CREVICULAR FLUID CONTENT DURING WOUND HEALING
COMPARISON BETWEEN TOOTH AND IMPLANT

THESIS

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ABSTRACT

Background: Histological comparison of peri-implant and periodontal tissues reveals several similarities at both hard and soft tissue levels. However, various phases of peri-implant wound healing may differ from periodontal counterpart due to absence of periodontal ligament apparatus and related blood supply. The ultimate goal of this study was to develop diagnostic tools to evaluate peri-implant wound healing during osseointegration. The specific aims included: 1) determine the expression of several pro-inflammatory cytokines, growth factors and tissue byproducts within newly forming peri-implant crevicular fluid, 2) determine any possible differences in peri-implant and periodontal crevicular fluid content during early phases of wound healing and, 3) determine whether the release of specific biomarkers is correlated with clinical parameters classically used in evaluating wound healing.

Methods: Subjects who were seeking treatment for replacement of a missing mandibular posterior tooth were recruited. Inclusion criteria consisted of non-smokers with no known systemic and/or oral diseases that might affect wound healing. Teeth that were adjacent to the implant site and an anatomically similar tooth at cross-arch position were included as positive (T+) and negative tooth (T-) controls, respectively. Implant (I) was placed by using one stage surgical protocol. Implant and T+ sites had surgical
intervention while T-site did not. Plaque and gingival indices and implant stability (Resonance Frequency Analysis; RFA) were recorded at surgery and at 1, 2, 3, 6, 8 and 12 post-operative weeks. Peri-implant and gingival crevicular fluid (PICF and GCF) samples were obtained at baseline, and at weeks 1, 2, 3, 6, 8 and 12 with a calibrated electronic volume quantification unit. Multiplex bead based human cytokines/growth factors immunoassay and MMP fluorokine kits were used to study PICF and GCF concentration and total amount of IL-1β, IL-1ra, IL-6, IL-7, IL-8, IL-10, IL-12, bFGF, Eotaxin, MCP-1, MIP-1β, TNF-α, VEGF, MMP-8, MMP-9 and TIMPs. Data was analyzed using multiple model regression analysis corrected with Bonferonni adjustment and presented in two phases: early wound healing (weeks 1-3) and late wound healing (weeks 6-12). Statistical significance level was accepted at P=0.016.

Results: 40 patients (22F/18M and 21-74yo) completed the study. Plaque levels were low (PI<1; for all sites) and well controlled throughout the study. Gingival indices showed a statistically significant decrease around I and T+ sites (P≤0.01) during the early wound healing period and continued to decrease only around the I site during late wound healing phase. PICF volume decreased ~3 fold by the end of the study compared to week 1 (P<0.0001). A gradual increase in RFA was noted from surgery to 12 weeks (P<0.0001). Some pro-inflammatory biomarkers (IL-6, IL-8, MIP-1b, MMP-9, TIMP-1 and TIMP-2) had high concentrations and total protein amounts at I and T+ sites at first week. Statistically significant decreases were noted for these biomarkers at I and T+ sites by the end of early wound healing phase compared to week 1 (P<0.016). At the I site, the decrease was approximately 40 fold for IL-6, four fold for IL-8 and three fold for MIP-1b.
and TIMP-1 during the early wound healing. Crevicular fluid levels of IL-8, MIP-1β and TIMP-1 at T+ sites returned to baseline levels by the end of late wound healing period. T-sites did not present any statistically significant changes in biomarker levels throughout the study period.

**Conclusion**: Wound healing around dental implants presents variations in clinical and biological parameters compared to natural tooth site. Several biomarkers have the potential to be diagnostic in evaluating peri-implant wound healing.

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DEDICATED TO MY HUSBAND

SARANDEEP S. HUJA
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CHAPTER 1

INTRODUCTION

Implant supported dental restorations offer an alternative treatment option for partially or fully edentulous patients with better oral rehabilitation outcomes. Dental implants present advantages such as increased stability, prevention of alveolar bone loss and better esthetic results when compared to conventional removable or fixed dental restorations. Additionally, the long term survival rate of dental implant supported restorations is reported as similar or even higher than traditional crowns, bridges and/or dentures.\textsuperscript{1-3} In 2001, approximately 400,000 dental implants were placed only in USA and this number is estimated to grow by 12\% per year.\textsuperscript{4} Thus, there is an increasing need to better understand the mechanism(s) involved in short and long term peri-implant wound healing and stability of peri-implant health.

The short term peri-implant wound healing outcome primarily depends on wound stability e.g. dental implant stability. Dental implant’s strength in the alveolar bone is the result of vital bone approximating to the implant metal surface. This direct structural and functional connection between bone and the implant surface is described as osseointegration.\textsuperscript{5} Osseointegration is a histological term that defines the contact of bone and implant surface at the magnification of a light microscope. Clinically, osseointegration corresponds to rigid fixation which means the absence of vertical and horizontal mobility under forces of 500g.\textsuperscript{6} Clinical guidelines to achieve predictive
osseointegration are described as; sufficient bone support around the newly placed implant, insertion of implant device with minimal surgical trauma and rigid fixation of the implant at the time of the surgery (primary stability).\textsuperscript{7}

The original implant insertion protocol was suggested as two-stage (submerged) protocol since exposure to oral flora and possible epithelial migration around dental implant were considered as main factors causing fibrous encapsulation of implant device. Similarly, lack of micromovement in the implant-bone interface during wound healing was accepted as necessary to obtain osseointegration. Thus, implants were submerged under the gingiva 3-4 months for mandibles and 6-8 months for maxillae for complete tissue healing.\textsuperscript{5,8} Later on, Buser et al. proposed one stage surgical protocol where the polished collar of the implant was exposed to the oral cavity.\textsuperscript{9} A recent review paper concluded that, provided the implant primary stability is achieved, one stage protocol presented similar outcomes as two-stage protocol in partially edentulous patients.\textsuperscript{10} Furthermore, one stage approach provides several clinical advantages including avoidance of a second surgery, less chair time per patient and simplified prosthetic procedures for temporary restorations.\textsuperscript{11-12} Additionally, one stage protocol is appropriate for clinical investigation of wound healing process.

\textbf{Wound Healing around Natural Tooth and Dental Implants:}

Periodontal wound healing following simple periosteal flap elevation has been documented in depth. Wound healing following flap surgery generally occurs at both hard and soft tissue levels due to surgical trauma to both soft tissue and periosteum.\textsuperscript{13-16}
Phases of wound healing following flap surgery are consisted of inflammation, granulation tissue formation, matrix formation and remodeling. The inflammatory phase (0-3 days) includes blood clot formation, neutrophil migration and degradation of erythrocytes. The granulation tissue formation phase (1-10 days) is characterized by macrophages and formation of young collagen fibers. Lastly, the remodelling phase is dependent on the size of the wound but in general, collagen fibers physically attach to the dentin in two weeks, and cementum formation is observed in three weeks.\textsuperscript{13-14} As of any wound, blood supply plays a major role with main vascular sources originating from alveolar, supraperiosteal and periodontal ligament. It is demonstrated that within three-five days cut vessels form anastomoses and reach to normal length by day 11.\textsuperscript{15} Following flap surgery an increased osteoclastic activity is observed for three weeks whereas the earliest osteoblastic activity was observed by two weeks. Overall, the mean alveolar crestal bone loss is 0.62 mm following full thickness flaps.\textsuperscript{16}

Similar to periodontal wound, a full thickness flap has to be elevated for conventional dental implant placement surgery which is followed by osteotomy for implant bed site preparation. Thus, healing is observed at both hard and soft tissue levels. Controversy still exists on phases and duration of peri-implant wound healing.\textsuperscript{17-19} However, the following information is generally accepted:

The bone healing process for peri-implant interface is described in three distinct phases which are osteoconduction, \textit{de novo} bone formation and remodeling.\textsuperscript{17} Bone healing starts within the first week after implant insertion, matures in 3-6 months and continues to remodel with function. Osteoconduction defines the early phases of the implant healing where recruitment of osteogenic cells and their migration to implant
surface is observed. Blood clot, fibrin formation and electrochemical interaction between implant surface and blood are the determinants of this process. *De novo* bone formation is the initiation of bone matrix formation by osteoblastic cells. This initial bone matrix is arranged as woven bone and then replaced by lamellar bone. Peri-implant osteogenesis consisting woven and lamellar bone can proceed in two different directions from host to the implant (distance osteogenesis) and from implant toward the healing bone (contact osteogenesis). It is suggested that contact osteogenesis has more importance to strengthen osseointegration since new bone directly forms on the implant surface.\textsuperscript{17, 20} Remodeling of the mature bone around implants is by process of bone formation followed by bone resorption and provides self repair and adaptation of the implant to occlusal forces. Bone healing around the implant is known as osseointegration. The principal mechanisms of osseointegration process are considered similar to those occurring during bone fracture repair.\textsuperscript{18, 21}

