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Nitric oxide levels in saliva increase with severity of chronic periodontitis

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Abstract: The aims of this study were to compare nitric oxide (NO) levels in stimulated whole saliva from individuals with and without generalized chronic periodontitis (GCP), and to evaluate correlations between these levels with a clinical diagnostic parameter. According to specific criteria, 30 individuals were divided into three groups: one comprising individuals without periodontitis (GC), a second comprising individuals with moderate GCP (GM), and a third comprising individuals with advanced GCP (GA). Samples were collected and NO levels measured. NO in the GCP group (GM: 7.78 µM; GA: 15.79 µM) was higher than in the GC group (5.86 µM). NO levels in the GA group were significantly higher ($P < 0.0001$) than in the GC group, and could also differentiate ($P < 0.0001$) the moderate and advanced forms of the disease. In addition, positive correlations between NO level and the number of teeth with a probing depth of ≥ 4 mm (r = 0.54) and ≥ 7 mm (r = 0.68) were observed. In conclusion, NO levels are elevated in individuals with GPC and are correlated with a periodontal clinical parameter. These results reveal that this form of periodontal disease and its severity are related to salivary nitrite concentration, indicating that NO may serve as a potential biological marker for detection and/or monitoring of GCP. (J. Oral Sci. 49, 271-276, 2007)

Keywords: chronic periodontitis; saliva; nitric oxide; biological marker; diagnostic.

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Introduction

Periodontal disease is an inflammatory reaction of periodontal tissues in response to infection caused by a specific group of bacteria. The diagnosis and classification of periodontal diseases are based essentially on clinical parameters. However, advances in molecular biology and genetics are leading to a better knowledge of the pathways and mechanisms through which bacteria maintain the host immune/inflammatory response (1). New auxiliary diagnostic tools based on analysis of body fluids, such as saliva and gingival crevicular fluid (GCF), as well as studies of subgingival microflora and genetic susceptibility, are useful and should be further developed (2). Saliva has been extensively studied in relation to periodontal disease because it is easily collected, and allows analysis of several local and/or systemic biological markers such as proteins, enzymes, host cells, hormones, bacterial products, volatile components and ions (3).

Nitric oxide (NO) has been intensively studied in the medical field (4). In addition, NO has been linked to the etiopathogenesis of periodontal disease (5,6) and is expressed in salivary glands as well as in their product (7,8). NO is both a gas and a free radical that reacts with many biological molecules, and was known previously as "endothelial-derived relaxing factor" of smooth muscle (9). NO is synthesized from L-arginine by a family of isoenzymes called NO-synthases (10). Two of these are expressed constitutively, and a third is induced by immunological stimuli. The NO released by this inducible NO synthase (iNOS) is generated for long periods by cells of the immune system among others. Cytokines and other bacterial products stimulate the expression of iNOS and interfere with periodontal disease progression (5,11,12). More specifically, bacterial lipopolysaccharide stimulates NO expression in bone, as well as in other tissues (13,14). The aim of the present study was to measure salivary NO levels in individuals with chronic generalized periodontal disease, in its moderate and advanced forms, and to compare these levels with those in a group of individuals without periodontitis. In addition, we evaluated the possible correlation between NO level and probing depth as a representative periodontal diagnostic parameter. It was anticipated that these data would help to determine the potential usefulness of NO measurements as a biological marker of periodontal disease.

Materials and Methods

Sample selection

Approximately 200 individuals attending the Dental School of Pontiphical Catholic University of Minas Gerais received a complete dental examination during a 6-month period. Periodontal examination included assessment of probing depth, probing attachment level and recession, as well as evaluation of bleeding and/or suppuration on probing.

Exclusion criteria included current smokers, pregnancy, recent (3 months) use of antibiotics or recent trauma. Inclusion criteria included participants who had not received periodontal treatment during the previous year. Probing depths were measured at six sites per tooth (mesiobuccal, midbuccal, distobuccal, distolingual, midlingual and mesiolingual), and the specific inclusion criteria used for differentiating the three groups were:

Control group: all sites with probing depths < 4 mm;

Moderate GCP: > 30% of sites involved; probing depths 4-6 mm and clinical attachment loss (CAL) 3-4 mm;

Advanced GCP: > 30% of sites involved; probing depths ≥ 6 mm and CAL ≥ 5 mm.

These criteria were based on previous reports (15-17).

Therefore, a total of 20 individuals with generalized chronic periodontitis (GCP) were selected. These individuals had an age range of 30 to 45 years, and an equal female to male ratio. They were subdivided into two groups of 10 individuals, one with moderate GCP (GM), and the other with advanced GCP (GA). A third group with 10 individuals without periodontitis (GC) was included.

This study was independently reviewed and approved by the Research Ethics Committee of Pontifical Catholic University of Minas Gerais, informed consent was obtained from all individuals prior to their participation and subjects' rights were protected at all times.

