Spreading vasodilation and vasoconstriction in human gingiva, the significance of the nitric oxide and the epinephrine

Doctoral thesis

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1. INTRODUCTION

Proper blood supply is essential for wound healing. The blood supply is damaged in different degrees after flap surgery, depending on the type of the flap. Periodontal inflammation can also be associated with morphological changes in the blood vessels, which affect the regulation of gingival blood flow.

As early as 1933, it was observed that during the infusion of a vasodilator, vasodilation also occurs upstream of the blood supply, where the vessel was not stimulated by a vasodilator. In our earlier studies, prolonged hyperaemia of the gingiva was observed after short-term strangulation, not only in its close vicinity but also in the surrounding distant parts. This suggests that a so-called *spreading vasodilation* mechanism is present in the gingiva. Flow-mediated vasodilation, in which flow and shear force induce vascular muscle relaxation in the blood vessels, may play a role in this mediation.

In contrast to the fact, the *spreading vasoconstriction* may be a determining factor in the extent of the ischemic area in the gingival tissue. The ischemic effect of epinephrine has been previously demonstrated with laser Doppler flowmetry in human gingiva, but its possible spread cannot be determined by this method. Our team has applied the Laser Speckle Contrast Imager (LSCI) for gingival microcirculation for five years. Several observations were made by our research group with the

device about the spatial change of blood microcirculation in the mucogingival flap and the reliability was also showen.

The biotype and thickness of the gingiva are decisive in the prognosis in the case of several periodontal and minor oral surgical treatments, as well as in the case of gingival retraction. However, it is not known what physiological differences there are between thin and thick biotypes. We hypothesized that spreading vasodilation and vasoconstriction are present with different intensities depending on their thickness. A large number of physiological human studies can be performed only by non-invasive thickness measurements. Therefore, a new previously unvalidated ultrasonic gingival thickness (GT) determination device was used for our studies and reliability studies were performed. In addition to objective measurement methods, we also used a subjective classification based on gingival transparency (TRAN method).

2. OBJECTIVES

To determine the accuracy of the gingiva thickness measurement of PIROP ultrasonic biometer.

What is the temporal and spatial course of vasodilation induced by the nitric oxide donor?

What is the extent the spatial and temporal effect of epinephrine-induced vasoconstriction?

Is there any correspondence between microcirculation and gingiva thickness?

3. METHODS

In the *in vitro* group, 3 slices of pork ham were placed on a flat glass plate for thickness measurements.

According to the inclusion criteria of the remaining 5 studies, healthy women and men with healthy periodontium were involved. In the PIROP *in vivo* group, 25 volunteers have participated in the study, of whom 16 were women. In the vasodilation studies of the 20 volunteers, 15 were women. In the NitroPohl group there were 7 women out of 10 volunteers and in the Nitromint group, there were 8 women out of 10 volunteers. In the vasoconstriction group, 21 volunteers (11 women and 10 men) were enrolled. In the keratinized gingiva group, 6 of 11 volunteers were women. In the gingival sulcus group, 10 patients (5 females and 5 males) were included.

I. *In vitro* measurements

10 measuring points were marked on each ham slice by selecting random areas but taking care of covering the widest possible range of thicknesses. Measurements were performed 5 times at each measurement point. At each measurement point they were measured 5 times with a PIROP device, with an average of 10 values per measurement. This was followed by a direct measurement method. In this method, the ham tissue was pierced using an ISO 15 spreader and the deepness was detected.

II. *In vivo* measurements

The measurement point of the gingival thickness test was located in the midbuccal line of the right upper second incisor (12) tooth, 2 mm from the marginal gingiva. First, the gingiva was coated with a gel containing 20% benzocaine, which served as a conductive medium for ultrasound measurement, while anaesthetizing the mucosa. After that we measured with PIROP device 5 times, holding it perpendicular to the measuring point by indirect method (PIROP GT), then with ISO 15 spreader invasive, direct measurement (spreader GT) followed 5 times similarly to the *in vitro* study.

