## BLOOD FLOW, TISSUE THICKNESS, AND MOLECULAR CHANGES DURING CONNECTIVE TISSUE GRAFT EARLY HEALING

## THESIS

Presented in Partial Fulfillment of the Requirements for The Degree Master of Science in the Graduate School of the Ohio State University

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#### ABSTRACT

The subepithelial connective tissue graft (CTG) is a common and predictable modality for the treatment of gingival recession. While CTG is widely used, little is known about its early healing, considered critical for success. The aim of this prospective study was to assess CTG revascularization, tissue thickness, and molecular changes during early healing.

Adult (18-55 year old) healthy non-smokers scheduled to receive CTG were recruited. Stents were fabricated for repeatable probe positioning during blood flow and tissue thickness measurements. Buccolingual tissue thickness was recorded at 6 sites over the grafted area using a probe inserted into a custom stent until contact with the tissue surface was made. Blood flow was determined using Laser Doppler Flowmetry (LDF) at the surgical recipient site and at a control site outside of the surgical area. Gingival crevicular fluid (GCF) was collected using filter paper strips at mesial and distal interproximal sites of CTG recipient and control sites. Blood was obtained from the antecubital vein pre-operatively and at 3 days post operatively and used to prepare serum samples. LDF, thickness measurements, and GCF samples were collected pre-operatively, immediately postoperatively (except for GCF), and at 3, 7, 14, and 21 days postoperatively. After processing, GCF and serum samples were used to measure angiogenin (ANG) levels by a commercially available ELISA assay.

13 subjects completed the study. Immediate postoperative graft thickness averaged 1.5 mm. Compared to immediate postoperative thickness, tissue thickness increased 102% on day 3, returning to baseline by day 21. LDF measurements showed 87% reduction in tissue perfusion immediately post-surgery compared to preoperative values. At postoperative day 3, tissue perfusion was reduced by 74%, recovering by day 7. At 3 days postoperatively, GCF ANG levels increased to 201% of baseline in CTG sites, and remained unchanged in control sites and serum. GCF ANG levels showed no significant change from pre-operative levels on postoperative days 7, 14, and 21. Significant differences in blood flow and ANG levels were observed when subjects were subgrouped by age. Subjects aged >35 years (n=6) showed an 80% reduction in blood flow at day 7 and GCF ANG levels were 248% of baseline at day 3, while the corresponding changes in subjects aged <35 years (n=7) showed a 120% increase in blood flow at day 7 and GCF ANG levels were 161% of baseline at day 3. CTG treated sites undergo significant thickness changes during early healing, with tissue thickness doubling at day 3 and requiring 2 weeks of healing to return to baseline. CTG perfusion is significantly reduced until one week postoperatively, with older subjects exhibiting reduced perfusion until 2 weeks postoperatively. GCF ANG concentration was significantly increased at post operative day 3 with a return to baseline levels at one week, with older subjects exhibiting greater increases.

CTG treated sites undergo significant thickness changes during early healing, with tissue thickness doubling at day 3 and requiring 2 weeks of healing to return to baseline. CTG perfusion is significantly reduced until one week postoperatively, with

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older subjects exhibiting reduced perfusion until 2 weeks postoperatively. GCF ANG concentration was significantly increased in the very early healing period, post operative day 3, with a return to baseline levels at one week, with older subjects exhibiting greater increases. This is the first study to document the early tissue thickness changes following CTG treatment and to suggest that age may impact early CTG healing in terms of tissue perfusion and local molecular changes.

## DEDICATED TO MY GRANDPARENTS

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### CHAPTER 1

### INTRODUCTION

Originally, mucogingival surgery was defined as "surgical procedures designed to preserve gingiva, remove aberrant frenulum or muscle attachment, and increase the depth of the vestibule."<sup>1</sup> While relevant, this definition, created in 1953, is primitive when compared to the scope of mucogingival therapy today. With the advent of new techniques, mucogingival therapy evolved into what is now known as periodontal plastic surgery (PPS), a term first coined by Miller.<sup>2-6</sup> PPS broadened the scope of periodontal therapy to include prosthetic considerations, ridge augmentation, esthetic surgery, root coverage, papilla reconstruction, soft tissue augmentation around implants, and the surgical exposure of unerupted teeth.<sup>7</sup> The catalyst behind the growth of PPS procedures was the autogenous soft tissue graft, specifically the free gingival graft (FGG) first described by Björn in 1963 and the subepithelial connective tissue graft (CTG) described by Langer and Langer in 1985.<sup>8,9</sup> Both FGG and CTG have been shown to increase gingival tissue thickness, increase the width of keratinized gingiva, and repair gingival recession defects. However, CTG has become the method of choice in treating gingival recession due to better esthetic outcomes, minimal palatal morbidity, and increased root coverage as compared to FGG.<sup>10-12</sup>

Marginal gingival tissue recession is defined as the apical migration of the soft tissue margin leading to the exposure of root surfaces.<sup>13</sup> This tissue loss can lead to

root sensitivity, root caries, root abrasion, esthetic concerns, and, if left untreated, eventual tooth loss.<sup>14-17</sup> Recession defects are guite common among dental patients. Among American adults over the age of 30, 58% are estimated to have at least 1 mm of gingival recession. In the same population, an average of 22.3% of teeth show some degree of root exposure.<sup>18</sup> It is up to the clinician to advise or attempt to treat these defects since many patients present with chief complaints of esthetic concerns or root sensitivity. Prior to any surgical therapy, the etiology behind the root exposure must be identified. Both pathologic and non-pathologic factors have been found to be the culprit behind gingival recession, and it is not uncommon for the true etiology to be multifactorial.<sup>17</sup> Once the contributing factors have been identified and eliminated, a practitioner can choose from a variety of treatment options to correct gingival recession defects. CTG has become the most common modality in the treatment of root exposure, especially in Miller class I and II defects, mainly due to pleasing esthetic outcomes and the ability to maximize both root coverage and gains in keratinized tissue.<sup>19-21</sup> Moreover, the palatal harvest site tends to heal with far less morbidity and discomfort as compared to that of FGG.<sup>22</sup> While several CTG techniques exist, the common factor they share is a reliance on bilaminar blood flow to the graft tissue from host site periosteum and the underlying surface of the overlaying gingival flap.<sup>9, 11, 23, 24</sup> Revascularization originating from these sources, allows for graft tissue survival.

Angiogenesis and blood flow is of the utmost importance to the success of CTG. Histologic studies of FGG have shown that the graft tissue survives mainly on avascular plasmatic circulation from the surrounding recipient site for 2 to 3 days. At

7 days, a dense network of smaller vessels is observed, and by 14 days, the number of vessels is reduced and the vessels take on a more normal appearance.<sup>25-29</sup> Despite the popularity of CTG in PPS, information is sparse concerning early healing events and angiogenesis of graft tissue. In an in vivo study using dogs, Guiha et al. performed histological analysis of CTG. The study data concluded that, in dogs, revascularization of the graft tissue originates from the periodontal plexus in addition to the host site periosteum and overlying flap.<sup>30</sup> In this study, regeneration and maturation of graft vasculature was complete by 14 days.<sup>30</sup> While this study represented the first mention in the literature of CTG revascularization, it was only a descriptive histologic analysis, rather than a functional assessment. The only human study examining re-vascularization used fluorescent angiography to measure angiogenesis. In the study, vascularization was measured immediately following graft placement and at 3 and 7 days post operatively. Findings determined that the grafted tissue had only re-vascularized 65% at 7 days, thus the time to complete revascularization in humans has yet to be established.<sup>31</sup> In contrast to the popularity of CTG in periodontal treatment, little is known about early healing events of CTG, especially in terms of revascularization. The present study proposes the use of a novel approach to monitor revascularization of CTG, laser Doppler flowmetry.

