Nitrite and Nitrate Levels of Gingival Crevicular Fluid and Saliva in Subjects with Gingivitis and Chronic Periodontitis

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ABSTRACT

Objectives: Nitrosative stress plays an essential role in the pathogenesis of periodontal disease. The aim of this study is to analyze the gingival crevicular fluid and saliva nitrite and nitrate levels in periodontally healthy and diseased sites.

Material and Methods: A total of 60 individuals including, 20 chronic periodontitis and 20 gingivitis patients and 20 periodontally healthy controls participated in the present study. Probing depth, clinical attachment level, bleeding on probing, gingival index and plaque index were assessed, gingival crevicular fluid (GCF) and saliva samples were obtained from the subjects, including 480 GCF samples and 60 unstimulated whole saliva samples. Nitrite and nitrate were analyzed by Griess reagent.

Results: Total GCF nitrite levels were higher in gingivitis and periodontitis groups (1.07 [SD 0.62] nmol and 1.08 [SD 0.59] nmol) than the control group (0.83 [SD 0.31] nmol) (P < 0.05) but did not differ significantly between gingivitis and periodontitis groups (P > 0.05). The difference in GCF nitrate level was not significant among the control, gingivitis and periodontitis groups (7.7 [SD 2.71] nmol, 7.51 [SD 4.16] nmol and 7.38 [SD 1.91] nmol). Saliva nitrite and nitrate levels did not differ significantly among three study groups. Saliva nitrate/nitrite ratios were higher in periodontitis and gingivitis groups than the control group. A gradual decrease in nitrate/nitrite ratio in GCF was detected with the presence of inflammation.

Conclusions: It may be suggested that nitrite in gingival crevicular fluid is a better periodontal disease marker than nitrate and may be used as an early detection marker of periodontal inflammation, and that local nitrosative stress markers don’t show significant difference between the initial and advanced stages of periodontal disease.

Keywords: gingival crevicular fluid; inflammation; nitric oxide; periodontitis; saliva.
INTRODUCTION

Nitric oxide (NO) is a diatomic free radical synthesized from the conversion of L-arginine to L-citrulline by nitric oxide synthase (NOS) [1]. NO is relatively unstable in the presence of oxygen and quickly auto-oxidized to produce nitrogen oxides. Because of NO’s reactivity and short-life, direct measurements of NO in cells and tissues are very difficult [2]. Although NO metabolites have a very short life, nitrate and nitrite are the relatively stable end products of NO oxidation [2,3]. The total levels of nitrite and nitrate in biological fluids are generally used for adequate monitoring of the NO synthesis [4]. Traditional diagnostic procedures for periodontal diseases are limited for evaluation of current disease status [5]. In order to improve clinical management of periodontal patients, there is a need for the development of new diagnostic tests that can detect the presence of active disease, future disease progression, and evaluate the response to periodontal therapy. Advances in oral and periodontal diagnostic research are moving toward methods by which periodontal risk can be identified and quantified by objective measures such as biomarkers [6]. Biomarkers of disease play an important role in diagnosis, assessing the treatment outcomes, and drug discovery [7]. In order to understand their role in routine practice, their relation to the mechanism of disease progression and therapeutic intervention must be understood [8].

Gingival crevicular fluid (GCF) is an exudate originating from serum and can be collected from the gingival sulcus surrounding natural teeth [9,10]. The flow of this biological fluid is an important determinant for the status of periodontal tissues, which reflects the cellular response in the periodontium by the constituents of serum and contributions from the gingival crevice [10-12]. Inducible NO synthase (iNOS) is produced by immune system cells such as macrophages infected with bacteria and is involved in the regulation of inflammatory reactions [13]. NO has beneficial and harmful effects and it is involved in the regulation of many physiological issues including platelet aggregation, immune regulation and vascular relaxation [14,15]. The activation of iNOS, inflammatory mediators in cells and nitrosative stress are reported to be increased in inflammatory periodontal tissues [16,17]. It has been shown that NO takes part in the etiopathogenesis of many diseases, including periodontal disease [18-20]. Overproduction of NO can cause destruction of periodontal tissues [18]. Matejka et al. [21] obtained gingival tissue from patients with moderate periodontitis and from healthy controls in their study and they reported that NO production is increased in inflamed periodontal tissue. In another study, it has been suggested that the expression of iNOS from macrophages in high levels may damage the periodontal tissues. iNOS activity in macrophages was reported to have the potential to inhibit leukocyte recruitment by acting on leukocytes that increase the inflammation in localized aggressive periodontitis (LAP) patients [22]. However, conflicting results of NO metabolites, either decreased or increased in saliva from patients with periodontitis have also been reported [23-26]. In a recent study by Andrukhov et al. [27] significantly lower serum and saliva levels of NO metabolites were found in periodontitis patients.

