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Evidence of spreading vasodilation in the human gingiva evoked by nitric oxide

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Background and Objective: Spreading vasodilation is an important means of increasing local blood flow effectively during increased metabolic demands or in case of vascular injury. Our aim was to develop a technique proving the presence of spreading vasodilation in the human keratinized gingiva.

Methods: Local vasodilation was evoked by the application of nitric oxide (NO) donor nitroglycerin into a well, fixed 2 mm above the marginal gingiva, in 20 subjects with healthy periodontal tissue. Either 1 or 8 mg/mL nitroglycerin solutions were dropped into the test well at the upper right second incisor, and saline was applied into the control well at the upper left first incisor. The gingival blood flow (GBF) was recorded for 15 minutes by a laser speckle contrast imager below the well and in the surrounding area in the mesial, distal, apical and coronal directions. Gingival thickness was measured by an ultrasonic biometer.

Results: Peak GBF increase was similar after 1 mg/mL and after 8 mg/mL nitroglycerin application in the well (51% ± 12% vs 42% ± 8%) and in the apical region (33 ± 9% vs 55% ± 13%). While the lower dose of nitroglycerin increased GBF only in the apical region around the well, the higher dose induced significant elevations in all surrounding regions, with apical prominence. Hyperaemia lasted 10-14 minutes in the low-dose group whereas it extended beyond the observation period in the high-dose group. Neither the baseline nor the NO-induced peak GBF were correlated with gingival thickness.

Conclusion: The role of the direct effect of NO in the regulation of perfusion was demonstrated in the human gingiva as well as the propagation of local vasodilation to distant, especially apical areas, probably by the mechanism of flow-mediated dilation. This mechanism may have a clinical importance for flap survival or wound healing.

KEYWORDS

ascending vasodilatation, blood flow, gingiva, gingival blood flow, gingival thickness, laser speckle contrast imager, microcirculation, nitric oxide, nitroglycerin

1 | INTRODUCTION

Proper vascular supply is essential for wound healing but blood supply is compromised after flap surgery, depending on the flap design.[1] The vessels in a mucosal flap regain their baseline level somewhere between day 11 and day 14 by angiogenesis.[2-4] Until the completion of angiogenesis, the flap survives by vasodilation and arteriogenesis of the remaining collaterals.[5] Arteriogenesis is activated by increased shear stress in the collateral vasculature after occlusion of the main vascular supply.[6] Our recent findings in the human gingiva[7] showed that after short-term strangulation, long-lasting hyperaemia develops not only in the close vicinity of strangulation but also further off in the surrounding areas. This suggests that the so-called spreading vasodilation mechanism may be present in the gingiva. Spreading vasodilatation opens the upstream vessels to ensure adequate microcirculatory blood supply in case of increased local demand [8] and it is partly mediated by nitric oxide (NO).[9,10] It is observed in several tissues such as the lymphatics,[11] the intestines,[12] the skeletal muscles,[13-15] the brain,[16,17] the kidneys[18,19] and the mesentery.[20]

There are several animal studies demonstrating that the endothelial or neuronal release of NO has a fundamental role in both the maintenance and the regulation of gingival blood flow (GBF)[21-24] in reactive hyperaemia[25,26] as well as in the pathophysiology of periodontitis.[27-29] Endothelial and inducible nitric oxide synthases were found in human gingival tissue,[27,29-31] but direct evidence of the contribution of NO to the regulation of human GBF has not been demonstrated yet.

Until recently, only single-point laser Doppler flowmetry was available to study human gingival circulation, which is blind to any remote effects. Spatial resolution was enhanced by using a laser Doppler imager[32] to assess the size of the area of capsaicin-evoked vasodilation. It was found that this localized stimulus could affect the gingiva of two neighbouring teeth but does not propagate beyond the midline.

The laser speckle contrast imager (LSCI) is a more recent advancement in microvascular imaging, which proved to be useful in measuring the microcirculation of the human gingiva with high spatial and temporal resolution simultaneously.[33-35] Taking the opportunity provided by the high spatial resolution, a new method was designed to study the remote effects of various drugs when applied to a small restricted area on the human gingiva. Our aim was to use this method to investigate the remote effect of local vasodilation evoked by the topical application of NO donor to ascertain the presence of spreading vasodilation in the human gingiva.