In the current medical and dental literature, laser Doppler flowmetry (LDF) has become a reliable means to measure blood flow in tissue.<sup>6, 32-45</sup> This FDA approved technique represents a simple, painless, and non-invasive means of measuring functional blood flow. LDF is comprised of two fibers mounted within a small probe that is positioned above the examined tissue. The transmitting fiber emits

a 780 nm wavelength laser with a penetrating depth of about 1 mm that is scattered as it encounters moving red blood cells. The scattered light undergoes a shift in wavelength and this shift in wavelength (Doppler shift) is recorded by the receiving fiber within the probe. The signal is then analyzed and data is recorded as perfusion units (PU).<sup>37, 46, 47</sup> In the dental literature, LDF has been used to study blood flow in the dental pulp following trauma and following orthodontic therapy.<sup>33, 36, 48-50</sup> In the periodontal literature, LDF has been used to examine the blood flow in different locations in the oral cavity and monitor the effects of smoking, aging, and inflammation on soft tissue perfusion.<sup>32, 34, 39-41, 45</sup> Focusing on periodontal surgery, only three studies exist that observe blood flow changes using LDF. Recently, LDF was used in order to compare the blood flow in gingival tissue of smokers and nonsmokers following periodontal regeneration surgery showing marked differences in healing between the two groups.<sup>42</sup> The two other studies examined perfusion within the gingival flap following periodontal flap surgery. Their results showed that immediately following surgery, the gingival tissue becomes ischemic; however, by day 1 post operatively, there is a spike above baseline flow that does not resolve until about 14 days.<sup>35, 44</sup> While LDF has been used successfully to monitor post operative blood flow changes, there are no studies in published, peer-reviewed literature employing this technique to study the healing of CTG. Since initial blood flow and revascularization are of such importance to the clinical outcomes of CTG, LDF represents a valuable tool in the study of graft healing.

As previously mentioned, the key to success of root coverage procedures is the re-establishment of blood flow to the soft tissue graft. Angiogenesis during graft

healing is a multifaceted process involving different molecules, such as growth factors, and cells, such as endothelial cells. Moreover, while revascularization is taking place, both pro and anti-inflammatory cytokines are released as a reaction to surgical injury.<sup>51-55</sup> Due to expanding knowledge of molecular mediators during healing, the current trend in wound healing research is focused on understanding both the relative concentrations and timed expression of these factors in order to improve therapeutic conditions and overall healing.<sup>56-58</sup> Several studies have examined the effects of using exogenous growth factors in combination with root coverage procedures; however, there is no data at this point on the actual endogenous molecular changes that occur during surgical repair of gingival recession.<sup>59-65</sup> ANG is a 14 kDA polypeptide that has undergone extensive research due to its role in new vessel formation in malignant tumors. This protein is also a normal component of GCF that can be detected in higher levels in tissues affected by chronic periodontitis, a chronic inflammatory process.<sup>66</sup> Recent studies suggest that ANG has both angiogenic and antimicrobial properties.<sup>67, 68</sup> In mice, cigarette smoke has been found to increase ANG levels in lung tissue.<sup>69</sup> Intercellularlly, ANG stimulates mRNA transcription, an essential rate limiting step of ribosome synthesis, protein synthesis, and overall cell growth. Downregulation of ANG has been found to decrease both endothelial cell growth and proliferation, confirming its importance in blood vessel formation.<sup>70</sup> To date, there exist no published studies observing levels of GCF ANG and serum ANG during oral soft tissue graft healing. In order to be able to improve early healing, such as angiogenesis, in CTG, it is important to understand the molecular changes occurring during this time and correlate them to clinical outcomes.

Long term clinical measures of CTG are typically reported as root coverage and increases in keratinized tissue; however, there are no reports of early clinical changes during healing.<sup>71</sup> This study aimed to examine tissue thickness changes of CTG in order to provide a clinical parameter to monitor during early healing. Only two studies exist examining the tissue thickness changes of CTG. Their findings showed that at 6 months post operatively, tissue thickness increased about 0.5 mm; however, there are no reports on corresponding changes that occur during the early healing period.<sup>31, 72</sup>

The overall objective of this research study was to examine early healing of CTG in terms of blood flow, molecular, and tissue thickness changes. Establishing a temporal guide to these events and understanding the correlation between them will provide knowledge that could help improve clinical outcomes in treating gingival recession. Furthermore, with this knowledge, interventions may be able to be performed in patients with known risk factors for healing, such as smoking and diabetes, in order to improve the predictability of PPS procedures.<sup>73-76</sup> The present study is based on and designed to test the following **hypothesis**: site specific and systemic levels of molecular mediators are related to the revascularization process and tissue thickness changes of the subepithelial connective tissue graft.

## **Specific Aims:**

The specific aims are to:

- establish the revascularization time of the subepithelial connective tissue graft in humans.
- determine local ANG changes during the course of early subepithelial connective tissue graft healing.
- determine systemic ANG changes during the course of early subepithelial connective tissue graft healing.
- determine changes in tissue thickness during the course of early subepithelial connective tissue graft healing.

### CHAPTER 2

## MATERIALS AND METHODS

#### Study Population and Experimental Design

The overall design was a prospective longitudinal observational cohort study. Subjects were recruited from The Ohio State University College of Dentistry Graduate Periodontology Clinic. Only subjects who had already been treatment planned for CTG as part of their overall periodontal treatment plan were recruited. Inclusion criteria for the study included: 1) systemically healthy non-smoking individual from 18 to 55 years of age, 2) patient of record at The Ohio State University College of Dentistry, 3) signed treatment plan for CTG of Miller I or Miller II gingival recession defect on a maxillary or mandibular incisor, canine, or pre-molar, 4) signed consent for gingival augmentation procedure, and 5) ability to provide informed consent. Exclusion criteria for the study included: 1) current use of calcium channel blockers, antiseizure medication, or cyclosporine, 2) current use of tobacco products, 3) Diabetes (type I and type II), 4) Pregnancy, 5) caries or a faulty restoration on the tooth receiving the CTG, and 6) a reported acrylic allergy.

All data gathering took place at the Graduate Periodontology Clinic at The Ohio State University. The total duration of the study was 6 visits, spanning 21 days from the day of the surgical procedure (Figure 1). At the first study visit, interested subjects were given a detailed explanation about the study timeline and procedures that would be required at all study visits. Interested subjects that met the inclusion criteria were then required to provide informed consent. Subjects were also given the option to participate in a blood draw, which had a separate consent form.

Following informed consent, one alginate impression of the arch containing the planned CTG site was taken in order to fabricate stone models. A pre-operative questionnaire (Figure 2) was then completed by the subject. The questionnaire included a series of questions to determine age, smoking status, and medications. From the stone casts, 2 acrylic stents were fabricated. Red rope wax was placed onto the stone cast at the planned surgical site and covered with aluminum foil prior to stent fabrication in order to simulate tissue swelling. One stent was fitted with miniholders to hold the LDF probe at a repeatable, stable position while measuring blood flow. The other stent was fitted with 6 aluminum tubes. The tubes were hollow and of sufficient size to intimately fit the diameter of a UNC-15 periodontal probe. These tubes were placed at a mesial coronal, mid coronal, distal coronal, mesial apical, mid apical, and distal apical site at the anticipated CTG soft tissue location in order to produce repeatable tissue thickness measurements.

Each subject contributed one test site (CTG) and one control site to the study. The control site was designated as a tooth in the same arch as the planned CTG; however, outside of the surgical field.

The second study visit included the surgical procedure, CTG. Vital signs, clinical photographs, LDF recordings, blood draw, gingival crevicular fluid (GCF) samples, and tissue thickness measurements were collected in the above order prior to local anesthetic administration. LDF and GCF were recorded at both the surgical site and control site, while tissue thickness was recorded only at the surgical site.