III. 1mg/ml NO donor solution (NitroPohl group)

In this group, 3 µl of a 1 mg/ml NitroPOHL® solution (Pohl-Boskamp GmbH, Germany) with a body temperature was dropped to the test side (12 tooth) into well at the keratinized gingiva with a Hamilton syringe. 3 µl of physiological saline, also at body temperature, was dropped to the control side (21 tooth) correspondingly. A total of 0.003 mg of active ingredient was applied topically to the gingiva during the examination. 5-5 measurement points (region of interests, ROIs) were designated at the teeth: at the site of examination in the well (“w”), coronally (“c”), mesially (“m”), distally (“d”) and apically ("a"). The length of the Time of Interest (TOI) periods were 10 seconds. One was designed in the baseline period, then

immediately after dropping, for 14 minutes in each minute, giving additional fourteen.

IV. 8 mg/ml NO donor solution (Nitromint group)

In this group, 3 µl of 8 mg/ml Nitromint® solution (Egis Pharmaceuticals PLC, Hungary) at body temperature was dropped at the test side (12 tooth) into the well on the keratinized gingiva with a Hamilton syringe. 3 µl of physiological saline, also at body temperature, was dropped to the control side (21 tooth) in a similar way. During the examination, a total of 0.024 mg of active ingredient was applied topically to the gingiva. 5-5 measurement points (ROIs) were designated at the teeth: at the site of instillation in the well ("w"), coronally ("c"), mesially ("m"), distally ("d") and apically ("a"). The length of the TOI periods was 10 seconds. We designated one in the baseline period, then immediately after the dropping, for 14 minutes per minute, giving an additional fourteen.

V. Effect of epinephrine in the keratinized gingiva

In the keratinized gingiva group, two wells were created 2 mm apically from the marginal gingiva with orthodontic rubber ligature and liquid dam in the midbuccal line on the test side (12 tooth) and on the control side (21 tooth). After recording the

initial microcirculation values, 3 μl of epinephrine solution (1 mg/ml) was dropped into the well on the test side and 3 μl of physiological saline was added to the control side. The changes in blood flow were recorded for 14 min according to the ROIs in the stimulated well ("w") and in the non-stimulated regions around it, mesially ("m") from the well, distally ("d"), apically ("a") and coronally ("c").

VI. Effect of epinephrine in the gingival sulcus

In the gingival sulcus group, the wells were formed on the labial surface of the teeth, one if their sides was provided by the marginal gingiva. The wells were formed on the surface of the teeth by a liquid dam, one of their sides was formed by the sulcus and the other side by a semicircular arc made of this light-cured resin material 2 mm from the deepest point of the arch of the sulcus. We created two wells, at tooth 12 on the test side and at tooth 21 on the control side. After recording baseline blood flow values, 3 μl of epinephrine solution was dropped into the well on the test side and 3 μl of physiological saline was dropped into the well of the control side, followed by monitoring the change in blood flow according to the ROIs for 14 min with LSCI.. 4-4 ROIs were generated on the test side for tooth 12, tooth 21 and tooth 11 without stimuli. For each tooth, the location of the ROIs from the marginal gingiva in the apical

direction by 1 mm: coronal, intermediate1, intermediate2 and apical region.

4. RESULTS

I. In vitro measurements

The mean thickness measured with PIROP (1.73±0.06 mm) was significantly lower than in case it was measured with the direct method (1.89±0.05 mm, $p<0.01$). The correlation between the two methods was strong ($r=0.61$) when only one measurement was performed per method. If the average of the 5 measurements was taken per method, this value increased significantly ($r=0.69$).

Bland-Altman's analysis showed the upper and lower limits of agreement (LoA+, LoA-), which is the difference between PIROP and the spreader (-0.15 mm), i.e. the average statistical constant between the two methods meant a bias. The set of points did not widen on the x-axis to the right, so the scatter of the points did not increase with the thickness. By statistical terms, the pattern of the point set did not suggest heteroscedasticity. Mean value (green dashed line) did not correlate with thickness ($r=0.218$, $p=0.248$), so there was no proportional bias. The agreement was LoA+ 0.34 mm, while it was LoA- -0.65 mm when the measurements were averaged. For one measurement, LoA+ was 0.43 mm, while LoA- was -0.74 mm. These limits show the range of differences between two random measurements with a 95% probability.

