Pre-Wounding and Free Gingival Grafts: A Pilot Investigation

Thesis

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

By

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Abstract

**Objectives:** The free gingival graft (FGG) is a well-established periodontal plastic surgery procedure. Although simple and predictable, the FGG is fraught with complications, especially postoperative morbidity associated with the donor site. Pre-wounding is a surgical technique based on the concept of inducing ischemic conditions (wounding) that results in increased blood supply, which helps improve surgical outcomes. The present study aims to examine the effects of pre-wounding on FGG donor site outcomes and microbiological community profiles.

**Methods:** Ten systemically healthy, nonsmoker, adults completed the study. One side of the palate was randomly chosen for pre-wounding by initial surgical incisions. Five days later standardized dimension FGGs were harvested from both the pre-wounded site and the contralateral site (routine FGG). Complete wound healing (epithelial closure), postoperative (PO) pain and healing complications were assessed on PO day 3, 7, 14, and 21 by direct examination and questionnaires. Microbiological samples were collected from FGG sites preoperatively and postoperatively at baseline, 0, 3, 7, 14, and 21 days. The samples were analyzed by terminal restriction fragment length polymorphism (t-RFLP) for bacterial community profiling.

**Results:** On PO day 3 and 7, none of the FGG donor sites exhibited epithelial closure. On PO day 14, 40% of pre-wounded FGG donor sites and none of the
routine FGG sites were completely healed; the corresponding values for day 21 were 89% and 78%. Pain (visual analog scale; VAS) scores on PO day 3 for pre-wounded and routine FGG donor sites were 3.9 ± 2.2 and 4.5 ± 1.8, respectively (p<0.4; paired t-test). The corresponding VAS scores for PO day 7 were 1.1 ± 2.1 and 2.8 ± 2.7 (p<0.001), while for PO day 14 were 0.5 ± 1.3 and 0.7 ± 0.7 (p=0.8), and PO day 21 were 0 and 0.2 ± 0.2 (p=0.19). Subjects consistently reported that pre-wounded FGG donor sites resulted in quicker healing and less PO bleeding than the routine FGG donor sites. Microbial profiles differed significantly between routine and pre-wounded sites on day of harvest, PO day 3, and PO day 7 (Tukey-Kramer, p<0.02, p<0.05, respectively). There was an increase in the number of bacterial species from surgical intervention to epithelial closure. The number of bacterial species in pre-wounded sites stayed the same from Day 0 to PO day 7, while the number of bacterial species in routine sites increased. Nonmetric multidimensional scaling showed a distinct difference in the bacterial species and abundance between routine and pre-wounded sites on PO day 3 and PO day 7.

**Conclusions:** Pre-wounding FGGs prior to harvest is well tolerated by systemically healthy nonsmoker patients, appears to improve the FGG donor site postoperative healing course and to reduce the associated morbidity, and it alters the palatal mucosa-associated oral biofilm.
Dedication

Dedicated to my family
Acknowledgments

I wish to thank my advisor Dr. Dimitris Tatakis for his insight, help and guidance throughout my Masters. I also thank Dr. Purnima Kumar for her help and guidance through the bacterial portion of the study. I also wish to thank Matt Mason and Esther Chien for their assistance during the laboratory portion of the study and Laura McCallister and Deb Hooper for their help in patient recruitment and supply ordering. Lastly, I would like to thank all of the Graduate periodontology residents for their support. This study was supported by the Division of Periodontology, The Ohio State University College of Dentistry.
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2010.................................................................D.D.S., The Ohio State University, School of Dentistry
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Publications


Fields of Study

Major Field: Dentistry
Periodontology
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CHAPTER 1

INTRODUCTION

*The Free Gingival Graft (FGG): Applications and Outcomes.*

The FGG is a procedure where a graft, consisting of gingival connective tissue and epithelium, is harvested from the palate and is used to treat either the lack of keratinized tissue (gingiva) or gingival recession (exposed root surface). To increase the width of keratinized tissue, the harvested epithelialized graft is placed on a recipient periosteal bed previously covered by mucosa.\(^1\)\(^-\)\(^5\). To treat recession defects, the harvested FGG is placed either in the area of a recession defect,\(^6\)\(^-\)\(^9\) or apical to the recession and later (after healing has taken place) coronally positioned over the denuded root.\(^10\),\(^11\).

Several parameters have been identified as causing poorer treatment outcomes when FGG is used; they are related to the graft itself, the recipient bed and other technical aspects related to graft placement, and the patient. Two of the most important factors associated with poorer outcomes are graft thickness <1 mm;\(^6\),\(^9\) and smoking.\(^5\),\(^8\),\(^9\)

Of particular interest are the patient-centered outcomes, specifically postoperative pain and donor site bleeding. When it comes to postoperative pain experience, FGG procedures result in greater pain prevalence and severity compared to CTG procedures.\(^12\),\(^13\) Patients undergoing FGG procedures also have a higher likelihood of experiencing postoperative bleeding.\(^12\),\(^14\)
The fact that FGG is predictable and relatively easy to perform are two of the reasons why it is still widely used to increase keratinized tissue dimensions in thin or absent gingiva. The fact that FGG is associated with greater prevalence of postoperative pain and bleeding, along with its frequent use, are reasons to try to improve the patient-centered outcomes associated with this procedure.

*Plastic Surgery: the Surgical Delay or Pre-wounding approach.*

In order to address poorer postoperative outcomes following flap surgery for certain types of patients, e.g., smokers, or procedures, e.g., breast reconstruction, plastic surgeons have attempted various technique modifications. One such successful modification is the “surgical delay” or “ischemic preconditioning” technique, which has been used in large reconstructive flap procedures.15-18 For skin grafts, a similar approach, termed “pre-wounding” has been used.19 The concept behind these technique modifications is that exposure of a flap (or a graft) area to ischemic conditions (wounding) results in increased tissue blood supply,15 thus improving the odds of successful outcomes at time of flap elevation (or graft transplantation). The timing of the delay/pre-wounding procedure is typically 1-2 weeks before surgery.15,17,20 The pre-wounding (PW) approach was recently used with connective tissue grafts (CTGs) in a pilot research study from this institution; the results indicated that pre-wounding resulted in an accelerated healing response.21 To the best of our knowledge, the pre-wounding approach has not been used or studied in the context of the FGG procedure; thus, the proposed
study is the first one to pursue such an approach in an effort to ultimately improve surgical periodontal therapy outcomes.