Saliva collection

The average unstimulated salivary flow rate is 0.3 ml/min, whereas the stimulated flow rate is 4 ml/min (18). NO derived from cells of the immune system is a component of GCF (5). The GCF flow rate varies from 4 to 21 µl/min (19) and in states of inflammation can be as high as 50 µl/min (20). Therefore, in order to investigate NO produced primarily by the salivary glands, stimulated whole saliva was collected from participants. Individuals were asked to refrain from eating or drinking two hours prior to collection (i.e. between 10:00 a.m. and 11:00 a.m). Individuals rinsed their mouth with water, and to obtain stimulated whole saliva samples, masticatory stimulation was induced by chewing parafilm (1.0 g) at a rate of approximately 60 strokes / min for 5 min. Samples were collected into 50-ml ice-chilled Falcon screw cap tubes and immediately centrifuged at 4ºC for 10 min. Supernatants were aliquoted into 1.5-ml Eppendorf tubes and frozen at -20ºC until use.

NO quantification

Salivary nitrite levels were measured using the Griess colorimetric reaction (21). Griess reagent is a 1:1 mixture of 1% sulfanilamide and 0.1% N-naphthylethylene diamine dichloride in 5% orthophosphoric acid (v/v). This reagent reacts with nitrite and produces a purple azo dye end-product, which can be measured spectrophotometrically with a maximum absorbance at 570 nm. Triplicate samples of saliva (50 µl) were transferred to a 96-well ELISA plate, and an equal volume of the Griess reagent was added to each well. In order to obtain standard curves, triplicates of sodium nitrite ($NaNO_2$) in PBS (pH 7.2) at concentrations of 100, 50, 25, 12.5, 6.25, 3.12, 1.56 and 0.78 µM were also included. After 10 minutes, the optical density was measured using an ELISA plate reader (SpectraMax 340, Molecular Devices, Sunnyvale, CA, USA) with a 570-nm filter.

Statistical analysis

NO measurements among the three groups were compared for statistical significance by a one-way repeated-measures ANOVA (post hoc analysis - Tukey) at a probability level of < 0.05. Pearson's correlation coefficient (r) was determined to evaluate possible correlation between NO concentration and probing depth. The statistical evaluation was performed using SAS software (SAS Institute, Cary, NC, USA).

Results

The observed NO levels derived primarily from salivary glands of individuals in the GC, GM and GA groups are described in Table 1. Analysis of NO levels revealed that the mean concentration in the GM (7.78 μM) and GA (15.79 μM) groups was higher than in the GC group (5.86 μM). Moreover, the NO levels in the GA group were significantly ($P < 0.0001$) higher than in the GM and GC groups. This result indicates that the severity of GCP has a direct influence on NO expression.

Since individuals from the GC group did not have periodontitis, the investigation of correlations between probing depth and NO was conducted only in the GM and GA groups using Pearson's correlation coefficient. Individuals from each group were clustered to add more power to the statistical analysis. Interestingly, probing depths and NO levels confirmed the influence of GCP severity on NO expression, as there was a positive correlation between salivary NO levels and the number of teeth with a probing depth of ≥ 4 mm (r = 0.54) (Fig. 1) and the number of teeth with a probing depth of ≥ 7 mm (r = 0.68) (Fig. 2).

Discussion

Biochemical and immunological markers present in saliva or GCF can partially determine the extent of periodontal disease and even may predict its progression (8,22,23). Collection of salivary constituents is a simple, non-invasive procedure that can be performed by any individual without a dental degree. In addition, obtained samples can be frozen and sent to laboratories for analysis of markers such as NO. In particular, the Griess reaction has potential as an auxiliary diagnostic tool because it is a simple, highly specific and extremely sensitive method for measuring micromolar concentrations of nitrite. Therefore this method might be considered as an instrument for epidemiological research.

NO has a short life, and according to Moncada and Higgs (24), it decays into equal amounts of nitrite and nitrate in aqueous solutions, which can be used as indices of NO synthesis *in vitro*. In the present study, NO production was measured indirectly using the level of nitrite in saliva, and an increase in salivary NO levels was observed in individuals with GCP. This result is similar to other studies that have shown increased levels of amino acids related to NO in periodontal disease (11), increased expression

Table 1 Concentration of NO in saliva samples subjected to the Griess Reaction

Participant*	Age			NO Concentration (μM)		
	GC	GM	GA	GC	GM	GA
1	22	31	36	5.51	4.01	16.38
2	22	35	31	8.26	7.59	20.34
3	21	31	42	8.16	12.12	29.03
4	22	40	35	4.48	7.31	11.66
5	21	45	30	3.06	3.63	16.76
6	22	36	40	6.65	7.22	12.79
7	21	33	39	5.53	10.33	11.94
8	21	33	45	6.27	8.07	15.81
9	22	38	37	5.04	5.42	9.67
10	23	41	44	5.61	12.14	15.79
Mean	21.6	36.3	37.9	5.86	7.78	15.79
SD	± 0.55	± 6.07	± 4.76	± 1.58	± 3.02	± 5.59
95%CI				4.88 - 6.84	5.91 - 9.65	12.3 - 19.2

* 1-5 female and 6-10 male; SD: standard deviation; CI: confidence interval

of iNOS in periodontal disease biopsy samples (25-27), as well as in gingival fibroblast cell cultures (12,28). In contrast, one report has described a reduction of salivary NO levels in individuals with adult periodontitis and with aggressive periodontitis (29). This report is in disagreement with the vast majority of the literature that describes increased levels of NO in periodontal disease (5,30-32).