2 | MATERIAL AND METHODS

Twenty volunteers (age range: 21-42 years) with a healthy periodontium were involved in the study. Inclusion criteria were as follows: at least 5 mm keratinized gingiva at the upper front teeth and general health. Excluding factors were pregnancy, breast-feeding, any medication, smoking, gingivitis, caries and coronal restoration with insufficient marginal integrity. The study was carried out in accordance with the Declaration of Helsinki Ethical approval was granted by the Hungarian National Public Health and Medical Officer Service (20104/2017/EÜIG). The study was registered with ClinicalTrials.gov (NCT03605095). Each subject received written information about any possible risk and details of the measurement. Signed informed consent was obtained from all subjects.

Patients were asked not to brush their teeth, gargle, rinse or eat and drink anything for 60 minutes prior to the measurements. Patients were restrained in a dental chair with the position of their neck and head fixed by a vacuum pillow (Spandex®, Hager & Werken, Germany). The lower jaw and lips were stabilized by OptraGate (Ivoclar Vivadent AG, Lichtenstein) and by a silicone bite. Elastic orthodontic ligatures were fixed by a light-cured resin barrier (OpalDam Green, Ultradent Products Inc., USA) on the keratinized gingiva at the upper left first incisor (tooth 21 following the FDI tooth notation system, control site) and at the upper right second incisor (tooth 12, test site) at the midbuccal line, 2 mm apical to the gingival margin (Figure 1).

In order to check the proper sealing of the resin barrier, a caries indicator (Caries Marker, VOCO GmbH, Germany) was applied into the well and the investigated area was videorecorded via a surgical microscope (Schmidt and Bender Hungária Optika Kft, Hungary).

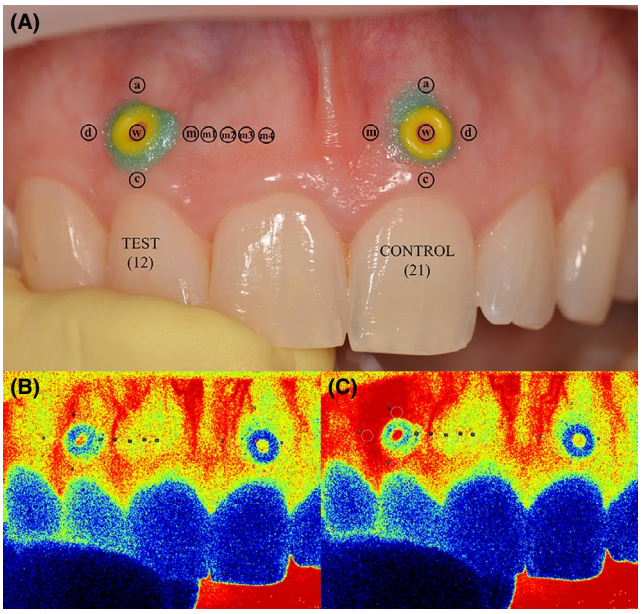

FIGURE 1 The setup of the measurements. Position of the two wells (test and control), fabricated from elastic orthodontic ligatures and fixed by light-cured resin barrier material, and the position of regions of interest: "a" apical, "w" well, "m" mesial, "d" distal, "c" coronal (A). Colour-coded perfusion images of the LSCI in a representative measurement at baseline (B) and at peak-flow following the application of Nitro POHL into the well. Blue indicates areas with reduced blood flow, and red indicates areas with increased blood flow

At the beginning and at the end of the measurement, gingiva temperature was recorded by an infrared thermometer (Rextra Kereskedelmi Kft., Hungary) in the midline of the captured area, at the right first incisor. Blood pressure was measured at the beginning and at the end of the experiment by an automatic blood pressure monitor (Omron M4, Omron Healthcare Inc., Kyoto, Japan) on the left upper arm.

Gingival blood flow was measured by LSCI (PeriCam PSI HR System, Perimed AB, Stockholm, Sweden). The measured area covered about 2 × 3 cm and 2 images/second were recorded. After 15 minutes' stabilization of the circulation, baseline GBF was recorded for 1 minute and then the solutions were applied into the wells with a Hamilton syringe (Model 75 RN SYR, Hamilton, Switzerland). At the test site, 10 patients (7 female) received based on previous unpublished results 3 μL 1 mg/mL nitroglycerin solution (Nitro POHL®, Pohl-Boskamp GmbH, Germany) and 10 patients (8 female) received 3 μL 8 mg/mL nitroglycerin solution (Nitromint®, Egis Pharmaceuticals PLC, Hungary). At the control site, physiological saline was applied in all patients. Every solution and syringe was preheated to body temperature (36.5°C) by a block heater (Dry Block Thermostat DBI-100, Boeckel GmbH, Hamburg, Germany).

