Investigation of ischemia induced in human gingiva

Ph.D. Thesis

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INTRODUCTION

The multiple interconnections between the different plexuses through numerous anastomoses and native collateral pathways of circulation establish an adequate blood supply in the gingiva; however, the extent of the contribution of the various collaterals in maintaining resting blood flow has not been evaluated yet. Most evaluations of the distribution patterns and course of the vascular system in the gingiva have been carried out under morphological studies in human cadavers and in animals. Therefore, we have little knowledge of the direction of blood flow in the various areas of the attached gingiva. Understanding the functional morphology of the gingival vasculature may have even more importance in clinical practice. Incision design during periodontal and oral surgical interventions aims to take the vascular anatomy of the oral mucosa into account in order to impair postoperative blood supply and consequently wound healing to the least possible extent. However, during flap elevation the circulation of the gingiva is challenged, and collateral recruitment is needed. In addition, the attached gingiva is continuously affected by mechanical stimuli (chewing, tooth brushing, etc.) which challenge the vasodilator capacity of the vessels. Vascular reactivity may also be influenced by conditions such as diabetes, periodontitis or smoking. Assessment of the functional capacity of the gingival vasculature may help during surgical interventions in making decisions at individual level, taking into account the vascular and systemic conditions of the patient.

Vestibuloplasty is periodontal surgery to the oral vestibule, aiming to restore vestibular depth with simultaneous enlargement of the zone of keratinized gingiva and soft tissue thickness for enhanced aesthetics and function. The modified apically repositioned split thickness flap (MARF) combined with a collagen matrix (CM) is a frequently used vestibuloplasty procedure. Xenogenic CM is highly recommended in several studies as a viable alternative to the autogenous free gingival graft for augmenting keratinized gingiva. Geistlich Mucograft consists of two structural layers: a superficial compact macro-structure, which provides stability while allowing open healing, and an underlying spongy micro-structure, which supports blood clot stabilization and the ingrowth and repopulation of fibroblasts, blood vessels and epithelium from the surrounding tissues. The horizontal incision and the separation of muscle attachment in the alveolar mucosa during the MARF procedure severe important blood supply to the attached gingiva. It is not known how the non-vascularized collagen membrane on the exposed periosteum becomes vascularized and whether the ingrowth of blood vessels occurs either vertically, from the recipient bed originating from the periosteum, or laterally, with vessels arising from adjacent tissues. A better understanding of graft incorporation could help clinicians implement more sophisticated surgical techniques for more favorable clinical outcomes

Dental practitioners are often caught in a dilemma when they have to decide between immediate and early implant placement after tooth extraction. It is prudent to plan early implant placement when the morphology of the sight prevents an optimal immediate implant placement, or a thin soft tissue biotype and also a thin bone wall phenotype would make the implant therapy unpredictable due to the resorption of the buccal bony plate. The early implant placement type-2 protocol is also recommended in the case of inflammation of the bone associated with the extracted tooth. According to the proposal at the 3rd ITI Consensus Conference in 2003, early implant placement type 2 refers to the placement of an implant after substantial soft tissue healing has taken place, but before any clinically significant bone fill occurs within the socket. Based on empirical evidence, complete soft tissue healing at the extraction site takes 4 to 8 weeks. Obviously, that broad time interval provides for deviation due to the method of tooth extraction and individual differences in pace of healing. Only a few studies placed post extraction soft tissue healing under scrutiny. In humans, complete re-epithelialization of the extraction wound takes place at 24-35 days. At the same time, it is not known when the connective tissue of the submucosa with an adequate vasculature emerges, which is a prerequisite for the survival of a subsequent surgical flap. Any objective method of measurement of the time of soft tissue healing adjacent to the extraction wound would allow for administration of the subsequent treatment steps without any time wasted and for better predictability of treatment outcomes.

Laser Speckle Contrast Imaging (LSCI) is a non-invasive, optical method, that has emerged over the past three decades as a powerful tool for imaging of blood flow dynamics in real time. It combines dynamic response and high spatial resolution, providing two dimensional color coded map of microcirculation in time in a human subject. In previous studies, our working group has found that LSCI is suitable also for mapping microvascular blood flow in the gingiva.

My Ph.D. research in clinical studies was conducted to reveal spatial and temporal changes in gingival blood flow under challenge, mainly after the induction of ischemia using the non-invasive laser speckle method. By application of different direction of transient compressions and different incisions during surgical procedures we aimed to study the function and orientation of the collateral network in the attached gingiva. With the clinical adaptation of the examination method and the use of the obtained circulatory regulation observations, we wanted to provide data for the prognosis of periodontal and implant aesthetic surgical curative procedures and for the objective assessment of their success.