Evaluation of nitrite and nitrate concentrations which are produced by the biochemical events in immunological response during host-pathogen interaction will play an important role in understanding the etiopathogenesis of periodontal disease. In recent years, exponential data on the involvement of NO and nitrosative stress in chronic periodontitis has emphasised the need to improve diagnostic parameters [28]. The localization and effects of nitrosative stress may be assessed by an analysis of nitrosative damage biomarkers isolated from tissues and biological fluids [28]. The aim of this study was to measure nitrite and nitrate levels in total saliva and gingival crevicular fluid in patients with gingivitis and chronic periodontitis and to assess the value of these nitrosative stress parameters of periodontal disease.

MATERIAL AND METHODS

Ethical aspects

The study protocol was approved by the Institutional Review Board of the Hacettepe University in accordance with the Helsinki Declaration of 1975, as revised in 2000. All voluntary participants were informed about the outline, purpose of the study and signed an informed consent form. When necessary, periodontal treatment was performed by periodontists (AOT and KBS) after sampling.

Study protocol

The present study was conducted in the Department of Periodontology between February 2011 and January 2012. A total of 60 individuals, which included,
20 chronic periodontitis and 20 gingivitis patients and 20 periodontally healthy controls participated in the present study. Ages, gender, number of teeth and smoking habit variables were recorded.

Clinical parameters

Clinical periodontal status was determined by assessing the probing depth (PD), clinical attachment level (CAL), gingival index (GI), [29], gingival bleeding time index (GBTI) [30] and plaque index (PI) [31] scores. Full-mouth recordings was performed by one examiner (AOT) during the first visit and assigned one of the following study groups.

Inclusion criteria

Gingivitis group

The existence of gingival inflammation (GI values > 0), with PD values < 3 mm and CAL ≤ 2 mm at more than or equal to 90% of teeth and no radiographic signs of alveolar bone loss due to periodontal disease (i.e., distance between the cemento-enamel junction and bone crest at > 90% of the proximal tooth sites ≤ 3 mm).

Chronic periodontitis group

Subjects with presence of sites presenting CAL values ≥ 3 mm and GI values > 0 and PD of ≥ 6 mm in multiple sites in all four quadrants of the mouth and moderate-to-severe alveolar bone loss (more than 3 mm) present in radiographs.

Control group

Clinically healthy periodontal status, that is, GI values of 0, PD ≤ 3 mm, no gingival recession and CAL ≤ 2 mm at more than or equal to 90% of the measured tooth sites as well as bleeding on probing in less than 10 % of the probing sites at examination and no radiographic evidence of alveolar bone loss in these subjects (i.e., distance between the cemento-enamel junction and bone crest ≤ 3 mm at > 90% of the proximal tooth sites).

In addition to the general periodontal status of the subjects, individual periodontal status of the sampled teeth was also considered.

Exclusion criteria

Exclusion criteria included the following: pregnant or lactating women; subjects used any antibiotics or received periodontal treatment within the last 6 months; subjects on anti-inflammatory therapy, or vitamin/nutritional supplements; and subjects with systemic diseases such as diabetes mellitus, hypertension, renal disease, rheumatoid arthritis, periapical infection of any tooth, or any other form of systemic inflammatory involvement.

