NERVE FIBER PATTERNS IN PERIODONTITIS

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Dedication

This Thesis is dedicated to my parents and my brother who have always been there for me and whose love and support have given me the strength to pursuit all my goals in life.

It's also dedicated to my beloved Triantafyllos for always giving me a reason to smile during our journey together..

Abstract

Background: Inflamed periodontal tissues exhibit widened intracellular epithelial spaces, increased vascularity and leukocytes when compared to healthy gingiva. This study was intended to describe nerve fiber patterns at periodontitis and control papillae.

Methods: Ten adults with moderate/severe periodontitis were enrolled. Gingival index (GI), probing depth (PD) and clinical attachment loss (CAL) were measured at periodontitis (test) and healthy (control) sites. Buccal interdental papilla from these same sites were biopsied, placed in Zamboni's fixative, sectioned at 60μm, immunostained with antibody to PGP 9.5 and with fluorescent secondary antibodies to visualize nerve fibers for quantification by confocal microscopy. Cumulative prevalence of nerve fibers was compared between periodontitis and control sites.

Results: Mean age of the sample was 53.8 years. All five females and two of the five males smoked cigarettes. Mean PD and CAL were significantly greater for periodontitis sites than control sites (4.3 vs 2.3 and 4.2 vs 1.2 respectively, $p < 0.0001$). Nerve fibers were seen in junctional, sulcular and col epithelium in 18%, 58% and 74% of the periodontitis sites and 0%, 64% and 52% of the control sites, respectively. Cumulative percentage of intraepithelial nerve fibers at periodontitis was 51% vs control sites 44%.

Conclusions: Nerve fibers penetrated to the stratum granulosum of gingival epithelium. Nerve fibers were more frequently in sulcular and col epithelium than junctional epithelium and tend to be more frequent and extensive at periodontitis than control sites. Further research is needed to determine if variations in intraepithelial nerve patterns are associated with inflammation and periodontitis.

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1. BACKGROUND AND SIGNIFICANCE

The periodontium constitutes a developmental, biologic and functional unit. Its main purpose is to anchor the teeth to the maxilla and mandible while maintaining an intimate attachment with the masticatory mucosa of the oral cavity. Morphological alterations of the periodontium relate to functional and environmental conditions[1].

Periodontal tissues are extensively innervated by nerve fibers that typically run parallel to the course of blood vessels. The nerves of the gingiva are found superficial to the periosteum whereby they disseminate as small branches and fibers that travel towards the free marginal gingiva and oral epithelium[1].

Immunohistochemical methods have been developed that provide a reliable way of viewing and differentiating various subpopulations of nerves that supply the oral tissues[2]. Protein gene product 9.5 (PGP 9.5) is a pan neuronal marker often used in skin and other organ specimens because it provides excellent visualization of the finest terminal nerve fibers that are not consistently stained by other markers. Ramieri et al. (1990) used PGP 9.5 and detected free nerve endings in lamina propria and lamina epithelialis as well as Merkel-neurite complexes, Meissner's corpuscles and simple coiled corpuscles in the human gingival mucosa[3]. PGP 9.5 offers unparalleled visualization of neural structure in epidermis and dermis. PGP 9.5 has been utilized as a total neuronal marker to investigate variations in nerve fibers associated with growth and development, aging and disease processes, as well as sequellae to pharmacotherapeutic or surgical treatment[4].

Hilliges et al. (1996) utilized this immunohistochemical marker to observe single intraepithelial nerve fibers in healthy human gingiva. The fibers were described as very thin yet frequently exhibiting round varicosities with only occasional branches. A dense network of small immunoreactive nerve bundles could be observed within the middle of the lamina propria that run parallel to the epithelial surface. Nerves fibers from this sub-epithelial plexus travel perpendicular from this region towards the overlying epithelial

surface. Nerve fibers and small blood vessels typically follow a similar pathway within connective tissue papillae. Nerve glomeruli are located in the top of the papilla as thin nerve fibers emanating from these structures and penetrating into the epithelium where they reach the level of the stratum granulosum[5]

The typical distribution of nerve fibers has been well documented in normal periodontal tissues. However, the nerves fiber distribution in inflamed gingiva merits further investigation. Inflammatory periodontal diseases are initiated and potentiated by bacterial plaque and its metabolic by-products that trigger the infiltration of inflammatory cells which are associated with the resultant degradation of extracellular matrix macromolecules[6]. Many studies have reported enhanced vascularization within the gingiva as inflammation develops in association with periodontal disease[7].

It is generally accepted that the nervous system contributes to the pathophysiology of peripheral inflammation and a neurogenic component is involved in many inflammatory diseases, including periodontitis[8]. Several studies have demonstrated that sensory neurons play a role in vascular inflammation whereby the term "neurogenic inflammation" has been coined to define the contribution of the nervous system to the local inflammatory responses[9]. Neurogenic inflammation may be considered as a protective mechanism that forms the first line of defense to protect tissue integrity. Alternatively, prolonged or intense neurogenic stimulation may result in an overzealous inflammatory response leading to injury rather than facilitating repair[8].

2. HYPOTHESIS AND PURPOSE

Based on the assumption that periodontal inflammation alters the cellular and vascular components of the gingiva, the purpose of this investigation was to determine if different nerve fiber patterns exist at periodontitis sites as compared to healthy control sites.