Soft tissue healing around dental implant is described as formation of a gingival seal. During early phases of healing, inflammatory, proliferative and remodeling phases are observed.\textsuperscript{22-23} Hemostasis has to be followed by coagulation and chemotaxis of pro-inflammatory host cells. The proliferative phase is characterized with granulation tissue formation and epithelium migration. With fibroblast activation and release of extracellular matrix components, connective tissue matrix is formed which matures into mucoperiosteal matrix during the remodeling phase of soft tissue healing.\textsuperscript{19, 23} Histologic analysis of dental implant soft tissue interface reveals sulcular and junctional epithelium and an underlying connective tissue which has similar characteristics to biologic width around natural tooth.\textsuperscript{22} The dimensions of peri-implant gingival seal are reported as 2 mm
for epithelial attachment and 1 mm connective tissue attachment. Once formed these
dimensions are confirmed to be stable and are not influenced by loading of implant.\textsuperscript{24} The
function of the gingival seal is to protect the underlying alveolar bone. Soon after implant
insertion, epithelium near the wound site grows over the fibrin network, reaches to
implant surface and starts migrating apically. The downgrowth of the epithelium is
limited by the presence of granulation tissue where it undergoes morphological and
functional changes to form epithelial seal. The epithelial attachment can occur as early as
1-2 week after healing and mature epithelial barrier is observed within 6-8 weeks of
healing.\textsuperscript{25}

Maturation of granulation tissue into connective tissue prevents epithelial
migration to the implant surface.\textsuperscript{26} The collagen fibers in the connective tissue mature
within 4-6 weeks post-operative healing. Unlike the tooth, these fibers do not attach to
implant surface. They are organized parallel to the implant surface generating a cuff and
thus, suggested to have less mechanical resistance compared to natural tooth.\textsuperscript{24} The
vascular supply of the peri-implant tissue also presents differences compared to a tooth.
Supraperiosteal blood vessels are the only source of blood supply of peri-implant tissue
due to the absence of periodontal ligament and its vascular anastomosis to periosteum
and the alveolar bone.\textsuperscript{19} Therefore, it is suggested that peri-implant tissues may have
impaired defense system compared to a natural tooth.\textsuperscript{27}
Diagnostic tools to evaluate Peri-Implant Wound Healing

Evaluation of the dental implant with conventional clinical tools such as probing depth, bleeding on probing, clinical mobility and with radiographic films is not sensitive enough to detect complications at early time points. The current trend of immediate or early loading practices with shorter healing intervals creates a necessity for chairside diagnostic tools that would improve evaluation of peri-implant wound healing. One stage surgical protocol (e.g. placement of implant device with healing abutment exposed to oral cavity) would allow such new tools to be used during osseointegration period.

Resonance Frequency Analysis and Implant Stability:

Primary implant stability at the time of implant placement is a requirement to achieve osseointegration.\textsuperscript{28-30} This is especially important for implant devices placed using one stage surgical protocol. Implant stability can be described as the absence of clinical mobility of the implant at titanium surface-bone interface.\textsuperscript{31} Several methods were used to test implant stability such as insertion torque measurements, Periotest\textsuperscript{a} and Resonance Frequency Analysis (RFA). Periotest\textsuperscript{®} and RFA could determine implant stability at the surgery and during the post-operative appointments whereas insertion torque measurements can only be applied at the time of the surgery. Periotest\textsuperscript{®} was originally designed to evaluate tooth mobility and has major limitations when used for dental implants.\textsuperscript{32} Initial dental implant stability is higher in the direction it is inserted. Resonance frequency analysis (RFA) determines stability to lateral forces by applying small bending force that would mimic occlusal forces at a minimal magnitude. The

\textsuperscript{a} Periotest, Medizintechnik Gulden, Modautal, Germany
resonance frequency instrument (Osstell Mentor\textsuperscript{b}) directs minimal bending load by sending magnetic pulses. The transducer abutment (SmartPeg\textsuperscript{c}) attached to the implant has a small magnet at the top portion and vibrates in two directions. The instrument reads the frequency from abutment and presents information about the stiffness of the implant-bone interface.\textsuperscript{31} The frequency information is converted from Hertz and introduced as a standardized unit of stability known as implant stability quotient (ISQ) reading. ISQ ranges from one to hundred as one representing the least possible stability where as hundred representing the highest possible stability of the implant.\textsuperscript{33}

Primary implant stability measured by RFA is found to be associated with gender, bone density, implant diameter, implant length and anatomical location of the implant.\textsuperscript{34-36} A recent study reported that implant stability ranged 57-70 ISQ levels at the time of placement.\textsuperscript{37} It has been also concluded that the initial ISQ values are kept and/or changed to higher values by twelve weeks of healing. The lowest ISQ measurements are generally recorded during 2\textsuperscript{nd} and 3\textsuperscript{rd} week of healing, most probably due to bone remodeling process.\textsuperscript{37-38} A continuous decrease of ISQ values is detected around failing implants until the instability is clinically evident.

Multiple assessments of RFA appear to be a valuable tool to observe implant stability changes overtime. Although the threshold values for failure is not identified, the high ISQ levels represent successful implant placement while low ISQ readings may be sign of future risk for failure. Most importantly, decreasing ISQ levels could be associated with implant disintegration or marginal bone loss. RFA technique is also

\textsuperscript{b} Osstell\textsuperscript{TM}, Mentor, Straumann USA, LLC, Andover, MA, USA
\textsuperscript{c} Smartpeg\textsuperscript{TM}, Straumann USA, LLC, Andover, MA, USA
suggested to serve as criteria for one stage versus two stage surgical protocols and, can be similarly used in decision making for immediate versus delayed implant placement protocols.\textsuperscript{31}

**Wound Fluid Volume and Content at Early Phases of Implant Healing.**

Gingival crevicular fluid (GCF) is a unique fluid composition which arises from the gingival plexus of blood vessels in the gingival connective tissue subjacent to epithelium lining the dentogingival space.\textsuperscript{41} Under healthy conditions, GCF is suggested to represent a transudate fluid formed as a result of osmotic gradient in the crevice. However, the production of GCF could be stimulated by chemical and mechanical irritation such as trauma and bacterial plaque accumulation. It can become an inflammatory exudate upon injury and pathologic process.\textsuperscript{41-42} Therefore, its presence and content is considered protective due to its flushing effect and transportation of host derived substances into the crevice.\textsuperscript{43}

GCF consists of a complex mixture of substances derived from serum, leukocytes, structural cells of the periodontium and oral bacteria.\textsuperscript{43} The host-derived substances in GCF include antibodies, cytokines, enzymes and tissue degradation products. Peri-implant crevicular fluid (PICF) is originated from the gingival vessel plexus of the implant. Its composition is similar to GCF, consisting of host derived enzymes and their inhibitors, inflammatory mediators, host response modifiers and tissue breakdown products.\textsuperscript{44-45}
GCF and PICF could provide a unique source to analyze periodontal/peri-implant conditions. Both fluids present the potential to serve as indicators to detect and classify periodontal or peri-implant diseases at its initial stages and evaluate healing of periodontal/peri-implant tissues following different types of therapies. In addition, non-invasive methods of collection and relatively simple analyzing procedures are considered as advantages of working with GCF and PICF.\textsuperscript{46}

**Inflammatory content within GCF and PICF:**

Gingival crevicular fluid contains a wide spectrum of serum proteins, inflammatory mediators, host cell degradation products and bacterial proteins. Enzymes, specifically proteinases, play a crucial part in the control of periodontal tissue turnover both in health and in the tissue destruction. Matrix metalloproteinase -8 (MMP-8) and -9 (MMP-9) are the two main collagen degrading enzymes in GCF and saliva.\textsuperscript{47} These enzymes are secreted from neutrophils during phagocytosis and are responsible for the degradation of interstitial collagens and extracellular matrix components including basal cell membrane.\textsuperscript{46} Based on their vital role in tissue remodeling, they are considered important indicators for periodontal inflammation. Since MMPs can degrade tissues, their activity is firmly controlled by tissue inhibitor of matrix metalloproteinases (TIMPs) which modulate the extracellular activity of MMPs. TIMP-1 is considered as the major inhibitor of MMPs and reported to be released at higher levels in inflamed gingiva.\textsuperscript{48} Elevated levels of MMP-8 and TIMP-1 are suggested to reflect the increased
Surgical trauma induces an acute phase response which controls tissue damage and infection and stimulates repair process for normal host function. The release of proinflammatory cytokines, specifically interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-α) from macrophages introduce the initial acute phase response and stimulate secretion of other cytokines such as interleukin-6 (IL-6).\textsuperscript{51-52} Interleukin-6 is a proinflammatory cytokine that is associated with elevated levels of C-reactive protein and neutrophil elastase (MMP-9).\textsuperscript{53} Additionally, it is shown to be effective in stimulating neutrophils.\textsuperscript{54} However, IL-6 is also important in activating anti-inflammatory responses by indirectly causing the release of cytokines such as Interleukin-10 (IL-10) and interleukin- (IL-4).\textsuperscript{55} The role of IL-6 in health and diseased periodontal tissues and peri-implant tissues has been investigated by several studies.\textsuperscript{56-59} Local level of IL-6 is reported as over-expressed at implant sites with peri-implantitis.\textsuperscript{56} In addition, fibroblasts isolated from peri-implantitis sites show increased secretions of IL-6 compared to healthy fibroblasts \textit{in vitro}.\textsuperscript{57}

Interleukin-8 (IL-8) is known as a potent chemotactic agent for neutrophils. It is documented to play a significant role in periodontal diseases due to its proinflammatory and chemotactic properties. IL-8 mediated neutrophil activation can advance tissue destruction in inflamed tissues.\textsuperscript{60-61} Macrophage inflammatory factor-1 (MIP-1) is another strong chemokine that leads selective migration of lymphocytes and monocytes to the site of inflammation during wound healing process.\textsuperscript{62} In the presence of early peri-implant mucositis, IL-8 and MIP-1 levels are positively correlated.\textsuperscript{62} This positive
correlation is suggesting that these inflammatory chemokines can be useful as markers for implant failure.\textsuperscript{62}