Regions of interests (ROIs) were defined on the LSCI intensity image (Figure 1). Five regions were selected at both sites (test and control) within the well ("w"), apical to the well ("a"), coronal to the well ("c"), mesial to the well ("m") and distal to the well ("d"). Time points were defined before the application of drugs (baseline), just after application (10 seconds) and at each of the subsequent minutes through the 14-minute observation period. In order to assess the distance of spreading vasodilation, further ROIs were defined. Horizontal distances were 2 mm for the "m," 4 mm for the "m1," 6 mm for the "m2," 8 mm for the "m3" and 10 mm for the "m4" ROI.

The thickness of the gingiva was measured by a PIROP® ultrasonic biometer (Echo-Son, Puławy, Poland) with a small transducer head (diameter: 1.7 mm) [36-38] at each region used for LSCI measurement.

2.1 | Statistical analysis

Changes of GBF in the ROIs were expressed as the difference between the laser speckle perfusion unit value at a specific observation time point and its baseline value (dLSPU). Data are presented in the text and the figures as mean ± standard error. The statistics on GBF changes were calculated by linear mixed model, with patient and ROI as the units of analysis. Changes at the test site were statistically compared to changes at the control site in the respective regions, except in the "m1"-"m4" regions, as the limit of the captured area prohibited the selection of ROIs further away to the "m" control region. Therefore, for these regions only the respective baseline values at the test site could be used for statistical comparison. A *P*-value of less than 0.05 was considered statistically significant after Bonferroni correction. The relationship between gingival thickness and GBF value was investigated through Pearson's product-moment correlation. The analysis was carried out by IBM SPSS Statistics, Version 25 (Armonk, NY: IBM Corp., USA).

3 | RESULTS

Mean arterial blood pressure slightly but significantly decreased in the Nitromint group (from 84 ± 1.1 to 81 ± 1.6 mm Hg, $P < 0.01$), but not in the Nitro POHL group (from 83 ± 2.2 to 82 ± 2.9 mm Hg, $P = 0.374$). The temperature of the gingiva dropped slightly (Nitro POHL: from 34.4 ± 0.35 to 32.9 ± 0.30°C, $P < 0.05$ and Nitromint: from 33.0 ± 0.41 to 32.3 ± 0.56°C, $P = 0.096$) during the entire measurement period. The proper sealing of the wells was demonstrated by a caries indicator outflow test under surgical microscope (see the Video S1).

After application of Nitro POHL, GBF at the test site was significantly higher compared to the control site in the "w" region during the whole investigation period (14 minutes), and for 10 minutes in the "a" region (Figure 2). No significant change was observed in

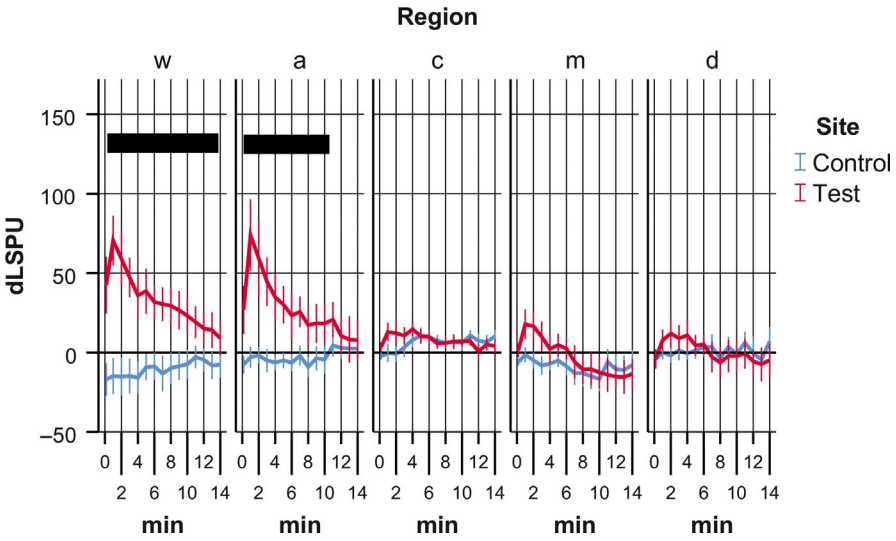

FIGURE 2 Changes in blood flow from the baseline (time point zero) after application of Nitro POHL at the control (blue line) and the test (red line) site in the 5 regions (well (w), apical (a), coronal (c), mesial (m) and distal (d)). Significant difference ($P < 0.05$ to $P < 0.001$) between the control and the test site is marked by a black bar spanning the corresponding time points

the "c," "m" and "d" regions. The highest change was observed at 1-minutes; therefore, this peak value was compared across the regions (Figure 3A). The peak values were similar in the "w" and "a" regions, and they were both significantly higher than the peak values in the "c," "m" and "d" regions.