3. MATERIALS AND METHODS

A. Study Population

Screening and patient care was completed within the Advanced Education Program in Periodontology at the University of Minnesota. Prior to initiating any therapy all patients received 1) medical and dental history evaluations, 2) clinical and radiographic periodontal examinations, 3) periodontal diagnosis and prognosis and 4) a periodontal treatment plan.

Ten individuals constituted the sample consisting of five males and five females with a mean age (SD) of 53.8 (10.5) years and a range of 39 – 74 years. The major prerequisite to participate in this study was an established diagnosis of localized moderate to severe periodontitis (sites with $\geq$ 3mm of clinical attachment loss and probing depth $\geq$ 4mm)[10]. Potential participants with moderate to severe periodontitis and in need of a tooth extraction and/or periodontal surgery were informed of the study's purpose and procedures. Presence of intact interdental papillae was also a prerequisite to participation in the study. The study protocol was verbally reviewed with potential volunteers prior to their surgery date and they were asked to read and sign the consent form as approved by the University of Minnesota Committee for the Protection of Human Subjects.

Adult volunteers whose health status conformed to American Society of Anesthesiologist (ASA) medical risk classification I, II or limited III and between 18 to 85 years old were selected. Individuals who were immunocompromised, pregnant, had taken antibiotics within 30 days, or were carriers of a transmissible disease that may expose laboratory personnel to unnecessary risks were excluded. Each participant served as his/her control and test site by providing a biopsy from both a healthy site and a periodontitis site according to the previous established treatment plan for tooth extraction and/or periodontal surgery.

B. Clinical Periodontal Measurements

Clinical measurements of the gingival index (GI)[11], bleeding on probing (BOP)[12], probing depth (PD) and clinical attachment loss (CAL) were recorded at the proximal surfaces of adjacent teeth where the two biopies were collected. Probing depth (PD) and clinical attachment loss (CAL) were measured utilizing a standardized North Carolina periodontal probe, according to the method previously described by Philstrom[13].

In each patient, one site was selected as a "test/periodontitis" site on the basis of probing depth $\geq$ 4mm, clinical attachment loss $\geq$ 3mm, and evidence of gingival inflammation represented by a color change, texture change and bleeding on probing, GI = 2 or 3[11]. Within that same quadrant an additional site was selected as a "healthy/control" site according to the clinical criteria of $\leq$ 2mm of clinical attachment loss, probing depth $\leq$ 3mm and mild or no evidence of clinical gingival inflammation, GI = 0 or 1[11].

C. Calibration Trial

Prior to the recruitment of study participants, a calibration trial was completed to determine intra-examiner and inter-examiner reproducibility for the clinical parameters to be measured. Seven patients who had previously been treated for moderate to severe periodontitis were measured for the clinical indices of GI, PD and CAL at six sites per tooth (mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual and disto-lingual) of the Ramfjord teeth (# 3, 9, 12, 19, 25, 28) for the calibration exercise.

D. Collection of Gingival Biopsies

Clinical measurements and biopsies were obtained in conjunction with the pending periodontal surgery in order to minimize inconvenience to the participant and periodontal surgeon. After the clinical parameters were measured, local anesthetic (2% Lidocaine

with 1:100,000 epinephrine) was administered and two small (~ 3mm or 1/8 inch) triangular-shaped biopsies were collected from each patient in conjunction with the surgical procedure that was subsequently completed according to standard surgical protocol.

E. Immunohistochemistry & Immunofluorescence

Individual biopies were placed in coded specimen bottles containing Zamboni's fixative solution (2% formaldehyde with picric acid) for 24h, then cryoprotected with 20% sucrose in 0.1M phosphate buffered saline (PBS) for at least 24h or until sectioned with a freezing sliding microtome[#] at 60µm intervals.

Floating sections were stained using rabbit polyclonal antibody to PGP 9.5[§] with donkey anti-rabbit IgG labeled with a cyanine 3.18 fluorescent probe[¶]. The same sections were also stained with mouse monoclonal antibody to type IV collagen[±] reacted with donkey anti-mouse IgG labeled with a cyanine 5.18 fluorescent probe[¶], and biotinylated *Ulex europaeus* agglutinin I (UEA-I)[ɔ] reacted with streptavidin labeled with cyanine 5.18 fluorescent probe[¶] to visualize the epithelium.

A solution of 0.1M PBS with 0.3% Triton X-100[†] and 1% normal donkey serum[¶] was used as a diluent and a wash solution. Sections were adhered to cover slips with agar, dehydrated with alcohol, cleared with methyl salicylate and mounted in DPX[‡][14,15].

Leica, Nussloch, Germany
§ Ultraclone, Wellow, UK
¶ Jackson ImmunoResearch, West Grove PA
± Chemicon, Temecula, CA
Ɔ Vector labs
† Sigma, St. Louis, MO
‡ Fluka, Ronkonkoma, NY

F. Descriptive Analysis of Gingival Innervation

Sections were visually evaluated with a Nikon Microphot-SA fluorescent microscope$^{\infty}$ at 10X while selective areas were viewed at 20X when image clarification was required (15). Each section was divided into five areas (A, B, C, D and E). Areas A and E depicted the apical part of the gingival sulcus on the periphery of the specimen (junctional epithelium), while areas B and D represented the coronal part of the sulcus (sulcular epithelium) and C was identified as the col area between B and D. The presence of nerve fibers in the five epithelial or sub-epithelial areas were quantified according to their morphological pattern as specified in Figure 1 (Appendix). Examples of epithelial and sub-epithelial nerve fiber patterns are shown in Figures 2a, 2b and 2c (Appendix) .