Immediately following the surgery, LDF and tissue thickness measurements were made once again. Subjects were given post operative instructions and prescribed analgesics and a chlorhexidine gluconate 0.12% mouth rinse (CHX). Study visits were completed at 3 days, 7 days, 14 days, and 21 days post operatively. At all study visits, vital signs, clinical photographs, LDF recordings, GCF samples, and tissue thickness measurements were collected in the above order. On the third study visit (Day 3), subjects who had previously consented for a blood draw had the procedure performed. A post-operative questionnaire was also completed by subjects at each one of study visits 3 through 6 (Figures 3-6). The questionnaire evaluated the subjects' post operative pain and discomfort using visual analog scale (VAS) and the location of the pain (donor vs. recipient site). The VAS was present on all post operative questionnaires. A score of 0 represented no pain while a score of 10 represented the most pain that a subject had ever experienced. The questionnaire was also used to determine the frequency of use of prescribed medications and to confirm smoking status. The final post-operative questionnaire asked subjects to also rate their overall experience of being in the study.

#### Clinical Study Procedures

## Vital Signs:

Blood pressure was measured with a standard blood pressure cuff and stethoscope. Heart rate was measured from the radial artery pulse. Temperature was measured using a disposable thermometer.

### Photographs:

Standard intraoral photographs were taken of the graft recipient site using a digital camera with cheek retractors at a 1:1 magnification and intraoral photographic mirrors when necessary.

### Laser Doppler Flowmetry Recordings:

LDF was recorded using the Periflux 5000 PF 5010 LDPM unit (Perimed AB, Sweden). Prior to each study visit, the LDF unit was calibrated according to the manufacturer's recommendation using the included motility standard and zeroing disk (Perimed AB, Sweden). LDF measurements were taken prior to local anesthesia delivery (baseline), immediately following surgery, and at each of the post-operative study visits. Recordings were performed at the surgical site and at the control site. The LDF probe was stabilized by inserting it into the miniholders held by the stent. The stent also provided repeatable positioning at each study visit. With the patient in a 45 degree reclined position with instructions to remain still, the probe readings were performed for 120 seconds in order to ensure stabilization of the readings. For the pre-operative reading, the LDF probe was positioned 1 mm apical to the existing gingival margin on the control and test tooth. For the post-operative recordings, care was taken to ensure that the LDF probe was positioned above exposed graft tissue. All recordings were collected by one examiner.

### Gingival Crevicular Fluid Samples:

A total of four GCF samples were collected from teeth receiving CTG and from control teeth at study visit 2 through 6. Two samples were taken from the mesial aspect and two from the distal aspect of the buccal surface of the teeth of interest. Cotton rolls were used to isolate the teeth, supragingival plaque was removed using a curette, and the tooth/soft tissue surface was gently air dried using an air-water syringe. Care was taken not to disturb the soft tissue with either the curette or the air-water syringe. GCF samples were taken one at a time with a one minute interval between samples. GCF was obtained using standardized filter paper strips (PerioPaper strips OraFlow Inc., Plainview, New York) placed with a nontraumatic technique in the entrance of the gingival sulcus until mild resistance was felt. Filter paper strips were left in place for 30 seconds. Samples contaminated with blood or saliva were discarded. Immediately following the 30 second sample collection, GCF volume was determined using the Periotron 8000 (electronic micromoisture meter, OraFlow Inc., Plainview, New York). GCF volume was recorded and the strip was placed into a sterile  $500\mu$ l polypropylene centrifuge tube. The tube was kept on ice until the end of the study visit. At that time the tube was transferred into storage at -80°C. All GCF samples were stored at -80°C until processing. All samples were collected by one examiner.

Tissue Thickness Measurement:

Tissue thickness measurements were performed using an acrylic stent inserted with six aluminum tubes that intimately fit the diameter of a UNC-15 periodontal probe. Measurements were made prior to local anesthesia delivery and immediately following the surgery (baseline). Tissue thickness was also recorded at each postoperative study visit. Measurements were recorded at six sites: mesial coronal, mid coronal, distal coronal, mesial apical, mid apical, and distal apical. A UNC-15 periodontal probe was inserted into the aluminum tube until contact with the underlying soft tissue was made. All measurements were collected by one examiner. *Serum Collection:* 

Blood draws were only performed on subjects who had consented to a blood draw at the initial study visit. Blood draws were performed after vital signs were recorded on the day of surgery and at three days post-operatively. Samples were collected via venipuncture on either the left or right antecubital fossa. Ten milliliters of peripheral blood was collected in a serum preparation tube (clot activator and gel) (BD<sup>©</sup>). Following the study visit, the collection tube was spun down and samples were aliquoted and stored frozen at -80°C.

## Gingival Crevicular Fluid Sample Processing

Gingival crevicular fluid samples were analyzed for the presence of ANG. Periopaper strips were thawed on ice and GCF was eluted from each Periopaper strip using a previously described method.<sup>77</sup> An extraction buffer containing 50 mM Tris/HCl with 5 mM CaCl<sub>2</sub>, 0.2 NaCl. pH 7.6 containing 1 mg/L antipain, 1 mg/L aprotinin, 1 mg/L leupeptin, 125 mg N-ethylaleimide and 50 mg Zwittergent 3-12 (inhibitor cocktail) was used. The 4 Periopaper strips collected from each site (CTG or control) were placed into 1.5 ml centrifuge tubes along with 225 µl of extraction buffer. The Periopaper strips, in combination with the GCF extraction buffer, were vortexed vigorously three times every fifteen minutes over a period of one hour. A hole was created at the bottom of a 400 µl Eppendorf tube using a 25-guage needle. Periopaper strips were then placed into the 400  $\mu$ l tube, and that tube was then fitted on top of the 1.5 ml centrifuge tube. The tubes were centrifuged (10,000 g, 3 min, 4°C) forcing excess elution fluid from the Periopaper strips housed in the Eppendorf tube down into the 1.5 ml centrifuge tube. A 200 µl sample was stored at -80°C until analysis.

## Molecular Analysis

GCF fluid and serum samples were analyzed for the presence of Angiogenin (ANG) using a commercially available double antibody sandwich enzyme-linked immunosorbent assay (ELISA) following manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). Previously prepared GCF and serum samples were diluted 10 fold and 200 fold respectively. Phosphate buffered saline was used to dilute the GCF samples while a calibrator diluent, provided by the manufacturer, was used to dilute the serum samples. A standard curve was created by serial dilution of a provided standard and plotting the absorbance at 450 nm versus the log of recombinant human ANG concentration. Based on this curve, ANG concentrations in GCF and serum were calculated. GCF ANG concentrations were calculated taking into account the volume of GCF on each Periopaper strip.



Figure 1. Study Design.

Connective tissue graft healing in smokers and non-smokers: LDF study 2008 (Rotenberg, S.A., Tatakis, D.N.)

STUDY SUBJECT #

#### **QUESTIONNAIRE (PRE-OPERATIVE)**

Date Completed

Please answer as best you can. If you have difficulty answering a question, please leave it blank. If you would like an explanation, please feel free to ask. Your answers are strictly confidential and will remain anonymous.

- 1. How old are you? \_\_\_\_years \_\_\_\_months
- 2. \_\_\_\_Male \_\_\_\_Female
- 3. Are you currently receiving dental treatment in addition to your periodontal treatment?