*The Microbiology of Healing Wounds and Flaps.*

The dominant bacterial phyla found in the oral mucosa include *Firmicutes* (*Streptococcus* and *Veillonella*), *Proteobacteria* (*Neisseria*), *Bacteroides* (*Prevotella*), and *Actinobacteria* (*Micrococcineae*). Bacteria have been shown to be important in wound healing by influencing healing responses through effects on epithelial cell migration, proliferation and apoptosis and endothelial motility, and by contributing to complicated postoperative outcomes through high bacterial loads. Bacterial collagenase promotes wound healing by stimulating post-injury cellular responses within the epidermal and dermal components, which enhances re-epithelialization, wound healing angiogenesis, and wound closure in vitro. It has also been found that antimicrobial peptides or proteins, part of the innate defense mechanisms, are produced by keratinocytes, neutrophils, and endothelial cells and are expressed constitutively or after an inflammatory stimulus. Pre-wounding, which results in an inflammatory response and altered expression of several healing-associated proteins in the palate, may affect the microflora of the healing palatal wounds (FGG donor sites). One study found that intestinal pre-wounding protects the intestine and reduces bacterial translocation. Although the above studies hint at a possible difference between the pre-wounded and conventional grafting techniques in the microbiological profile during early donor site wound healing,
there are currently no studies that have assessed the microbiological profile of oral wounds pertinent to periodontal plastic surgery procedures.

Working hypotheses:
The present study was designed to test the following hypotheses: i) a pre-wounding approach will improve the postoperative healing and patient outcomes for FGG donor sites, and ii) a pre-wounding approach will result in altered microbial profile for healing FGG donor sites.

Specific aims:

1) To assess and compare the effects of a pre-wounded and a routine FGG harvesting approach on FGG donor site healing.

2) To assess and compare the effects of a pre-wounded and a routine FGG harvesting approach on post-operative patient-centered outcomes.

3) To assess and compare the effects of a pre-wounded and a routine FGG harvesting approach on the microbiological profiles of the donor site during early healing.
CHAPTER 2
MATERIALS AND METHODS

Experimental methods

- Study Population and Experimental design.

This study was a prospective, split-mouth clinical trial that examined the effects of pre-wounding on free gingival grafts (Table 1). Healthy volunteers, non-smokers, were consented for the study, to serve as donors of standardized FGGs. One FGG was routinely harvested (routine FGG; rFGG) from a randomly chosen side of the palate, while a second FGG of same dimensions (12mm by 8mm) was harvested from the contralateral side of the palate following a pre-wounding procedure (pre-wounded FGG; pwFGG) (Figure 1). Portions of the harvested grafts will be processed for gene expression, histology and immunohistochemistry. The healing of the respective donor sites was assessed clinically. Subject pain experience was assessed by questionnaires. Bacterial samples were collected with microbrushes.

Study participants provided signed informed consent and were reimbursed for participation. The study protocol, informed consent, study forms and questionnaires were approved by the OSU IRB. All clinical procedures were performed at the Graduate Periodontology Clinic, The Ohio State University College of Dentistry.
Periodontally and systemically healthy adult (aged 21-50 years) non-smokers were included. Non-smokers were never smokers. Former smokers were excluded. Smoking status was confirmed by expired air carbon monoxide. Subjects aged over 50 were not included because they are much more likely to meet the numerous exclusion criteria (see below).

At screening, subjects were assessed for the following additional exclusion criteria (Figures 2-4):

a) Systemic/general: uncontrolled systemic disease; history of systemic disease affecting healing; obesity; medications affecting the gingiva, the immune system, the cardiovascular system, the wound healing process; pregnancy; allergy to impression materials, surgical materials, topical or local dental anesthetic; unable/unwilling to adhere to study visit schedule; and unable/unwilling to provide informed consent.

b) Oral/dental: history of soft tissue graft harvested from palate; history of other periodontal surgery involving palate; cleft palate; periodontitis; thin periodontal phenotype (inadequate palatal thickness); shallow palatal vault (inadequate palatal height); gingival enlargement; extensive calculus deposits; maxillary removable appliances (orthodontic, restorative); mucosal disease (e.g., candidiasis); lack of maxillary premolars on either side; and subjects with significant gag reflex (unable to easily tolerate maxillary impression procedure).

Subjects were recruited through flyers, postings and advertisements in the OSU Health Sciences Colleges bulletin boards, the CCTS website, the OSU
Lantern, the OSU On Campus, OSU Today (email), local suburban weekly newspapers and various local retail outlets.

For each eligible and enrolled (obtained informed consent) subject maxillary arch impressions were made at the consent appointment to construct custom templates (stents) to use postoperatively (Figure 6). Each subject underwent the pre-wounding procedure (1st surgery) prior to harvesting of a FGG (pwFGG) in a randomly chosen palatal side over the first/second premolar palatal area. All surgical procedures were performed by one highly experienced surgeon (D.N.T.). The contralateral corresponding palatal area served as within-subject control and the site from which a routine FGG (rFGG) was harvested, at the same appointment as the pwFGG (2nd surgery). The time lapse between 1st and 2nd surgery visit was five (5) days. Therefore, the pwFGG was “delayed” by 5 days.

The harvested grafts (rFGG and pwFGG) were stored for future histological, immunohistochemical, and gene expression analyses. The donor sites were clinically assessed at end of surgery, and at postoperative (PO) days 3, 7, 14, and 21(±1 day). Subject experiences were assessed by questionnaires.

- Smoking status assessment. Expired air carbon monoxide (ECO) analysis.

ECO analysis, a rapid, accurate, non-invasive procedure that quantifies CO (byproduct of smoke exposure) in end-expired alveolar air, provided an indirect measure of blood carboxyhemoglobin. ECO analysis was performed at day of 1st procedure, at day of 2nd procedure, and at PO days 3, 7, 14, 21(±1 day). ECO, a valid and reliable index of smoking, with sensitivity and specificity >90%, has a
short half-life (4-6 h) and detected acute changes in smoking status. The cut-off point for distinguishing smokers from non-smokers was 8 parts per million (ppm). Subjects were instructed to exhale completely, draw a deep breath, hold for 15 seconds, and slowly exhale into the instrument (Figure 5, Bedfont Smokerlyzer). A digital readout immediately displayed the CO level in ppm. Subjects underwent ECO analysis at the screening and all other appointments.

- Dental impressions.

Dental impressions of the maxillary arch were used to fabricate a healing stent for use following graft harvesting. An alginate impression was made at the screening appointment after consent is signed. From this impression, stone casts were made, and custom healing palatal stents were fabricated and disinfected (Figure 6). After graft harvest, patients were given the palatal stent to wear during wound healing. The second stent was kept in storage, to be used in case of loss or damage of the first stent.

- Surgical procedures.