Intra-examiner reproducibility for scoring nerve fiber patterns as shown in Figure 1 was tested via a blind calibration trial prior to scoring the study images. Exact agreement or +/- 1 increment was achieved in 97.3% of the two hundred and twenty-six (n=226) regions of sections scored.

G. Smoking Data

Smoking status was assessed retrospectively by reviewing the participant's chart and questioning their periodontist. Smokers were defined as individuals who had smoked at least 100 cigarettes in their lifetime. Participants classified as current smokers utilized cigarettes within the month that the examination and biopsies were collected. Former smokers were individuals who had abstained from smoking cigarettes for at least one month prior to the examination and biopsy procedures. A smoking cessation date was recorded for former smokers. Smoking exposure was expressed in term of packyear, which is calculated by multiplying the number of packs of cigarettes smoked per day by the number of years smoked[16].

∞ Lake Success, NY

H. Statistical Analysis

Mean values for all clinical indices (GI, BOP, PD and CAL) were calculated for both the periodontitis and control groups. Standard deviations were calculated for PD and CAL. Significant differences for categorical and continuous measures were evaluated by the McNemar's test (GI, BOP) and the paired Student's *t* test (PD, CAL), respectively. *P*-values < 0.05 were considered significant[d].

Frequency distributions and cumulative percentage of nerve fiber patterns observed at five epithelial and five sub-epithelial locations were used to contrast the findings between control and periodontitis papillae.

4. RESULTS

A. Calibration Results

An intra-examiner and inter-examiner reproducibility trial for clinical measurements was completed on periodontal maintenance subjects prior to recruitment of study participants. Intra-examiner agreement within +/- 1mm for PD was 99% and 97-98% for CAL while exact agreement for GI ranged between 90-94%. Inter-examiner reproducibility between the primary examiner and secondary back-up examiner within +/- 1mm was 95-96% for PD and 94-95% for CAL while exact agreement between the two examiners for the GI ranged from 70-72%.

B. Clinical Findings

Nine of the ten participants were Caucasian while one individual was African-Somali.

[d] SAS V9 1.3 was used for the analysis

One participant had been previously diagnosed with Type II diabetes with an HbA1c of 6-7. Seven out of 10 (70%) study participants were classified as current cigarette smokers or former smokers with a mean pack-years of 14.4 (4.4). All five females were current cigarette smokers at the time biopsies were harvested. On the contrary, only 1 out of the 5 male participants was utilizing cigarettes at the time the biopsies were collected, while another male had quit smoking 3 months prior to his biopsies. All interproximal papillary biopsies where obtained from posterior teeth with 80% harvested from the mandible.

Table 1 (Appendix) gives the mean values for all clinical parameters (GI, BOP, PD, CAL) as measured on the day of biopsy. All 10 of the periodontitis papillae (100%) exhibited a GI of 2.0, while 100% of the control papillae had a GI of 1.0. A p-value could not be calculated for the GI using the McNemar's test due to no discordant pairs. BOP was more frequent at periodontitis than at control papillae ($p=0.0027$). Ten out of ten papillae categorized as periodontitis exhibited bleeding on probing, while only one of the ten papillae categorized as control exhibited bleeding on probing.

A statistically significant difference ($p < 0.0001$) and clinically relevant difference in the mean probing depth was observed between periodontitis and control sites in accordance with the study's design. Periodontitis associated papillae had a mean (SD) probing depth of 4.3 mm (0.4), while control papillae exhibited a mean (SD) probing depth of 2.3 mm (0.5). Similarly, mean clinical attachment loss (SD) distinctly separated periodontitis associated papillae from control papillae with a mean value of 4.2 mm (1.0) and 1.2 mm (0.3) respectively ($p < 0.0001$).

C. Analyses of Quantitative Nerve Fiber Patterns

The cumulative presence of quantitative nerve fiber patterns in junctional, sulcular and col epithelium is presented in Table 2 (Appendix). Nerve fibers were more frequently observed in the junctional epithelium and col of periodontitis associated papillae than of

control papillae (18% verses 0% for P = 0.025 and 74% versus 52% for P = 0.141, respectively). The presence of nerve fibers within sulcular epithelium was approximately equal at periodontitis sites (58% versus 64%) compared to control sites. Almost half (60 of 118 regions of sections or 51%) of the periodontitis biopsies exhibited intra-epithelial nerve fibers compared to 44% (52 of 119) of the control samples. While a trend was noted for nerve fibers to be more prevalent at periodontitis associated papillae than control papillae, this difference was not statistically significant (P = 0.299). It should also be noted that the junctional epithelium exhibited only a very small portion of the nerve fibers observed in the col and sulcular epithelium irrespective of the clinical periodontal disease status.

Table 3 (Appendix) depicts the frequency of nerve fibers in sub-epithelial gingival connective tissue. In general, nerve fibers (category 1 thru 5) were more plentiful in both groups within sub-epithelial tissue (130 of 133 regions in periodontitis sections = 97.7% while 100% of healthy regions exhibited nerve fibers) as compared to epithelial tissue (approximately 50% of regions). Moreover, sub-epithelial nerve fibers were noted more frequently adjacent to sulcular and col epithelium than near the junctional epithelium. This distribution was similar to nerve fiber endings penetrating the epithelium. However, the nerve fibers in the sub-epithelial connective tissue were present to a greater extent than in epithelium.

