final product extension or brief storage. The temperatures used and the lengths of time they are applied in each cycle depend on a variety of parameters. These include the enzyme used for DNA synthesis, the concentration of divalent ions and dNTPs in the reaction, and the melting temperature (T_m) of the [primers]⁸⁴.”

Below details a step by step analysis of the PCR process as outlined by Sharkey⁸⁵:

Initialization step: This step consists of heating the reaction to a temperature of 94–96 °C, which is held for 1–9 minutes. This step is only required for DNA polymerases that require heat activation by hot start hot-start PCR.

Denaturation step: This step is the first regular cycling event and consists of heating the reaction to 94–98 °C for 20–30 seconds. This causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules.

Annealing step: The reaction temperature is lowered to 50–65 °C for 20–40 seconds allowing annealing of the primers to the single-stranded DNA

template. Typically the annealing temperature is about 3-5 degrees Celsius below the T_m of the primers used. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA formation.

Extension/elongation step: At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified. As a rule-of-thumb, at its optimum temperature, the DNA polymerase will polymerize a thousand bases per minute. Under optimum conditions, i.e., if there are no limitations due to limiting substrates or reagents, at each extension step, the amount of DNA target is doubled, leading to exponential (geometric) amplification of the specific DNA fragment.

Final elongation: This single step is occasionally performed at a temperature of 70–74 °C for 5–15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.

Final hold: This step at 4–15 °C for an indefinite time may be employed for short-term storage of the reaction.

Table VI. (below) lists the primers and probes that were used for the analysis of local inflammatory mediators in both the periodontal and control subjects' biopsied tissue.

TABLE VI.
PCR PRIMERS AND PROBES USED

Mediators	DNA Primers and probes
IL-1β	F: 5'-cactgatgatgacctggaggc-3' R: 5'-tcacattgctcaggaagctaaaag-3' P: 5'FAM-ctcagaggaagaaatctacaagcctaggtcatc-3'TAMRA
IL-1α	F: 5'-ggaggcagttaacatcactgacct-3' R: 5'-ctgagcggatgaaggcga-3' P: 5'FAM-agcgagaacagaaagcaggacaagcg-3'TAMRA
IL-6	F: 5'-ccaggagcccagctatgaac -3' R: 5'-cccagggagaaggcaactg -3' P: 5'FAM-ccttctccacaagcgcttcggt -3'TAMRA
IL-8	F: 5'-gccttctgatttctgcagc -3' R: 5'-tgcactgacatctaagttcttagcac -3' P: 5'FAM-tgtgtgaagggtgcagtttgcgaagg -3'TAMRA
MCP-1	F: 5'-agaagctgtgatcttcaagaccatt -3' R: 5'-tggaatcctgaaccacttctg -3' P: 5'FAM-ccaaggagatctgtgctgacccaa -3'TAMRA
MIP-1α	F: 5'-gcatcacttgctgctgacac -3' R: 5'-tctgtggaatctgccggg -3' P: 5'FAM-accgcctgctgcttcagctacacc -3'TAMRA
TNF-α	F: 5'-atcttctcgaaccccgagtga -3' R: 5'-ggttcagccactggagctgc -3' P: 5'FAM-cccatgtttagcaaaccctcaagctga -3'TAMRA
VEGF	F: 5'-acgaagtggatgaagttcatggat -3' R: 5'-tggaagatgtccaccagggt -3' P: 5'FAM-tctatcagcgcagctactgcatccaat -3'TAMRA

I-CAM	F: 5'-gcagacagtgaccatctacagctt -3' R: 5'-cttctgagacctctggcttcgt -3' P: 5'FAM-ccggcgcccaacgtgatt -3'TAMRA
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Table VI. The table above lists the Primers and Probes used to analyze the local inflammatory mediators in both the periodontal (N=10) and control (N=10) groups through real time-PCR.

III. METHODOLOGY

PARTICIPANTS

This study was a pilot study for a potentially larger scale study that will include up to 60 adult volunteers. This pilot study involved 20 male subjects aged 24-45 years old. 10 clinically healthy individuals with a mean age of 34.7 and 10 individuals with a mean age of 34.6 who suffer from active chronic moderate periodontitis (prior to treatment) were recruited for this study. The test group in this pilot study was limited to moderate periodontitis patients due to IRB stipulations. On average the periodontitis group had clinical attachment loss of $3.28\text{mm} \pm 0.74$, with probing depths of anywhere from 2-7mm with an average of 3.37 ± 0.74 . The deeper probing depths were often the result of pseudo-pocketing due to inflammation and edema. Recruitment of this initial study group ended once complete data was obtained for 10 subjects in the periodontitis group and 10 healthy controls.

The target population included patients, students and employees of the College of Dentistry. The periodontitis group was composed of patients screened or examined in either the postgraduate periodontics clinic or the pre doctoral dental school clinic.

These individuals were solicited with study flyers in areas frequented by patients, students and university employees.

All participants in the study gave written informed consent. Monetary compensation was provided for participation. Sets of standardized questionnaires were utilized to obtain specific personal information including health history, demographics, behaviors, and any medications currently in use. Exclusion criteria included patients who had an oral disease needing emergency intervention or high-risk medical conditions that could impact surgical procedures or cause a delay in wound healing (e.g. unstable angina, diabetes, or any infectious disease such as AIDS, or TB). Wounding of the palate was performed by a licensed periodontist (P.G). All procedures were completed within the University of Illinois at Chicago (UIC) College of Dentistry post-graduate periodontal clinic and the analysis of biological samples was performed at UIC College Of Dentistry in Dr. Engeland's research lab. Approval for the study was granted by the institutional review board and ethics committee at the University of Illinois at Chicago.

WOUNDING PROTOCOL

Participants arrived and were escorted into the dental chair between 7:30 and 9:30 AM. Subjects were then asked to fill out a 10-minute questionnaire to determine mood and psychological status (e.g. depression). After completion of the questionnaire, subjects were asked to let the saliva in their mouth pool and expectorate into a 50ml polypropylene tube for 5 minutes. Once saliva collection

was complete, subjects had 2 vials of blood drawn by a nurse or phlebotomist from the clinical research center: a total of 15 ml of blood was drawn in injectable EDTA (10ml) and Heparin sulfate (5ml) coated tubes, and blood plasma was used to measure inflammatory markers.

A coin toss was done to randomly assign either the left or right side of the palate for wounding. The surgical site was first anesthetized with a 2% lidocaine solution with 1:100K epinephrine. Three excisional wounds were made into the hard palate between the first premolar and second molar approximately 3 mm from the marginal gingiva. The most posterior wound was created using a 3.5 mm tissue punch for symmetry and standardization. Surface epithelium and superficial connective tissue were removed with a 15-blade scalpel to create wounds with 1.5 mm in depth. The wound site was left exposed to the oral cavity; no surgical or periodontal dressing was placed. Participants were instructed to resume their standard oral hygiene regimens but were asked to abstain from using alcohol-based mouthwash. Post-operative instructions were given to outline limitations on diet including no chips, hard breads, or anything sharp that might compromise the wound area. Two longitudinal wounds (1 x 5 x 1.5 mm) were then made anterior to the initial round wound. Later a 2 x 5 x 1.5 mm biopsy of these wounds was acquired at first 6 h and then at 24 h after initial wounding. Tissues were flash frozen in liquid nitrogen at time of collection for gene expression analysis. Real-time PCR was used to measure gene expression for inflammatory mediators. Gene expression for TNF- α , IL-1 β , IL-1 α , I-CAM, IL-6, IL-8, MCP-1, MIP-1 α , and VEGF was

determined in all tissues obtained at 0, 6, and 24 hours.

WOUND SIZE ASSESSMENT

The 3.5mm round wounds were then video graphed at 24-hour intervals for the next 7 days. A standard 6mm round guide was placed around the wound as a template to achieve standardization and account for variations in magnification and angulation of the video graph. Computer screen shots were taken from these video graphs and later blind coded for unbiased measurement of wound surface area.

Two separate blinded assessors (A.M., M.M.) measured all the wounds and these values were averaged. The 6mm standard label was used to express final measurements as a ratio of wound size to standard. Comparisons and ratios were then made of daily changes in wound size to the original (initial day of wounding). This objective wound closure measurement method was derived for and has been used extensively in both dermal⁸⁶ and mucosal^{87,14} wound healing studies in humans.