Blood pressure and heart rate measurements were taken at the initial screening appointment and before all surgical procedures. Immediately prior to harvesting, subjects received topical and local anesthesia in the palate. FGGs were obtained in the following dimensions: thickness (buccolingual dimension) = 1.25-1.5 mm; length (mesio-distal dimension) = 12 mm; width (apico-coronal dimension) = 8 mm (Figure 5). Prefabricated sterilized metal templates were used to ensure consistency of FGG size (length, width) during pre-wounding and harvesting procedures (Figure 7).
The pre-wounding procedure, which was performed first (1st surgery visit), consisted of all the steps included in the routine harvesting of a FGG, except the complete removal of the graft. The graft was only elevated 2/3 and the apical 1/3 of the graft left intact for blood supply. The pwFGG was secured in situ with a single absorbable suture (chromic gut) until the next appointment (2nd surgery visit). At the 2nd surgery visit, both the pwFGG and the rFGG were harvested and then processed as below. The rFGG, of the same dimensions as the pwFGG, was harvested from the contralateral side of the palate. Subjects received their custom-made stent to minimize postoperative discomfort. If necessary, donor sites had hemostatic agents (Surgicel®, Ethicon, Inc., Somerville, NJ, USA and Collatape, Zimmer Dental, Inc., Carlsbad, CA, USA) and CoePak™ (GC America, Alsip, IL, USA) dressing applied. Postoperative instructions included modified oral hygiene, analgesics (acetaminophen first and, if necessary, ibuprofen), as per routine clinical protocol for such procedures.

- Processing of harvested FGG.

The harvested FGGs were immediately placed in sterile saline. After completion of the donor site assessment (see below), the harvested grafts were sectioned in two even parts (~ 6 mm in length). The first (mesial) section was immediately placed in buffered-formalin (Protocol®, Fisher Scientific Company L.L.C., Kalamazoo, MI, USA) and processed for future routine histology and immunohistochemistry; the second (distal) section was dried with sterile gauze,
stored frozen (RNAlater®, Ambion, Inc., Austin, TX, USA) and used for future gene expression studies.

- **Digital intraoral photographs.**

  Intraoral photographs of the wound sites were taken with a digital camera prior to each procedure, at the end of each procedure and at each postoperative visit. Photographs were used to record the healing of the wound sites.

- **Donor site assessment.**

  Donor sites were assessed for two parameters: Immediate bleeding and wound closure (complete epithelialization). Immediate bleeding was recorded at the donor site, immediately after graft harvesting. Presence or absence of bleeding was assessed after 2 min of gentle external (digital) pressure was applied with a sterile gauze, intended to stop the hemorrhage; lack of immediate bleeding was recorded when no active bleeding is seen and a clinical photograph of the wound can be taken without need for suction. Immediate bleeding was recorded as a dichotomous variable (yes/no). Immediate bleeding was recorded only at the end of FGG harvesting (2nd surgical visit).

  Complete epithelialization was scored clinically, following visual inspection. As the wound approached closure, the area was also blotted dry with a gauze sponge and 3% hydrogen peroxide (H₂O₂) (Walgreen Co., Deerfield, IL, USA) was applied to the wound, seeking to observe bubbling (oxygen liberation) in the wound area. If the epithelial barrier was intact, then the H₂O₂ does not diffuse into the connective tissue, it was not acted upon by catalase, and oxygen
was not liberated. Absence of bubble formation following hydrogen peroxide application was scored as positive complete epithelialization. Complete epithelialization was recorded as a dichotomous variable (yes/no). Complete epithelialization was recorded at the postoperative visits on day 3, 7, 14, and 21 following harvesting.

- Pain assessment.

Questionnaires (Figures 8 to 21) that included a visual analog scale (VAS) were used to assess pain experience and analgesic use by the subjects, as previously described. Questionnaires were given to the subjects at the end of 1st and 2nd surgical visit and at postoperative days 3, 7, 14, and 21(±1).

- Microbiological samples.

Microbiological samples were collected from each side of the palate prior to each procedure, on the day of the 1st surgery visit and of 2nd surgery visit, and at post-operative visits on days 3, 7, 14, and 21. Collection was performed via one pass of sterile swab (Microbrush®, Grafton, WI, USA) over each surgical donor area (preoperatively) or donor site (postoperatively) (Figure 22). Samples were stored frozen at -80°C until processing and further qualitative and quantitative analysis of cultivated and as-yet-uncultivated microbiota by terminal restriction fragment length polymorphism (t-RFLP) analysis (see below).

-Microbiological sample processing.

Bacteria were removed from the microbrushes by the addition of 200 uL of phosphate-buffered saline and vortexing. DNA will be isolated with a Qiagen
DNA MiniAmp kit (Qiagen, Valencia, CA), and amount of DNA measured using a spectrophotometer (NanoDrop 1000; Thermo Scientific, Wilmington, DE, USA) and stored frozen until 16S amplification of rRNA genes for t-RFLP analysis (see below).

-t-RFLP Analysis. Bacterial 16S rRNA genes were amplified with 25 cycles of PCR with fluorescent-labeled broad-range bacterial primers A18 (5’-TTTGATCCTGGCTCAG - VIC-3’) and 317 (5’-FAM-AAGGAGGTGATCCAGGC- 3’) (Applied Biosystems, Foster City, CA, USA). The cycling conditions have been described previously. The amplicons were purified with the use of a Qiaquick kit (Qiagen, Valencia, CA, USA). A 10-mL of purified PCR product normalized to 100ng was digested with either 20 U of either Hha I or Msp I in a total volume of 20 -mL at 37ºC for 3 hrs. A 100ng quantity of the restriction digestion product was purified by Millipore PCR plate purification system (Millipore, Billerica, MA, USA). A 5-mL quantity of the purified product was denatured with 10 uL or de-ionized formamide and mixed with 0.2 mL GeneScan 1200 LIZ size standard (Applied Biosystems, Foster City, CA, USA). Fragment lengths were determined on an AB 3730 DNA Analyzer in GeneScan mode. The number of peaks and the height and area of each peak were determined with the use of GeneMapper 4.0 Software (Applied Biosystems, Foster City, CA, USA).
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Table 1. Study timeline.
Figure 1. Standardized size (8mm by 12mm) of grafts taken from premolar area on palate.
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<td></td>
<td></td>
</tr>
<tr>
<td>Maxillary removable appliances</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maxillary premolars missing on either side</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Significant gag reflex</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
## Telephone Screening Checklist

<table>
<thead>
<tr>
<th>Question</th>
<th>YES</th>
<th>NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Have you completed your 21\textsuperscript{st} birthday?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Have you completed your 51\textsuperscript{st} birthday?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Are you a smoker?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>If no, have you ever been a smoker?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Do you have any systemic disease, such as diabetes, high blood pressure, etc.?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Are you obese?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Are you taking any medications?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Females only) Is there any chance you might be pregnant?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Are you allergic to any dental materials or dental anesthetic?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Would you be available for 6 study visits over 30 days?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Did you ever have a soft tissue graft (skin graft) from the roof of your mouth?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Do you have or did you have cleft palate?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Has any dentist/hygienist ever told you that you have gum disease?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Do you have your upper teeth?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Do you have any removable appliances on the upper jaw (e.g., denture)?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Do you easily gag? Is it difficult for you to have a mold made of your upper jaw?</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4. Preoperative questionnaire.
Figure 5. Smokerlyzer used to measure CO levels in subjects’ breath samples to ensure nonsmoking status.