\_\_\_\_Yes \_\_\_\_No

If yes, please describe\_

- 4. Have you been diagnosed with diabetes mellitus?
  - If yes: a) what type of diabetes mellitus? (please circle) Type I Type II
    - b) when were you diagnosed? \_\_\_\_\_
- 5. Are you currently taking any medications? \_\_\_\_\_Yes \_\_\_\_\_No

If yes, please list the medication, reason for taking it, and dosage (if known)

Medication	Reason	Dosage

- 6. Do you have any allergies? \_\_\_\_Yes \_\_\_No If yes, what are you allergic to? \_\_\_\_\_Yes
- 7. Do you currently smoke cigarettes or use other tobacco products? \_\_\_\_Yes \_\_\_\_No

If yes: a) what do you use? (circle all that apply) cigarettes pipe cigar chewing tobacco b) If you smoke cigarettes, how many do you smoke each day?

c) If you smoke cigarettes, how long have you been smoking? \_\_\_\_years \_\_\_\_months

- If you currently do not smoke, have you ever smoked cigarettes or used other tobacco products?
  Yes \_\_\_\_No
  - If yes: a) what did you use? (circle all that apply) cigarettes pipe cigar chewing tobacco b) If you smoked cigarettes, how many did you smoke each day?
    - c) If you ever smoked cigarettes or used other tobacco products, when was the last time you

smoked or used tobacco? \_\_\_\_\_year \_\_\_\_\_month

Thank you for completing this questionnaire

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Connective tissue graft healing in smokers and non-smokers: LDF study 2008 (Rotenberg, S.A., Tatakis, D.N.)

STUDY SUBJECT # \_\_\_\_

**QUESTIONNAIRE DAY 3 (Post-Operative Visit 1)** 

#### Date Completed

Please answer as best you can. If you have difficulty answering a question, please leave it blank. If you would like an explanation, please feel free to ask. Your answers are strictly confidential and will remain anonymous.

1. Did you have any pain since the end of the surgery? \_\_\_\_Yes \_\_\_\_No

If yes, please describe the pain as best you can:

2. How much pain did you have? Please circle number, with '0' being no pain and '10' being the most severe pain imaginable



- Please circle the number that best describes the pain that you experienced and how it affected your activities
  - 0 = No pain
  - 1 = Tolerable and pain does not prevent any activities
  - 2 = Tolerable and pain prevents some activities
  - 3 = Intolerable and pain does not prevent use of telephone, TV viewing, or reading
  - 4 = Intolerable and pain prevents use of telephone, TV viewing, or reading
  - 5 = Intolerable and pain prevents verbal communication.

If you experienced pain that you rated 2 or higher, please list or describe all the activities that were prevented by the pain:

4. Where was the pain localized? (please circle all that apply)

Donor Site (roof of mouth) Surgery (graft) site Elsewhere

If elsewhere, please indicated the location \_

#### Please continue the questionnaire on the next page

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Figure 3. First Post Operative Questionnaire (continued)

### Figure 3: continued

Connective tissue graft healing in smokers and non-smokers: LDF study 2008 (Rotenberg, SA., Tatakis, D.N.)

STUDY SUBJECT # \_\_\_\_

5. Did you take any pain medication since the end of the surgery? \_\_\_\_Yes \_\_\_\_No

If yes, please indicate what pain medication, when you took it, how much you took, and how often you took it:

Pain medication	When taken	Amount taken	How often taken

 Did you use the prescribed mouth rinse since the end of surgery? \_\_\_\_Yes \_\_\_\_No If yes, how much and how often did you use it?

7. Have you smoked since the end of surgery? \_\_\_\_Yes \_\_\_\_No

If yes: a) how long after the surgery did you begin smoking? \_\_\_\_\_Days \_\_\_\_\_Hours

b) how many cigarettes did you smoke per day, on average, since you began smoking after the end of surgery? \_\_\_\_\_

Thank you for completing this questionnaire

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Connective tissue graft healing in smokers and non-smokers: LDF study 2008 (Rotenberg, S.A., Tatakis, D.N.)

STUDY SUBJECT # \_\_\_\_

#### QUESTIONNAIRE DAY 7 (Post-Operative Visit 2)

#### Date Completed

surgery?

Please answer as best you can. If you have difficulty answering a question, please leave it blank. If you would like an explanation, please feel free to ask. Your answers are strictly confidential and will remain anonymous.

1. Since the first visit after the surgery (DAY 3 after the surgery), have you had any pain related to the graft

Yes No

If yes, please describe the pain as best you can:

2. How much pain did you have? Please circle number, with '0' being no pain and '10' being the most severe pain imaginable



- Please circle the number that best describes the pain that you experienced and how it affected your activities.
  - 0 = No pain
  - 1 = Tolerable and pain does not prevent any activities
  - 2 = Tolerable and pain prevents some activities
  - 3 = Intolerable and pain does not prevent use of telephone, TV viewing, or reading
  - 4 = Intolerable and pain prevents use of telephone, TV viewing, or reading
  - 5 = Intolerable and pain prevents verbal communication.

If you experienced pain that you rated 2 or higher, please list or describe all the activities that were prevented by the pain:

4. Where was the pain localized? (please circle all that apply)

Donor Site (roof of mouth)	Surgery (graft) site	Elsewhere	
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If elsewhere, please indicated the location \_\_\_\_

#### Please continue the questionnaire on the next page

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### Figure 4. Second Post Operative Questionnaire (continued)

#### Figure 4: continued

Connective tissue graft healing in smokers and non-smokers: LDF study 2008 (Rotenberg, S.A., Tatakis, D.N.)

#### STUDY SUBJECT # \_\_\_\_\_

5. Did you take any pain medication since the first visit after the surgery (DAY 3 after the surgery)?

\_\_\_Yes \_\_\_No

If yes, please indicate what pain medication, when you took it, how much you took, and how often you took it:

Pain medication	When taken	Amount taken	How often taken

6. Did you use the prescribed mouth rinse since the first visit after the surgery (DAY 3 after the surgery)?

Yes	No
100	

If yes, how much and how often did you use it?

7. Have you smoked since the end of surgery? \_\_\_\_Yes \_\_\_\_No

If yes: a) how long after the surgery did you begin smoking? \_\_\_\_\_Days \_\_\_\_\_Hours

b) how many cigarettes did you smoke per day, on average, since you began

smoking after the end of surgery? \_\_\_\_

#### The following questions are only for subjects who had a biopsy performed on DAY 3

8. Did you have any pain associated with the biopsy area? Please circle number, with '0' being no pain and '10' being the most severe pain imaginable



9. Did you have to take pain medication only because of the biopsy-related pain? \_\_\_\_\_Yes \_\_\_\_\_No

#### Thank you for completing this questionnaire

VERSION 1.0 Page 2 of 2

Connective tissue graft healing in smokers and non-smokers: LDF study 2008 (Rotenberg, S.A., Tatakis, D.N.)

STUDY SUBJECT #

#### **QUESTIONNAIRE DAY 14 (Post-Operative Visit 3)**

#### Date Completed \_

3.

Please answer as best you can. If you have difficulty answering a question, please leave it blank. If you would like an explanation, please feel free to ask. Your answers are strictly confidential and will remain anonymous.

1.