OBJECTIVES

The present thesis reports on three series of clinical studies aimed at learning about the following.

I. Functional characterization of collaterals in the human gingiva

- We aimed to determine the rate of blood flow between the various areas of the gingiva in resting position,
- to reveal the dominant directions of blood flow in the attached gingiva, and
- to map spatial and temporal changes in gingival blood flow after transient compression.

II. Blood flow kinetics of a xenogeneic collagen matrix following a vestibuloplasty procedure in the human gingiva

- Our primary aim was to determine the time course of CM vascularization in exposed conditions.
- Our secondary aim was to determine relative contribution of the recipient wound bed's environment to the graft's neovascularization, corroborated by simultaneous quantitative determination of Vascular Endothelial Growth Factor (VEGF) expression.

III. A proposed method for assessing the appropriate timing of early implant placements: a case report

- We aimed to reveal whether the LSCI method is suitable to determine the individual healing periods (ischemia, hyperemia, rebound) of surgical flaps in clinical conditions, and based on it to determine the time of a required second surgery.

MATERIALS AND METHODS

The studies involved healthy patients aged 18 to 45 years with intact periodontium. Exclusion criteria were pregnancy, smoking, general diseases and any medication, except for contraceptives.

Blood flow measurements were performed by LSCI (785 nm PeriCam PSI HR System, Perimed AB, Stockholm, Sweden) in all series of the clinical studies. Specific measurement zones called ROIs (region of interest) were determined on the two-dimensional images provided by the PimSoft software (Perimed AB, Stockholm, Sweden). Perfusion values of pixels within a ROI were averaged and defined as the blood flow value of the ROI, expressed in an arbitrary value called Laser Speckle Perfusion Unit (LSPU). In the case of video recording, specific time periods of interest (TOI) were defined on the graph recording the measurement. This allowed for averaging perfusion in a ROI over a definite period of time. Blood pressure was measured with an automatic blood pressure monitor (Omron M4, Omron Healthcare Inc., Kyoto, Japan).

I. Seventeen volunteers (10 females and és 7 males; 21 to 40 years) were recruited. After one minute of resting blood flow measurement (baseline) on the buccal side of the maxillary attached gingiva, horizontal (at tooth 12, perpendicular to the long axis of the tooth, 2 mm far from the gingival margin), vertical (parallel to the long axis of tooth 12, at the distal third of the tooth projection), and papilla base (at the base of the mesial papilla of tooth 12) compression was applied using a manual instrument specially developed for this purpose by the authors. The pressure applied on an area of 10 mm^2 was 100 grams and lasted for five seconds. When five seconds elapsed, the pressure was released and blood flow was monitored continuously for 20 minutes. ROIs defined at each session of measurements were representative sub-samples of anatomical regions. For statistical analyses the following TOIs were selected: TOI1: within the resting period; TOI2: during compression; TOI3: at the hyperemic peak of reperfusion, within one minute after the release of pressure; TOI4 to TOI7: at 5, 10, 15 and 20 minutes after the release of pressure. Except for TOI2 the duration of each TOI was 30 seconds. Blood pressure and blood flow changes were analyzed with a mixed-model. This enabled us to adopt a complex design where time, ROI and gender were the main factors, with their interactions integrated into the model. Pairwise comparison was made with a Least Significant Difference post hoc test. The p values were adjusted using the Benjamini–Hochberg method. Statistical evaluation was carried out by SAS 9.4 (SAS Institute Inc., Cary, USA).

II. Five patients (2 males and 3 females, aged 18 to 45 years) with inadequate width (< 2 mm) of keratinized gingiva (KG) at least on two teeth at the buccal aspect in the anterior mandible were recruited. Patients in this prospective observational study underwent a periodontal plastic surgery intervention to augment KG at selected teeth, involving an apically repositioned flap and the application of a CM (Mucograft®, Geistlich Pharma AG, Wolhusen, Switzerland).

Immediately before and 1 and 6 months after the surgical intervention *KG* was measured. *Keratinized gingival thickness* (KGT*)* was recorded with a Sonoscape A6V (Providian Medical Equipment, Cleveland, USA) ultrasonic device preoperatively and 1, 3 and 6 months postoperatively.