DETAILED CLINICAL PROTOCOL

This study was coordinated by investigator (A. M.) and consisted of a total of 10 visits, including the initial screening visit:

Initial screening session - (Approximately 30 minutes) Was completed over the phone and/or at the patient's initial visit. This session was held to: 1) ensure subject eligibility through a health questionnaire 2) explain the study; 3) take a

measurement of the subject's height and weight; and 4) obtain the subject's agreement (informed consent) to participate.

Day 0 – Subjects (1-4 per day) arrived at the periodontal clinic at the College of Dentistry between 7:00-9:30am and were seated in a dental chair. First, subjects were asked to fill out health questionnaires and psychosocial surveys as well as vendor forms to allow for study participation payment. Next, subjects were asked to passively expectorate into a pre-weighed, ice chilled polypropylene test tube over a timed 5 minute period. The collected saliva was vortexed for 30 seconds and clarified by centrifugation (5000 xg, 6 min, 4°C). The supernatant was aliquoted (6 x 400µl) and stored at -80°C until assayed. This portion of the study was modeled after previous unstimulated saliva collection first described by Navazesh in 1993⁸⁸. An oral examination, including a periodontal evaluation and charting was then conducted by a certified periodontist (PG) before any procedures were performed on the subject by the CRC staff. Subjects were then asked to fill out a small batch of questionnaires regarding their current health, along with an assessment of psychosocial indices (e.g., depressive symptoms, stress) which may impact healing rates. If subjects expressed thoughts of suicidal ideation (as determined by questionnaire) they were referred to their primary care physician or the psychiatric services clinic at the UIC medical center for immediate follow up.

The wounding protocol was initiated between 7:30-10:00 am. Participants were asked to eat breakfast at least 1 h prior to their arrival time and to refrain from any food or beverage after that except for water until the wounding was completed.

Subjects were comfortably seated for 30 min prior to blood draw. A clinical research center (CRC) nurse then obtained blood pressure and inserted an intravenous (iv) catheter with a heparin lock into the subject's non-writing arm. Two vials of blood: A total of 15 ml (one 10ml EDTA coated tube and one 5 ml heparin coated tube) were drawn for cytokine assessment. Collected blood sat for no more than 45 minutes, the EDTA tube sat on ice and the Heparin tube sat at room temperature. Samples were then centrifuged at 1500 g for 15 min at 4° C, the supernatant (plasma) was then removed by pipette, and 6 x 400ul aliquots were stored at -80°C. A subset of heparinized plasma was treated with LPS to assess immune responses (see detailed blood work protocol below). Blood samples were used to measure levels of inflammatory mediators and growth factors in both normal and periodontitis groups through multiplex bead arrays.

A licensed periodontist (P.G.) performed all oral wounding procedures. Three mucosal wounds were placed on the day of wounding (Day 0). The first wound was precisely placed between the first and second molars, 3 mm from the gingival margin (to edge of wound). Care was taken to avoid close proximity to the greater palatine nerve and artery. The side of the mouth in which the wounding was performed was randomized by a coin toss. If the subject was identified to have

periodontal pocketing in the region, or anatomical variations, that would impact the wound site, the wounds were placed slightly anterior to this position. Wounds were not placed in a site adjoining a tooth with loss of attachment of greater than 8mm, or a tooth with class III mobility, or in an area where any periodontal abscess was present.

Prior to wounding the site was anesthetized with 2% lidocaine used to block the greater palatine nerve. Infiltration around the site was also used. A 3.5 mm tissue punch was then used to outline the initial wound site. Surface epithelium and superficial connective tissue was then removed using a 15-blade scalpel, to make a relatively uniform 1.5mm deep wound. Two additional wounds were placed to assess gene expression during healing. These were placed with a double bladed scalpel in a line anterior to the first wound and measured 1x5 mm, and again were 1.5 mm deep.

Wounds were not dressed and, with the exception of avoiding the use of alcohol-based mouthwash, subjects were instructed not to change their normal oral hygiene procedures.

Day 0/Day 1 – The longitudinal (1x5mm) wounds were biopsied, one at 6 hours and one at 24 hours post-wounding. The biopsy was harvested by removing the entire wound and the surrounding tissue; again this was performed with a double bladed scalpel for precision. The biopsy sites measured 2 x 5 mm and 1.5mm in depth so as

to include the wound edge. 2% lidocaine was administered at the times of biopsy to anesthetize the surgical area. An intra-oral videograph of the 3.5mm wound site was taken at this time as well as at the 24 hour time point (approx 9 am).

To date over 700 of these oral mucosal wounds have been placed in four previous studies. No participant has exited the protocol because of discomfort after wound placement or due to sequelae of the wounds.

Day 2-7 – Intra-oral video graphs of the circular wound were taken at 24-hour intervals for 7 days. Most wounds were close to fully healed by the end of the first week. A 6mm round standard sized stent was positioned over the 3.5mm round wound to correct and standardize any angulation or magnification errors. The wound images were taken on a computer video graph and later coded for blinded interpretation and analysis. The still screen shots taken from the video graphs were then measured and recorded as a ratio of the circular wound area to circular standard stent area by two separate blinded research team members (A.M. and M.M.). The two groups of measurements were then averaged and those final values were statistically analyzed using Statistical Package for the Social Sciences (SPSS, Chicago, IL.).

DETAILED BLOOD WORK PROTOCOL

Blood samples:

Once the blood was drawn on Day 0, a subset of blood (1ml) it was stimulated with 1ng LPS ex-vivo to induce an inflammatory response so that inflammatory mediators of interest (IL-6, IL1- β , IL-8, TNF- α , GMCSF, IL-4) could be measured in a dynamic fashion. Samples were then incubated at 37C in 5% CO₂ for 4 hours. All tubes were then spun at 1500 g for 15 minutes at 4°C and two aliquots were then prepared. All samples were divided equally between two freezers (-80C) and stored in sets based on collection time and blood treatment. A procedure flowchart is illustrated below (Source: Invitrogen website; Figure 3.)

Figure 3.

**Procedure
Flowchart**

The following diagram provides an overview of the procedure described in this protocol.