				_								
How muc	h pain	did you	have	Plea	se cir	cle num	iber, w	ith 'O' I	being n	o pai	n and '10' being th	ne mos
severe pa	ain imaç	ginable		r.	т	T	т	r	E		T	
		+	+	+	+	+	+	+		+	_	
	0 No Pair	1	2	3	4	5 Iodarat	6	7	8	9	10 Worst Pain	
	NO Fail	1.			IV	Pain	6				Imaginable	
Please ci	ircle the	numb	er that	best d	escrib	es the	pain th	at vou	experi	ence	d and how it affect	ed vo
activities									1			,
0 = No pa	ain											
1 = Toler 2 = Toler	able an	d pain	does n	ot pre	vent a	ny activ	/ities					
3 = Intole	erable a	nd pair	does	not pre	event	use of	elepho	one, T\	/ viewin	ng, or	reading	
4 = Intole	erable a	nd pair	n preve	nts us	e of te	elephon	e, TV v	viewing	g, or rea	ading		

If you experienced pain that you rated 2 or higher, please list or describe all the activities that were prevented by the pain:

4. Where was the pain localized? (please circle all that apply)

Donor Site (roof of mouth) Surger	y (graft) site	Elsewhere
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If elsewhere, please indicated the location \_\_\_\_\_

#### Please continue the questionnaire on the next page

VERSION 1.0 Page 1 of 2

## Figure 5. Third Post Operative Questionnaire (continued)

### Figure 5: continued

Connective tissue graft healing in smokers and non-smokers: LDF study 2008 (Rotenberg, S.A., Tatakis, D.N.)

#### STUDY SUBJECT # \_\_\_\_\_

5. Did you take any pain medication since last week's visit (DAY 7 after the surgery)?

\_\_\_Yes \_\_\_\_No

If yes, please indicate what pain medication, when you took it, how much you took, and how often you took it:

Pain medication	When taken	Amount taken	How often taken

6. Did you use the prescribed mouth rinse since last week's visit (DAY 7 after the surgery)?

Yes	No
100	110

If yes, how much and how often did you use it?

7. Have you smoked since the end of surgery? \_\_\_\_Yes \_\_\_\_No

If yes: a) how long after the surgery did you begin smoking? \_\_\_\_\_Days \_\_\_\_\_Hours

b) how many cigarettes did you smoke per day, on average, since you began

smoking after the end of surgery? \_\_\_\_\_

#### The following questions are only for subjects who had a biopsy performed on DAY 3

8. <u>Since last week's visit</u> (DAY 7 after the surgery), did you have any pain associated with the biopsy area? Please circle number, with '0' being no pain and '10' being the most severe pain imaginable



9. <u>Since last week's visit</u> (DAY 7 after the surgery), did you have to take pain medication <u>only</u> because of the biopsyrelated pain? \_\_\_\_\_Yes \_\_\_\_\_ No

Thank you for completing this questionnaire

VERSION 1.0 Page 2 of 2

Connective tissue graft healing in smokers and non-smokers: LDF study 2008 (Rotenberg, S.A., Tatakis, D.N.)

STUDY SUBJECT # \_\_\_\_

**QUESTIONNAIRE DAY 21 (Final Post-Operative Visit)** 

Date Completed \_

Please answer as best you can. If you have difficulty answering a question, please leave it blank. If you would like an explanation, please feel free to ask. Your answers are strictly confidential and will remain anonymous.

1. Since last week's visit (DAY 14 after the surgery), have you had any pain related to the graft surgery?

\_\_\_Yes \_\_\_\_No

If yes, please describe the pain as best you can:

2. How much pain did you have? Please circle number, with '0' being no pain and '10' being the most severe pain imaginable



- 3. Please circle the number that best describes the pain that you experienced
  - 0 = No pain
  - 1 = Tolerable and pain does not prevent any activities
  - 2 = Tolerable and pain prevents some activities
  - 3 = Intolerable and pain does not prevent use of telephone, TV viewing, or reading
  - 4 = Intolerable and pain prevents use of telephone, TV viewing, or reading
  - 5 = Intolerable and pain prevents verbal communication.

If you experienced pain that you rated 2 or higher, please list or describe all the activities that were prevented by the pain:

4. Where was the pain localized? (please circle all that apply)

Donor Site (roof of mouth) Surgery (graft) site Elsewhere

If elsewhere, please indicated the location

Please continue the questionnaire on the next page

VERSION 1.0 Page 1 of 3

Figure 6. Final Post-Operative Questionnaire (continued)
#### Figure 6: continued

Connective tissue graft healing in smokers and non-smokers: LDF study 2008 (Rotenberg, S.A., Tatakis, D.N.)

#### STUDY SUBJECT # \_\_\_\_\_

5. Did you take any pain medication since last week's visit (DAY 14 after the surgery)?

\_\_\_\_Yes \_\_\_\_No

If yes, please indicate what pain medication, when you took it, how much you took, and how often you took it:

Pain medication	When taken	Amount taken	How often taken
		7	

6. Did you use the prescribed mouth rinse since last week's visit (DAY 14 after the surgery)?

1	1	h la
1	res	1/10

If yes, how much and how often did you use it?

7. Have you smoked since the end of surgery? \_\_\_\_Yes \_\_\_\_No

If yes: a) how long after the surgery did you begin smoking? \_\_\_\_\_Days \_\_\_\_\_Hours

b) how many cigarettes did you smoke per day, on average, since you began smoking after the end of surgery? \_\_\_\_\_

8. How would you rank the experience of being in this study? (please circle)

Fantastic Great Average Poor Never Again

9. Please tell us what, if anything, was worst about this study:

10. Please tell us what, if anything, was best about this study:

#### Please continue the questionnaire on the next page

VERSION 1.0 Page 2 of 3

## Figure 6: continued

Connective tissue graft healing in smokers and non-smokers: LDF study 2008 (Rotenberg, S.A., Tatakis, D.N.)

STUDY SUBJECT # \_\_\_\_\_



Thank you for completing this questionnaire

VERSION 1.0 Page 3 of 3

## CHAPTER 3

## DATA MANAGEMENT AND STATISTICAL ANALYSIS

Collected data were organized into a computer program spreadsheet. Data entered were proofed for entry errors. The site (CTG or control) was the unit of analysis for site-specific data, while the subject was the unit of analysis for serum data.

Descriptive data were analyzed using frequency distributions (numbers and percentages), measures of central tendency (means), and measures of dispersion (standard deviation and standard error).

Data were analyzed using mixed model repeated measures ANOVA. Frequency data were analyzed by Chi-square test. Statistical significance was set at alpha = 0.05.

#### CHAPTER 4

## RESULTS

#### General Observations

A total of 18 subjects who fulfilled the inclusion criteria were recruited into the study. Of these 18 subjects, 13 returned for the surgical visit and completed the study. All 13 subjects who completed the study underwent the planned CTG with no post operative complications noted. All CTG were considered successful. According to post operative questionnaires, subjects obeyed post operative instructions and used the prescribed analgesics. Neither smoking nor the use of any tobacco related products were reported by any of the subjects. Subjects did not report any self described post operative complications, and discomfort relating to surgery was consistent with previous reports in the literature.<sup>22</sup>

#### Subject Reported Outcomes

The mean VAS values were 3.23 on day 3, 2.15 on day 7, 0.15 on day 14, and 0.46 on day 21. At 3 days post operative, 12 out of 13 subjects reported pain. At day 7, only 10 subjects reported pain. At day 14 and 21, the number of subjects reporting pain was reduced to 2. No significant difference was seen in patient reported pain locations between the palatal donor site and the graft recipient site.

#### Laser Doppler Flowmetry

At pre-operative baseline, the mean  $\pm$  standard error (SE) perfusion units (PU) reported by the laser Doppler flowmeter for control and CTG treated sites were 192.8  $\pm$  39.0 PU and 192.9  $\pm$  25.9 PU, respectively. In control sites, LDF perfusion was  $209.4 \pm 30.7$  PU immediately post operatively,  $217.5 \pm 26.4$  PU at day 3,  $231.3 \pm$ 39.2 PU at day 7, 220.7  $\pm$  27.2 PU at day 14, and 181.2  $\pm$  28.8 PU at day 21. CTG treated sites showed LDF measured perfusion at  $24.4 \pm 7.3$  PU immediately post operatively,  $62.9 \pm 33.0$  PU at day 3,  $160.6 \pm 43.4$  PU at day 7,  $228.7 \pm 24.3$  PU at day 14, and  $174.1 \pm 23.0$  PU at day 21. In control sites, blood flow was 115.4% of baseline immediately post operatively, 128.1% at day 3, 136.8% at day 7, 145.2 % at day 14, and 112.3% at day 21 (Figure 7). In CTG treated sites, mean blood flow was 13.0% of baseline immediately post operatively, 26.2% at day 3, 127.6% at day 7, 177.1% at day 14, and 135.7% at day 21 (Figure 7, Table 1). Non-treated control sites showed no significant change from baseline throughout the study. CTG treated sites exhibited a significant change from baseline blood flow immediately post operatively and at day 3 (p < 001). By day 7, there was no significant mean change from baseline blood flow.