Interleukin-1 (IL-1) is a proinflammatory polypeptide that presents wide range of activities including inflammation, tissue breakdown and tissue hemostasis.\textsuperscript{63} IL-1 can induce production of cytokines causing destruction of tissues. In addition, it plays a major role in pathogenesis of many bone related diseases including periodontitis.\textsuperscript{61} Studies which investigated the association of IL-1 levels suggested increased levels both in GCF and PICF due to plaque accumulation and in response to peri-implant disease.\textsuperscript{62,64-65} However, when compared to natural tooth higher levels of IL-1 were found around clinically healthy implants implicating some intrinsic factors related to implants.\textsuperscript{58-59}

Tumor necrosis factor-alpha (TNF-\(\alpha\)) is another well documented proinflammatory cytokine responsible for production of adhesion molecules, proinflammatory cytokines and chemokines such as IL-1, IL-6, IL-8 and MMPs, for destruction of bone and cartilage through the stimulation of IL-1 and PGE\(_2\) secretion. It presents synergistic effects similar to IL-1 for local bone resorption by inducing osteoclastogenesis.\textsuperscript{66-67} The presence of TNF-\(\alpha\) was shown both in GCF and PICF by several reports.\textsuperscript{58-59, 62, 64-65, 68-69} Elevated TNF-\(\alpha\) levels were detected in peri-implantitis lesions and high levels of TNF-\(\alpha\) were successfully reduced with anti-infective therapy in 12 month period.\textsuperscript{62, 68-69}

Several inflammatory cytokines, chemokines, tissue enzymes and enzyme inhibitors as well as growth factors were investigated to identify potential markers
specific for periodontal and/or peri-implant health and disease conditions. However, studies evaluating the healing response/outcome following implant surgery do not exist. The purpose of this study is to evaluate wound healing response/outcome following implant surgery by comparing clinical parameters and biological markers in periodontal and peri-implant crevicular fluid.

**Null Hypothesis:** Wound healing patterns around newly placed dental implant devices and surgically manipulated adjacent periodontal sites are similar.

**Specific Aims:**

1. To determine changes in clinical parameters (e.g. crevicular fluid volume, gingival and plaque indices) around newly placed dental implant and adjacent teeth during early wound healing.

2. To detect local levels of wound healing markers specific for inflammatory process and tissue degradation/remodeling within PICF and GCF of newly forming peri-implant and surgically manipulated adjacent gingival crevices.

3. To determine possible associations between clinical parameters, local levels of various healing markers and implant stability during early wound healing.
Significance: The ultimate goal of this study is to determine whether various phases of early wound healing around dental implants placed with one-stage surgical protocol can be detected by using peri-implant crevicular fluid content as a possible diagnostic tool. The data generated in this study may help to better understand physiological peri-implant wound healing and modify existing treatment modalities and post-operative care protocols to prevent and/or control healing related early complications.
CHAPTER 2

MATERIALS AND METHODS

Study population and study design:

The overall study design was prospective longitudinal observational cohort study. Patients who were treatment planned for one stage single implant placement surgery on the mandible posterior sextant were recruited from the patient pool of Graduate Periodontology Clinic at the Ohio State University between November 2008 and January 2011. The inclusion criteria included: 1) adult patients, age 18-75 years old; 2) patients treatment planned for a single implant placement on the mandibular posterior sextant; 3) patients who are non-smokers; 4) patients who are able and willing to provide informed consent for the surgery and the study protocol. Exclusion criteria were: 1) patients with untreated periodontal disease; 2) patients with systemic diseases affecting their periodontal health or healing process, e.g.; diabetes; 3) absence of natural teeth adjacent to the implant surgery site; 4) pregnant women; 5) unable or unwilling to provide informed consent forms. Exit criteria were: 1) voluntary withdrawal; 2) non-compliance with study protocol; 3) the implant surgical protocol changed to two-stage protocol due to lack of primary stability at the time of implant placement; 4) development of systemic or oral
diseases that are in the exclusion criteria. The study protocol and informed consent forms were approved by the Institutional Review Board of the Ohio State University. A written consent form was obtained from all participants before entry to study.

The study design consisted total of eight visits (Figure 1). At the initial visit, participants’ periodontal and medical exams were reviewed. Initial bone grafting at the implant surgery site was noted. All participants were given oral and written informed consents for their implant treatment and also for the study protocol. The signed informed consent forms were collected. The second visit was the baseline visit for the data collection and the time for implant surgery. Prior to surgery, plaque (PI) and gingival indices (GI) were recorded and GCF samples were collected from the teeth adjacent to implant surgery site (T+) and from an anatomically similar tooth on the opposite site of the jaw (T-). After implant placement, stability of the implant was measured by using RFA and ISQ was recorded. At weeks 1, 2, 3, 6, 8, and 12, PI, GI measurements and GCF collection were performed at three sites, T+, implant (I) and T-. The stability measurements were repeated at the I site at weeks 1, 3, 6, 8, 12. (Figure 1)

**Surgical protocol:**

Surgical incisions including sulcular incisions around natural teeth and a crestal incision at the edentulous site were performed. Full thickness flaps were elevated. Implant osteotomy sites were prepared with guidance of a custom made surgical stent.
Screw type, root form implants\textsuperscript{defg} were placed using a standard one stage protocol, following manufacturer’s recommendations with specified instruments (eg; burs). Following implant placement, a polished transgingival healing abutment, 2-4 mm in height was screwed to the implant body and soft tissues were sutured around it. Post-operative instructions included refraining plaque control for one week at the site of the surgery, use of 0.12% chlorhexidine rinse and use of analgesics as needed. Sutures were removed at 1 week postoperatively. If analgesics and/or antibiotics were used the dose and frequency were recorded at the second visit.

**Clinical Data Collection:**

*Clinical Parameters:*

Initial documentation including probing depths, recession, clinical attachment levels on all six sites per tooth, furcation involvement and tooth mobility was completed to reach periodontal diagnosis.\textsuperscript{70} Participants who enrolled to the study were either periodontally healthy or completed required periodontal treatment and were under periodontal maintenance care. PI and GI measurements were recorded from two sites, namely T+ and T- sites at the first visit. PI, GI and modified GI and PI measurements were repeated at tooth sites and I site respectively, at the following appointments, visits 2 through 8. Original PI and GI were used for teeth related sites. Modified PI and GI were

\textsuperscript{d}Astra Tech, Mondial, Sweden
\textsuperscript{e}Straumann USA, LLC, Andover, MA, USA
\textsuperscript{f}Zimmer Dental, Carlsbad, CA, USA
\textsuperscript{g}Biohorizons, Birmingham, AL, USA
used for implant related sites due to presence of healing abutment.\textsuperscript{71-72} Gingival index evaluates the gingival soft tissue characteristics such as gingival color, edema, consistency and bleeding. A GI of 0 indicates the gingival health where gingival tissues are pink in color, firm in consistency and does not bleed on probing. GI of 1 indicates mild inflammation with color change; GI 2 indicates inflammation with edema and color change and/or presence of bleeding on probing. Lastly, GI 3 indicates spontaneous bleeding. A PI of 0 represents absence of plaque, index 1 represents presence of plaque recognized by running a probe. Index 2 represents presence of visual plaque and index 3 is characterized by abundant plaque covering the clinical crown of the tooth.

At the time of the baseline appointment, before the implant placement is performed, the buccal tissue thickness, buccal and lingual bone thickness was measured using a caliper.

\textit{Gingival and Peri-implant crevicular fluid collection:}

A total of four GCF samples were collected both from T+ and T- site starting from visit 2 to 8, whereas four PICF samples was collected from I site from visit 3 to 8. Cotton rolls were used to isolate the site. Supragingival plaque was removed using a periodontal probe and soft tissue was gently air dried using an air/water syringe without disturbing the gingival margin. GCF and PICF samples were obtained one at a time using sterile paper strips (Periopaper\textsuperscript{®})\textsuperscript{h} (Figure 2). Paper strip was gently introduced into the newly forming gingival sulcus until a mild resistance was felt. GCF/PICF collection time

\textsuperscript{h} Periopaper, Proflow Inc. Amityville, NY, USA
was 20 seconds for each strip. Samples that were contaminated with blood or saliva were discarded. Immediately following crevicular fluid collection the paper strip was placed into a previously calibrated electronic volume quantification unit (Periotron 8000®) to determine the collected volume. The GCF/PICF samples from each site (T+, I, T-) were pooled and placed into 3 sterile cyrovials. Vials were kept on ice until the end of study visit. Later, samples were transferred and stored at -80°C until processing. All samples were collected by one examiner.

**Resonance frequency analysis:**

Based on the manufacturer’s guidelines, a transducer specific for implant system and diameter was hand torqued into the implant fixture (Figure 2). Stability of the implant was measured using Resonance Frequency Analysis (Osstell Mentor®). Three measurements were taken both from buccal and lingual aspects of the transducer and averaged ISQ values were recorded for buccal and lingual aspects. All measurements were recorded by single examiner.