5. DISCUSSION

In recent years, the nervous system has been identified as a critical regulator of periodontal inflammation. Despite our knowledge of the sensory innervation of periodontal tissues[1, 3, 5], the effect of inflammation on gingival nerve fibers or the role that gingival nerve fibers play in the pathogenesis of periodontal disease is unknown. In the present study, we attempted to determine the presence of nerve fibers and their

morphological pattern at periodontitis associated papillae as compared to control/health associated papillae.

Our findings suggest that intra-epithelial nerve fibers appear to be slightly more prevalent at periodontitis papillae (51%) than at control papillae (44%). However, no statistically significant difference was detected between the two groups (Table 2). This lack of statistical significance might be due to the small sample size for each group. Yet, the presence of nerve fibers appears to increase in the epithelial structures as signs of periodontal disease intensify. This trend agrees with Hall et al. (2001) who speculated that periodontal neural tissue can survive during rapid remodeling of the surrounding tissues and be actively involved in the inflammatory process including the release of various neuropeptides[17]. Sarin et al. (2006) examined the role of the nervous system in rhinitis and demonstrated that nerve function can be chronically upregulated in the presence of mucosal inflammation[18]. Our findings suggest that nerve fibers tend to be more prevalent into epithelial tissues as clinical signs of periodontitis become more obvious.

Intraepithelial nerve fiber patterns never exceeded category 3 at periodontitis or at control sites in our study. Category 3 represents a moderate number of nerve fibers without clusters (see Figure 1). This finding coincides with Hilliges et al. (1996) who reported very thin single intraepithelial nerve fibers with only occasional branching in healthy human gingiva. However, they noted a denser nerve network containing nerve bundles that ran parallel to the epithelial surface within the gingival connective tissue[5]. We found a similar prominent pattern of sub-epithelial nerve fibers. Our data depicted sub-epithelial nerve fibers to be approximately double that number observed within epithelium irrespective of the clinical disease status (sub-epithelium: periodontitis papillae 130 of 133=97.7% and control papillae 139 of 139=100% versus intra-epithelium: periodontitis sites 60 of 118=51% and control sites 52 of 119=44%). Kennedy et al. (1996) reported an abrupt termination of many nerve fibers at or near the basement membrane of the dermal-epidermal junction in diabetic subjects with neuropathy. They related the phenomenon to

failed attempts at reinnervation due to an inability of nerves to penetrate basement membrane[14].

Saglie et al. (1982) described widened intracellular epithelial spaces within periodontal sulcular epithelium and the penetration of bacteria into the underlying connective tissue[19]. The sulcular epithelium within our specimens depicted nerve fibers at levels of fifty-eight percent (58%) at periodontitis sites and sixty-four percent (64%) at control sites. We observed intra-epithelial nerve fibers within the junctional epithelium 18% of the time at periodontitis sites while none of the control sites exhibited nerve fibers. This implies that the intra-epithelial fibers might be an early sign of periodontal disease. The intra-epithelial nerve fibers appear to be independent of the sub-epithelial nerve fiber patterns since the sub-epithelial nerve fiber patterns were almost identical at the control and periodontitis sites. Therefore, it can be theorized that neuropeptides released via the fibers may act as neural inflammatory mediators and thereby potentially enhance the inflammatory reaction and pathogenesis of periodontal disease[8].

It is well documented that periodontitis patients perceive greater tenderness at diseased sites than at clinically healthy sites during periodontal probing. Heft et al. (1993) demonstrated that the degree of periodontal inflammation is related to the pain and discomfort associated with periodontal probing[20]. Armitage et al. (1977) described a consistent relationship between the degree of gingival inflammation and the extent of periodontal probe penetration into sulcular epithelium and underlying connective tissue in terms of the apical border of the junctional epithelium (AJE). The probe penetration was 0.39 mm coronal to the AJE in healthy specimens, 0.10 mm coronal to the AJE in gingivitis specimens, and penetrated 0.24 mm into the connective tissue at periodontitis specimens[21]. Our study noted the presence of nerve fibers beneath the junctional epithelium in 96% of the specimens examined while 18% of the periodontitis sites demonstrated intra-epithelial nerve fibers within the junctional epithelium. Therefore, the minor discomfort experience by some patients during periodontal probing can be partly

explained by the degree of probe penetration and the presence of nerve fibers within the sulcular and junctional epithelium as well as the underlying connective tissue.

This was a pilot study intended to observe patterns of nerve fibers at papillae associated with periodontitis and control specimens. Inferences of the study must be taken with caution due to the small sample size as well as potential selection bias. Further evaluation of gingival neural patterns observed within a larger number of subjects should be completed to verify our findings and to determine their clinical significance. A trend for more prevalent nerve fibers in the junctional and col epithelium was detected at periodontitis as compared to control sites while the quantity of nerve fibers was similar in the sulcular epithelium. Among our small sample size there appears to be a tendency for more nerve fibers to be observed at periodontitis sites than control sites. The effect of inter-individual variability is undoubtedly a confounder to establishing any relationship between clinical status and the presence of nerve fibers. Offenbacher et al. (2008) reported that there are dramatic differences between individuals who may have very similar clinical presentations. A similar clinical picture or diagnostic category may be produced by different interactions of biofilms with the individual-dependent host inflammatory and immune responses[22].