The between-subject variance with PIROP thickness (SD=0.33 [0.26-0.43] mm) and spreader thickness (SD=0.28 [0.22-0.37] mm) was not statistically different. The repeatability (within-subject variance) for PIROP 0.12 [0.11–0.14] mm did not differ significantly from that measured by the spreader method 0.12 [0.11–0.14] mm (p=0.82). The CV values were good in both cases and did not differ significantly between the two methods (PIROP, 7.2% vs. spreader, 6.5%, p=0.82). The correlation between repeated measurements was very strong for both PIROP (r=0.88) and spreader (r=0.84).

II. In vivo measurements

No systematic bias was observed between ultrasound and spreader measurements (mean PIROP GT 1.13 ± 0.07 vs. mean spreader GT 1.04±0.03 mm, p=0.218). The correlation between the two methods was weak (r=0.26) considering one measurement per method. However, the average of the 5 measurements improved significantly (r=0.39). In clinical practice, if a given point is measured once, the person performing the measurement may get a more inaccurate measurement result. Our aim was to mimic what happens when this is done in clinical practice. Unfortunately, in the clinic, if the user is not aware of the errors of the device, it may get an incorrect measurement result.

Bland-Altman's LoA analysis showed no heteroscedasticity as the standard deviation did not increase with increasing thickness. However, the mean deviation increased significantly with the thickness (proportional bias), as indicated by an increase in the regression line ($r = 0.68$, $p < 0.001$). This means that PIROP showed a higher value than the spreader method for higher gingival thickness. In case of smaller gingival thicknesses, approximately below a thickness of 1 mm, the spreader determined the thickness at the bottom compared to the PIROP method. The agreement was LoA + 0.75 mm, while LoA- -0.58 mm when the measurements were averaged. For one measurement, LoA + was 0.88 mm, while LoA- was -0.71 mm. Between-subject variance was greater for PIROP GT (SD=0.36 [0.27-0.48] mm) than for spreader GT measurements (SD=0.14 [0.10-0], 21] mm, $p < 0.001$). The between-subject variance does not refer to precision (repeatability). The within-subject variance provides information on this issue. It shows the standard deviation within a subject, in the same location, measured in the same way. The between-subjects variance shows the biological diversity of the subjects from each other. It is best to measure the precision of a new method if it has a wide limit. In our case, we measured both methods on the same population. Therefore, significantly greater variability between

subjects may indicate a wider measurement range of the method.

The repeatability (within-subject variance) of ultrasonic gingival thickness measurements was found to be better than the direct thickness measurement (SD=0.14 [0.13–0.17] mm vs. 0.20 [0.17– 0.23] mm, p <0.001). CV values were moderate for both methods (13% and 19%). The correlation between repetitions was strong for PIROP GT (r=0.86) and weak for spreader GT (r=0.34).

In the case of TRAN gingival thickness classification, 12% of the volunteers belonged to a thick biotype, while 88% belonged to a thin biotype. In the case of PIROP measurement, the mean GT was 1.66±0.15 mm for thick gingiva and 1.06±0.07 mm for thin (p<0.001). In the case of spreader method, the mean GT was found to be 1.23±0.08 mm for thick biotype, while 1.02±0.03 mm in the thin biotype group (p=0.160). For quantitative measurements (PIROP and spreader), using a value of 1 mm as a threshold, those above were classified into thick GT groups and those below into thin GT groups. Accordingly, in the case of the PIROP method, 64% of the subjects were in the thick and 36% in the thin category and in the case of parallel spreader GT measurements, 60% in the thick and 40% in the thin biotype. These ratios showed no significant difference in the chi-square test. However, the biotype ratio determined by

both quantitative measurements was significantly different from the ratio obtained by the TRAN method (12%/88%, $p<0.001$).