## Laser Doppler Flowmetry by Age

Subjects under the age of 35 years (n=7) were compared to subjects over the age of 35 (n=6) in terms of tissue perfusion as measured by the laser Doppler

flowmeter. At control sites, the mean  $\pm$  SE baseline blood flow was  $162.7 \pm 27.3$  PU in subjects under 35 and  $228.2 \pm 44.5$  PU in subjects over 35. Over the span of the study, subjects under the age of 35 showed perfusion values of  $190.2 \pm 30.6$  PU, 209.57 ± 37.6 PU, 197.3 ± 46.0 PU, 253.3 ± 44.1 PU, and 210.8 ± 47.0 PU immediately post operatively, at 3 days, 7 days, 14 days, and 21 days respectively at control sites. Blood flow recorded at control sites in subjects over the age of 35 measured  $231.7 \pm 58.2$  PU,  $226.7 \pm 40.3$  PU,  $271.0 \pm 66.5$  PU,  $192.8 \pm 24.0$  PU, and  $146.7 \pm 27.8$  PU immediately post operatively, at 3 days, 7 days, 14 days, and 21 days. As a mean  $\pm$  SE percent baseline measurement at control sites, subjects under the age of 35 recorded 125.6%, 135.2%, 133.7%, 129.1%, and 126.0% immediately post operatively, at day 3, day 7, day 14, and day 21, respectively. In subjects over the age of 35, blood flow at control sites was 103.4%, 119.8 %, 140.4%, 102.8%, and 74.3% immediately post operatively, at day 3, day 7, day 14, and day 21, respectively. No significant difference was found between subject age groups in control sites. In CTG treated sites, mean  $\pm$  SE baseline blood flow was  $157.3 \pm 37.8$ PU in subjects under 35 and  $234.2 \pm 72.6$  in subjects over 35. Subjects under 35 exhibited tissue perfusion of  $18.6 \pm 10.9$  PU,  $33.6 \pm 7.7$  PU,  $239.9 \pm 61.6$  PU,  $248.9 \pm 10.9$  PU,  $33.6 \pm 7.7$  PU,  $239.9 \pm 61.6$  PU,  $248.9 \pm 10.9$  PU,  $33.6 \pm 7.7$  PU,  $239.9 \pm 61.6$  PU,  $248.9 \pm 10.9$  PU,  $33.6 \pm 7.7$  PU,  $239.9 \pm 61.6$  PU,  $248.9 \pm 10.9$  PU,  $33.6 \pm 7.7$  PU,  $239.9 \pm 61.6$  PU,  $248.9 \pm 10.9$  PU,  $33.6 \pm 7.7$  PU,  $239.9 \pm 61.6$  PU,  $248.9 \pm 10.9$  PU,  $33.6 \pm 7.7$  PU,  $239.9 \pm 61.6$  PU,  $248.9 \pm 10.9$  PU,  $33.6 \pm 7.7$  PU,  $33.6 \pm 7.7$  PU,  $33.6 \pm 7.7$  PU,  $239.9 \pm 61.6$  PU,  $248.9 \pm 10.9$  PU,  $33.6 \pm 7.7$  PU,  $33.6 \pm 7.$ 32.3 PU, and  $183.3 \pm 29.3$  PU immediately post operatively, at day 3, day 7, day 14, and day 21 respectively. Subjects over 35 exhibited tissue perfusion of  $31.1 \pm 9.6$ PU, 97.1 ± 71.6 PU, 68.0 ± 36.3 PU, 205.0 ± 37.5 PU, and 163.4 ± 38.9 PU immediately post operatively, at day 3, day 7, day 14, and day 21, respectively. When examining the re-establishment of tissue perfusion in CTG treated sites in terms of percent baseline, subjects under the age of 35 returned to baseline blood flow levels sooner than subjects over 35. Subjects under 35 showed blood flow at 9.7% of baseline immediately post operatively, 25.0% at day 3, 217.9% at day 7, 219.6% at day 14, and 150.1% at day 21 (Figure 8, Table 1). The tissue perfusion in subjects over 35 was 16.9% of baseline immediately post operatively, 27.6% at day 3, 22.4% at day 7, 127.4% at day 14, and 119.0% at day 21 (Figure 8, Table 1). Subjects under the age of 35 experienced a significant change from baseline blood flow immediately post operatively and at 3 days while subjects over the age of 35 experienced a significant change from baseline blood flow immediately post operatively and at 3 days while subjects over the age of 35 experienced a significant change from baseline blood flow immediately post operatively and at 3 days while subjects over the age of 35 experienced a significant change from baseline immediately post operatively, at day 3, and at day 7. The difference in blood flow level at day 7 between the two groups proved to be significant (p<0.05).

	CTG (n=13)	CTG <35 years (n=7)	CTG >35 years (n=6)
Immediately Following CTG	$13.0 \pm 3.27$	$9.7 \pm 3.59$	$16.9 \pm 5.67$
3 Days Post-Op	$26.2 \pm 5.32$	$25.0 \pm 4.75$	$27.6 \pm 10.72$
7 Days Post-Op	$127.6 \pm 39.61$	$217.9 \pm 53.57$	$22.4 \pm 5.20$
14 Days Post-Op	$177.1 \pm 31.85$	$219.6 \pm 47.67$	$127.4 \pm 34.32$
21 Days Post Op	$135.7 \pm 28.21$	$150.1 \pm 32.34$	$119.0 \pm 50.67$

Table 1. Mean ( $\pm$ SE) percentage of baseline blood flow at surgical site





Figure 7. Mean (±SE) percentage of baseline blood flow at control and surgical site



Figure 8. Mean (±SE) percentage of baseline blood flow at surgical site by age

## **Tissue Thickness**

All tissue thickness measurements were collected by one calibrated examiner with a 94.0% exact agreement on repeated measurements. Immediately following surgery, an average of all 6 measurement sites showed mean buccolingual tissue thickness increase of 1.5 mm (baseline). At day 3, mean tissue thickness increased 102.3% compared to baseline. At day 7 and day 14, mean tissue thickness was increased 52.3% and 5.3% respectively. At 21 days post operatively, mean tissue thickness had decreased 11.5% compared to baseline (Figure 9). This corresponded to a 1.29 mm mean increase in tissue thickness compared to pre-operative measurements. When comparing site specific tissue thickness measurements, apical and coronal measurements show very similar patterns of change over the post operative time frame. Comparisons between apical and coronal measurements did not show any significant difference in tissue thickness changes (Figure 10). Interproximal and mid buccal measurements also showed similar patterns of tissue thickness changes over time. Again, no significant difference was noted between the groups (Figure 11).