After application of Nitromint, GBF at the test site was significantly higher compared to the control site in all regions during the whole 14-minute investigation period (Figure 4). The highest change was observed at 3 minutes; therefore, this peak value was compared across the regions (Figure 3B). The peak value was highest in the "a" region, and it was similar in the "w," "c," "m" and "d" regions. GBF was significantly elevated in the "m1" region at 3, 4, 5, 7 and 9 minutes (Figure S1). A gradually decreasing tendency of spreading vasodilation was observed in the "m2," "m3" and "m4" regions, but these changes were not significant.

Gingival thickness ranged from 0.42 to 2.08 mm in the Nitro POHL group and from 0.44 to 2.29 mm in the Nitromint group. No correlation was found between baseline GBF and gingival thickness ($r = -0.114$, $P = 0.633$). No correlation was found between peak GBF and gingival thickness either ($r = 0.394$, $P = 0.086$).

4 | DISCUSSION

The laser speckle contrast imager has a high spatial and temporal resolution at the same time, and it is a non-invasive and non-contact method. These features allowed us to develop a new method for investigating the local and remote effect of various drugs on the microcirculation of the human gingiva. However, there are some limitations to the method. The maximum area that could be captured was only approximately 3-4 cm wide due to technical reasons (CPU and FireWire speed limit). The direct view of the regions of interest—especially the wells—was also limited by the curvature of the dental arch and previously it was found that the blood flow values is influenced by the angle of the view.[34] Due to these restrictions, the homologous tooth could not be used as control site (left second incisor). Therefore, remote effects could not be investigated further distally only to the other directions. But the midline and an intermediate tooth between the control and test sites was provided enough distance to eliminate any possible crosstalk between the sites.[32]

In some regions, there was a tendency of reduction in GBF at the control site, which may be explained by the slight drop in gingival temperature and in mean arterial blood pressure during the blood flow measurement. A slight drop (marginally significant) in blood pressure was also observed in another LSCI study where the patient was in relaxed position for 30-40 minutes and no vasoactive drug was applied [7] suggesting that the observed decrease in blood pressure may not due to the systemic effect of Nitromint. The drop is probably caused by the prolonged relaxed position with an open mouth. This highlights the importance of recording changes in blood flow at a reference site simultaneously with the test site, which allows us to control any systemic change affecting the oral mucosa. The possibility for such simultaneous measurement is an important advantage of LSCI compared to single-point laser Doppler measurements. Another limit of the LSCI is the sensitivity of measurement to the movement of tissues in relation to the LSCI camera. Sensitivity to movement strongly limits the investigation period due to the intolerance of immobility of most patients beyond 30-45 minutes. As a further criticism of the LSCI, its limit of tissue penetration may be mentioned. The measurement depth of LSCI is about 300-700 μm based on some in vitro measurement [39-41] but it depends on the optical feature and the vessels architecture of the tissue. The