**Laboratory Analysis:**

Extraction of crevicular fluid from paper strips was performed as following: PCR microtubes with small holes at the bottom were prepared and sterilized. These tubes were placed into sterile 1.5 ml Eppendorf tubes. Paper strips were separated from waxed

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1 Periotron 8000, Ora Flow Inc, Plain View, NY, USA
portion by using a sterile scissor and were transferred from cyrovials into PCR microtubes. They were soaked in 200 µl cold sterile PBS and incubated on ice for 15 minutes with occasional vortexing. Samples then were centrifuged at 13,000 rpm for 10 min. Supernatant was collected from the bottom of the tubes and centrifugation was repeated. Approximately 160 µl elution volume was recovered from each tube. Forty µl of this volume was loaded into multiplex bead-based assay plate.

*Multiplex bead-based assay*

The multiplex bead-based assay\(^{1}\) was used to detect the presence and the amount of several cytokines within crevicular fluid samples including IL-1\(\beta\), IL-1ra, IL-6, IL-7, IL-8, IL-10, IL-12, Eotaxin, FGF-b, MCP-1, TNF-\(\alpha\), MIP-1b, VEGF, MMP-8, MMP-9, TIMP-1, TIMP-2, TIMP-3 and TIMP-4. Briefly, the assay uses Luminex multi-analyte profiling (xMap) technology. This technology uses digital signal processing capable of classifying polystyrene beads (microspheres) dyed with distinct proportions of red and neared fluorophores. The spectral addresses for different bead populations where up to hundred different detection reactions can be performed in small volume of samples. The cytokine reagent kit includes assay buffer, antibody diluent, streptavidin-PE, and a 96-well filter plate. Specific kit\(^{k}\) designated as human cytokine group (Group I) was used for cytokines and growth factors. Plates and reagents used for MMP and TIMP detections were purchased from another manufacturer\(^{l}\).

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\(^{1}\) Bioplex\(^{TM}\) Cytokine Assay. Bio-Rad Laboratories, Inc.

\(^{k}\) Bio-Rad laboratories, Life Science Research, Hercules, CA,

\(^{l}\) R & D Laboratories Ltd. Antrim, Northern Ireland
**Statistical analysis:**

The statistical analysis was completed by a statistical consultant at the OSU Center of Biostatistics using Statistical Analysis Software (SAS; version 9.2). Data was analyzed to detect possible changes that may occur during early wound healing (week 1 through week 3) and, differences that may become significant during late wound healing (week 6 through week 12). A mixed model regression analysis was used for repeated measures within and between groups. Initially a random effect (intercept and slope) regression analysis was conducted to estimate the slopes of the outcome over continuous time for I, T+ and T- groups. The variability of the outcome within and between subjects was used to estimate standard error that tests the regression coefficients. The comparisons were considered significant if the p-value is ≤0.0167 (0.005/3 using a Bonferroni correction) in order to conserve the overall type I error at the 0.05 level. Secondary analyses compared the first observation time to the last observation time for the I, T+ and T- groups for each outcome. The results were considered significant if the p-value was ≤0.0167.

One week post-operative observation time was chosen to analyze associations between various variables around implant, T+ and T- sites. Spearman Correlation Analysis corrected with Bonferonni adjustment was used when variables are parametrics in nature. Kruskal-Wallis Correlation Analysis corrected with Bonferonni adjustment

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^m SAS Institute Inc. Cary, North Carolina, USA
was used when variables are non-parametric in nature. The results for each correlation were considered significant if the p-value was \( \leq 0.0167 \).
CHAPTER 3

RESULTS

Study population

A total of 57 subjects have fulfilled the inclusion criteria and recruited to the study. Each patient had one implant in the mandible posterior sextant that qualified for the study. Of the 57 subjects, 40 patients successfully returned to follow-up appointments and completed the study. Of the 17 patients/implants that were excluded from the study, six had implant failure (e.g. loss of stability by third week of healing). Surgeon had to modify surgical protocol for four cases and preferred to use two-stage surgical approach instead of one-stage implant placement. Five subjects failed to comply with the follow up appointments and lastly 2 patients had surgical intervention during the study period and were excluded.

The clinical and patient demographics of 40 subjects are given in Table 1. Briefly, study population included 22 female and 18 male subjects with mean age of 54.4 ± 2 yrs. The youngest patient was 21 yrs old and the oldest was 74 yrs old with no significant differences in age distribution between genders. Among the 40 implant sites, 14 sites had to receive either alveolar socket preservation immediately following tooth extraction or guided bone regeneration due to already missing tooth and lack of sufficient bone volume
at the edentulous site. Freeze Dried Bone Allograft alone or mixed with autogenous bone graft was used with or without collagen membrane for these sites. Minimum of four months of healing was necessary for these patients prior to implant placement surgery. Among the total of forty sites, 26 implants were placed for a missing first or second molar while 14 implants replaced missing first or second premolars. Mean implant diameter was 4.7±0.07 mm while mean implant length was 11.5±0.2 mm. Routine post-operative care was not modified due to study but systemic antibiotic and analgesic uptakes were recorded by asking the subject to complete a diary. Based on this record, 10 subjects took systemic antibiotics and 30 subjects took some type of analgesics. All subjects were asked to use chlorhexidine mouthrinse for the first week since they were not able to brush surgical site.

**Clinical Measurements:**

Plaque accumulation was well controlled and PI levels stayed below 1 during the study period (Figure 3). Initial plaque index prior to surgery was 0.8±0.07 for surgical site and 0.71±0.08 for negative control site. Plaque accumulation at 1 week post-operative control was slightly higher for surgical site compared to non-surgical site (0.6±0.08 for I, 0.73±0.09 for T+ and 0.43±0.07 for T-; P>0.05 for between group differences, mixed model regression analysis with Bonferonni adjustment). There was no statistically significant changes in T+ (P=0.03 and P=0.9 for early and late wound healing phases, respectively), T- (P=0.6 and 0.4 for early and late wound healing phases, respectively) and I (P=0.08 and P=0.07 for early and late wound healing phases,
respectively) sites throughout the study period. The group comparisons have shown no statistically significant differences between groups at any time (P>0.016). The decrease observed in plaque index at implant site between week 1 and week 12 was statistically significant (P<0.0001, mixed model regression analysis with Bonferroni adjustment) (Figure 3).

Gingival inflammation was high at first week for T+ and I sites compared to baseline levels for surgical site (Figure 4). The decrease observed in GI at implant sites between week 1 and week 12 was statistically significant (P<0.0001). This was more noticeable for implant site during early wound healing (from 1±0.09 at week 1 to 0.4±0.06 at week 3; P<0.0001) and continued to significantly decline during the late wound healing period (0.2±0.05 at week 6 to 0.08±0.03 at week 12, P<0.01). A similar decrease was observed in surgically modified adjacent periodontal sites when index values were compared between week 1 and week 12 (0.9±0.08 at week 1 to 0.3±0.05 at week 12, P<0.0001). This decrease mainly occurred during early wound healing phase (up to 3 weeks of healing, P<0.0001). GI changes in T- site was not statistically significant at any time point (P>0.05). Comparisons between sites showed statistically significant differences between I and T- sites (P<0.0001) and T+ and T- sites (P<0.0001) only during the early weeks of wound healing (Figure 4).

The data analysis in crevicular fluid changes was based on total GCF and PICF volume (Figure 5). In general, the decrease in crevicular fluid volume between week 1 and week 12 was statistically significant for both I and T+ sites (1.8±0.15 µl at week 1 to 0.6±0.07 µl at week 12 for I site and, 1.7±0.12 µl at week 1 to 1.3±0.13 µl at week 12 for T+site, P<0.0001). When the data was analyzed as early and late wound healing phases,
a statistically significant decrease in PICF volume at I site (P<0.0001) and GCF volume at T+ site (P<0.0001) were only detected in early weeks. No difference was observed within T- site during the study period which stayed similar to baseline values without major fluctuations. During early weeks of healing, there were statistically significant differences between sites I and T-(P=0.0001) and T+ and T- sites (P=0.002). Although the decrease in PICF volume continued up to 12 weeks, the difference between periodontal and peri-implant sites was not statistically significant in late healing period (P>0.05).

**Resonance Frequency Analysis**

Resonance Frequency Analysis (RFA) revealed an initial ISQ value of 69.5±1.3 at the time of implant placement. This value stayed stable at week 1 of healing (69.4±1.1 ISQ) and started increasing by week 3 (70.4±1.3 ISQ). This increase continued throughout weeks with a final reading of 75±1.3 ISQ at 12 weeks (Figure 6). The difference between initial ISQ values and values obtained at 12 weeks was statistically significant (P<0.0001).

**Biomarker concentrations in GCF and PICF**

All biomarkers except TIMP-3 and TIMP-4 (IL-1β, IL-6, IL-7, IL-8, IL-12, TNF-α, MCP-1, MIP-1b, MMP-8, MMP-9, TIMP-1, TIMP2, VEGF and b-FGF) were detected using the multiplex bead-based assays. However, IL-7, IL-12, IL-10, Eotaxin, bFGF and
MCP-1 were expressed at low levels at all time points. Related data is presented in Table 2 but not included into the current session.