## Tissue Thickness by Age

In subjects under the age of 35, mean post operative tissue thickness increased 1.43 mm from pre-operative tissue thickness while subjects over the age of 35 experienced a 1.63 mm increase. This difference in immediate post operative tissue thickness between age groups did not prove significant. In subjects under 35, tissue thickness increased 2.86 mm, 2.26 mm, 1.71 mm, and 1.45 mm from pre-operative

35

measurements at day 3, day 7, day 14, and day 21, respectively. This corresponded to 217.2%, 175.4%, 131.6%, and 112.5% percent of immediate post operative tissue thickness (baseline). Subjects over the age of 35 showed mean tissue thickness increases of 3.14 mm, 2.22 mm, 1.36 mm, and 1.11 mm at day 3, day 7, day 14, and day 21, respectively. This corresponded to 203.6%, 145.8%, 80.9%, and 70.3% of baseline. In both groups, tissue thickness was significantly different from baseline at day 3 (p<.001), yet no significant difference was seen between age groups.

# Mean Change in Tissue Thickness



Figure 9. Mean (±SE) change in tissue thickness of CTG





Figure 10. Mean (±SE) % baseline tissue thickness of CTG at coronal and apical sites





Figure 11. Mean (±SE) % baseline tissue thickness of CTG at mid buccal and interproximal sites

#### Gingival Crevicular Fluid Angiogenin

GCF ANG concentration at control sites was measured at day 0 and day 3. Mean  $\pm$  SE GCF ANG concentration was  $130.9 \pm 19.5$  pg/µl pre-operatively (baseline) and  $154.3 \pm 25.0$  pg/µl at day 3 corresponding to 118.0% of baseline. GCF ANG concentrations at CTG treated sites showed the majority of change at 3 days post operatively. Baseline ANG concentration was 146.23 pg/µl. At day 3, mean ANG concentration increased to 243.3 pg/µl. Post operative day 7, 14, and 21 showed ANG concentration resembling baseline levels at 132.37 pg/µl, 123.0 pg/µl, and 144.28 pg/µl, respectively.

Relative to baseline concentrations measured pre-operatively, GCF ANG was 201.4% of baseline at day 3, 102.6% at day 7, 98.5% at day 14, and 115.5% at day 21 (Table 2, Figure 12). Only post operative day 3 GCF ANG concentrations proved to be significantly different than the mean baseline level (p<.005).

#### Gingival Crevicular Fluid Angiogenin by Age

In subjects under 35 years of age, mean  $\pm$  SE GCF ANG concentration at control sites measured 138.4  $\pm$  24.1 pg/µl pre-operatively and 176.9  $\pm$  36.3 pg/µl at day 3. In this group, mean day 3 GCF ANG at control sites was 125.3% of baseline. In subjects over 35, mean  $\pm$  SE control site GCF ANG was 122.2  $\pm$  33.7 pg/µl preoperatively and 127.8  $\pm$  33.7 pg/µl at day 3. When examining CTG treated sites, both groups followed the same pattern of ANG concentration change; however, subjects over the age of 35 expressed higher concentrations of GCF ANG at day 3. In subjects under the age of 35, GCF treated sites showed ANG concentrations of  $141.3 \pm 21.5$ pg/µl,  $218.9 \pm 23.2$  pg/µl,  $154.0 \pm 29.0$  pg/µl,  $136.9 \pm 19.9$  pg/µl, and  $148.9 \pm 13.3$ pg/µl pre-operatively, at 3 days, 7 days, 14 days, and 21 days, respectively. This corresponded to 161.5%, 106.5%, 103.1%, and 114.3% of pre-operative baseline GCF ANG concentrations at day 3, day 7, day 14, and day 21, respectively (Table 2, Figure 13). In subjects over the age of 35, the measured concentrations of GCF ANG at CTG treated sites were  $151.9 \pm 47.4$  pg/µl,  $271.8 \pm 65.7$  pg/µl,  $107.2 \pm 19.2$  pg/µl,  $106.8 \pm 26.5$  pg/µl, and  $138.9 \pm 49.3$  pg/µl pre-operatively, at day 3, day 7, day 14, and day 21. This corresponded to 248.1%, 97.9%, 93.1%, and 117.0% of baseline GCF ANG at day 3, day 7, day 14, and day 21 (Table 2, Figure 13). In both age groups, GCF ANG showed a significant difference from baseline at day 3. The difference between GCF ANG concentrations at day 3 was significant between age groups as well (p<.05).

	CTG (n=13)	CTG <35 years (n=7)	CTG >35 years (n=6)
3 Days Post-Op	$201.4 \pm 27.62$	$161.5 \pm 12.35$	$248.1 \pm 54.28$
7 Days Post-Op	$102.6 \pm 9.63$	$106.5 \pm 6.80$	$97.93 \pm 20.24$
14 Days Post-Op	$98.5 \pm 10.21$	$103.1 \pm 14.27$	<b>93.1</b> ± 15.69
21 Days Post Op	$115.5 \pm 19.84$	$114.3 \pm 15.26$	$117.0 \pm 41.47$

Table 2. Mean (±SE) percentage of baseline ANG concentration at surgical site





Figure 12. Mean (±SE) % baseline GCF ANG at CTG treated sites





Figure 13. Mean (±SE) % baseline by age of GCF ANG at CTG treated sites

## Serum Angiogenin

Serum was collected from a total of 10 subjects who had consented for the blood draw. No complications arose from venipuncture. Serum ANG was compared in pre-operative samples and samples obtained at day 3 post operatively. The mean pre-operative ANG concentration was 297.5 pg/µl while day 3 concentration was 297.3 pg/µl. At day 3, mean % baseline ANG serum concentration was 104.9% showing no significant serum ANG change between pre-operative levels and 3 day post operative concentrations.

#### CHAPTER 5

## DISCUSSION

The current study sought to examine early healing in CTG in terms of blood flow, molecular changes, and tissue thickness changes. The results showed that the first week of healing following CTG is critical for revascularization of graft tissue. Within this time period, ischemia with an eventual return of blood flow is seen with corresponding changes in GCF ANG levels. Previous studies on revascularization in CTG have only been in the forms of a descriptive histological analysis in dogs and an angiography study in humans.<sup>30, 31</sup> The present study represents a functional analysis of revascularization. The data presented in this study shows a marked drop of 87.0% in CTG blood flow immediately post operatively. Previous studies on the effect of periodontal flap surgery have shown similar results as well.<sup>35,44</sup> However, in those studies, it was unclear what role local anesthesia played in the observed ischemia since blood flow was shown to be significantly reduced following local anesthesia administration, prior to surgery. Local anesthesia containing a vasoconstrictor has been shown in previous LDF studies to cause ischemic effects on gingival tissue.<sup>78, 79</sup> Also, in flap surgery, blood flow is still maintained from the base of the flap in contrast to CTG where the graft tissue is totally removed from the donor site, severing all previous vascular connections. In the study of CTG blood flow using fluorescent angiography by Burkhardt and Lang, the only previously published study

quantifying revascularization of CTG in humans, CTG blood flow was reduced 92.0% immediately following surgery, which is consistent with the current study.<sup>31</sup>

Overall, the revascularization trend in this study does not follow the same pattern as those presented in the animal study by Guiha *et al.* and the human angiography study by Burkhardt and Lang.<sup>30, 31</sup> The data presented in the Burkardt and Lang study show blood flow at 44.5% and 63.9% of baseline at day 3 and day 7. In the present study, the mean of blood flow of all subjects was reduced to 26.2% of baseline at day 3, a similar finding; however, blood flow at day 7 surpassed baseline blood flow, 127.6% of baseline. In a similar study by our group LDF recorded CTG blood flow did not reach baseline levels until day 14. In that study, blood flow was 7.6%, 34.9%, 67.5%, 105.2%, and 114.9% of baseline levels immediately post operatively, at day 3, day 7, day 14, and day 21.<sup>80</sup> The current study showed a return to baseline blood flow by day 7.