III. NitroPohl group (1 mg/ml NO donor solution)

On the test side, blood flow was significantly increased compared to the control side in the "w" region for the whole duration of the measurement (min 14) and in the "a" region for min 10. There was no significant change in regions "c", "m" and "d". The highest change in the microcirculation was observed in the first minute when the regions were compared. The peaks were similar in the stimulated "w" and adjacent "a" regions, which were significantly higher than the peaks in the "c", "m" and "d" regions.

IV. Nitromint group (8 mg/ml NO donor solution)

Following the application of Nitromint, there was a significant increase in blood flow microcirculation on the test side in all observed regions compared to the control side that persisted throughout the measurement period. The largest change occurred at 3rd min, so we compared these peaks. The peak change of region "a" was the highest, and the microcirculation of other regions ("w", "m", "d" and "c") increased similarly.

V. Effect of epinephrine in the keratinized gingiva

No significant difference was observed for either the three-way interaction (page*region*time, p=0.999) or the two-way interactions (page*region, p=0.352, page*time, p=0.244 and region*time, p=0.956). According to the main aspects, there was no significant difference in gingival blood flow on either side (p=0.203), so epinephrine did not affect the test side and physiological saline on the control side. However, the "region" (p <0.001) and "time" (p <0.001) factors in blood flow were significant. Thus there was a difference in microcirculation values between regions and a slight and slow decrease occurred in each region.

VI. Effect of epinephrine in the gingival sulcus

Gingival microcirculation significantly reduced on the test side in all 4 regions compared to control teeth 12 and 21. The extent and time course of the decrease on the test side showed significant differences between regions. In the coronal region, blood flow was significantly reduced from the first minute to the end of the measurement. The peak value was 87 dLSPU in the 14^{th} minute from the baseline (baseline: 162±1.9 LSPU final: 75±5.0 LSPU). Similarly, there was a significant decrease in blood flow in the intermediate1 region from the first minute to the end of the measurement, where the maximum - 89 dLSPU - was reached compared to the initial value (baseline: 174±2.0

LSPU final: 84±5.9 LSPU). In the intermediate2 region, blood flow decreased from 176±2.4 LSPU to 101±8.7 LSPU at 14th min and this change was significant from 2nd min compared to the control regions. In the apical region, it decreased from 186 ± 2.1 LSPU to 125 ± 9.4 LSPU at the end of the measurement, which was the smallest change among the regions (-60 dLSPU) on the test side. Blood flow in this region was significantly lower from the 4th minute compared to the adjacent control region.

CORRELATION BETWEEN GINGIVAL CIRCULATION AND GINGIVAL THICKNESS

There is no agonist effect at baseline blood flow. Therefore, the correlation between gingival thickness and blood flow was calculated by summing the baseline values of the four groups grouped by region and site. Significant correlation was not observed in any region, even without applying a correction for multiple comparisons.

NitroPohl reached its maximal effect at 1st min, but the extent of change did not correlate with gingival thickness. Nitromint reached its maximal effect at 3rd min, but the extent of the change did not show a significant correlation with gingival thickness, except for the distal region. In the case of epinephrine, only the sulcus application was studied, as the tension had no effect. Epinephrine had a maximal effect at 14th

minute after application into the sulcus, but the extent of the change did not correlate with gingival thickness.

5. CONCLUSIONS

1. The reproducibility of the PIROP ultrasonic gingival thickness determination is more effective than that of the invasive piercing method.

2. We developed a new method to study the microcirculation of the gingiva. It is designed with a plastic hoop and liquid case and attached to the gingiva, which is suitable for testing precisely dosed bioactive materials.

3. We were the first to demonstrate the retrograde vasodilator effect of nitric oxide in human gingiva in our research.

4. Our studies showed that the strong vasoconstriction caused by epinephrine propagates upstream direction.

5. According to our studies, the microcirculation of the gingiva does not depend on the thickness under resting conditions.

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