Our findings should be interpreted in light of the notion that clinical phenotype does not always reflect the underlying biologic processes. Subject-level factors such as race, gender, smoking and the presence of diabetes have been proven to be the strongest contributors to periodontal disease expression. Therefore, the clinical expressions of periodontitis would be the consequences of host predisposing factors and environmental insult[22]. From the proceeding statement it is clear that our pilot study was balanced for gender but not for smoking, race or diabetes. Our subjects were equally divided with five males and five females. However, smoking status was not uniform among study subjects. Two of the five men (40%) were smokers while all five of the women (100%) were smoking at the time the biopsies were collected. Numerous epidemiological studies that have controlled for confounding variables, such as age, plaque, calculus, gender, and

socioeconomic status, have provided strong evidence that smoking is a risk factor for periodontitis. Grossi et al. (1994) reported that cigarette smokers are about two to five times more likely to develop periodontitis than individuals who have never smoked[23]. Future research should include biopsies of non-smokers at periodontitis and control sites to be evaluated in terms of our findings. Chronic toxicity of tobacco smoke has been shown to alter myelinated peripheral nerves of the visual system of rats[24] and it may potentially also affect gingival innervation. Moreover, nine out of the ten participants in this study were Caucasian while one was African-Somali. One participant had been diagnosed with Type II diabetes prior to the biopsy collection and exhibited an HbA1c of ~6-7.

The age of our population ranged from 39 to 74 years, with a mean of 53.8 years. Matheny et al. (1992) reported a reduction in gingival vascular perfusion as human subjects age[25]. On the other hand, Wendelschafer et al. (2009) demonstrated in children ranging in age from 3 months to 18 years with idiopathic chronic constipation that nerve density was not dependent on age[15].

Differences in nerve patterns were observed within the same sextant of a study subject. This site independence within the same host is also illustrated by clinical parameters reported by Lindhe et al. whereas only 0.7% of sites underwent progressive periodontitis during a 2-year observation period[26]. Lack of attachment loss on sites immediate adjacent to teeth with advanced periodontitis and a hopeless prognosis also depicts disease variability within individuals[27].

Further research appears warranted to clarify the significance of differences in gingival nerve fiber patterns and their location regarding periodontal disease status. Evaluation of gingival nerve patterns may potentially aid in early diagnosis and longitudinal monitoring of periodontitis, while neural modulating drugs may someday be useful for regulation of periodontal inflammation. The efficacy of any potential periodontal treatment modalities

based on differences in intraepithelial nerve fibers patterns would have to be justified in terms of patient response as well as altered pattern of nerve fibers.

6. CONCLUSIONS

- This study demonstrated the presence of nerve fibers in both epithelial and sub-epithelial gingiva at control as well as at periodontitis associated papillae.
- A trend was observed for more intra-epithelial nerve fibers at periodontitis associated papillae compared to control papillae (51% versus 44%).
- Nerve fibers penetrate sulcular and col epithelium more frequently than junctional epithelium.
- Sub-epithelial nerve fibers (97.7% at periodontitis associated papillae and 100% for control papillae) are more frequent than intra-epithelial nerve fibers (~ 50%) irrespective of the clinical periodontal disease status.
- Sub-epithelial nerve fiber patterns are considerably more prominent than intraepithelial nerve fiber patterns in gingiva (category 3 with > 25 nerve fibers was only observed in the sub-epithelial area).
- Sub-epithelial innervation is more prevalent adjacent to sulcus and col tissue as compared to the junctional epithelium.
- Inter-individual variability must be considered when inferring any relationship between clinical periodontal status and the presence of nerve fibers.
- Further research is needed to determine if variation in gingival nerve fiber patterns is associated with neurogenic inflammation and periodontal pathogenesis.

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8 APPENDIX

A. Tables

Table 1. Summary of Clinical Parameters at Periodontitis and Control Papillae

	Periodontitis n=10	**Control n=10**
Gingival index (GI), n (%)*	GI=2 n=10 (100%) Mean=2.0	GI=1 n=10 (100%) Mean=1.0
Bleeding on probing index, n (%)*	BOP=1 n=10 (100%)	BOP=1 n=1 (10%)
Probing Depth, mean (SD)**	4.3 (0.4)	2.3 (0.5)
Clinical Attachment loss, mean (SD)**	4.2 (1.0)	1.2 (0.3)

* McNemar's test; GI, p=NC; BOP, p=0.0027

**Paired t-test p-value < 0.0001

Table 2. Frequency/Distribution of Nerve Fiber Patterns in Gingival Epithelium

Location	Nerve Fiber Pattern	Periodontitis	Control
Col			
Absence of nerve fibers	0	26% n = 8	48% n = 10
Presence of nerve fibers	1, 2, 3	74% n = 23	52% n=11
Sulcular			
Absence of nerve fibers	0	42% n = 22	36% n = 23
Cumulative presence of nerve fibers	1, 2, 3	58% n = 31	64% n = 41
Junctional			
Absence of nerve fibers	0	82% n=28	100% n=34
Cumulative presence of nerve fibers	1	18% n = 6	0%
Cumulative presence of nerve fibers in col, sulcular and junctional epithelium		51% n = 60 of 118	44% n = 52 of 119

Fisher's exact test (assuming site independence) p-value for: junctional = 0.025, sulcular = 0.571, col = 0.141 & overall = 0.299.