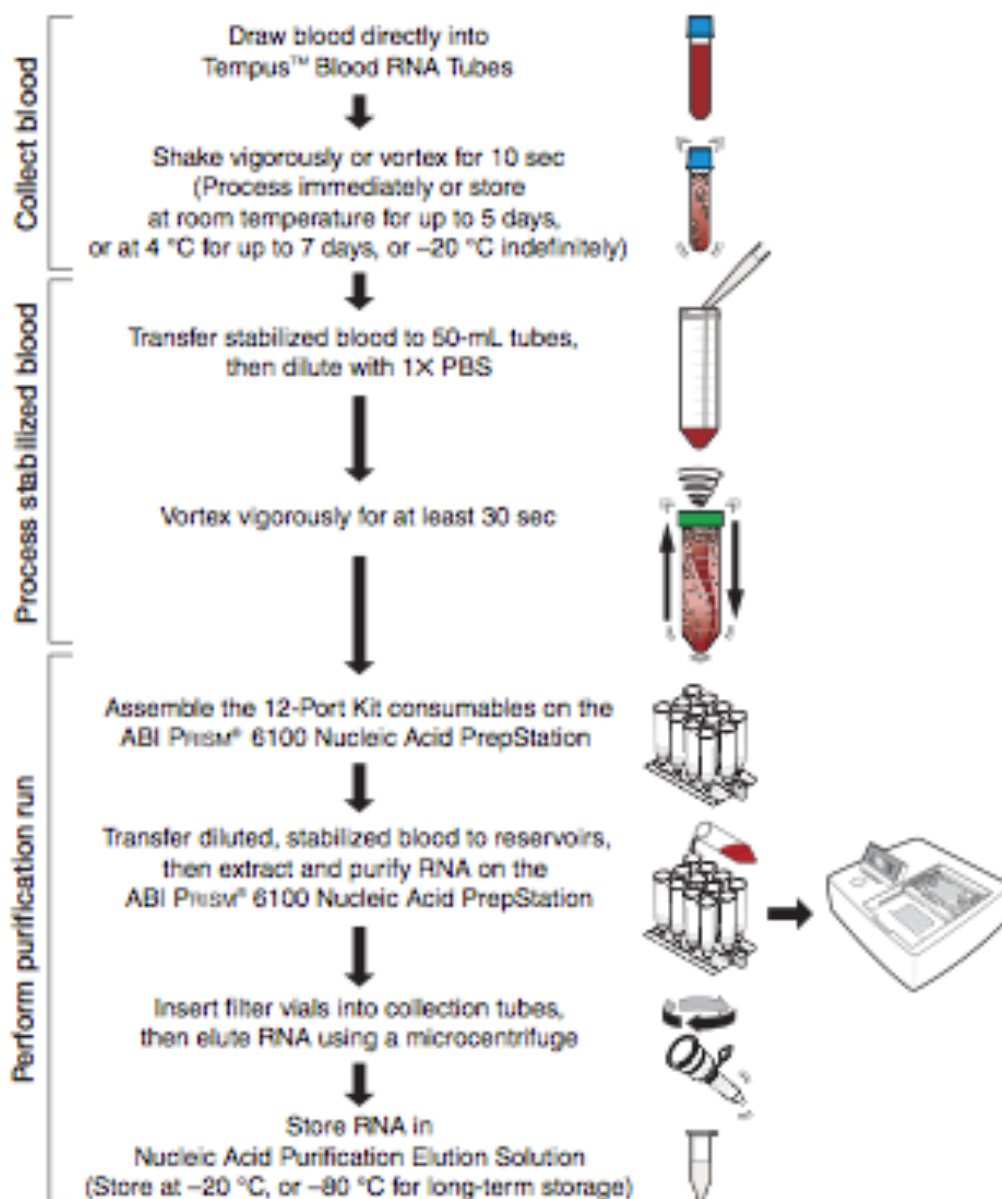


Figure 3. Outlines the steps of blood preparation and RNA storage.

DETAILED PCR PROTOCOL

TRI Reagent solution was ordered from Applied Biosolutions through Invitrogen Corporation in order to isolate RNA from the blood using the Trizol method. A brief summary of the process as provided via the Invitrogen website is outlined below:

[The Trizol method is a] highly reliable technique with samples larger than ~5 mg tissue or 5×10^5 cultured cells, [which can be used on human, animal, plant, bacterial, yeast and viral samples. The] TRI Reagent solution combines phenol and guanidine thiocyanate in a monophasic solution to rapidly inhibit RNase activity. [In this procedure the] biological sample [was] homogenized or lysed in TRI Reagent solution, and the homogenate was then separated into aqueous and organic phases by adding bromochloropropane (BCP) and centrifuging. [After centrifuging is complete] RNA partitions to the aqueous phase, DNA to the interphase, and proteins to the organic phase. Next the RNA [was] precipitated from the aqueous phase with isopropanol, and finally it [was] washed with ethanol and solubilized.

Once the RNA was isolated a poly(A) tail was added to the miRNA by diluting the Poly A Polymerase 1:1 with DEPC-treated water, mixing gently and centrifuging. The tube was then incubated in a heat block at 37°C for 15 minutes. After incubation first strand cDNA synthesis was conducted, by reverse transcribing the polyadenylated miRNA to generate first strand cDNA. This was completed by adding polyadenylated RNA, annealing buffer and Universal RT Primer to a polypropylene tube and incubating at 65°C for 5 minutes. The tube was then placed on ice for 1

minute before 2X First-strand Reaction Mix and SuperScript III RT/RNaseOUT Enzyme Mix were added to the tube. The contents were then spun briefly and transferred to a thermal cycler that was preheated to 50°C and incubated for 50 minutes. The aliquots were then incubated at 85°C for 5 minutes to stop the reaction as per the manufacturers instructions. At this point the aliquots can be stored at -20°C or can be run for real time PCR.

Real time PCR was then run on the aliquots by first diluting the cDNA 1:10 in DEPC-treated water. 5 µL of diluted cDNA was used per 50-µL reaction (i.e., 1% v/v cDNA). DNase/RNase was then added to the plate well. The real-time PCR instrument was then programed with the cycling program designed for Applied Biosystems® instruments. First the sample was incubated for 2 minutes at 50°C (UDG incubation) followed by 10 minutes at 95°C (UDG inactivation and DNA polymerase activation). After initial incubation was completed the RT PCR instrument ran 40 cycles of: 95°C for 15 seconds followed 57°C for 60 seconds. After cycling, the reaction was held at 4°C until further analysis of the cycle threshold (Ct) values, slope of the standard curve, Y-intercept, and correlation coefficient was analyzed and computed.

STATISTICAL ANALYSIS

Data analysis was performed using **Statistical Package for the Social Sciences** (SPSS) software (Chicago, IL. Version 19.0) Probing depths (PD), Clinical attachment loss (CAL), and Bleeding on probing (BoP) were assessed for significance between the untreated periodontal and control groups. The mean, standard deviation and

standard error were derived, and significance was determined using an independent T-test. Analysis of variance (ANOVA) with repeated measures was used to assess differences in healing rates between groups. Independent T-tests using a Bonferroni correction were used to assess between-group differences in plasma cytokine levels. Significance level (α) was set at 0.05 (5%) to determine significance. Error bars represent standard error of the mean (SEM).

IRB APPROVAL

This study methodology was reviewed and approved by the UIC Institutional Review Board (IRB Protocol: #2010-0187) SEE APPENDIX. All subjects gave informed consent for this study and received financial remuneration for their time.

IV. RESULTS

DIFFERENCES IN PERIODONTITIS VS. HEALTHY CONTROL GROUPS

The mean probing depth (PD) was 3.37 ± 0.74 mm in the periodontitis (test) group and 1.67 ± 0.78 mm in the healthy (control) group. The mean bleeding on probing (BoP) was $72.0\% \pm 31.5\%$ in the periodontitis (test) group and $1.2\% \pm 1.8\%$ in the healthy (control) group. The clinical attachment loss (CAL) was 3.28 ± 0.74 mm in the periodontitis (test) group consistent with moderate periodontal disease and 1.62 ± 0.76 mm in the healthy (control) group. It was found that all three of the measured periodontal criteria were significantly different ($P < 0.001$). Tables VII and VIII

below detail these measurements with corresponding standard deviation and standard error, respectively:

TABLE VII.
AVERAGE PROBING DEPTH, BLEEDING ON PROBING AND CLINICAL ATTACHMENT LOSS (WITH STANDARD DEVIATION)

Mean \pm S.D

	Periodontitis (n=10)	Control (n=10)	P-value
PD	3.37 \pm 0.74	1.67 \pm 0.78	<0.001
BoP	72.0% \pm 31.5%	1.2% \pm 1.8%	<0.001
CAL	3.28 \pm 0.74	1.62 \pm 0.76	<0.001

Table VII. T-Independent test results with mean and standard deviation between probing depth, bleeding on probing and clinical attachment loss between periodontitis group (N=10) and healthy controls (N=10)

TABLE VIII.
AVERAGE PROBING DEPTH, BLEEDING ON PROBING AND CLINICAL ATTACHMENT LOSS (WITH STANDARD ERROR)

Mean \pm S.E.

	Periodontitis (n=10)	Control (n=10)	P-value
PD	3.37 \pm 0.23	1.67 \pm 0.25	<0.001
BoP	72.0% \pm 10.0%	1.2% \pm 0.6%	<0.001
CAL	3.28 \pm 0.23	1.62 \pm 0.24	<0.001

Table VIII. T-Independent test results with mean and standard error between probing depth, bleeding on probing and clinical attachment loss between periodontitis group (N=10) and healthy controls (N=10)

These data support that significant differences existed in the periodontal health group (control) and the moderate periodontitis (test) group. From these groups we set out to gather information on wound healing rates and local inflammatory markers in the wounded tissues; the findings are outlined below.

WOUND HEALING RATE

Using standardized video-graphic measurements it was found that over a 7 day follow up period, wound healing in healthy young men with periodontitis was similar to that in healthy young men without periodontitis when wounds were placed in an unaffected area of the mouth. These results are portrayed below.

Figure 4.