Blood flow measurements were obtained before the operation (baseline) and postoperatively on the following days: 1, 2, 3, 4, 5, 7, 9, 11, 14, 21, 28, later, during the first 6 months of healing monthly, and lastly at the 12-month control. The measurement area covered the whole surgical field. The instrument was set to take 30 s shots. Multiple ROIs were determined at different distances from the center of the implanted graft, marked as zone F. Zone A and B were defined in the ‘peri’ region (in the surgically affected surrounding mucosa) and zone C, D, E in the graft. Each of these zones was identified separately in all four directions from the graft: laterally (left and right), apically and coronally. The data of the two lateral sides were aggregated.

The relative volume of *wound fluid* (WF) was assessed by Periotron 8000 (OraFlow Inc., NY, USA) on the first 14 postoperative days. WF was collected at the coronal, lateral and apical sides of the graft with methylcellulose strips for 10 seconds. Relative volume values were expressed in Periotron Scores (PS).

VEGF was determined from the WF collected for 60 s at the coronal, lateral and apical sides of the graft on day 2 and day 4 postoperatively. VEGF expression was quantified with a sandwich enzyme immunoassay technique (Human VEGF Quantikine ELISA Kit, R&D Systems, USA).

The presence of *scar formation* was evaluated at each ROI based on the intraoral photographs taken in the sixth month.

Data in the text are presented as mean ± standard error of the mean (SEM). Factors affecting changes in blood flow, WF, KG and KGT were analyzed by a mixed-model approach. Pairwise comparison was made based on the Least Significant Difference post-hoc test. The p values were adjusted by the Bonferroni method. VEGF expression in the WF was categorized into four classes according to quantity. Differences in VEGF expression across the regions were tested by non-parametric Friedman's Two-Way Analysis of Variance by Ranks followed by pairwise comparison. The abundance of scar tissue was assessed by calculating the proportion of scarred ROIs for each side of the graft. Significant differences between the sides were evaluated by chi-square statistics. Statistical evaluation was carried out with SPSS 24 (IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp).

III. Following surgical tooth extraction of the unrestorable maxillary left second premolar of a 28-year-old male patient, healing of the gingival area close to the extraction site was monitored in order to determine the optimal time for surgery in preparation for type-2 implant placement. The implant was placed two months later, after the formation of soft tissue over the extraction wound and partial bone fill of the alveolar socket.

Wound healing and soft tissue alterations was monitored visually and by the method of tissue blood flow measurement. Blood flow was measured at the surgical site before extraction (baseline) and on days 1, 3, 5, 7, 11, 14, 20, 31, 42 and 62 post-extraction, then after implant placement on days 1, 4, 7, 20 and 498 (i.e. on days 63, 66, 82 and 562 post-extraction). Buccal and occlusal snapshots were taken by the LSCI device at an interval of two seconds, the former directly, the latter using a dental photographic mirror.

During evaluation, identical ROIs were defined on all LSCI recordings. On occlusal images, ROI A covers the extraction wound, ROI B and C represent concentric regions of a width of 1 mm each around the extraction site, and ROI D the areas more distant to the wound. On direct buccal images, 2 mm high regions, ROI E, F and G were defined from the marginal gingiva to the vestibule.

When comparing blood flow values measured on different days, we relied on our previous observation that a change in blood flow between two measurements performed on two different days may be established with 95% confidence if the later measurement represents a decrease to 79% or an increase to 127% of the earlier measurement. Soft tissue healing time was estimated based on the regularity of two subsequent blood flow measurements, or, when baseline blood flow was known, based on blood flow values' return to the baseline.

RESULTS

I. The mixed-model analysis gave similar results in the three experiments in terms of the main factors and their interaction. All the main factors (gender, ROI and time) had a significant influence on blood flow after transient compression. The gender x ROI x time interaction was not significant, but the ROI x time interaction was significant. Therefore, the effect of time on ROI was analyzed separately, comparing each time-point to its respective baseline. The gender x ROI interaction was not significant, but the gender x time interaction was significant. Consequently, the effect of gender on changes by time was analyzed by comparing the differences between the genders at each respective time-point using the grouped values of the ROIs.

Mean Arterial Pressure (MAP) did not differ significantly between the genders in any of the study series, either before or after the blood flow measurements, and did not change significantly throughout the observation periods.

Resting blood flow was higher in the papilla than in the attached gingiva in the midbuccal area of the teeth but lower than in the attached gingiva apical to the papilla in both genders.

Short-term localized horizontal pressure significantly reduced blood perfusion both coronal and apical to the occlusion line, apical to the mesial and distal papilla as well as at the mesial and distal papilla ($p<0,001$). Blood flow reduction was larger coronal to the occlusion than apical ($p<0,001$).