Figure 6. Plastic palatal healing stents were fabricated and given to subjects after graft harvest.
Figure 7. Metal template fabricated to standardize graft size. A raised lip on the template creates an indentation when pressed onto the palate.
Figure 8. Study visit 1 postoperative questionnaire. Pre-wounding procedure.
1. Did you have any pain since the end of the 1st procedure? _____ Yes _____ No
   If yes, please describe the pain as best you can (for example: throbbing, stabbing, sharp, dull, duration, etc...)

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

<p>| | | | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td>8</td>
</tr>
</tbody>
</table>

3. 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:

   ______________________________________________________
   ______________________________________________________

Please continue the questionnaire on the next page

VERSION 1.0   Page 1 of 3

Figure 9. Preoperative questionnaire on 2nd surgical visit (graft harvest), page 1.
Figure 10. Preoperative questionnaire on 2nd surgical visit (graft harvest), page 2.
STUDY SUBJECT #_____

8. Did you experience any bleeding from the wound?  ____Yes  ____No
   If yes, please describe when and how often you experienced it:
   ____________________________________________________

9. Did you have any swelling in the wound area?  ____Yes  ____No
   If yes, please describe when did it start and whether it prevented you from any activities:
   ____________________________________________________

Thank you for completing this questionnaire
Figure 12. Postoperative questionnaire on 2nd surgical visit (graft harvest), page 1.
Figure 13. Postoperative questionnaire on 2nd surgical visit (graft harvest), page 2.
Figure 14. Postoperative questionnaire on Day 3, 7, and 14 visits, page 1.
THE QUESTIONS IN THE BOX BELOW ARE REGARDING THE LEFT SIDE OF YOUR MOUTH

5. Did you have any pain since your last visit? Yes No
   If yes, please describe the pain as best you can (for example: throbbing, stabbing, sharp, dull, duration, etc...)
   _____________________________________________________________

6. How much pain did you have? Please circle number, with '0' being no pain and '10' being the most severe pain imaginable
   | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
   | No Pain | Moderate Pain | Worst Pain Imaginable |

7. 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:
   _____________________________________________________________

Please continue the questionnaire on the next page

VERSION 1.0  Page 2 of 4
Figure 16. Postoperative questionnaire on day 3, 7, and 14 visits, page 3.
12. Did you have any swelling in the wound area?  ____Yes  ____No
    If yes, please describe when did it start and whether it prevented you from any activities:
    __________________________________________________________
    __________________________________________________________
    If yes, the swelling you experienced in the wound on the roof of your mouth was (Please circle one):
    Only on the RIGHT side    Only on the LEFT side    On BOTH sides

Thank you for completing this questionnaire
Figure 18. Postoperative questionnaire on day 21 visit, page 1.
Figure 19. Postoperative questionnaire on day 21 visit, page 2.
Figure 20. Postoperative questionnaire on day 21 visit, page 3.
Figure 21. Postoperative questionnaire on day 21 visit, page 4.
Figure 22. Use of microbrush to collect biofilm on graft sites.
CHAPTER 3
DATA MANAGEMENT AND STATISTICAL ANALYSIS

The proposed study includes sufficient number of subjects to determine differences in post-operative outcomes between rFGG and pwFGG, based on several studies in the literature where gene expression during wound healing has been assessed in humans.39-41

Experimental data collected from questionnaires, clinical measurements, and imaging software analysis were organized onto a spreadsheet format. The harvest sites on the palate and the FGGs were the unit of analysis for site-specific data.

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

Comparisons between rFGG and pwFGG sites were performed by paired (non-independent) tests. Data were analyzed using paired t-test, repeated measures ANOVA or nominal logistic analysis were used as appropriate to test for intra- and inter-group differences. All of the values represent mean +/- SD.

The statistical significance of differences among the means was analyzed by one-way ANOVA using the JMP R 10.0 statistical software package (SAS Institute, Inc., Cary, NC) or paired t-test and Wilcoxon signed-rank test using Excel (MicrosoftR, Redmond, WA, USA). Differences were considered significant if P was <0.05.
Fragments with a peak height < 50 fluorescence units were excluded from analysis of the t-RFLP data. Peak areas were standarized by converting the raw values to a proportion of the total area, as previously described (Rees et al., 2004). Peaks representing <1% of the total area were assigned a value of zero, and the percentages of the remaining peaks were recalculated. A variance stabilizing transformation was used to create normal distribution of the data. Repeated-measures analysis of variance (ANOVA) was used to compare the means of this transformed variable X over the time points between rFGG and pwFGG. All statistical analyses were performed with statistical software (JMP software, SAS Institute, Cary, NC, USA). Shannon diversity and equitability indices were computed from s-OTU data.42

Nonmetric multidimensional scaling was conducted with SPSS statistics (IBM SPSS Statistics Version 19;IBM Corporation, Armonk, NY, USA) with the percentages of peaks and total areas with the multidimensional scaling based upon proximity.
CHAPTER 4

RESULTS

General Observations

Study Population and Demographics

Twelve subjects were recruited for the study. Of the 12 recruited subjects, 2 did not complete the study because of failure to comply with the study protocol or started taking systemic medications that excluded the subject from the study. The 2 subjects who dropped out after pre-wounding and before grafts were harvested did not experience any untoward complications and had no complaints. All the study procedures were completed uneventfully. Therefore, ten subjects (6 males and 4 females; aged 26.4 ± 1.7 years; age range: 24 to 29 years) complied with and completed the study protocol.

According to the PO questionnaires, subjects followed PO instructions, used the prescribed analgesics, and refrained from using mouthrinses. Six of the subjects had the right side of the palate pre-wounded and 6 subjects had the left side pre-wounded. As for the 10 subjects that completed the study, 4 subjects had the right side of the palate pre-wounded and 6 subjects had the left side pre-wounded.

One subject developed a late (~60 days) complication. This subject had a non-painful ingrowth of epithelium on the margin of the routine FGG side. Gingivoplasty was done, and the area healed without any further complications.
Fifty percent of the subjects rated the study experience as great and 50% rated the study experience as average. Sixty percent of the subjects probably would re-participate and 40% of the subjects reported "maybe/not sure" about re-participation.

**Smoking Status Assessment**

Smoking or recent use of tobacco products was denied by all the study participants. The non-smoking status was confirmed by ECO analysis. The average ECO for the duration of the study was 1.9 ± 0.7 (range: 1 to 3) (Table 2).

**Subject Reported Outcomes**

**Donor Site Assessment**

Donor sites were assessed for two parameters: immediate bleeding and wound closure (complete epithelialization). At 2 minutes post-harvest, all of the rFGG and pwFGG sites were recorded as yes for immediate bleeding. The immediate bleeding assessed 2 minutes after digital pressure after graft harvesting was 100% for both the pwFGG and rFGG sites (Table 3).

Figure 23 shows the percentage of sites with complete epithelialization. Regarding complete epithelial closure, none of the 10 subjects had complete closure for the pwFGG and rFGG sites at the PO day 3 and PO day 7. By PO day 14 visit, 40% of the pwFGG sites and 0% of the rFGG sites had complete epithelial closure. At the PO day 21 visit, 89% of the pwFGG sites and 78% of the rFGG sites had complete epithelial closure.