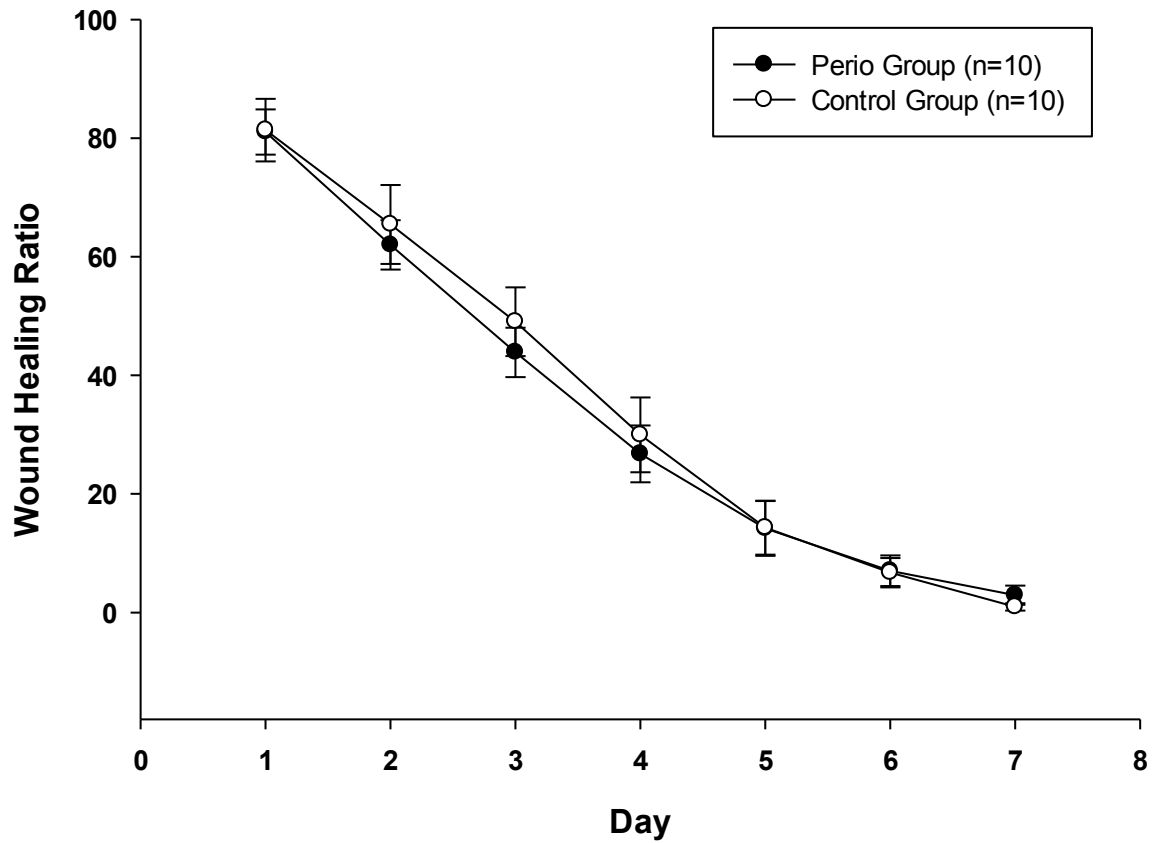


Figure 4. Wound Healing Rate: No statistically significant differences were found between the periodontitis (N=10) and control (N=10) groups for healing rates (wound sizes) on any day.

LOCAL INFLAMMATION

Gene expression for Local inflammatory mediators (TNF- α , IL-1 β , IL-1 α , IL-6, IL-8, MCP-1, MIP-1 α , VEGF, and I-CAM) were measured at the time of wounding (0 hours), and at 6 hours and 24 hours post-wounding through palatal tissue biopsies. These tissue samples were processed and mRNA measurement was performed using real-time PCR as described in the methods section. The results show that local inflammation at baseline (0 hours) and after wounding (6 and 24 hours) was similar between healthy young men with periodontitis and healthy young men without periodontitis in an unaffected area of the mouth. At baseline, 0 hours (time of wounding) and 6 and 24 hours post wounding (time points chosen to depict early and late inflammatory response), results indicate that inflammatory mediator gene expression is relatively the same at all time points (no statically significant differences were detected). The figures below (figures 5. and 6.) convey these findings for each individual inflammatory mediator measured. These inflammatory mediators varied considerably amongst individuals, accounting for the large error bars noted in the figures below.

Figure 5.

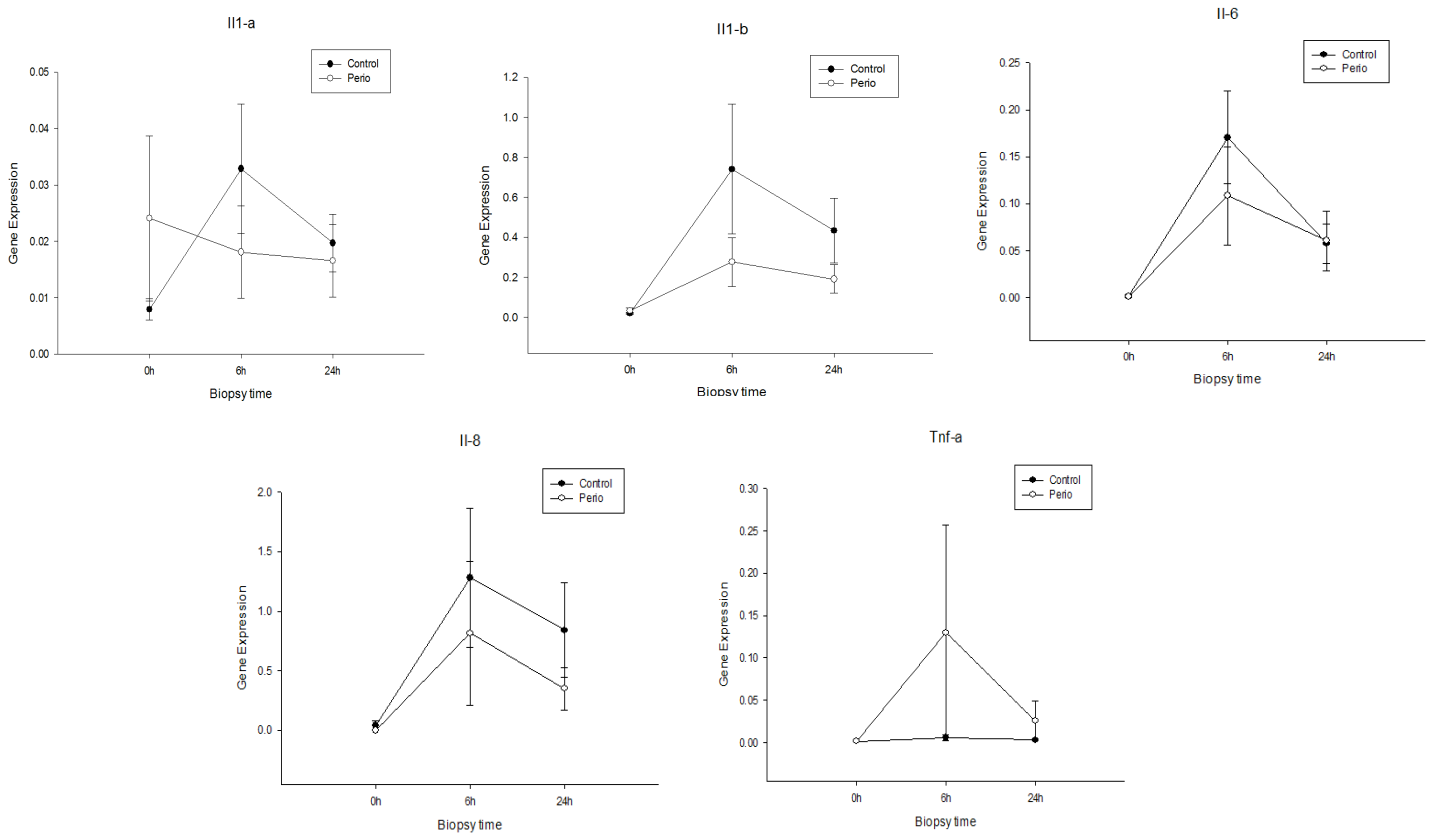


Figure 5. Denotes local inflammatory cytokines that were measured through real time PCR. No statistically significant differences were found at any of the time points 0h, 6h and 24h between control (N=10) and Periodontitis (N=10).

Figure 6.