Post-occlusion hyperemia propagated into a much wider area in all types of compression than ischemia itself, covering approximately a three teeth sized unit ($p<0,05$). Blood flow increase was smaller coronal to the occlusion line than apical ($p<0,001$). By distance from the occlusion line the degree of hyperemia decreased.

Five minutes after the occlusion, blood flow was not significantly different from the baseline and it remained unchanged during the rest of the measurement in all ROIs, except apical to the compression line, where blood flow remained significantly elevated 5, 10, 15 and 20 minutes after the release of pressure compared to the baseline.

Short-term localized vertical pressure significantly reduced blood perfusion both mesial and distal to the occlusion line ($p<0.001$), however, there was no significant difference in the degree of ischemia. Post-occlusion blood flow was significantly elevated in the ischemized regions and also farther away ($p<0,05$). There was no significant difference in the hyperemic peak of reperfusion in a mesial and distal direction by the occlusion line. Moving away horizontally from the line of the occlusion, the degree of hyperemia gradually decreased, both mesially and distally.

Five minutes after the occlusion, blood flow was not significantly different from the baseline, and remained unchanged during the rest of the measurement in all ROIs, except at the line of compression, and mesially from it, where hyperemia remained significant 5, 10 and 15 minutes after the release of pressure compared to the baseline.

Short-term compression on the papilla base significantly reduced blood perfusion, both coronal and apical to the occlusion line as well as in a mesial and distal direction in the attached gingiva, i.e. in the midbuccal area of the central incisor and the lateral incisor ($p<0,001$). There was no significant difference in blood flow reduction either coronal and apical or mesial and distal to the occlusion.

One minute after the release of compression, blood flow increased significantly and propagated into a larger area than ischemia itself ($p<0,05$). Blood flow increase was significantly larger apically in the attached gingiva than coronally in the papilla ($p<0,05$), however, in a mesial and distal direction by the occlusion line no significant difference was found.

Five minutes after the occlusion, blood flow was not significantly different from the baseline and remained unchanged during the rest of the measurement in all ROIs.

There was no significant difference in blood flow reduction between the genders in any of the series. Post-occlusion blood flow increase in males was more pronounced and prolonged than in females at almost all time-points in the case of all types of compression. ($p<0,05$).

II. All patients healed uneventfully. The width of KG increased at all the tested incisors in all cases ($p<0.001$), mean KG was 2.7±0.28 mm at baseline and 4.2±0.28 mm 6 month postoperatively. After six months, KGT was similar to baseline (0.98±0.04 mm vs 0.91±0.07 mm, $p=0.89$).

There was no significant change in MAP during the investigation and only a slight difference was found in MAP before (83.5±2.8 mmHg) and after (85.4±2.8 mmHg, $p<0.01$) the blood flow measurements within a session. Blood flow did not change significantly (it ranged from 188 to 223, $p=0.088$) in the papillae during the entire procedure. In the first 5 days, post-surgical ischemia was observed in all regions of the grafted area regardless of side. From day 6, blood flow increased at different rates depending on side and zone. The day of peak-flow also differed by side and zone. After day 64, blood flow stabilized until the end of the observational period in all regions. No ischemia was observed in the 'peri' region where blood flow began to increase from day 6 and remained elevated until day 64.

The comparison of the regions situated at the incision line showed that perfusion in the 'peri' zone was significantly higher than in the outermost zone of the graft for 9 days at the coronal side, for 7 days at the lateral side and for 14 days at the apical side ($p<0.001$).

The rates of increase in graft perfusion in zone C to E were significantly higher than in the central zone F until day 11 at the coronal side and until day 14 at the lateral side ($p<0.05$). At the coronal side, blood flow in zone F exceeded perfusion values in the 'peri' regions on day 22. At the apical side, the perfusion of zones within the graft increased at a similar rate.

In order to assess the direction of revascularization of the graft, blood flow changes in the coronal, lateral and apical zones C – the outermost zones of the graft – were compared. Perfusion at the coronal and lateral sides was significantly higher than at the apical side, until day

11 coronally and until day 14 laterally. Blood flow was significantly higher in the lateral zone from day 9 to day 29 (except on day 22) than at the coronal side ($p<0.05$).

Wound fluid production was significantly higher at the apical side than either at the coronal or at the lateral side of the graft from day 2 to day 5 after surgery ($p<0.05$).

VEGF expression was significantly more abundant at the apical side than at either of the coronal or lateral sides ($p<0.05$ and $p<0.01$).