The data presented in this study show an overall return to baseline blood flow by 7 days post operatively, while the previous human study and a previous study from our group concluded that blood flow levels do not reach baseline levels until after day 7.<sup>31, 80</sup> A possible explanation for the differences seen in this study compared to the two previous studies may be the effect of age on revascularization. When comparing subjects under the age of 35 and those over the age of 35 showed similar blood flow reductions at day 3, 25.0% and 27.6% of baseline respectively. However, subjects under the age of 35 return to baseline blood flow levels much earlier than those over 35. At 7 days post operatively, subjects over the age of 35 are at 22.4% of baseline blood flow and do not reach baseline levels until day 14, which is consistent with the Burkhardt and Lang study and the study by Wessel.<sup>31, 80</sup> On the contrary, subjects under the age of 35 show blood flow levels over 200% of baseline levels at day 7, surpassing baseline levels one week earlier than subjects over 35. In a study by Matheny *et al.* the effect of aging on the microcirculation of marginal gingiva was assessed.<sup>39</sup> In that study, no difference in LDF measurements were found between subjects under 35 and those over 35, which is consistent with the baseline values in this study. However, age was shown to significantly decrease blood flow velocity and reduce the number of active blood vessels when comparing the two age groups.<sup>39</sup> The data presented in this study provides further evidence that aging can affect the microcirculatory system especially in terms of angiogenesis.

There is limited information in the literature concerning early healing of CTG in terms of molecular changes. ANG represents a key growth factor in new blood vessel formation and is identifiable in GCF.<sup>57, 66, 67, 70, 81</sup> The results of this study show increases in GCF ANG compared to baseline values in response to an ischemic event in CTG. The current study shows that GCF ANG levels were highest at day 3, about two times baseline levels. This corresponds to the drop in perfusion seen at day 3 from the LDF data. The results further suggest that by 1 week, GCF ANG levels return to baseline. This study reports a mean baseline GCF ANG concentration of 146.23 pg/µl increasing to 243.3 pg/µl at 3 days following CTG. The mean baseline GCF ANG concentration appeared to be approximately half of baseline serum ANG concentration which was 297.5 pg/µl. The GCF ANG concentrations that were recorded in this study are consistent with levels seen in a previous study by our group. In that study, baseline GCF ANG was found to be 156.3 pg/µl, and at 3 days

following CTG, GCF ANG rose to 295.6 pg/µl.<sup>31, 80</sup> When comparing this study to the study by Wessel, differences are seen in GCF ANG at 7 days and 14 days. In the current study, GCF ANG returned to baseline by day 7 and remained at that level until day 21; however, in the Wessel study, GCF ANG concentration is still significantly greater than baseline at day 7 (243.8 pg/µl) and day 14 (213.2 pg/µl).<sup>80</sup> One possible explanation for this disparity may be the difference in blood flow seen between the two studies. In the current study, tissue hypoxia was only encountered immediately post operatively and at day 3 while the Wessel study reported blood flow levels below baseline until day 14.<sup>80</sup> The delayed tissue revascularization encountered in the CTG in that study may have attributed to the prolonged elevated GCF ANG concentrations.

In terms of serum ANG levels, the findings from this study show that there is no change in serum ANG concentration from Day 0 to Day 3 in CTG post operative healing, unlike GCF ANG. This study is the first to measure serum ANG during not only CTG, but any periodontal surgery procedure. Moreover, the serum concentration measured was comparable to levels found in the medical literature.<sup>82, 83</sup>

In the current study, the relationship between GCF ANG and revascularization is strengthened further when comparing the relationship between the concentration of GCF ANG at day 3 and LDF measured at day 7 between subjects under the age of 35 and those over the age of 35. At post operative day 3, GCF ANG concentration is 161.5% of baseline for subjects under the age of 35 while subjects over the age of 35 have GCF ANG concentrations increased to 248.1% of baseline. While subjects over the age of 35 show higher concentrations of GCF ANG at day 3, those subjects do not return to baseline LDF values by day 7, unlike those subjects under 35. At this point it is not clear why older subjects did not attain baseline blood flow values as early as younger subjects even though GCF ANG concentrations were higher in subject over the age of 35. One possibility may be an inefficiency in GCF ANG mediated angiogenesis as an individual ages. Many metastatic tumors are reliant on ANG and it is well documented in the medical literature that some non-hormone related tumors grow faster in younger individuals.<sup>84-86</sup> An alternative explanation for the age related changes seen in this study may be that tissue in older subjects experience a greater hypoxic state than tissue in younger individuals inducing correspondingly greater ANG release. In the medical literature, ANG has been shown to be up-regulated by Hypoxia Inducible Factor-1 alpha, a factor released in response to hypoxia.<sup>87-89</sup>

In addition to blood flow and molecular changes during early CTG healing, this study is the first to report on tissue thickness changes. The earliest reports of tissue thickness changes in previous studies have been at 6 month post operatively. These studies utilized endodontic files fitted with rubber stoppers to penetrate the surface tissue until contact was made with underlying bone or tooth in order to measure buccal tissue thickness. Both of these studies showed that at 6 months after CTG, soft tissue thickness increased around 0.5 mm.<sup>72, 90</sup> Only one other method of measuring tissue thickness following surgery is mentioned in the literature. In a study comparing free connective tissue grafts to guided tissue regeneration for root coverage, Müller *et al.* used an ultrasonic device pre-operatively and at 6 months post operatively. At 6 months, tissue thickness increased 0.6 mm compared to preoperative dimensions.<sup>91</sup> The present study employed a novel technique using an acrylic stent outfitted with aluminum cylinders that intimately surrounded the diameter of a UNC-15 periodontal probe. The results from this study showed that the majority of tissue thickness changes occur at day 3 post operatively with tissue thickness almost doubling in dimension from immediate post operative dimension. In this study, no differences were found between increases in tissue thickness between apical and coronal or mid buccal and interproximal sites. Moreover, no correlation was found between tissue thickness changes and blood flow and GCF ANG levels. The lack of correlation between post operative tissue thickness and angiogenesis is to be expected since soft tissue swelling post surgery is mainly a result of edema, an event that cannot be recorded via LDF.<sup>92</sup> The results of this study show that the majority of significant tissue thickness changes occur rapidly over the first 21 days of healing. Due to the greatest increase in tissue thickness occurring during the most critical time of CTG healing, 1 week, proper suturing and flap design allowing for significant tissue thickness increase is vital for CTG success.

#### CHAPTER 6

## CONCLUSIONS

The results of the current study demonstrate that revascularization of CTG reaches pre-operative levels by post operative day 7; however, age may affect revascularization with older individuals taking up to 14 days to regain baseline. Nevertheless, both younger and older patients undergoing CTG show a dramatic decrease in graft tissue blood flow immediately post operatively until day 7, proving that the first week following surgery is the most critical regarding graft survival and care must be taken during that time not to disrupt the process of revascularization.

Angiogenesis seen in CTG is accompanied by changes in ANG levels in GCF that are not seen in circulating serum. This increase in GCF ANG is short lived, observed at day 3 but not at 1 week. In individuals over the age of 35, GCF ANG concentration increases to levels higher than those seen in younger individuals; however, revascularization still takes 7 days longer to reach baseline blood flow. This discrepancy in GCF ANG and revascularization may be due to an inefficiency of ANG mediated revascularization in older individuals. In terms of tissue thickness, CTG thickness almost doubles in size by day 3 returning to immediate post operative dimensions by day 21. Overall, this study emphasizes how critical the first week of healing is, especially the first 3 days, in terms of blood flow, release of angiogenic factors, and changes in tissue thickness which can all influence graft success; and how age may delay revascularization. Future studies should re-examine the effect of aging more closely. By understanding the relationship between blood flow and angiogenic factors in CTG, novel approaches may be able to be taken to improve early healing events and overall success rates of periodontal plastic surgery.

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