Generally, there was a visible difference in healing between the pwFGG and rFGG sites. The rFGG sites consistently were markedly more erythematous
than the pwFGG sites, although not objectively analyzed. One subject developed a late (~60 days) complication involving ingrowth of epithelium, but after gingivoplasty, the area healed uneventfully.

Pain Assessment

According to the PO day 0 questionnaires, after the pre-wounding procedure on the day 0 (harvest), 8 of the 10 subjects had experienced postoperative pain since the surgery. The mean VAS pain score for the pre-wounding procedure was $1.4 \pm 0.97$ (range: 0 to 3) and the pain effect score was $0.9 \pm 0.74$ (range: 0 to 2) (Figure 24 and 25). Based upon analgesic consumption, the discomfort occurred mainly immediately after the pre-wounding procedure and lasted approximately 24 hours.

At PO day 3 after harvesting, 9 of 10 subjects reported experiencing pain on the pwFGG site, and all 10 subjects reported experiencing pain on the rFGG site. The mean VAS pain and pain effect scores for the pwFGG site was $3.9 \pm 2.2$ (range: 0 to 7) and $1.4 \pm 0.7$ (range: 0 to 2), respectively, while the scores for the rFGG subjects were $4.5 \pm 1.9$ (range: 2 to 8) and $1.8 \pm 0.7$ (range: 1 to 3), respectively. The differences between the pwFGG and rFGG scores did not reach statistical significance (t-test: p=0.39 and p=0.16 for VAS and pain effect scores, respectively).

At PO day 7 after harvesting, 3 of 10 subjects reported experiencing pain on the pwFGG site, and 9 of 10 subjects reported experiencing pain on the rFGG site. The mean VAS pain and pain effect scores for the pwFGG site was $1.1 \pm 2.1$ (range: 0 to 6) and $0.5 \pm 0.9$ (range: 0 to 2), respectively, while the scores for the
rFGG subjects were $2.8 \pm 2.7$ (range: 0 to 9) and $1.4 \pm 1.4$ (range: 0 to 5), respectively. The differences between the pwFGG and rFGG scores were statistically significant (paired t-test: $p<0.002$ and $p<0.010$ for VAS and pain effect scores, respectively; Wilcoxon signed rank test; $p<0.05$ and $p<0.05$ for VAS and pain effect, respectively).

At PO day 14 after harvesting, 2 of 10 subjects reported experiencing pain on the pwFGG site, and 5 of 10 subjects reported experiencing pain on the rFGG site. The mean VAS pain and pain effect scores for the pwFGG site was $0.5 \pm 1.3$ (range: 0 to 4) and $0.2 \pm 0.3$ (range: 0 to 1), respectively, while the scores for the rFGG subjects were $0.7 \pm 0.7$ (range: 0 to 2) and $0.5 \pm 0.5$ (range: 0 to 1), respectively. The differences between the pwFGG and rFGG scores did not reach statistical significance (paired t-test: $p=0.77$ and $p=0.19$ for VAS and pain effect scores, respectively).

At PO day 21 after harvesting, none of the subjects reported experiencing pain on the pwFGG site, and 2 of 10 subjects reported experiencing pain on the rFGG site. The mean VAS pain and pain effect scores for the pwFGG site was $0.0 \pm 0.0$ (range: 0 to 0) and $0.0 \pm 0.0$ (range: 0 to 0), respectively, while the scores for the rFGG subjects were $0.2 \pm 0.2$ (range: 0 to 1) and $0.2 \pm 0.4$ (range: 0 to 1), respectively. The differences between the pwFGG and rFGG scores did not reach statistical significance (t-test: $p=0.19$ and $p=0.16$ for VAS and pain effect scores, respectively).

The 10 subjects received acetaminophen (500mg) analgesic prescriptions. After the pre-wounding procedure, subjects reported having taken $1.5 \pm 1.3$
(range: 0 to 4) pills (Figure 26). At PO day 3 after harvesting, subjects reported
taking 11 ± 7.2 (range: 1 to 24) pills. Between PO day 3 and PO day 7, subjects
reported taking 3.2 ± 3.9 (range: 0 to 10) pills. And for both PO day 7 to PO day
14 time periods, subjects reported taking 0.2 ± 0.6 (range: 0 to 2) pills. There
was a statistically significant increase in pain pills taken at the PO day 3 visit
(One-way ANOVA; p<0.0001). There were a few subjects who had taken another
analgesic other than acetaminophen to control postoperative pain. At PO day 3,
one subject took 1 pill vicodin (5/500). Between PO day 3 and PO day 7, a
separate subject took 9 pills ibuprofen (600mg) and 2 pills vicodin (5/500).

At the PO day 3 visit, all subjects reported taking analgesics for pain. Six
subjects reported experiencing more pain on the rFGG side, 2 subjects reported
experiencing more pain on the pwFGG side, and 2 subjects reported the same on
both the rFGG and pwFGG sides. Pain prevalence decreased in subsequent PO
visits. Eight subjects reported experiencing more pain on the rFGG side and 1
subject reported the same on both the rFGG and pwFGG sides on the PO day 7
visit. Six subjects reported taking pain medications. By the PO day 14 visit, 2
subjects reported taking pain medications, with 1 subject only taking for leg
pain. Five subjects reported experiencing more pain on the rFGG side, 1 subject
experienced more pain on the pwFGG side, 3 subjects the same on both the rFGG
and pwFGG sides and 1 subject did not experience pain with any side. Two
subjects reported experiencing more pain on the rFGG side, 6 subjects reported
both rFGG and pwFGG sides felt the same, and 2 subjects experienced no pain at
the PO day 21 visit. Only 1 subject took pain medication, but for leg pain.
Digital Intraoral Photographs

The intraoral photographs showed faster healing with the pwFGG sites compared with the rFGG sites (Figure 27).