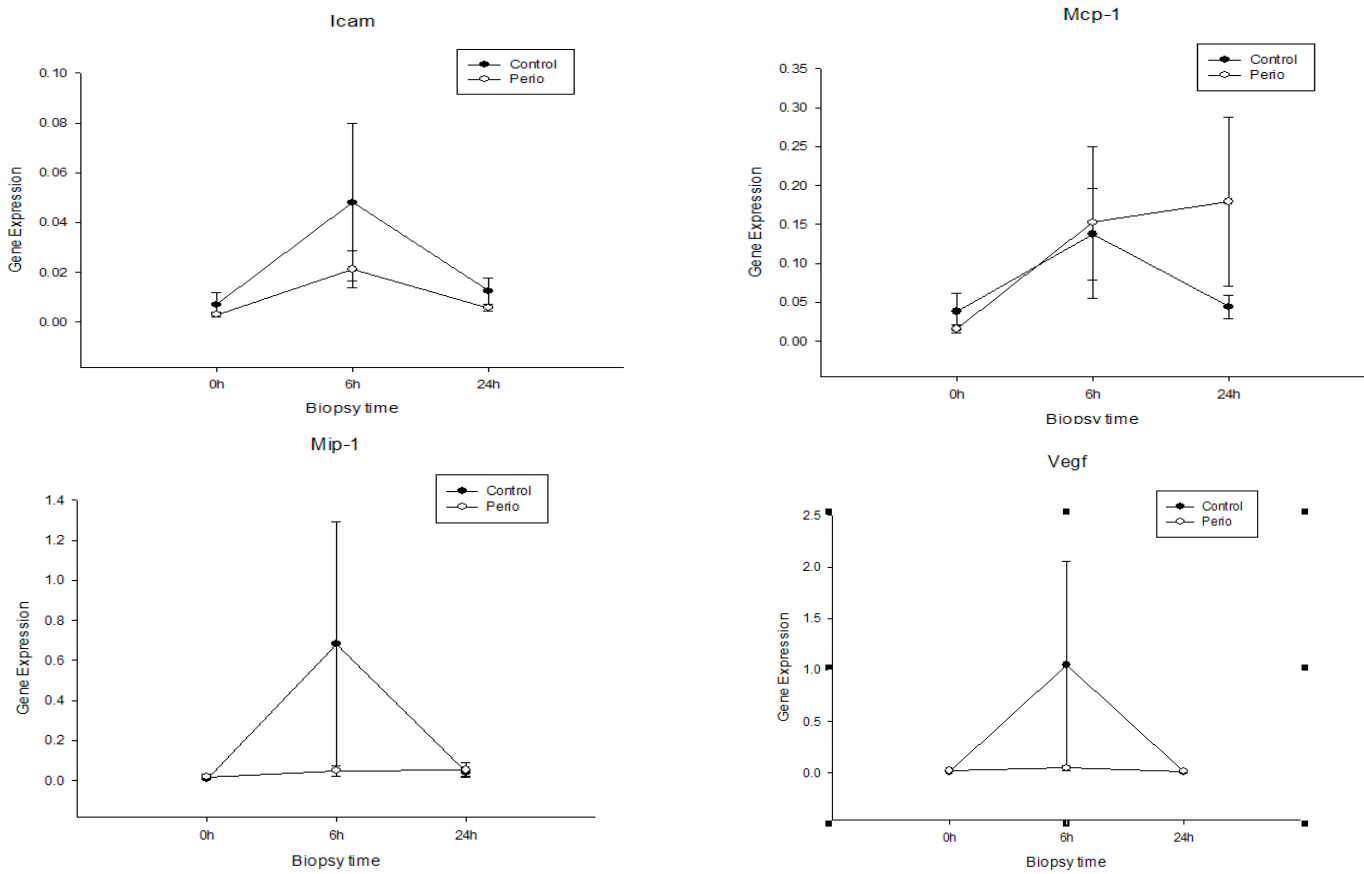


Figure 6. Denotes local inflammatory chemokines, adhesion molecules and growth factors that were measured through real time PCR. No statistically significant differences were found at any of the time points 0h, 6h and 24h between control (N=10) and Periodontitis (N=10).

SYSTEMIC INFLAMMATION

Analysis of systemic inflammatory mediators was completed by using a multiplex micro-bead array. First in non-stimulated (baseline) blood samples, protein levels were determined for a subset of common inflammatory mediators including IL-1 β , IL-6, TNF- α , IL-8, GMCSF, and IL-4. No significant differences existed in baseline systemic inflammation between the two groups, as noted in Figure 7. Similarly, as shown in Figure 8 below, no significant differences were found in the inflammatory responses (levels of the same inflammatory mediators) to *ex vivo* LPS stimulation between these young healthy, males with periodontitis (n=10) and age matched controls (n=10). Systemic inflammation (in blood) was similar among young healthy non-smoking men with periodontitis and control.

Figure 7.

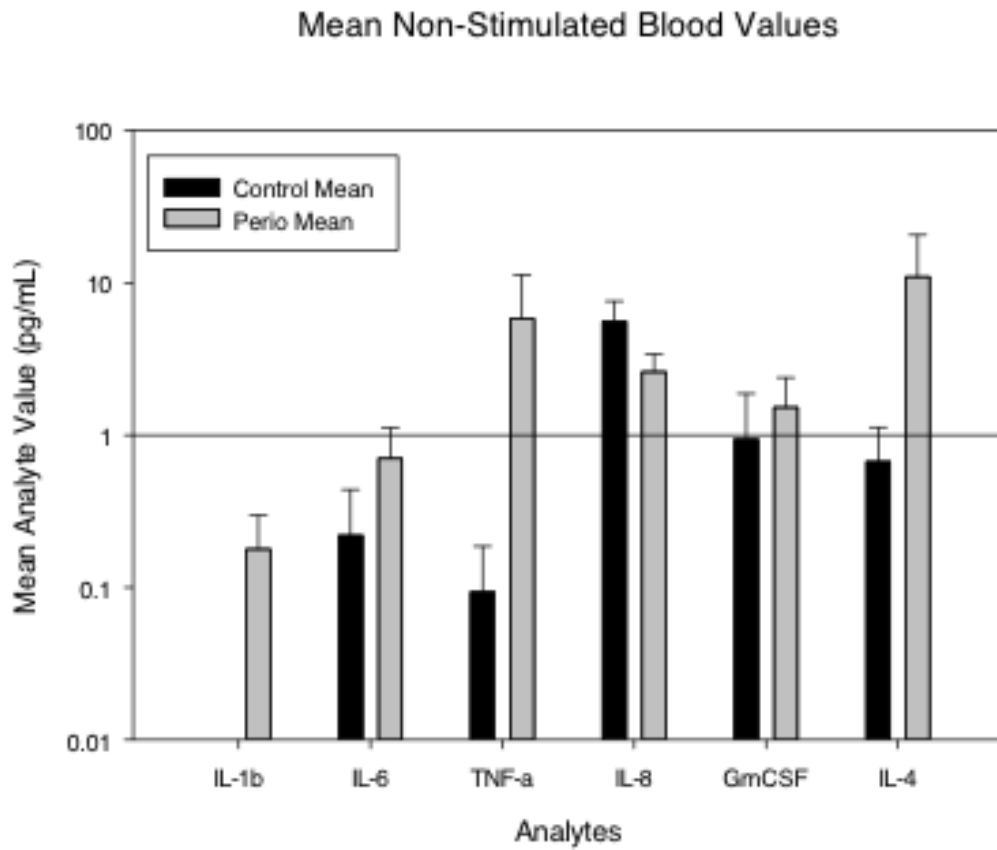


Figure 7. Cytokine analysis at baseline (non-stimulated blood): No significant differences existed in systemic inflammatory markers at baseline, prior to stimulation with LPS ex-vivo, between individuals with periodontitis (n=10) and controls (n=10).

Figure 8.