Among the studied cytokines and chemokines, IL-8 and MIP-1b were differentially released at surgical site with higher levels during early wound healing phase. IL-8 total protein expression was high at I and T+ sites at week 1 (208±36 pg for I and 109±17 pg for T+ sites) and declined fivefold at the I site (39±7 pg, p=0.002) and almost three fold at the T+ site (43±6 pag, p=0.0002) by week 3 where as T- site did not present statistically significant changes in IL-8 total protein expression overtime (Figure 7). Between group differences in IL-8 protein expression were statistically significant between I and T- groups and T+ and T- groups during early weeks (P=0.002 and 0.001, respectively). In late wound healing period, a slight increase was detected in T+ site (P=0.03) and the difference observed in IL-8 total protein expression between I and T+ sites was statistically significant (P=0.01). When week 1 and week 12 values were compared, the decrease in IL-8 total protein expression around implant sites was statistically significant (208±36 pg versus 33±7 pg, P=0.001). By 12 weeks, IL-8 levels returned to baseline values at both newly placed implant site (33±7 pg final total amount) and surgically manipulated periodontal sites (96±29 pg final total amount compared to 61±8.3 pg pre-surgical total amount) (Figure 7).

Similar to interleukin-8, a sharp increase at implant site at one week post-operative period followed by a statistically significant decrease by week 12 observed for MIP-1b total protein expression (15.5±3.7 pg versus 3±0.4 pg, P<0.0001) (Figure 8). This was a twofold decrease in I site between week 1 and week 3 (15.5±3.7 pg to 6±1.4 pg, P=0.02). The decrease observed at T+ site was moderate but still statistically significant
at early weeks (6±0.6 pg to 3.3±0.6 pg, P=0.01). Comparisons between groups revealed statistically not significant differences between I and T+ sites and, T+ and T- sites (P=0.02 and P=0.05, respectively). Late wound healing have demonstrated some fluctuations in MIP-1b total protein expression only at the T+ site (a slight increase, 3.5±0.9 pg at 6 weeks to 5.2±1.5 pg at 12 weeks, P=0.03). Between group differences in MIP-1b total protein expression at late weeks was statistically significant between I and T+ sites (P= 0.01) (Figure 8).

Interleukin-1 beta, IL-1ra, TNF-α and IL-6 were chosen for their important pro-inflammatory functions during wound healing. IL-1β total protein expression was higher at surgical site compared to non-surgical site even prior to surgery (18±3 pg versus 9±1.5 pg, T+ and T- at baseline, respectively) (Figure 9). This expression pattern stayed stable until 12 weeks at which time an increase in IL-1β total protein expression was noted at T+ site compare to T- and I sites (26±4 pg, 11±3 pg and 6.5±2 pg for T+, T- and I sites, respectively). The increase observed at T+ sites between week 1 and week 12 was statistically significant (P=0.003). Similarly, the decrease observed at I sites for the same time periods was statistically significant (19.5±3.2 pg to 6.5±2 pg, P=0.0002). Most of this decrease in peri-implant IL-1β total protein expression occurred during the first three weeks of healing (P=0.004). Between group difference was statistically significant for I and T- sites for early wound healing since T- sites did not show much changes (P=0.01). However, the difference between I and T+ sites became statistically significant for late wound healing phase due to the increase levels of IL-1β at T+ sites (P=0.002) (Figure 9).
Similar to interleukin-1β, IL-1ra total protein expression was higher at surgical site compared to non-surgical site even before surgery (16,732±5,757 pg versus 7,839±1,880 pg for T+ and T- sites, respectively) (Figure 10). Surgical manipulation was followed by lower IL-1ra total protein expression at surgical site (e.g IL-1ra levels close to baseline T- control site levels for all three groups) during early wound healing phase. Peri-implant IL-1ra total protein expression at 1 week was similar to baseline T- control site levels (5,956±1,505 pg for I versus 9,026±1,456 pg for T-). An increase in IL-1ra total protein expression was noted in late wound healing phase for all three groups. However, this was not statistically significant (P=0.05). (Figure 10)

Tumor Necrosis Factor-alpha was expressed at very low levels throughout study with maximum total protein amount detected at implant site at one week (1.7±0.22 pg) (Figure 11). A steady but not statistically significant decrease in peri-implant TNF-α total protein amount from week 1 to week 12 was detected (1.7±0.22 pg versus 0.9±0.12 pg, P=0.02) (Figure 11).

Similar to tumor necrosis factor-alpha, interleukin-6 is a potent pro-inflammatory cytokine and should be released at very low levels in health. IL-6 baseline total protein expression was very low for both surgical and non-surgical sites (0.7±0.2 pg for T+ and 0.4±0.07 pg for T- sites prior to surgery) (Figure 12). Surgical manipulation induced significant IL-6 protein release into crevicular fluid at one week for both implant and tooth sites while T- site maintained low levels of IL-6 protein expression (42±8.4 pg for I, 16±4 for T+ and 0.3±0.05 for T- sites). The decrease observed in IL-6 total protein expression at I and T+ sites during early wound healing was statistically significant (P<0.0001 and P=0.0001, respectively). Similarly, a statistically significant decrease was
observed for I and T+ sites when week 1 values were compared with week 12 levels (P<0.0001 and P=0.004, respectively). During early wound healing, peri-implant sites had higher levels of total IL-6 protein expression compared to T+ sites (P=0.006) and T- sites (P<0.0001). Similarly, the difference between T+ and T- sites was statistically significant at early weeks of healing (P=0.0001). IL-6 total protein levels went back to low baseline values by week 12 (0.4±0.09 pg for I, 0.53±0.1 pg for T+ and 0.52±0.24 pg for T- sites) (Figure 12).

Vascular Endothelial Growth Factor plays major role in wound healing. Peri-implant VEGF total protein levels reached similar baseline level by week 1 (41±7 pg) while surgically manipulated T+ and non-surgical T- sites had stable levels of VEGF (Figure 13). I site had a final VEGF total protein expression of 23±3 pg by 12 weeks and the decrease between week 1 and week 12 was statistically significant (P=0.0006). Interestingly, there was no statistically significant changes within I group for early wound healing (P=0.11) and late wound healing periods (P=0.06). On the other hand, an increase in VEGF total amount was noted around T+ site between week 6 and week 12 while VEGF levels stayed stable at T- site (P=0.0166 for T+, P=0.187 for T-). The differences between I and T+ was statistically significant due to mainly increasing VEGF levels at T+ sites, respectively (P=0.004) (Figure 13).

Matrix metalloproteinases 8 and 9 are highly expressed in gingival crevicular fluid even in health (Figure 14 and 15). Baseline MMP-8 total protein expression was in the range of 20,000-25,000 pg for T+ and T- sites (Figure 14). Peri-implant levels easily reached this range by week 1 of healing (27,338±6,694 pg) and started decreasing at the early and late healing process (13,859±4,858pg at 12 week, P=0.02). Similarly, a
statistically significant decrease at T+ values was noted up to 3 weeks healing (16,315±2,625 pg, P=0.001). The difference between T+ and T- groups was statistically significant during early wound healing phase (P=0.003). However, this treatment effect disappeared by 12 weeks since there was an increase in MMP-8 total protein expression around T+ sites by the final time period (P>0.05) (Figure 14).

Matrix Metalloproteinase-9 had higher baseline levels compared to MMP-8 (48,000-53,000 pg) (Figure 15). Peri-implant MMP-9 total protein amount was 61,328±6411 pg at week 1 and gradually decreased throughout the study. The difference between 1 week and 12 week was statistically significant (27,197±4,634 pg at 12 week, P<0.0001) as well as the decreases noted during early wound healing (P=0.0002) and late wound healing (P=0.008). Due to this continuous decrease in MMP-9 total protein amount at peri-implant crevice, between group differences for I and T- and, for T+ and T- groups were statistically significant at early wound healing phase (P<0.0001 for both comparisons, P=0.43 for I vs T+). Similar but statistically not significant decrease in I group was also noted during late wound healing phase (P=0.03 for I vs T+, P=0.11 for T+ vs T-, and P=0.53 for I vs T-) (Figure 15).

Tissue Inhibitor Matrix Proteinase-1, TIMP-2, TIMP-3 and TIMP-4 were investigated as possible inhibitors of MMP-8 and MMP-9 within crevicular fluid. TIMP-3 and TIMP-4 were not detected (data not shown). Baseline TIMP-1 total protein amount was 332±63 pg for T+ and 301±44 pg for T- sites (Figure 16). A significant increase at surgical site was noted by week 1 (1,241±150 pg for I and 731±113 pg for T+) compared to stable T- site levels. A fivefold decrease in peri-implant TIMP-1 total protein amount was noted by week 3 while this was almost two fold decrease for surgically manipulated
adjacent periodontal sites (248±42 pg for I and 423±247 pg for T+ by 3 weeks, P<0.0001 for both I and T+ groups). Similarly, when week 1 values were compared with week 12 values, observed decrease in TIMP-1 total protein amount was statistically significant for both I and T+ sites (P<0.0001 for I and P=0.002 for T+). During the early wound healing period, the differences were statistically significant between I and T- (P<0.0001) and T+ and T- (P=0.003). Final TIMP-1 protein expression around implant was significantly lower than T+ values although tooth sites maintained baseline levels (P=0.01; I vs T+) (Figure 16).