n = Number of regions scored

Table 3. Frequency/Distribution of Nerve Fiber Patterns in Sub-Epithelial Area

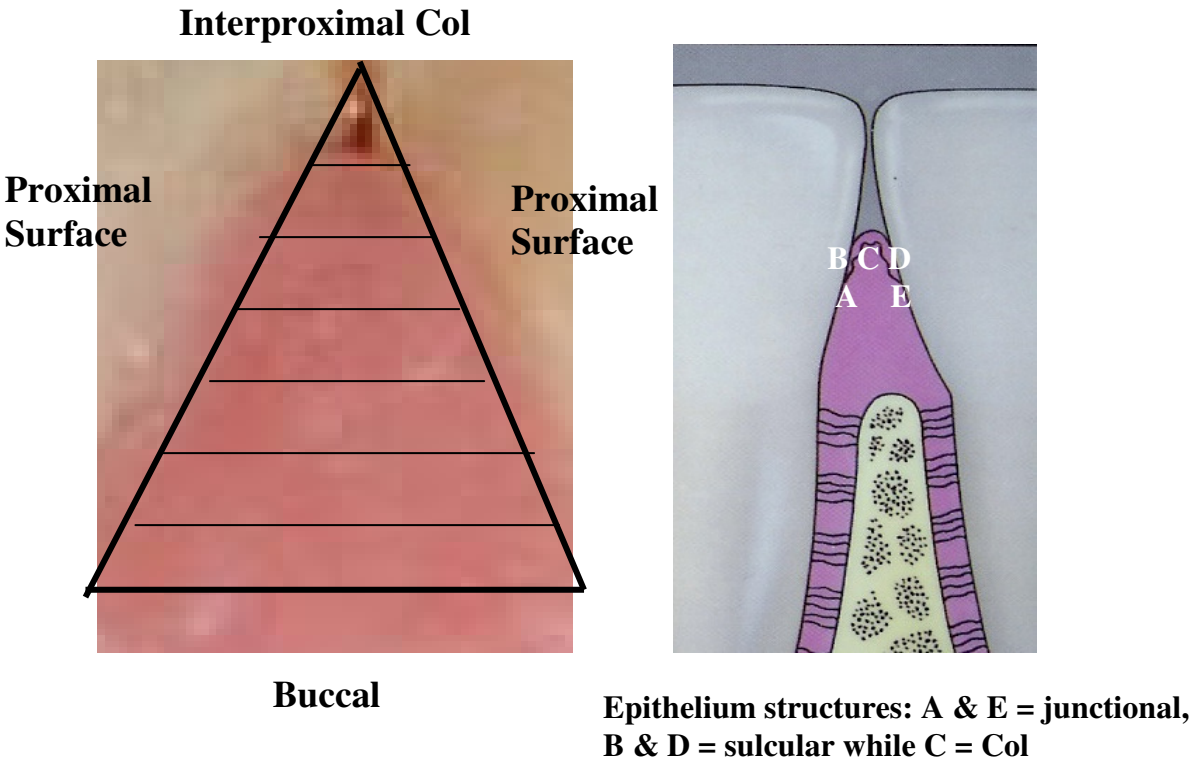
Location	Nerve Fiber Pattern	Periodontitis	Control
Sub-Col			
Absence of nerve fibers	0	3% n = 1	0% n = 0
Cumulative presence of nerve fibers	1 - 5	97% n = 30	100% n = 21
Sub-Sulcular			
Absence of nerve fibers	0	0% n = 0	0% n = 0
Cumulative presence of nerve fibers	1 - 5	100% n = 54	100% n = 64
Sub-Junctional			
Absence of nerve fibers	0	4% n = 2	0% n = 0
Cumulative presence of nerve fibers	1 – 5	96% n = 46	100% n = 54

Fisher's exact test (assuming site independence) p-value for: junctional = 0.219, sulcular = NC, col = 1.000 & overall = 0.116

n = Number of regions scored

B. Figures

Figure 1. The photograph on the left depicts where the biopsies were obtained from buccal interproximal papillae. Specimens were horizontally orientated for sectioning with col epithelium superior while mesial and distal sulcular epithelial surfaces were at lateral margins. Specimens were sectioned in a coronal to apical (superior to inferior) fashion at 60 microns thickness. The schematic diagram on the right illustrates where nerve fibers were quantitatively scored at five locations using criteria shown below.



Epithelium structures: A & E = junctional, B & D = sulcular while C = Col

Scoring Criteria of Nerve Fiber Patterns in Epithelial and Sub-epithelial Areas

0=none

1=sparse (1-5 linear fibers)