Microbiological Outcomes

Figure 28 shows the total number of peaks in the rFGG and pwFGG samples by study visit. In the samples collected (7 subjects analyzed), the total number of peaks on day 0 (harvest) for the pwFGG and rFGG were 38.7 ± 4.4 (range: 34 to 46) and 30.9 ± 8.2 (range: 21 to 43), respectively. However, this difference did not reach statistical significance (Tukey-Kramer, p<0.08). At PO day 3, the total number of peaks for the pwFGG and rFGG were 38.7 ± 2.1 (range: 37 to 42) and 42.6 ± 1.6 (range: 41 to 46), respectively. This difference was statistically significant (Tukey-Kramer, p < 0.02; Wilcoxon signed-rank test, p<0.007). At PO day 7, the total number of peaks for the pwFGG and rFGG were 38.0 ± 4.4 (range: 31 to 43) and 42.9 ± 3.2 (range: 39 to 49), respectively with a statistical significance (Tukey-Kramer, p<0.05; Wilcoxon signed-rank test, p<0.08). At PO day 14, the total number of peaks for the pwFGG and rFGG were 28.9 ± 7.6 (range: 15 to 39) and 31.4 ± 4.1 (range: 28 to 40). This difference was not statistically significant (Tukey-Kramer, p= 0.32; Wilcoxon signed-rank test, p<0.70). At PO day 21, the total number of peaks for the pwFGG and rFGG were 24.7 ± 9.8 (range: 11 to 38) and 28.3 ± 7.1 (range: 13 to 34), respectively with no statistical significance (Tukey-Kramer, p<0.5; Wilcoxon signed-rank test, p<0.48). One-way ANOVA analysis found statistical significance with intergroup comparisons between the postoperative visits (p<0.001).
Figure 29 shows the nonmetric multidimensional scaling for the rFGG and the pwFGG sites. The nonmetric multidimensional scaling shows the trend for the microbial profiles of pwFGG and rFGG sites to group together for day 0 (harvest), PO day 3, and PO day 7. The microbial profiles of pwFGG and rFGG sites become more similar by the PO day 14 and PO day 21. The normalized raw stress (NRS) values for the nonmetric multidimensional scaling for each PO visit ranged from 0.02 to 0.03.

The Shannon diversity and equitability indices for the rFGG sites show a significant increase in diversity and equitability from day 0 (harvest) to PO day 3 (Figure 30 and 33). By PO day 14 and PO day 21, there is a significant decrease in both rFGG and pwFGG diversity and equitability (One-way ANOVA; p<0.0001 and p<0.0001 for diversity and p<0.003 and p<0.007 for equitability, respectively) (Figures 30-31, 33-34). Comparing pwFGG and rFGG sites in figures 32 and 35, there were statistical significant differences in diversity and equitability for the PO day 3 and PO day 7 (Tukey-Kramer analysis; p<0.0008 and p<0.001, respectively for diversity and p<0.002 and p<0.0003, respectively for equitability).
Table 2. Expired air carbon monoxide analysis (Smokerlyzer) by study visit.

<table>
<thead>
<tr>
<th>Visit</th>
<th>Screening</th>
<th>Pre-wounding</th>
<th>Day 0 (harvest)</th>
<th>Day 3</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average</td>
<td>2</td>
<td>2</td>
<td>1.8</td>
<td>1.8</td>
<td>1.9</td>
<td>1.9</td>
<td>2</td>
</tr>
<tr>
<td>Std Dev</td>
<td>0.6</td>
<td>0.7</td>
<td>0.8</td>
<td>0.6</td>
<td>0.6</td>
<td>0.6</td>
<td>0.8</td>
</tr>
<tr>
<td>Range</td>
<td>(1-3)</td>
<td>(1-3)</td>
<td>(1-3)</td>
<td>(1-3)</td>
<td>(1-3)</td>
<td>(1-3)</td>
<td>(1-3)</td>
</tr>
</tbody>
</table>

Table 3. Immediate bleeding on rFGG and pwFGG sites as assessed by dichotomous (yes/no) scale after 2 minutes of digital pressure with wet gauze.

- rFGG: 100%
- pwFGG: 100%
Figure 23. Percentage of subjects with complete epithelial closure as assessed by hydrogen peroxide test.

Figure 24. Average pain scores (VAS) by study visit.
Figure 25. Average pain effect scores (0 to 5) by study visit.
Figure 26. Average analgesic (acetaminophen 500mg) use by study visit.
Figure 27. Subject 7 intraoral photographs by study visit. (A) Preoperative (day -5), (B) after pre-wounding procedure (day -5), (C) preoperative before graft harvesting (day 0), (D) after graft harvest (day 0), (E) day 3 postoperative visit, (F) day 7 postoperative visit, (G) day 14 postoperative visit, (H) day 21 postoperative visit.
Figure 28. Total number of peaks by study visit. One-way ANOVA, p<0.001. Tukey-Kramer analysis, p<0.01 for PO day 3; p<0.04 for PO day 7.
Figure 29. Nonmetric multidimensional scaling by study visit. Orange dots denote rFGG sites and purple dots denote pwFGG sites. Normalized raw stress values range 0.02 to 0.03.
Figure 30. Shannon diversity index for rFGG sites by study visit. One-way ANOVA analysis, p<0.0001. Horizontal line denotes mean between study visits.
Figure 31. Shannon diversity index for pwFGG sites by postoperative visit. One-way ANOVA analysis, p<0.001. Horizontal line denotes mean between study visits.
Figure 32. Shannon diversity index pwFGG and rFGG sites by postoperative visit. Tukey-Kramer analysis, p<0.0008 for PO day 3; p<0.001 for PO day 7.
Figure 33. Shannon equitability index for rFGG sites. One-way ANOVA analysis, p<0.003. Horizontal line denotes mean between study visits.
Figure 34. Shannon equitability index for pwFGG sites. One-way ANOVA analysis, p<0.0055. Horizontal line denotes mean between study visits.
Figure 35. Shannon equitability index for pwFGG sites and rFGG sites by postoperative visits. Tukey-Kramer analysis, $p<0.0002$ for PO day 3; $p<0.0003$ for PO day 7.
CHAPTER 5

DISCUSSION

In order to justify the introduction of a new surgical technique, the benefits of the procedure should outweigh the costs. For pre-wounding (PW) to be a valid treatment option, it must be acceptable for patients to tolerate an extra visit and the postoperative outcomes. The minimal pain (1.4 ± 0.9 VAS, range 0-3) supports the practicality of incorporation of PW into certain periodontal procedures. This VAS was similar to the VAS found in the CTG study previously reported (1.2 ± 1.4 VAS).\textsuperscript{21} The pain reported for the pre-wounding procedures (1st surgical procedure) was much less than the pain experienced after the FGG harvesting procedure (2nd surgical procedure) in the same subjects. Subjects also reported taking minimal analgesics, mostly on the day of the pre-wounding procedure.

The present study differed from the previous CTG pre-wounding study in that all the rFGG and pwFGG sites were categorized as having immediate bleeding. The previous study reported 60.4% of the sites as immediate bleeders, with 15 of the 24 PW sites and 14 of the 24 R harvest sites.\textsuperscript{21} The study also found that no statistically significant differences were observed between the smokers and the nonsmokers at any time/group (Fisher’s exact test, p>0.05).\textsuperscript{21} There was a non-statistically significant trend for greater immediate bleeding in the PW group.\textsuperscript{21} Immediate bleeding was only assessed after 2 minutes of digital
pressure. It was observed that it would take about 20-30 minutes of digital pressure on the rFGG and pwFGG sites in order to achieve no active bleeding and a clinical photograph of the wound can be taken without the need for suction. But this was only an observation, and no records were taken as to how many minutes this was achieved. A future study could increase the minutes of digital pressure to evaluate the presence or absence of immediate bleeding between rFGG and pwFGG sites.