Tissue Inhibitor Matrix Proteinase-2 showed similar expression compared to TIMP-1 (Figure 17). Baseline TIMP-2 total protein amount was in the range of 2,300-1,700 pg. Peri-implant TIMP-2 total protein expression was 2,971±440 pg at 1 week and this was parallel to an increase observed in surgically manipulated adjacent periodontal crevice (2,543±353 pg). A statistically significant decrease was observed for both I and T+ groups by week 3 (P<0.0001 for I and P=0.0004 for T+ sites). The differences between I and T- groups and, between T+ and T- groups were statistically significant up to 3 weeks (P<0.0001 and P=0.0009, respectively). The decrease observed between week 1 and week 12 was also statistically significant for implant site (P<0.0001). A slight but statistically significant increase back to baseline levels was noted for T+ site during late wound healing phase (P=0.01) (Figure 17).
Possible Correlations between Initial Tissue Thicknesses and Week 1 Outcomes:

Peri-implant tissue thicknesses following final osteoctomy has been reported as an important factor affecting implant stability and long term success of the implant. The amount of keratinized tissue at future implant site was measured prior to surgery while flap thickness, buccal and lingual bone thicknesses were determined during surgery right before implant placement with the help of a caliper. Spearman Correlation Analysis corrected with Bonferonni adjustment was used to determine possible association between these initial measurements and peri-implant clinical parameters obtained at week 1 (Table 3). Based on this analysis, there was a statistically significant negative correlation between week 1 RFA readings and week 1 PI values (P=0.003). Also, a strong correlation was noted between PI and GI (P=0.006). Although a statistically significant correlation was not detected at P≤0.016 level, the following associations may be clinically significant since coefficient correlations were high with P values close to become statistically significant: Implant diameter versus week 1 RFA values (P=0.019); Surgical flap thickness versus week 1 PICF volume (P=0.05); Surgical flap thickness versus week 1 GI (P=0.05) and, implant diameter versus week 1 PI (P=0.033; negative correlation). We also studied the possible correlation between these initial measurements and total amount of protein for certain cytokines detected at week 1 healing at implant site (Table 4). There were no statistically significant correlations at P≤0.016. However, it seems that week 1 PICF volume may play an important role in total amount of IL-8 (P=0.02), MIP-1b (P=0.03) and IL-6 (P=0.04) at least for the week 1. Similarly, several measurements related to initial soft and hard tissue thickness around dental implant as well as implant diameter, may have direct effect on peri-implant IL-6 total protein.
amount obtained at week 1 (Table 4). Correlations between post-operative prescription usage, previous grafting of the site, anatomical location and week 1 peri-implant outcome are presented in Table 5. There was no statistically significant association at $P \leq 0.016$ level. Similarly, correlation analyses were repeated for week 1 T+ and T- outcomes (data not shown). There were no statistically significant correlations and P values were at $\geq 0.05$ level.
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<td>GENDER (FEMALE/MALE)</td>
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<td>AGE (mean ± s.e.)</td>
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**Table 1-** Patient demographics

* Zimmer Dental, Carlsbad, CA, USA  
** Straumann USA, LLC, Andover, MA, USA  
*** Astra Tech, Mondial, Sweden  
**** Biohorizons, Birmingham, AL, USA
Visit 1
Initial visit
Exam
Consent

Visit 2
Study
Baseline
Surgery

Visit 3
Post-op
Week 1
PI, GI
GCF
(T+ T- I)
RFA (I)

Visit 4
Post-op
Week 2
PI, GI
GCF
(T+ T- I)
RFA (I)

Visit 5
Post-op
Week 3
PI, GI
GCF
(T+ T- I)
RFA (I)

Visit 6
Post-op
Week 6
PI, GI
GCF
(T+ T- I)
RFA (I)

Visit 7
Post-op
Week 8
PI, GI
GCF
(T+ T- I)
RFA (I)

Visit 8
Post-op
Week 12
PI, GI
GCF
(T+ T- I)
RFA (I)

Figure 1- Study Design
Figure 2- Clinical Pictures Presenting PICF and GCF Sampling Procedure and Resonance Frequency Analysis transducer.
Figure 3- Plaque Accumulation at Surgical Site during Wound Healing

Mean ± s.e.; *P<0.0001 (I wk1 vs wk12)

**Early Wound Healing**

- P=0.03 (T+ over time)
- P=0.08 (I over time)
- P=0.04 (T+ vs T-)
- P=0.85 (I vs T+)
- P=0.08 (I vs T-)

**Late Wound Healing**

- P=0.07 (I over time)
- P=0.58 (T+ vs T-)
- P=0.33 (I vs T+)
- P=0.66 (I vs T-)
Figure 4- Soft Tissue Characteristics at Surgical Site during Wound Healing

Mean ± s.e.
*P<0.0001 (I wk1 vs wk12)
*P<0.0001 (T+ wk1 vs wk12)

**Early Wound Healing**
*P<0.0001 (I over time)
**P<0.0001 (T+ over time)
***P<0.0001 (I vs T-)
****P<0.0001 (T+ vs T-)

**Late Wound Healing**
******P=0.01 (I over time)
**Figure 5**- Changes in Crevicular Fluid Volume at Peri-Implant and Periodontal Sites during Wound Healing

Mean ± s.e.; *P<0.0001 (I wk1 vs wk12); *P<0.0001 (T+ wk1 vs wk12)

**Early Wound Healing**

*P<0.0001 (I over time);
*P<0.0001 (T+ over time);
P=0.0001 (I vs T-); P=0.002 (T+ vs T-)

**Late Wound Healing**

P=0.35 (I vs T-); P=0.86 (I vs T+)
Figure 6- Resonance Frequency Analysis during Wound Healing

Mean ± s.e. P<0.0001 (I over time)
Figure 7- IL-8 Total Amount within PICF and GCF during Wound Healing

Mean ± s.e.; *P=0.001 (I 1 wk vs 12 wks)

**Early Wound Healing**

***P=0.002 (I over time)***

***P=0.0002 (T+ over time)

P= 0.03 (I vs T+) 

P= 0.002 (I vs T-)

P= 0.001 (T+ vs T-)

**Late Wound Healing**

P= 0.03 (T+ over time)

P=0.01 (I vs T+)

P=0.02 (I vs T-)
Figure 8- MIP-1b Total Amount within PICF and GCF during Wound healing

Mean ± s.e.; *P<0.0001 (I wk 1 vs wk 12)

**Early Wound Healing**

**P=0.016** (I over time)

***P=0.007** (T+ over time)

P=0.018 (I vs T-)

P= 0.048 (T+ vs T-)

**Late Wound Healing**

P=0.03 (T+ over time)

P=0.01 (I vs T+)

P=0.02 (I vs T-)
Figure 9- IL-1β Total Amount within PICF and GCF during Wound Healing

Mean ± s.e.; *P=0.0002 (I wk1 vs wk12); **P=0.003 (T+ wk1 vs wk12)

**Early Wound Healing**
***P=0.004 (I over time)
P=0.01 (I vs T-)

**Late Wound Healing**
P=0.048 (T- over time)
****P=0.001 (T+over time)
P=0.002 (I vs T+)
Figure 10- IL-1ra Total Amount within PICF and GCF during Wound Healing

Mean ± s.e.; P= 0.046 (T+ wk1 vs wk12)
Figure 11- TNF-α Total Amount within PICF and GCF during Wound Healing.

Mean ± s.e.; P=0.02 (I wk1 vs wk12)
Figure 12- IL-6 Total Amount within PICF and GCF during Wound Healing.

Mean ± s.e.; *P<0.0001 (I wk1 vs wk12); **P=0.004 (T+ wk1 vs wk12)

Early Wound Healing
*P<0.0001 (I over time)
***P=0.0001 (T+ over time)
P=0.006 (I vs T+)
P<0.0001 (I vs T-)
P=0.0001 (T+ vs T-)
Figure 13- VEGF Total Amount within PICF and GCF during Wound Healing

Mean ± s.e.; *P=0.0006 (I wk1 vs wk12)

**Late Wound Healing**
P=0.06 (I over time)
**P=0.016 (T+ over time)
P=0.004 (I vs T+)
P=0.031 (I vs T-)
Figure 14- MMP-8 Total Amount within PICF and GCF during Wound Healing.

Mean ± s.e.; P=0.02 (I wk1 vs wk12)

Early Wound Healing
*P=0.001 (T+ over time)
P=0.22 (I over time)
P=0.003 (T+ vs T)
Figure 15- MMP-9 Total Amount within PICF and GCF during Wound Healing

Mean ± s.e.; *P<0.0001 (I wk1 vs wk12)  

**Early Wound Healing**  
***P=0.0002 (I over time)  
****P=0.0003 (T+ over time)  
P<0.0001 (I vs T-)  
P<0.0001 (T- vs T+)  
P=0.43 (I vs T+)  

**Late Wound Healing**  
***P=0.0008 (T+ over time)  
P=0.03 (I vs T+)  
P=0.11 (T+ vs T-)  
P=0.53 (I vs T-)  

[Graph showing MMP-9 total amount within PICF and GCF during wound healing with statistical significances indicated]
**Figure 16**- TIMP-1 Total Amount within PICF and GCF during Wound Healing

Mean ± s.e.; *P<0.0001 (I wk1 vs wk12); **P=0.002 (T+ wk1 vs wk12)

**Early Wound Healing**
*P<0.0001 (I over time)
*P<0.0001 (T+ over time)
P=0.02 (I vs T+)
P<0.0001 (I vs T-)
P=0.003 (T+ vs T-)

**Late Wound Healing**
P=0.033 (T+ over time)
P=0.01 (I vs T+)
P=0.26 (I vs T-)
Figure 17- TIMP-2 Total Amount within PICF and GCF during Wound Healing

Mean ± s.e.; *P<0.0001 (I wk1 vs wk12)

**Early Wound Healing**
- *P<0.0001 (I over time)
- **P=0.0004 (T+ over time)
- P<0.0001 (I vs T-)
- P=0.0009 (T+ vs T-)

**Late Wound Healing**
- ***P=0.01 (T+ over time)
- P=0.11 (I vs T+)
- P=0.49 (I vs T-)
- P=0.34 (T+ vs T-)
Table 2 - Results of Multiplex bead-based assays for pro-inflammatory biomarkers with low detection levels

<table>
<thead>
<tr>
<th>IL-7</th>
<th>T+BL</th>
<th>T+1WK</th>
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<th>T+6WKS</th>
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Table 2: Results of Multiplex bead-based assays for pro-inflammatory biomarkers with low detection levels
Table 3- Correlation Between Surgical Measurements and Week 1 Peri-Implant Clinical Parameters

Spearman Correlation Analysis Corrected with Bonferonni at P≤0.016. Color pink presents statistically significant correlation. Color blue presents a correlation that is not statistically significant following Bonferonni adjustment. However, it may be a clinically significant correlation.