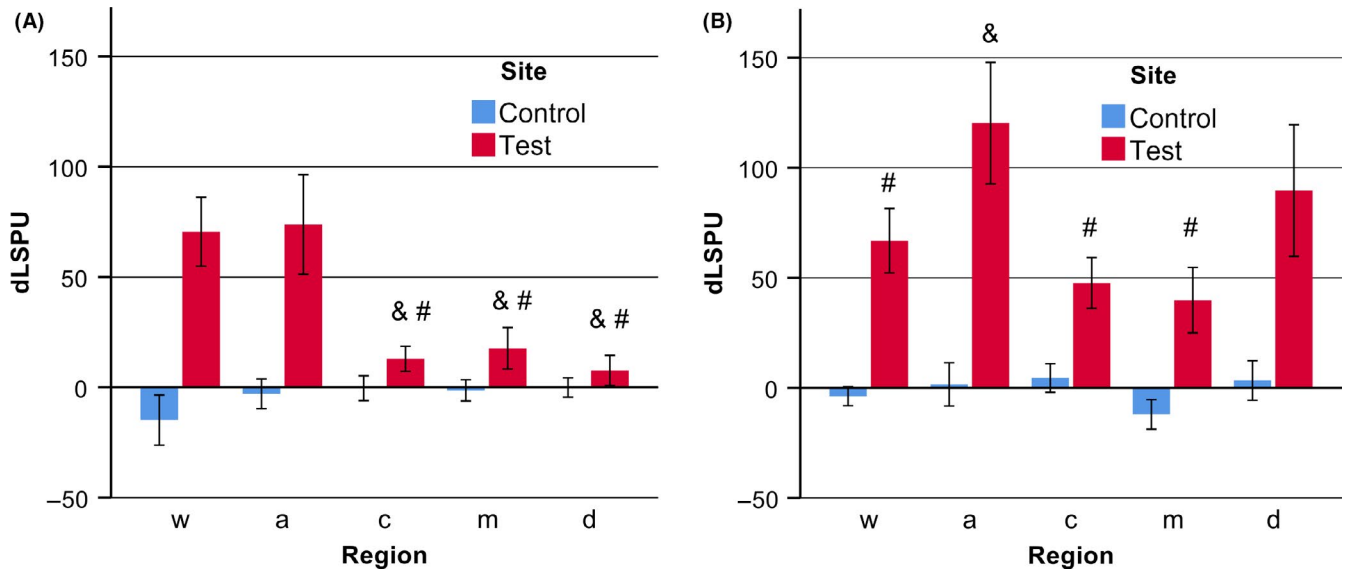

FIGURE 3 The highest changes in blood flow measured after the application of Nitro POHL (A, at 1 minutes) and Nitromint (B, at 3 minutes) at the control (blue column) and the test (red column) site in the 5 regions (well (w), apical (a), coronal (c), mesial (m) and distal (d)). Significant difference ($P < 0.05$ to $P < 0.001$) between "w" and other regions is marked by &, and by # for differences between "a" and other regions

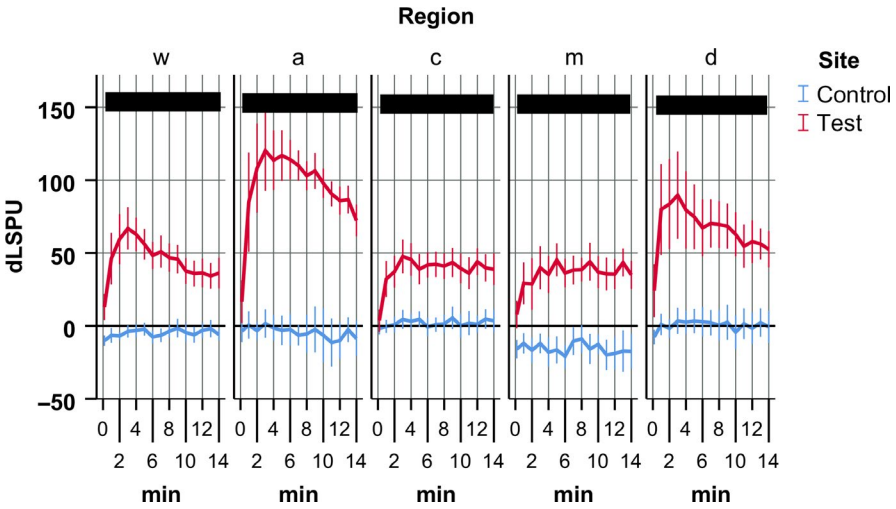

FIGURE 4 Changes in blood flow from the baseline (time point zero) after application of Nitromint at the control (blue line) and the test (red line) site in the 5 regions (well (w), apical (a), coronal (c), mesial (m) and distal (d)). Significant difference ($P < 0.05$ to $P < 0.001$) between the control and the test site is marked by a black bar spanning the corresponding time points

keratinized gingiva is rather thin and LSCI signals are a summation of the underlying tissue speckle pattern. Therefore, it was hypothesized that the gingival thickness smaller than the penetration depth may underestimates the blood flow, as the blood flow of the bone is about one magnitude less than the mucosa.[42] However, in our study, no positive correlation was found between gingival thickness and GBF indicating the gingival microcirculation was assessed correctly in our study.