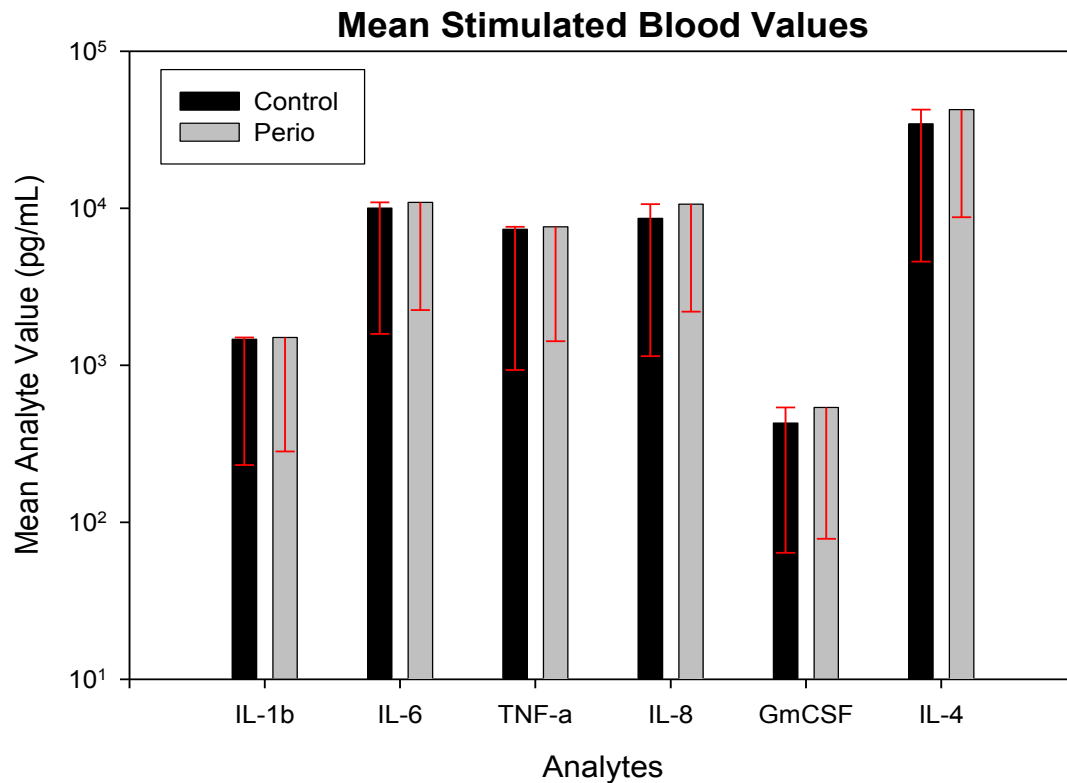


Figure 8. Cytokine analysis in stimulated blood: No significant differences existed in inflammatory responses to *ex vivo* LPS stimulation between individuals with periodontitis (n=10) and controls (n=10).

CORRELATIONS BETWEEN LOCAL AND SYSTEMIC INFLAMMATION

Statistical analysis was run on each of the inflammatory mediators in this study to determine if any correlations were evident when comparing local versus systemic levels of inflammation. Inflammatory levels in unwounded tissue (gene expression) obtained at 0h were compared to protein levels in baseline blood, and inflammatory responses in wounded tissue (gene expression) obtained at 6h and 24h post-wounding were compared to protein levels in LPS-stimulated blood. Analytes were

only compared to themselves (e.g., IL-6). No significant correlations were found for any of these analyses.

V. DISCUSSION

This study demonstrates that in young healthy male subjects with untreated periodontal disease there are no differences in mucosal wound healing and inflammation in unaffected sites as compared to age-matched healthy young males without periodontal disease. Palatal tissue samples, away from any sites of active periodontal infection, were excised and analyzed. Over the first 7 days of wound healing no visual differences in wound size between the control and perio group were detected by the blinded clinical examiners using a standardized wound measurement protocol (measuring wound diameter against a 6mm standard). Local inflammation was measured at 0 hours (baseline), 6 hours and 24 hours (early and late inflammatory points) by evaluating tissue biopsy samples. mRNA levels, for key inflammatory mediators, was measured through real time PCR. Gene expression in tissue at 0 hours was used to analyze unwounded tissue. There were no differences in inflammation found in the sampled tissues between groups. This is an important observation as it suggests that unaffected oral mucosal tissue does not appear different in the presence of periodontitis. This differs from the results of previous studies that have shown elevations in local inflammatory mediators, IL-1 (both IL-1 α and IL-1 β) and TNF- α , in active periodontal disease sites, i.e. sulcular and junctional epithelium of diseased teeth⁸⁹. To our knowledge, the results of this

study are the first to address unaffected sites in the mouth of periodontal subjects. From our data we determined that in these unaffected areas of the mouth, young healthy male periodontal subjects did not have increased tissue inflammation at unaffected sites of the mouth. These differences may be due to the analysis of an unaffected areas of the mouth and the exclusion of potential confounding variables such as the comorbidities of age, smoking, diabetes.

Gene expression at 6 hours, 24 hours (early and late points of inflammation) was used to analyze potential differences in inflammation in the tissue in response to injury. Again no differences in inflammatory responses were found in the tissue between groups. Although conflicting data exist, a number of papers have shown higher numbers of inflammatory mediators in saliva in the presence of periodontitis. Significantly higher levels of TNF- α , IL-6, and metalloproteinase-8 (MMP-8) have been observed in cases of periodontitis compared to gingivitis and healthy adults^{90, 91, 92}. Other studies, reported increased levels of salivary inflammation with increase severity of periodontitis; these studies reported a two-fold increase of IL-1 β in periodontal patients and demonstrated that IL-6, IL-1 β and calprotectin concentrations in saliva could reflect the degree of gingival inflammation^{93,94}.

From these findings and our data we conclude that, despite increases in circulating inflammatory mediators in surrounding saliva, inflammatory response in wounded oral tissues are not altered by periodontitis. This was found to be the case at both

early and late time points of inflammation (6 and 24 hours). It seems that both overall tissue inflammation and the rate of repair are not impaired in these subjects. This is a noteworthy finding; despite these wounds being open to the oral environment and being bathed in saliva shown to be high in inflammatory mediators, no compromises were seen in the local areas of wound repair.

Although more analysis and a greater population size is needed, preliminary data indicate that local inflammation and the resultant level of inflammatory mediators in this subset of the population are no different at baseline (0 hours), at 6 hours or at 24 hours between healthy male subjects with untreated moderate periodontal disease and subjects with healthy periodontium.

We also examined systemic inflammation in the blood of these subjects; it was found that blood measured at baseline (no stimulation with LPS) and stimulated blood with lipopolysaccharide (LPS) had no differences in inflammation between the periodontal (test) group and the normal (control group). In-vitro methods were used to analyze specific inflammatory markers (IL-1 β , IL-6, TNF- α , IL-8, GMCSF, and IL-4) and once again no differences were observed between the test and control groups in any of these analytes. Some studies have shown increased inflammatory levels at baseline, however our results contradict some of this current literature which states that “the total [number] of leukocytes [(neutrophils), IL-6,] and plasma levels of C-reactive protein (CRP) are consistently higher in periodontitis patients compared [to] healthy controls ^{95,96}.” “Kweider et al. were the first to report higher

numbers of [leukocytes] in periodontitis [(8.7 x 10⁹/l in periodontal patients in comparison to 6.0 x 10⁹/l in controls)]⁹⁷." Other studies have reported that subgingival red complex bacteria (*Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola*) and CRP levels were increased in untreated chronic moderate to severe periodontitis cases. Although a number of studies have reported on this subject matter, the data are still insufficient to support a true causal relation between periodontal infections and subsequent systemic inflammation leading to cardiovascular disease. These studies all state that further research is required^{98,81}. Although our research did not directly test for numbers of leukocytes and CRP, we did test for related inflammatory mediators that are instrumental in the recruitment and proliferation of these cells, we did find that the levels of these systemic inflammatory mediators, including IL-6, did not seem to be elevated in our untreated moderate periodontal disease group in comparison to our healthy control group. Therefore our data only contradicts results found for IL-6 levels in the blood, at this time we cannot speak to the levels of CRP and leukocytes because they were not directly measured in this study.

Similarly, systemic inflammation in blood stimulated with LPS was measured to assess any differences in these mediators. Based on previous periodontal research and the theory that periodontal disease is a hyper-inflammatory disease one would expect to see much higher levels of inflammatory responses in the periodontal group's blood stimulated with this bacterial endotoxin. This, however, was again not the case. No significant differences were found between the test and control

groups when inflammatory analytes were compared. Statistical analysis was also run on each of the inflammatory mediators in this study to determine if any correlations were evident when comparing local versus systemic levels of inflammation. As stated earlier these analytes were only compared to themselves (e.g., IL-6 to IL-6) and no significant correlations were found for any of these analyses as well.

This leads to the question of why group differences and local vs. systemic variations were not evident? It may be that other studies had more confounding variables (comorbidities) that may have lead to higher levels of inflammation in the blood of those periodontal patients. However there are studies, such as the Loos 2000 study, in which, IL-6, CRP and levels of leukocytes were elevated in both local and generalized periodontitis patients in which con-founding variables such as age, education, body mass index, smoking, or any other medical disorder were supposedly controlled for⁹⁶. That would lead one to believe that this discrepancy in findings may be due to the degree of periodontal disease in this study. Patients with moderate periodontal disease were chosen and it may be that this level of disease was not severe enough to cause inflammatory spill over into the systemic circulation.