Faster healing with the pwFGG sites (40%) vs. rFGG sites (0%) on PO day 14 was consistent with earlier findings with the pwCTG. The CTG study had a faster healing because the wound was smaller (2mm x 12mm) than the FGG wound (8mm x 12mm). The earliest complete epithelialization was noted at the PO day 7 visit in the CTG study and at the PO day 14 visit in the FGG study.21

For the CTG study, the mean ± standard deviation (SD) VAS scores for the PO day 3 visit were 3.0 ± 2.2 (range 0-8) and 2.8 ± 2.3 (range 0-8) for the R and PW sites, respectively.21 The mean VAS scores for the PO day 7 visit were 1.3 ± 1.6 (range 0-7) and 1.2 ± 1.7 (range 0-7) for the R and PW sites, respectively.21 All the subjects reported VAS of 0 at the PO day 14 and PO day 21 visits.21 No statistically significant differences were found between R and PW at different time points or between smokers and nonsmokers.21 The findings of the present FGG study are similar with 4.5 ± 1.8 (range 2-8) and 3.9 ± 2.2 (range 0-7) for the R and PW sites, respectively. The mean VAS scores for the PO day 7 visit were 2.8 ± 2.7 (range 0-9) and 1.1 ± 2.1 (range 0-6) for the R and PW sites, respectively. For the PO day 14 visit, the mean VAS scores were 0.7 ± 0.7 (range
0.5 ± 1.3 (range 0-4) for the R and PW sites, respectively. At the PO day 21 visit, subjects reported 0.2 ± 0.2 (range 0-1) and 0 for the R and PW sites, respectively.

The VAS score for the rFGG for the present study (4.5 ± 1.8 (range: 2 to 8)) are similar to the mean VAS pain score for FGG subjects in an earlier study (4.8 ± 1.2 (range: 3 to 6)). The pain in the palatal donor site PO day 3 was reported by 90% of subjects in the pwFGG site and 100% of subjects in the rFGG site, which is consistent with the earlier study of 90% of FGG subjects. A previous study found similar results and attributed the poorer patient outcomes following FGG to differences in donor site harvesting techniques. In an 8-week observational study using a verbal descriptor scale to assess postoperative discomfort, Del Pizzo et al. reported postoperative discomfort at palatal donor sites during the first postoperative week in 100% of subjects treated with an FGG harvesting technique and only 50% of subjects treated with a single-incision harvesting technique. Additionally, in a study on 228 subjects using questionnaires to assess postoperative pain, Griffin et al. reported FGG subjects were three times more likely than CTG subjects to develop post-surgical pain during the first postoperative week. In contrast to the earlier CTG study, a statistically significant difference in VAS pain score was found at the PO day 7 visit in this FGG study. Subjects also reported less bleeding with the pwFGG sites compared to the rFGG sites. The reduction in postoperative pain and bleeding makes the pre-wounding procedure an attractive consideration for use in painful FGG procedures.
The present investigation used an open-ended, quantitative molecular approach to comprehensively examine the pwFGG and rFGG microbial communities in systemically healthy nonsmokers. This allowed us to identify a unique microbial profile that included both cultivated and as-yet-uncultivated organisms. Sequence-specific digestion of the 16S rRNA gene results in terminal fragments of varying sizes, as the location of the restriction site is dictated by the nucleotide sequence of the gene for each species. Thus, the total number of t-RFs represents the number of unique species in a community, while the area of a terminal fragment measures the abundance of each species in the community.

t-RFLP has been used to examine the microbial profiles of complex communities, however, underestimation of the number of species is possible by fragment analysis, as closely related species may share common restriction sites. Further, species of low abundance may not be consistently represented in the t-RF profile. If pre-wounding leads to colonization by several closely related species or by species that are less abundant in the community, these results may not be apparent with t-RFLP. To account for these closely related species, more sensitive, targeted molecular approaches are needed for seeing the effects of pre-wounding on the prevalence and levels of specific bacteria.

There was an increase in the number of peaks, which are assumed different bacterial species, from the time of the first surgical intervention in the site to the early PO visits until the time of epithelial closure for both the rFGG and pwFGG sites. The early postoperative visits between the rFGG and pwFGG sites on PO day 3 and PO day 7 visits showed a significant difference in the
number of bacterial species. It is interesting to note that the number of bacterial species decreased as the wound sites achieved epithelial closure. It is likely that an open wound influences the microbial community in a biofilm through blood supply, host immune defenses, or ischemic conditions produced by the surgical procedure itself. It is also interesting to note that there is also an increase in the number of bacterial species on day 0 (harvest) on the pwFGG site compared with the rFGG site, despite epithelial closure 5 days after the pre-wounding procedure.

Multidimensional scaling (MDS) is a set of data analysis techniques for representing dissimilarity data (or similarity data) by spatial distance models. MDS represents a set of objects as points in a multidimensional space in a way that the points corresponding to similar objects are located close together, while those corresponding to dissimilar objects are located far apart. The nonmetric multidimensional scaling was produced by applying the differences between the bacterial species in the t-RF profiles and the abundance of each species to a model that could be applied to each sample. The PO day 3 and PO day 7 visits produced nonmetric multidimensional scaling bubble plots that showed distinct groups between the rFGG and pwFGG sites. This denotes the differences in the number, type, and abundance of bacterial species between the rFGG and pwFGG sites during early wound healing. Being a split-mouth study, the factors regarding the host are accounted, and so it can be assumed the differences in the rFGG and pwFGG are localized and site-dependent.
There are many factors that could be involved in altering the microbial profiles between the rFGG and pwFGG sites in the subjects. In addition to use in plastic surgery in skin grafts and breast reconstruction, pre-wounding or "ischemic preconditioning" has been used to help prevent brain cell death in the neonatal brain, and confer cardioprotection. It has been found that different phenotypes of neuroprotection are induced when different preconditioning stimuli (brief ischemia or endotoxin (LPS)). This suggests that there are many pathways that could be used for neuroprotection, and also that bacterial challenge could produce a preconditioning stimulus. A gastrointestinal study found that ischemic preconditioning protects the intestine and reduces bacterial translocation in rats and may help in preventing sepsis. Other studies are looking at other preconditioning stimuli, such as toll-like receptor (TLR) agonists. Stimulation of TLRs leads to the production of proinflammatory cytokines, type I interferons and other anti-inflammatory cytokines. Activation of certain TLRs prior to ischemia provides robust neuroprotection: TLR2, TLR4, TLR7 or TLR9.