The results from the present and previous studies indicate that the use of nitric oxide synthase inhibitors may have great value in the treatment of periodontal disease (28).

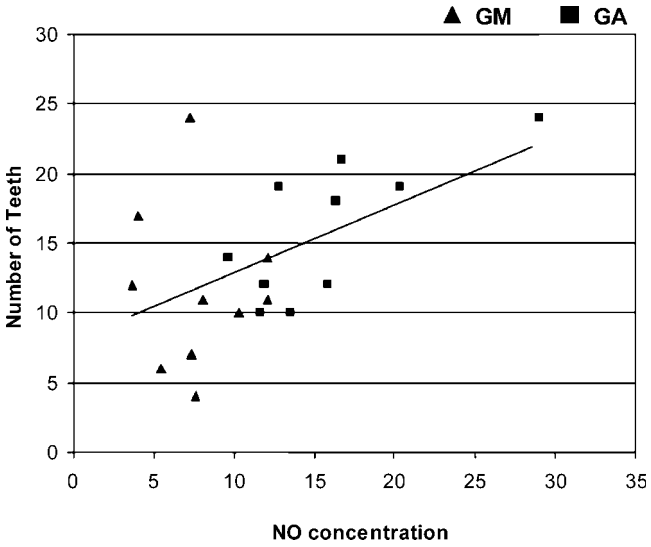

Fig. 1 Pearson's correlation coefficient of NO levels with number of teeth with a probing depth of ≥ 4 mm. NO values are expressed in μM. r = 0.54.

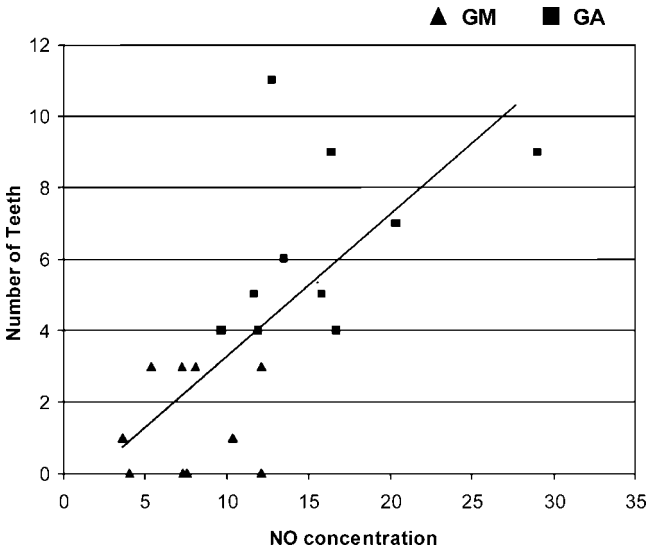

Fig. 2 Pearson's correlation coefficient of NO levels with number of teeth with a probing depth of ≥ 7 mm. NO values are expressed in μM. r = 0.68.

In this regard it has been reported that bone resorption in rats with induced periodontal disease could be prevented with isosorbid, an iNOS inhibitor (33,34), as well as with mercaptoethylguanidine (25). Other inhibitors used have included aminoguanidine (35) and the enzyme arginase, which competes with the substrate (L-arginine), thus reducing NO production (23).

The criteria used to select the study participants, in particular the absence of periodontal treatment during the previous year, led to a small sample size. Nevertheless, to our knowledge, this is the first study to show that salivary NO levels are associated with periodontitis severity, allowing differentiation between moderate and advanced GCP. NO levels were correlated with probing depth, a clinical diagnostic parameter. Greater probing depth (≥ 7 mm) yielded a higher correlation value, and the graphical distribution of GA and GM individuals (Figs. 1 and 2) differentiated between these groups and again confirmed the direct correlation between NO level and disease severity. The biological plausibility of the differences observed in this study may be partly explained by periodontal bacterial components triggering the host-immune response, and causing inflammation and activation of pro-inflammatory mediators (i.e. interleukin-1 and TNF-α). These molecules traveling via blood to other organs and tissues might influence a variety of mechanisms (36). Finally, prospective clinical trials should be conducted to confirm our findings, which indicate that NO might be useful for diagnosis and monitoring of advanced GCP, as well as leading to a better understanding of the mechanisms by which periodontal disease might modulate salivary gland function.

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