With continued research, an improved understanding of the relationship between periodontal disease and systemic inflammation may allow for more informed and proactive health care decisions. Although these previous studies have reported

systemic changes in inflammation, the discrepancy in this study's results again may have to do with the level of periodontal disease and inflammation or the lack of other confounding variables in these subjects such as co-morbidities or smoking. "It has been shown that, with increasing severity and extent of [periodontitis,] the peripheral numbers of white blood cells increase (dose response)⁹⁶." In the majority of previous studies, participants had untreated severe periodontal disease. This study's untreated periodontal disease group, however, included more moderate disease (rather than severe).

The results in this study contradict the current understanding of both periodontal inflammation and wound healing.

[The commonly agreed upon primary] etiology of periodontal diseases is bacteria. The human oral cavity harbors a [great number of] continuously evolving microbial species. The [pathogenesis of periodontal] diseases is mediated by the inflammatory [response] to bacteria in the dental biofilm. [The inflammatory interactions, mounted by the host defense upon these microbes,] determine the severity of the disease. Unlike many infectious diseases, periodontal diseases [appear] to be mediated by the overgrowth of commensal organisms, rather than by the acquisition of an exogenous pathogen. There is evidence that specific microbes are associated with the progressive forms of the disease; however, these microorganisms [have been observed] in individuals with no evidence of disease progression. [This] suggests that the disease is the net effect of the immune response and the

inflammatory processes, [and] not [merely due to] the presence of the [bacteria]¹².

In 1986, Theilade described and supported the Non-Specific plaque hypothesis theory. This paper postulated that in the absence of oral hygiene, indigenous oral bacteria colonize the gingival crevice to form plaque. This bacterial plaque colonization was further proposed to be the causative mechanism of inflammatory periodontal disease progression due to uncontrolled bacterial plaque proliferation above the threshold of host resistance⁹⁹. In 1988, Socransky and Haffajee supported a slightly different philosophy, the specific plaque hypothesis theory, in which a single pathogenic species or complex is theorized to cause inflammatory periodontal disease. They pointed to a particular group of bacteria including *Bacteroides forsythus*, *Porphyromonas gingivalis* and *Treponema denticola*¹⁰⁰.

Whether one chooses to support the specific or non-specific plaque hypothesis, it has been shown in numerous studies that the interaction between the host inflammatory response and pathogenic bacteria is what initiates and stimulates the progression of periodontal disease.

A number of studies, some conducted by our research group, have shown that discrepancies in inflammation can cause delayed wound healing. Factors as benign as examination stress have been found to alter inflammatory function of IL-1 β leading to differences in the rate of oral mucosal wound healing as great as 40%⁶⁷.

This cytokine up-regulates adhesion molecules on endothelium and induces the expression of chemokines and, thus, is important in inflammatory cell [recruitment]¹⁰¹. IL-1 also activates fibroblasts to produce keratinocyte growth factor, which induces keratinocyte proliferation and migration, key steps in reepithelialization of the wound. Furthermore, it induces the production and activation of metalloproteinases, which are required for [the] initiation of keratinocyte migration and in remodeling of the [wound]⁶⁷.

These previous findings on deregulation of the inflammatory process and altered wound healing, coupled with our acute awareness of inflammation and its effect on the periodontium, sparked our group's interest in the interaction between periodontal disease and wound healing.

As previously discussed, it has been shown in a number of studies that inflammation as a response to pathologic bacteria can cause periodontal disease. Further altered inflammatory responses can lead to impairment of wound healing. These results have typically been found in patients with severe periodontitis and have looked at inflammation and wound healing in directly affected tissue sites^{102,103}. These previous studies have investigated tissue response and inflammatory markers/wound healing within the gingival crevicular fluid (GCF) and/or sulcular epithelium around periodontally diseased teeth. To our knowledge no studies have looked at periodontal inflammation and its relation to unaffected tissues in the oral cavity (palatal gingiva). This study is the first to examine this.

Although this is a pilot study with a limited sample size and very specific inclusion criteria, the results suggest that the current beliefs and understanding of this subject matter need to be reassessed and re-evaluated.

Beliefs about wound healing and periodontal disease have been generalized over time to encompass the patient population as a whole. Based on the findings of the present study, these beliefs need to be re-evaluated on a more individual basis. Within the study, results indicate no statistically significant differences between the groups, thereby nullifying the hypothesis that the presence of untreated periodontal disease will relate to increases in inflammatory responses (either localized or systemic), resulting in impaired mucosal wound healing. These findings indicate that mucosal healing in areas of the mouth unaffected by periodontal pathogens heal at similar rates regardless of the presence or absence of periodontitis. Furthermore, we found that the level of inflammatory mediators in unaffected sites, both local and systemic, did not appear to be significantly different between young healthy non-smoking males with normal healthy gingiva and those with moderate periodontal disease. These levels were tested both at baseline (0 hours) and at early and late inflammatory time points (6 hours and 24 hours post wounding).

This being said, there are subsets of the population; older individuals, post-menopausal women, smokers and individuals with systemic conditions such as diabetes that have been found to have delays in mucosal wound healing and need to

be addressed individually^{14,63,104}. It is unknown if the present findings would apply to these specific groups.

LIMITATIONS OF THE STUDY

Due to time and resources this study was limited in its number of participants. This limited the power of the analysis of the study. This pilot study was intended to serve as a proof of concept. At its inception the thought was that patients with more advanced periodontal disease and inflammation would have dysregulated inflammation and slower wound healing than those that were unaffected by periodontal disease. The intention of this study was to eliminate as many confounding variables as possible, to deliver the most unbiased data possible. A number of confounding variables have been found to affect inflammation and wound healing including hormonal changes, smoking, advanced age and diabetes. Hormonal changes, specifically lower levels of testosterone have been related to faster healing times in both young men and young women¹⁴. “[Wounds in post-menopausal women also heal more slowly, and have been] characterized by increased neutrophil influx, protease production, decreased phagocytosis, and excessive inflammation [resulting in delayed re-epithelialization and reduced collagen deposition]^{14,105}.”

Strong epidemiological evidence indicates that smoking confers a considerably increased risk of periodontal disease and delayed mucosal healing¹⁰⁶. A number of studies have reported on age as another variable shown to influence wound healing,

as well as periodontal disease progression^{70,107}. As we age periodontal disease progression increases and our ability to heal is slowed. Systemic disease such as diabetes are frequently cited as contributing to the severity of periodontitis and delayed or inadequate wound healing¹⁰⁸.

For these reasons age, sex, smoking and systemic disease were eliminated from this pilot study to minimize confounding variables. Age and sex were limited to young males with a maximum age of 45 years old. Due to these limitations we had a very narrow population subset, which limits the generalizability of the current results.

FUTURE RESEARCH

Future studies could be conducted to expand these preliminary results by utilizing similar methodology with larger sample size populations. With the increase in sample size, individual variance would be reduced. These more diverse and larger population subsets would include a wider age range, women and more severe cases of periodontitis. Studies could even expand into looking into groups with systemic diseases such as diabetes or coronary artery disease. Post partum women and pregnant women in their second trimester with periodontal disease could be compared to healthy pregnant controls. Additional inflammatory mediators could be measured both locally and systemically; such as IL-2, IL-4, EGF, FGF, PDGF and IL-10 as they are other mediators commonly found in inflammation.

VI. CONCLUSION

The present results suggest that periodontal disease and its associated inflammation, even when untreated, does not affect healing rates in unaffected oral tissues in young healthy adult males. Despite higher levels of inflammation in periodontally diseased tissues, baseline inflammation and inflammatory responses to wounding in an unaffected site (in local biopsied tissue) appeared similar between young healthy male controls and young healthy male periodontitis subjects.

These results may have clinical implications in the management of surgical procedures and implant placement into unaffected tissue sites in this patient population. Although we do not recommend carte blanche implant and restorative treatment of patients with periodontal disease, some clinical situations may be addressed prior to periodontal therapy. If a patient with periodontal disease is in need of urgent care, a clinician can more prudently address the problem without the worry of impaired tissue healing in this population subset.