The proportion of scarred tissue 6 months after surgery was higher centrally and at the apical side than at the coronal side ($p<0.01$). Scar tissue proportion at the lateral side was in between.

III. The surgical extraction wound healed without complications. As healing of the extraction wound progressed, the area affected by ischemia after tooth extraction shrunk concentrically already after day 3 and evolved into a homogeneous hyperemic zone by day 20. It was also from day 20 that epithelialization of the extraction wound could be considered complete.

On the first day post-extraction, there was a significant drop in gingival blood flow around the extraction wound – i.e. the distal area of the mucoperiosteal flap – compared to the baseline. Ischemia lasted 7 days in ROI B and 5 days in ROI C, followed by a hyperemic period of 3 weeks in both regions. 2 mm apically from the edge of the flap no ischemia was observable and from day 5 hyperemia also occurred here for 3 weeks. In the area of the extraction wound, clearly significant blood flow was measured on day 14, comparable to the degree of hyperemia in the adjacent regions. In occlusal regions the blood flow values measured on day 62 post-extraction did not differ significantly either from the values measured on day 42 (ROI A: 88%, ROI B: 95%, ROI C: 103% and ROI D: 111%) or from baseline values (ROI B: 100%, ROI C: 89% and ROI D: 89%).

In buccal regions hyperemia was observable from day 4 for 3 weeks, without any ischemic period preceding it. There was no significant change in the blood flow values measured on day 62 in the buccal regions as compared to day 42 (ROI E: 85%, ROI F: 85%, and ROI G: 89%) or baseline values (ROI E: 97%, ROI F: 116% and ROI G: 116%). There was a clear increase in blood flow values from the edges of the flap towards its base. Hyperemia arose first at the base of the flap and spread gradually towards the edges of the flap, reaching the extraction wound the latest.

Wound healing after implant placement was also free of complications. Unlike in the case of the flap created for tooth extraction, no pronounced ischemia occurred in either of the occlusal regions (day 63 vs. day 62: ROI B: 84%, ROI C: 118% and ROI D: 166%). Instead, all regions were affected by hyperemia after implantation (day 63-82) with various time and degrees.

During the one-year follow-up no measurements were taken in ROI B and C as the crown obstructed the view. In ROI D and E, blood flow values did not differ significantly from those measured on day 20 postoperatively (day 82) (ROI D: 96%, ROI E: 89%), whereas in ROI F and G they fell below day 20 values (ROI F: 78%, ROI G: 78%). As compared to the results of the measurement taken immediately before implant placement (day 62), blood flow values were similar in all regions (ROI D: 111%, ROI E: 124%, ROI F: 124% and ROI G: 118%).

CONCLUSIONS

I.

- LSCI method clearly shows spatial differences of resting blood flow in the human gingiva.
- Blood supply has a flow pattern characterized by apico-coronal predominance in the attached gingiva, however, no mesio-distal orientation was found, at least in the anterior maxillary area.
- Intraseptal vessels at the papilla base could contribute to the blood supply of the coronal part of the attached gingiva both above the papilla and at the marginal gingiva.
- The attached gingiva has a very high vasodilator capacity; this may be due to ascending vasodilation.
- Gender difference in responses to transient ischemia is a new observation; its importance and explanation needs further investigation.

II.

- Following an apically repositioned flap procedure in combination with a CM graft reperfusion started at the earliest and most intensively from the lateral side.
- From the apical side, vascular ingrowth is inhibited due to deep vestibular preparation.
- The arising prolonged apical ischemia induces an overshot in VEGF expression, assumed to be triggered by hypoxia, and results in disturbed pruning and capillary maturation. This process, together with intermittent traction forces, may lead to unfavorable scar formation.
- All this implies that developing a new preparation technique at the alveolar mucosa and the horizontal limit of extension of the grafted area would be recommended, to result in more predictable healing.

III.

- Blood flow became stable in the entire area of the extraction wound and the mucoperiosteal flap elevated as part of the surgery after the 6th week post-extraction.
- This indicates that the minimum recommended healing period of 4 weeks for early implant placement would not have been sufficient in this case for soft tissue to heal completely.
- Blood flow measurements in the gingiva using LSCI, in combination with the statistical methodology we have developed, seem to be a promising tool for routine monitoring of surgical flaps under clinical conditions.
- Based on the changes in flap perfusion monitored by LSCI the optimal timing of early implant placements may be assessed individually. This allow for administration of the subsequent treatment steps without unnecessary time loss or delay for better predictability of treatment outcomes.

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