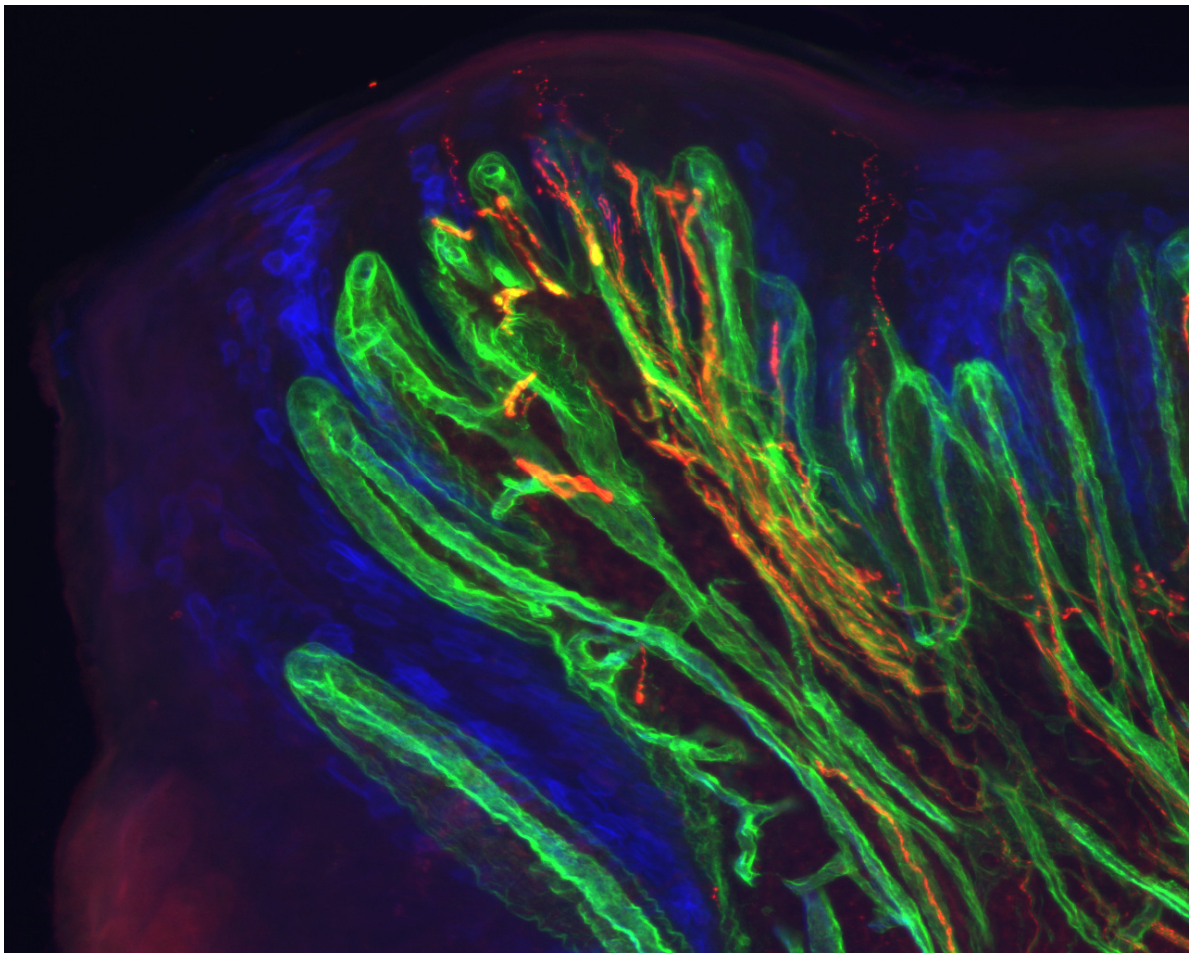
2=few (6-25 linear fibers)

3=moderate (25+ fibers)

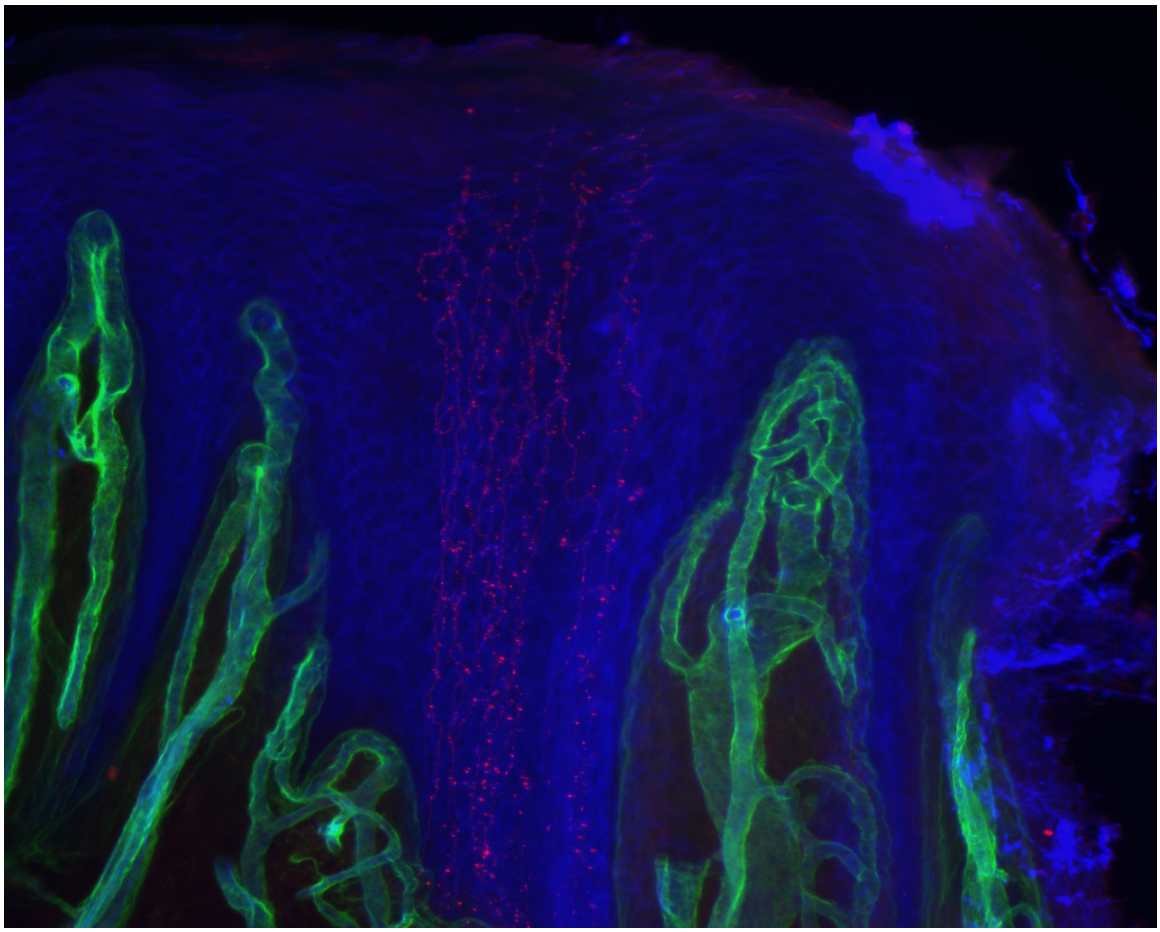
4=scattered densely innervated clusters (2+)