KG= Initial amount of keratinized gingiva at implant site; Buc. Bone= Buccal Bone thickness following osteoctomy; Ling. Bone= Lingual Bone thickness following osteoctomy; PI= Plaque Index; GI= Gingival Index; PICF= Peri-Implant Crevicular Fluid; RFA= Resonance Frequency Analysis
<table>
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<tr>
<th>IMPLANT WEEK 1</th>
<th>RFA</th>
<th>PICF</th>
<th>GI</th>
<th>PI</th>
<th>KG</th>
<th>FLAP THICK</th>
<th>BUC BONE</th>
<th>LING BONE</th>
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<th>IMP LENG.</th>
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<td>-0.298</td>
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<td>p-value</td>
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<td>-0.298</td>
<td>0.340</td>
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</tr>
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**Table 4**: Correlation Between Surgical Measurements and Week 1 Total Protein Amount for Some of the Cytokines and Chemokines.

Spearman Correlation Analysis Corrected with Bonferonni at $P \leq 0.016$. Color blue presents a correlation that is not statistically significant following Bonferonni adjustment. However, it may be a clinically significant correlation.

KG= Initial amount of keratinized gingiva at implant site; Buc. Bone= Buccal Bone thickness following osteoctomy; Ling. Bone= Lingual Bone thickness following osteoctomy; PI= Plaque Index; GI= Gingival Index; PICF= Peri-Implant Crevicular Fluid; RFA= Resonance Frequency Analysis.
TABLE 5- Correlation Between Prescription Usage, Site Specific Characteristics and 1 Week Peri-Implant Findings.

Kruskal-Wallis Non-parametric test. Color blue presents a correlation that is not statistically significant following Bonferonni adjustment. However, it may be a clinically significant correlation.
CHAPTER 4

DISCUSSION

One stage implant placement surgical protocol allows clinical evaluation of peri-implant wound healing during early phases of osseointegration. The research interest of our group has evolved around this surgical technique. The ultimate goal of our studies is to determine whether various phases of early wound healing around dental implants placed with one-stage surgical protocol can be detected by using peri-implant crevicular content as a possible diagnostic tool. We previously reported changes in clinical parameters together with subgingival flora formation during early peri-implant wound healing. We also investigated the clinical and biological markers of early soft tissue healing around dental implants placed at previously grafted and non-grafted edentulous sites using the same wound model. The later study allowed us to conclude that PICF concentration of several pro-inflammatory cytokines and growth factors involved in wound healing follows the changes observed in clinical parameters used to evaluate early soft tissue healing. One of the main conclusions from the previously conducted preliminary studies was that PICF volume and cytokine concentrations demonstrated significant changes in the course of postoperative healing of one-stage dental implants.
The current study aimed to develop a better wound healing model by limiting the size and the anatomical location of the wound. It also included surgically manipulated adjacent periodontal site as well as an anatomically similar but not treated periodontal site within the same oral cavity as positive and negative control groups. Finally, the size of the study population was increased with more strengthen power analysis to compensate already reported high variation ranges for various outcome parameters used to evaluate wound healing. Among the fifty seven recruited subjects, 17 subjects were excluded. The drop-off rate due to missing follow-up appointments was 9% (5 subjects). In addition, four cases (7%) had to be removed from the study due to necessary surgical protocol modifications. Actual failure rate was 13% (6 cases out of 48 cases with implant device loosening during early wound healing). In addition, two patients (4%) presented early complications (e.g. peri-implant infection, bone loss etc.) and had to be excluded from the study since they had to have second surgery to prevent implant failure. Thus, the survival and success rates of these mandible posterior implants following 12 weeks of healing were 87% and 83%, respectively. Success rate of dental implants placed into similar anatomical location is not well documented. However, reported survival rate for longer time periods are in the range of 95-96%. Recent studies are reporting an association between survival rate and surgeon’s experience level. Based on these reports, a failure rate of 9% is expected for implants placed in graduate training clinics settings. Similarly, there are three main implant surgical systems used in this study and learning curve for each system may vary especially if it is a new system for the training institution. Among the six failures reported, three were type II (Straumann) and two were
type III (Astra), and one implant was type IV (BioHorizons). Type II and type IV systems became available with the initiation of this study protocol.

The different age groups had similar distributions in the present study ranging from 21 to 74 years old. Several other studies on dental implants included subjects with similar age distributions (13-84yo, 20-68yo) and none of these reports found age as a contributing factor for implant failure or for complications related to wound healing of dental implants. Nevertheless, age- and gender-associated delays in both cutaneous wound closure and in oral mucosal wound healing have been reported in the literature. Thus, emphasis was given to have similar distributions of different age groups and two genders within the current study population.

Similarly, diabetes and smoking have been well-accepted patient related risk factors that may delay wound healing and/or cause wound healing related complications. Impaired leukocyte functions including decreased chemotaxis to the wound, diminished neuroinflammatory signaling, release of oxygen free radicals through increase in Advanced Glycosylation End Products, Arginase and NOS activities and, decrease in angiogenesis were all reported as possible mechanisms involved in complicated/delayed wound healing observed in diabetic patients. Thus, uncontrolled diabetes was one of the exclusion criteria for this study. In addition, the patients in this study were non-smokers. Smoking impairs the revascularization of the soft and hard tissues following surgical trauma and also, presence of nicotine alters oral flora. Alveolar bone loss in smokers can be 4 times as much as nonsmokers mainly due to over-expression of several potent pro-inflammatory cytokines. Thus, smokers were excluded from the current study population.
The current study design was a prospective longitudinal observation trial to determine early wound healing outcome. Actual treatment protocol related to implant placement surgery was not modified for research purpose. Patients who required antibiotic prophylaxis and/or NSAIDs as analgesics were excluded. However, post-operative prescriptions for antibiotics and analgesics were given if they were deemed necessary by the surgeon. Subjects were given a diary and asked to record medicine uptake for the first week. The effect of antibiotics and NSAIDs use at the time of implant surgery and following implant surgery was previously shown to have limited effect on early implant failures and wound healing.\textsuperscript{85-86} In the current study, ten patients (25\%) took systemic antibiotics during first week of healing while 30 subjects (75\%) used analgesics, especially for the first three days of healing. There was no statistically significant correlation between prescription usage and changes in various clinical parameters and biomarkers during first week (P>0.05). Post-operative instructions following implant surgery included soft diet, use of chlorhexidine mouth rinse (0.12\%) and to refrain from brushing the surgery site for the first week. This clinical approach was shown to effectively control plaque levels and inflammation.\textsuperscript{87} It is considered that this clinical approach would not cause any differential effect since all subjects were given the same instructions.

Implant stability is one of the main clinical outcomes that determines treatment outcome. Factors such as bone quality, anatomical location, implant surface characteristics, implant diameter and length and bone-implant interface for contact surface affect initial implant stability.\textsuperscript{34,36} Resonance Frequency Analysis (RFA) is a clinical tool that helps evaluate implant stability at the time of placement and at various
phases of healing. The available data on RFA reveals that a decrease in ISQ levels is expected during the first weeks of healing followed by an increase up to 12 weeks.\textsuperscript{37,38} The current results are in acceptance with what is already reported in the literature. The variation in ISQ readings was negligible during early wound healing however the increase by week 12 became statistically significant compared to initial values (P<0.0001). Several factors like anatomical locations, bone quality and implant length have been well controlled in this study and this may be the reason that there was no significant decrease in ISQ levels during early weeks of healing.

The current study design investigated both previously grafted and non grafted sites. The grafted sites had extraction and socket reconstruction 3-4 months prior to implant placement. Recent studies report that previously grafted maxilla and mandible have demonstrated similar ISQ levels as non grafted sites.\textsuperscript{88-89} This was explained by the fact that re-vascularized grafted bone can respond surgical trauma with similar healing and osseointegration qualities.\textsuperscript{90} A previous study from our research group concluded that the difference in RFA readings between grafted and non-grafted sites became statistically significant only at week 3 with grafted site having a lower reading compared to non-grafted site.\textsuperscript{74} In the current study, there was no statistically significant correlation between RFA readings and presence/absence of grafting at week 1. The previous study included both maxillary and mandible anterior and posterior sextants while current report only includes single anatomical location. This approach probably eliminates the variation seen in bone quality at different anatomical locations possibly detected in the initial work.