In young healthy adult males, tissue repair seems to occur normally in unaffected tissue despite these patients having untreated periodontal disease. That being said, after the more urgent needs of the patient are met and if implants have been placed, it is still recommended that the periodontal condition be addressed to minimize

bacterial contamination and future inflammation of restorative sites. This would include phase one periodontal therapy, including scaling and root planning with possible local or systemic antibiotic delivery followed by a 4-6 week recall in which the need for further, possible surgical periodontal therapy, including gingival flap and or osseous surgery would be evaluated. It is imperative that we as clinicians and researchers work together in collaborative efforts to improve these patients overall health.

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APPENDIX

UNIVERSITY OF ILLINOIS AT CHICAGO

Office for the Protection of Research Subjects (OPRS)
Office of the Vice Chancellor for Research (MC 672)
203 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612-7227

Approval Notice Continuing Review

November 6, 2013

Praveen Kumar Gajendrareddy, BDS, PhD
Periodontics
801 S Paulina St
M/C 859
Chicago, IL 60612
Phone: (312) 413-8405 / Fax: (312) 916-0943

RE: Protocol # 2010-0187
“The Role of Inflammation in Wound Healing and Periodontal Disease”

Dear Dr. Gajendrareddy:

Your Continuing Review was reviewed and approved by the Convened review process on November 6, 2013. You may now continue your research.

Please note the following information about your approved research protocol:

Protocol Approval Period: November 6, 2013 - November 6, 2014

Approved Subject Enrollment #: 60 (15 Enrolled to Date)

Additional Determinations for Research Involving Minors: These determinations have not been made for this study since it has not been approved for enrollment of minors.

Performance Sites: UIC

Sponsor: College of Dentistry Microarray Seed Grant

PAF#: Not Applicable

Research Protocol(s):

- a) Research Protocol: 12/20/2012 Version 5

Recruitment Material(s):

- a) Oral Wound Care Instructions, 05/19/2010, Version 2
- b) Research Participants Needed: Internet Announcement, Version 5, 12/20/2012

c) Research Participants Needed: Flyer Version 5, 12/20/2012

Informed Consent(s):

- a) Waiver of Informed Consent (Recruitment Only) [45 CFR 46.116(d)]
- b) The Role of Inflammation in Wound Healing and Periodontal Disease, Version #5, 12/20/2012

HIPAA Authorization(s):

- a) The Role of Inflammation in Wound Healing and Periodontal Disease, Version #2, 1/28/2011. Please continue to use the Authorization form, which was stamped and approved on October 7, 2011.
- b) Waiver of Authorization (Recruitment Only) [45 CFR 164.512(i)(1)(i)], as documented on October 26, 2011.

Please note the Review History of this submission:

Receipt Date	Submission Type	Review Process	Review Date	Review Action
10/22/2013	Continuing Review	Convened	11/06/2013	Approved

Please remember to:

→ Use your **research protocol number** (2010-0187) on any documents or correspondence with the IRB concerning your research protocol.

→ Review and comply with all requirements on the enclosure,
"UIC Investigator Responsibilities, Protection of Human Research Subjects"
(<http://tiger.uic.edu/depts/ovcr/research/protocolreview/irb/policies/0924.pdf>)

Please note that the UIC IRB has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

Please be aware that if the scope of work in the grant/project changes, the protocol must be amended and approved by the UIC IRB before the initiation of the change.

We wish you the best as you conduct your research. If you have any questions or need further help, please contact OPRS at (312) 996-1711 or me at (312) 413-1835. Please send any correspondence about this protocol to OPRS at 203 AOB, M/C 672.

Sincerely,

Jonathan W. Leigh, MPH
IRB Coordinator, IRB # 1
Office for the Protection of Research Subjects

Enclosure(s):

1. Informed Consent Document(s):

- a) The Role of Inflammation in Wound Healing and Periodontal Disease, Version #5, 12/20/2012

2. Recruiting Material(s):

- a) Oral Wound Care Instructions, 05/19/2010, Version 2
- b) Research Participants Needed: Internet Announcement, Version 5, 12/20/2012
- c) Research Participants Needed: Flyer Version 5, 12/20/2012

cc: Bruce S. Graham, Dean, Administration, College of Dentistry, M/C 621
Lauren S. Castro, CRC, 148 CSB, MC 596
OVCR Administration, M/C 672
Privacy Office, Health Information Management Department, M/C 772

VITA

Andrew P. Monestero, DDS

Education:

- Aug 2014 **University of Illinois Chicago, College of Dentistry, Chicago, IL**
Certificate in Periodontics
Masters in Science
- May 2011 **University of Illinois Chicago, College of Dentistry, Chicago, IL**
Doctor of Dental Surgery
- Dec 2006 **University of Illinois Chicago, Chicago, IL**
Concentration in Biological Sciences and Chemistry
- May 2001 **Miami University, Oxford, OH**
Bachelor of Science in Business Administration
Major in Management Information Systems
Major in Finance

Work Experience:

University of Illinois at Chicago College of Dentistry

- ❖ Post Graduate Periodontics Resident
- ❖ Master of Science Candidate
- ❖ Resident Faculty in Pre-Doctoral Periodontal Clinic

Hewitt Associates

- ❖ Project Manager for Verizon Defined Benefit Team
- ❖ Lead Pension Calculation system Analyst
- ❖ Defined Benefits Quality Assurance Analyst

Apartment People

- ❖ Licensed Real Estate Managing Broker
- ❖ Real Estate Leasing Agent

Volunteer/Leadership Experience:

American Association of Public Health Dentistry

- ❖ AAPHD Executive Board
- ❖ Goldie's Clinic Team Captain
 - In charge of first ever student run dental clinic
- ❖ Goldie's Clinic Dental Provider

American Student Dental Association

- ❖ ASDA Executive Board
- ❖ Legislative Liaison
- ❖ Licensure chair
- ❖ Service Chair
- ❖ Mentor and Liaison to underclassmen and pre-dental students

Research Experience:

The Role of Inflammation in Wound Healing and Periodontal Disease University of Illinois at Chicago, Department of Periodontics

- ❖ Master's thesis
- ❖ Presented at UIC COD Clinic and Research day
- ❖ Working to publish

Case Cat: ***Reconstruction methods for the atrophic mandible and other vertical and horizontal defects*** University of Illinois at Chicago, Department of Oral and Maxillofacial Surgery

- ❖ Presented critically appraised topic at clinic and research day and to group practice clinic

A systematic review of treatment modalities for impacted mandibular second molars University of Illinois at Chicago, Department of Oral and Maxillofacial Surgery

- ❖ Literature review of available treatment modalities for impacted second molars, currently under review
 - Andrew Monestero, Dr. Patricia A. Lukasavage, Dr. Michael Miloro, Dr. Antonia Kolokythas

Externships and Rotations:

Aug 2012	<u>University of Illinois at Chicago Hospital – Anesthesia Rotation (4 Weeks)</u>
April 2011	<u>Lawndale Christian Health Center – Chicago Illinois, General Dentistry Rotation (2 weeks)</u>
Sep 2010	<u>Washington Hospital Center, Department of Oral and Maxillofacial Surgery (1 week)</u>
Sep 2010	<u>Carle Foundation Hospital, Department of Oral and Maxillofacial Surgery (1 week)</u>
Aug 2010	<u>University of Iowa, Department of Oral and Maxillofacial Surgery (1 week)</u>
Aug 2010	<u>University of Colorado, General Practice Residency (1 week)</u>
June 2010	<u>Salud Family Health Centers - Longmont Colorado, General Dentistry Rotation (8 weeks)</u>
May 2010	<u>The Ohio State University, Department of Oral and Maxillofacial Surgery (2 weeks)</u>
Aug 2009	<u>University of Illinois at Chicago, Department of Oral and Maxillofacial Surgery (1 week)</u>

Additional Experience/Activities/Memberships:

ACLS certification

BLS certification

Illinois Society of Periodontology

Midwest Society of Periodontology

American Academy of Periodontology

American Dental Association

Illinois State Dental Society

Chicago Dental Society

Academy of General Dentistry

Delta Sigma Delta Dental Fraternity

Awards and Accomplishments:

Brian D. Stone Memorial Award

Odontographic Society of Chicago Dr Walter E. Dundon Memorial Award

Arcolian Dental Arts Society Scholarship Recipient

The Dr. Samuel J. Cascio, DDS Scholarship - Two Time Recipient

General Assembly Scholarship Recipient