5=dense innervation (70+) and/or clusters

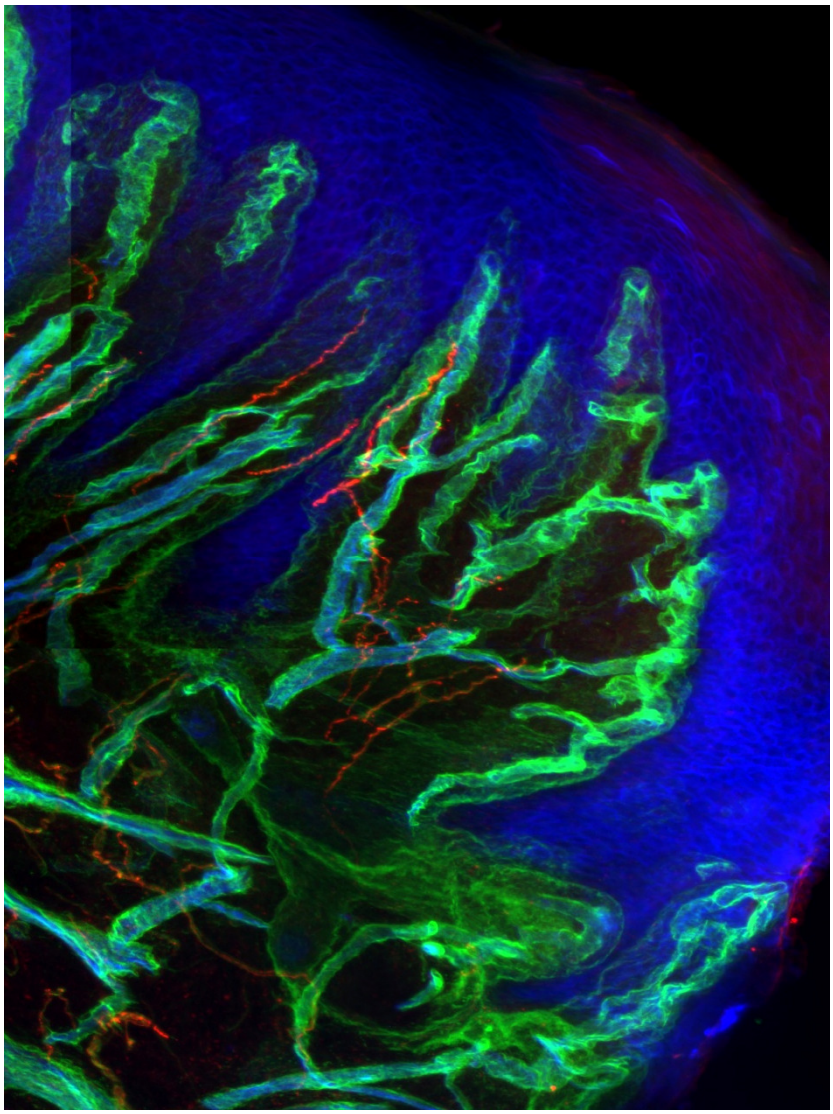
Images were scored at 10X magnification with selective images scored at 20X for clarification.



Multi-panel figure 2a. Immuno-fluorescent stain that depicts nerve fibers (pinkish-orange) accompanying capillary loops (green) through sub-epithelial connective tissue with individual nerve fibers (pinkish-orange) penetrating into epithelium (stained blue) up into stratum granulosum layer of biopsy showing location B. This picture is an illustration of an epithelial visual scoring of 2 and a sub-epithelial scoring of 4.



<u>Multi-panel figure 2b</u>. Col portion of biopsy illustrating nerve fibers (stained pinkish-orange) penetrating into the superficial epithelial layer of the stratum granulosum. This pictures typically illustrates an epithelial quantitative scoring of 2.



Multi-panel figure 2c. Immuno-fluorescent stain illustrating nerve fibers (stained pinkish-orange) penetrating into stratum spinosum epithelium (stained blue in top third of image) and sulcular epithelium (right lateral surface of image) of biopsy showing location D & E. This picture is an example of sub-epithelial visual scoring of 2 and an epithelial quantitative scoring of 1 in both locations.