Plaque and gingival indexes are frequently used in literature as diagnostic measurements to evaluate oral hygiene level and inflammatory status of teeth and
implants. In the current study, overall the plaque index was well controlled at all sites throughout the study period. There were no statistically significant differences in none of the three groups (I, T+ and T-). This could be explained by the frequent research visits where plaque index was taken and then plaque was removed to eliminate the contamination of plaque with PICF and GCF samples. However, the only statistically significant decline in PI was noted around implant when week 1 was compared with week 12 (P<0.0001). This is again expected since subjects resume oral hygiene home care practices following first week of healing. In terms of GI, the increase noted for I and T+ sites for the first week is expected due to surgical trauma and plaque accumulation. Correlation analysis revealed that PI and GI are strongly correlated at week 1 for I, T+ and T- groups (0.0067, 0.0078 and 0.00005, respectively). Similar to PI, GI showed a decrease at I and T+ sites throughout the study while it did not change at T- sites. This is a desired outcome for a well controlled wound healing.

Plaque accumulation (PI) and inflammation at the soft tissue level (GI) may have direct effects on the volume and content of crevicular fluid. The use of sterile absorbent filter strips and a calibrated electronic volume quantification unit are well accepted although they have several limitations. The simplicity, non-traumatic handling and the feasibility to collect site specific samples are the advantages. Possible contamination with blood, saliva or plaque and, collection time and its effect on protein concentration and evaporation are well documented disadvantages. Conventionally accepted collection time is 30sec/strip for periodontal disease related studies. We shortened this time to 20 sec/strip due to high blood contamination risk during early weeks of healing. Special effort was given to immediately transfer the sample papers to electronic unit and seal of
cryogenic vials after introduction of each sample to minimize evaporation. Also, strip number per site was kept same for each patient. An increase in GCF and PICF volumes at surgical sites during the first week of healing was noted which was gradually decreased throughout the study. GCF volume returned back to baseline levels by three weeks. However, PICF volume continued to decrease during late wound healing phase. This finding was consistent with previous studies that found correlations between GCF, PICF volume and gingival inflammatory status.\textsuperscript{64-65} Furthermore, this finding is implying the importance of GCF and PICF volumes as useful inflammatory markers. PICF collection by 8 and 12 weeks was challenging due to stronger peri-implant mucosal seal formation around healing abutment especially for those implants with healing abutments designed for platform switching concept.

A controversy still exists on whether to report site specific concentrations or total amount of proteins within GCF. While some researchers believe that total amount is more meaningful due to changes in collected volume, others prefer to report concentration together with collected amount.\textsuperscript{43} Our preliminary data was analyzed by using concentrations with significantly larger numbers and increased standard error bars. Thus, we chose to report our final data as total protein amount since it allowed eliminate noise created by volume changes throughout the weeks.

Wound healing process around dental implants is crucial step for implant survival and success. Capability of bone to develop load-bearing connection to the titanium surface and formation of stable connective tissue barrier that will protect underlying bone against challenges presented by oral environment are the two determinants of successful wound healing around dental implants.\textsuperscript{93} Therefore, understanding the molecular events
taking place at the early stages of implant integration is important to diagnose peri-
implant health status and to predict implant success. Several cytokines, chemokines,
growth factors which are related to inflammatory process, wound healing and tissue
remodeling of dental implants are investigated in the current study. It is found that IL-6,
IL-8, MIP-1b, and TIMP-1 in PICF are the dominant biomarkers specifically for implant
site during early wound healing period. In addition, an increased peri-implant response
was detected for IL-1β, VEGF, MMP-8 and MMP-9 during the first week of healing.
However, baseline periodontal levels of this second set of markers were high causing
some difficulty in the interpretation of wound healing related changes.

IL-6, IL-8 MIP-1b, and TIMP-1 have been investigated both in peri-implant and
periodontal health and in diseases.\textsuperscript{49, 58-59, 62,69, 86, 94-95} There is limited information in the
literature concerning molecular changes in GCF and PICF during wound healing. MIP-1b
and IL-8 are chemokines responsible for chemotaxis of monocytes and lymphocytes to
the inflammation site. These chemokines were found at high levels around the I and T+
sites during the early weeks and declined to baseline levels by week 3. In addition, the I
site showed statistically significant decrease between week 1 and 12. Previously, our
group has reported an increase in IL-8 concentrations at one week following the implant
surgery.\textsuperscript{86} Although the IL-8 levels are not comparable between studies due to different
techniques used in protein detection, both presented eight to five fold decreases in IL-8
levels between week 1 and 3. Similarly, others report an increase in IL-8 levels around
implants detected as early as 24 hours following surgery and also in peri-implant health,
mucositis and peri-implantitis conditions.\textsuperscript{62} Similar to our findings, Petkovic et al., have
shown difference in IL-8 concentrations around implants with inflammatory gingiva as
fivelfold higher than healthy implant sites. Based on these studies, IL-8 levels in PICF is associated with inflammatory status of implant and could be a prognostic factor for implant survival.\textsuperscript{62}

IL-1\(\beta\), TNF-\(\alpha\), IL-6 are proinflammatory cytokines that promote inflammatory response by stimulating bone resorption and protease production by many cell types such as fibroblast and osteoblasts.\textsuperscript{51-52, 63} One interesting finding of the present study is the differences in IL-1\(\beta\) levels between implant and natural tooth. While IL-1\(\beta\) continued to decrease at the I site throughout the study, T+ showed a decrease in the early wound healing times and an increase started during the late healing period demonstrating an inverse relationship with the I site. Recent studies reported IL-1\(\beta\) total amount ranging between 5-62pg around tooth and implants. This finding is comparable to the current study where amount of IL-1\(\beta\) ranged between 5-26pg.\textsuperscript{62, 65, 86} In addition, changes in the amount of IL-1\(\beta\) in GCF and PICF are reported to reflect the severity of inflammation in periodontal or peri-implant disease.\textsuperscript{62, 65, 86} The increase in IL-1\(\beta\) level at the surgically manipulated tooth site during late wound healing period observe in this current study requires further analysis which should include oral flora changes around surgically manipulated teeth.

TNF-\(\alpha\) is a proinflammatory cytokine with comparable functions like IL-1\(\beta\). In the current study, TNF-\(\alpha\) ranged between 0.9-1.7 pg which was similar to previously reported levels.\textsuperscript{64} Overall, TNF-\(\alpha\) did not show any differences in any of the sites or time points. This finding was similar to previous reports that are questioning the significance of TNF-\(\alpha\) as a biomarker of health or disease both around tooth and peri-implant sites.\textsuperscript{64}
One of the significant findings in this study is the detection of IL-6, IL-8 MIP-1b, and TIMP-1 at implant and T+ site at high levels in week one following surgery. Furthermore, during the early healing times (week 1 to 3), same cytokines have been detected in higher levels around implant site when compared to surgically manipulated T+ site and/or non-surgical T- control site. In the current study, elevated TIMP-1 synthesis at week 1 was followed by a decline around the implant site by week 3. The similar trend of TIMP-1 changes with time was observed in a recent study where TIMP-1, MMP-1, 8 and collagenase activity was investigated. Likewise, in that report the PICF level of TIMP-1 returned to GFC levels of TIMP-1 from healthy teeth by week 4 following implant surgery. The up-regulation of TIMP synthesis at week 1 could be important to inhibit and control MMP activity. It appears that elevation of TIMP-1 in PICF is protecting the integrity of the connective tissue at early times of healing.

There are a few studies in the literature that investigated the changes in the peri-implant crevicular fluid following implantation and compared the content of PICF to GCF of a natural tooth. To our knowledge this is the first report to investigate the wound healing process around dental implants and compares the differences in PICF and GCF following implant surgery during the first 12 weeks of healing. Based on our findings, it can be concluded that marked changes occur in biomarker expression at I and T+ site during early healing times following implant surgery. Furthermore, pronounced biomarker expression is observed at the I site compared to T+ site. Lastly, IL-1β, IL-6, IL-8, MIP-1b and TIMP-1 can be used as biomarkers to evaluate peri-implant wound healing due to low levels of expression at baseline and their significant increase at peri-implant sites during early phases of healing.
In summary, the results of this current study demonstrate that implant stability increased during the healing period around dental implants that are placed with one-stage surgery. Overall, the clinical parameters of inflammation and crevicular fluid volume were detected in high levels following implant surgery. These clinical markers returned baseline levels by the end of the study period as a sign of well-controlled peri-implant wound healing during osseointegration phase.

Crevicular fluid content, specifically IL-6, IL-8 MIP-1b, and TIMP-1 were detected at high levels both at I and T+ sites in week 1 following surgery. Furthermore, during the early healing times (week 1 to 3), same cytokines have been detected in greater amount around implant site when compared to surgically involved T+ site and/or T- control site where no surgical intervention was performed. This finding emphasizes the importance of events that take place at the wound healing site within the first week of healing. Overall, it is suggested that wound healing around dental implants presents variations in clinical and biological parameters compared to natural tooth site. Several biomarkers have the potential to be diagnostic in evaluating peri-implant wound healing.

With advanced understanding of wound healing process around dental implants, future studies could focus on developing diagnostic tools and treatment modalities to address peri-implant related conditions. In addition, the generated data could be used to improve healing events in compromised sites (maxillary posterior teeth) or patients (patients with smoking habits and diabetes) and prevent peri-implant healing related complications.
REFERENCES


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