Saliva is part of the immune system and includes IgA antibodies, lysozymes, aggregating factors, histidine-rich and anionic proteins, lactoferrin. There are innate immune system mechanisms that could be in play which include mammalian peptidoglycan recognition proteins (PGRP). These were first discovered in silkworms in 1996 and mammals were found to have four PGRP genes, Pglyrp1, Pglyrp2, Pglyrp3, and Pglyrp4. These proteins are present in PMN granules, epithelial cells, skin and mucous membranes,
salivary glands, oral cavity, intestinal tract, eyes, and liver and function in antibacterial defenses and innate immunity. Antimicrobial proteins and peptides (AMPs) are also present in salivary glands, oral epithelial cells and neutrophils. Over 45 AMPs have been indentified in the oral cavity and are present in gingival crevicular fluid as well as saliva and 13 of these were found to be upregulated in periodontal disease and 11 are downregulated. In addition to direct antimicrobial activity, they affect periodontal disease by inactivating bacterial or host proteases or bind bacterial toxins, and act as immune system alarmins to recruit and activate antigen-presenting cells to enhance innate and adaptive immune responses. It could be that AMPs are involved in mucosal wound healing as well and can be upregulated or downregulated in the healing process.

Toll-like receptors are a class of proteins that play a role in the innate immune system and the digestive system. Toll-like receptor 4 (TLR4) has been found to have a role in early skin wound healing. TLR4 has a role in initiation of innate immunity and the regulation of adaptive immune responses. The cellular location of TLR4 has been in keratinocytes at the wound edges. The inflammatory cytokine production by injured normal human epidermal keratinocytes is stimulated by the TLR4-p38 and JNK MAPK signaling pathways and these suggest a role for TLR4 at sites of injury and regulation of wound inflammation. TLR9 has also been shown to induce neuroprotection against ischemic brain injury when targeted as a preconditioning stimulus.
The inflammatory response following tissue injury has important roles in both normal and pathological healing. After activation of the innate immune system, recruitment of inflammatory cells from the circulation is initiated. The hypoxic environment of the wound promotes inflammation and stimulates macrophages to produce inflammatory mediators. Neutrophils infiltrate the wound quickly and are the dominant leukocyte in the early stages. Circulating monocytes enter the wound and differentiate into mature tissue macrophages, mast cells increase and then T cells appear in the wound bed. Many studies suggest the inflammatory phase has profound effect on the final wound outcome. It has been suggested that the pattern of macrophage function during the course of wound healing include monocytes and resident macrophages becoming activated, undertaking phagocytosis of microbes and early neutrophils and producing proinflammatory mediators and chemoattractants in the early wound. Then macrophages also assist in the induction of apoptosis in neutrophils, steering the wound towards a noninflammatory, reparative state. In the later phases of wound repair, macrophages ingest apoptotic neutrophils, producing growth factors to support tissue restoration. In the very late stages, as the wound resolves, macrophages may guide tissue remodeling by producing factors to promote capillary regression and collagen remodeling.

In addition to the role macrophages play, it is possible that neutrophils also play a role in early wound healing. Blood neutrophils were found to be primed by hypoxic preconditioning in the gut. The authors found elevated
production of superoxide and hydrogen peroxide on stimulation, increased membrane translocation of cytosolic p47 and p67 and augmented bacterial-killing and phagocytic activities.\textsuperscript{76} If neutrophils can be primed by preconditioning in the gut, it is possible that neutrophils can be primed by the pre-wounding procedure in the oral cavity.

Recently, a new family of immune cells were described, termed innate lymphoid cells (ILCs) that were found to contribute to inflammation, modulate adaptive immunity and regulate wound healing and tissue regeneration at barrier surfaces.\textsuperscript{77} ILCs comprise a novel class of immune effectors that are hypothesized to be of lymphoid origin and classified as innate immune cells because they lack antigen-specific receptors and are involved in the initial stages of an immune response.\textsuperscript{78,79} These cells, as part of the innate immune system, could also play a role in bacterial profile differences between the rFGG and pwFGG sites.

In addition to immune cells, platelets themselves could be a factor in differences between rFGG and pwFGG sites. Platelet (PLT) gels are increasingly used as adjuvant therapy for the treatment of ulcers \textsuperscript{80-84} or burns.\textsuperscript{85} PLTs may improve wound healing through release of growth factors \textsuperscript{86,87} and exhibiting antimicrobial properties.\textsuperscript{88,89} Components responsible for the antimicrobial activity within these platelet gels remain poorly understood because of the complex mixture of PLTs, white blood cells, and plasma.\textsuperscript{90} One study noted that the plasma components were found to be mostly responsible for the antimicrobial activity found in PLT gels after separating components of the
gels. Another study tested the use of pure platelet-rich plasma against oral microorganisms. Platelets may play multiple roles in antimicrobial host defense by generating oxygen metabolites, including superoxide, hydrogen peroxide and hydroxyl free radicals; binding, aggregating, and internalizing microorganisms, participating in antibody-dependent cell cytotoxicity functions to kill protozoal pathogens and releasing antimicrobial peptides. After a pre-wounding procedure, the healing process recruits platelets that play a role in influencing the microbiological profile of the site.

Lastly, the microbial community itself could influence the differences between the rFGG and pwFGG sites. Application of topical bacterial lipopolysaccharide (LPS) has been shown to affect the inflammatory response and promote wound healing. Murine skin wounds were treated with bacterial LPS, the main exogenous ligand of TLR4. The topical LPS treatment was found to upregulate the secretion of proinflammatory cytokines (IL-6, IL-1beta, and leukemia inhibitory factor) and CC-chemokines (CCL2/MCP-1, CCL7/MCP-3, CCL3/MIP-1alpha, and CCL5/RANTES), and growth factors (VEGF, TGF-beta1, and FGF-2). LPS treatment strongly affects the wound-healing process by accelerating the resolution of inflammation, increasing macrophage infiltration, enhancing collagen synthesis, and altering the secretion of a number of mediators that are involved in the skin regeneration process. The literature suggests that small amounts of bacteria in wounds may be beneficial in wound repair although larger amounts of bacteria could infect a wound and delay wound healing. In animal models, a number of authors have reported that
presence of microorganisms in wounds enhances the proinflammatory response and even leads to acceleration of wound healing.\textsuperscript{100-102} It seems that the pre-wounding procedure initiates a cascade of inflammatory and wound-healing events that influence the microbiota found in biofilm on mucogingival surgical sites.
CONCLUSIONS

In conclusion, subjects tolerated the PW procedure well and there were very few PO complications. There were statistically significant differences between the rFGG and pwFGG sites in terms of pain score (VAS) and pain effect scores for the PO day 7 visit. Subjects consistently reported that they felt less pain and less bleeding with the pwFGG site versus the rFGG site. Also, the pwFGG sites tended to have complete epithelial closure earlier than the rFGG sites. The reduction in postoperative pain and bleeding makes the pre-wounding procedure an attractive consideration for use in painful FGG procedures.

While both the rFGG and pwFGG sites had an increase in the number of bacterial species from the first surgical intervention of the site, there were statistically significantly more bacterial species in the routine sites on PO day 3 and PO day 7. The microbial communities were more diverse and had more even spread of species on PO day 3 and PO day 7. There was a significant difference in the microbial profiles between the routine and pre-wounded sites in the early days of healing. The pre-wounding technique resulted in wound sites with an altered microbiological profile with less species, less diversity, and
less equitability of bacterial species than the routine FGG sites. This is the first report of microbial profiling in periodontal plastic procedures.
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