The high sensitivity of the vasculature to NO was proved in our study for the first time in the human gingiva. In our study, NO donors caused rapid and notable vasodilation at the site of application (well) in the healthy human gingiva despite the fact that only a very low amount of nitroglycerin (3 μg and 24 μg per subject) was applied. Vasodilation in the well region had a very similar extent in both groups (Nitro POHL: 51 ± 12%, Nitromint: 42 ± 8%) despite the 8x dose of nitroglycerin in Nitromint. It is not known how much nitroglycerin is absorbed through the keratinized gingiva and from the absorbed nitroglycerin how much NO is released. But extent of the vasodilatory effect was very similar to the previous data. In these studies, thermal provocation of the gingiva [43] or post-occlusive reactive hyperaemia test [7] were applied in which NO has a significant role in the vasodilatory mechanism.[44,45] These indicate that the locally released NO from nitroglycerin in this study could be close to the physiological levels.

Both medicament containing NO donors evoked prominent vasodilation apical to the site of application, which demonstrates the presence of spreading vasodilatation in the human gingiva. Vasodilation mainly spreads upstream of the arteriolar network to effectively ensure blood supply to the active site.[8,46] The two known mechanisms of supplying upstream apical remote vasodilation are conducted vasodilation via retrograde propagated hyperpolarization of endothelial cells and vascular smooth muscle [8] and flow-mediated vasodilation via shear stress-induced endothelial NO release.[47] The former has an onset within a few seconds, while the latter has a lag of 10-40 seconds and is responsible for sustained vasodilation.[48] However, these time frames depend on tissue type and species.[12,48-50] The elevation of GBF was observed apically in most cases already at 10 seconds, in parallel with elevation in the focal (w) region, suggesting a relatively rapid response. We could take measurements only after 10 seconds, which was necessary due to manipulation with the drugs. Therefore, it is difficult to distinguish between the two mechanisms based on time lag. However, NO donors cannot induce conducted vasodilation.[9,51] Consequently, the observed remote vasodilation induced by the NO donor in the gingiva was mainly due to the flow-mediated mechanism. Vasodilation ends within 60 seconds in other tissues,[48] contrary to our study, in which it was sustained for more than 10 minutes in the Nitro POHL group and lasted longer than the observation period (14 minutes) in the Nitromint group. The extended hyperaemia is in line with our other observations in the human gingiva, where hyperaemia after short-time occlusion [7] or after heat provocation [43] lasted much longer than in other tissues, suggesting a high reactivity of the gingiva.

The apical direction of spreading vasodilation reaffirms the priority of the plexus of the alveolar mucosa in blood supply to the keratinized gingiva in physiological conditions [7,52] and during regular flap elevation or wounding.[53-55] It also concurs with the ascending feature of flow-mediated vasodilation. The apical part of the elevated mucogingival flap is hyperaemic one day after periodontal surgeries [53,56,57] in spite of severing many vessels supplying the flap during surgery. This early hyperaemia precedes the revascularization process. Accordingly, vasodilation of the remaining core vessels of the flap and/or the vasodilation of collaterals—so-called arteriogenesis—may account for it. The role of NO and flow-mediated dilation in arteriogenesis was demonstrated in an ischaemic skin flap,[5,58] which may therefore be a candidate mechanism for mucogingival flap survival.

Nitromint evoked similar elevation of GBF in the area of application ("w") as Nitro POHL; therefore, similar remote responses were expected. In contrast, Nitromint caused higher and longer apical vasodilation (55 ± 13% vs 33 ± 9%) and vasodilation spread into the lateral and coronal areas as well. This suggests that mechanisms other than flow-mediated dilation were also activated due to the higher nitroglycerin content or another ingredient. The ethanol content of Nitromint may be considered here. There is some sparse evidence [59,60] that ethanol may also possess some vasodilator activity, but the mechanism is not known. A more practical explanation for limited local vasodilation

may be the mesh-like property of gingival vessels, which may promote the stealing effect during the opening of multiple branches of the same feeding arteria.[61,62] Nitromint evoked vasodilation in a lateral direction, spanning the area of the same feeding artery. The dilation of the feeding vessels resulted in the homogenization of microcirculation in the supplied area.[63] Statistically significant vasodilation was detected up to 4 mm mesial to the site of stimulus. Lack of controlling systemic changes during the investigation in case of sites "m1" to "m4" may underestimate the statistical significance of vasodilation as the control sites showed a tendency of decreasing GBF. Therefore, it seems that remote vasodilator effects in the keratinized human gingiva may spread over a considerable distance.

As a conclusion, the method we developed could be a useful tool to investigate the remote effect of various vasoactive substances in the human gingiva. This study was the first to demonstrate the direct effect of nitric oxide on human gingival blood flow and to present evidence on the spreading of vasodilation prominently in an apical direction.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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