Saliva and GCF sampling

Maxillary teeth including first molars, second premolars, canines and central incisors (eight teeth of each subject) were selected to avoid possible saliva contamination during sampling. Sampled teeth were free of caries, prosthetic reconstruction and root canal therapy. All the samples were collected by one blind examiner (KBS), 48 hours following the clinical measurements in the morning (between 10 a.m. - 11 a.m.) following an overnight fast. Saliva samples were obtained prior to GCF samples. The participants were told not to eat or drink anything or chew gum that morning before sampling. They were asked whether they followed these instructions or not, before collection of the samples. Unstimulated whole saliva samples were obtained over 5 min. periods while they seated. Subjects were asked not to swallow any saliva for the duration of the collection to allow saliva to pool in the bottom of the mouth and collection tube was used to drain when necessary. Saliva samples were then placed in sterile Eppendorf tubes and stored at -80 °C until the laboratory analysis. GCF samples were obtained after saliva sampling, according to the method described by Rüdin and colleagues [32], using standardized commercial paper strips (Oraflow Inc., Amityville, NY, USA). Following the isolation of the sampling area with sterile cotton rolls, supragingival plaque was removed and experimental site was gently air dried to reduce any possible contamination with saliva. Extreme care was taken to minimize mechanical irritation during sampling. Paper strips were placed at a standardized depth of 1 mm at each sampling site independent from PD measurements and were left there for 30 seconds for each sampling. Samples with evidence of bleeding were excluded and sampling was replicated from another site of the natural tooth that was not sampled. To eliminate the risk of evaporation [33], paper strips with GCF were immediately transported to previously calibrated, Periotron 8000 (Oraflow Inc., Amityville, NY, USA) for volume quantification. Before sampling, the Periotron 8000 was switched on and allowed to warm up before a blank paper strip was placed in the device and the reading dial was set to zero [34]. The calibration of the device was checked with periodic intervals and performed

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by triplicate readings as previously described [35]. The GCF was measured electronically in Periotron units, which were converted to micro liters (µl) by MCCONVRT software (Software version 2.52, Oraflow Inc., Amityville, NY, USA). GCF samples were then placed in sterile Eppendorf tubes and stored at -80 °C until the day of laboratory analysis.

**Determination of nitrite/nitrate level of saliva and GCF**

The stable end products of NO, nitrite, and nitrate were analyzed by Griess reagent as described by Grisham et al. [36], which is based on determination of the nitrate reductase activity in samples. Saliva samples were centrifuged for 5 minutes at 10000 x g at 4 °C to remove any particulate matter. The supernatant was used to determine total nitrite levels. To each GCF sample in the Eppendorf Tube, 130 mL of distilled water was added. The samples were vigorously mixed for the extraction of nitrite into the water. For the determination of nitrite levels, 100 mL of the extract was mixed with 0.5 ml of freshly prepared Griess reagent, using a micro plate. After 10 minutes of incubation at room temperature, the absorption of each sample in microplate wells was determined at 540 nm [36]. A standard curve was prepared using sodium nitrite to calculate nitrite and nitrate concentration in saliva and GCF.

**Statistical analysis**

Categorical variables were evaluated by Chi-square and extension of Fisher’s exact test to 3 x 3 table. Parametric data were expressed as mean and standard deviation (M [SD]). Non-parametric Kruskal-Wallis test was used to compare the differences among healthy, gingivitis and periodontitis groups for continuous variables. Pair wise comparisons were done by Dunn test. Correlation between continuous variables was evaluated by Pearson correlation coefficient. For all parameters P values < 0.05 were considered to be statistically significant.

**RESULTS**

Age, gender, number of teeth present in mouth and smoking habit variables are listed on Table 1. Gender, number of teeth present in mouth and smoking habits did not differ significantly between the groups. On the other hand, mean age of the chronic periodontitis group was significantly higher than periodontally healthy controls (42.65 [8.04] years and 29.9 [2.17] years) (P < 0.05). No significant correlation was detected among for all parameters between the groups.

**Clinical periodontal parameters**

Data regarding full-mouth PD and CAL measurements and GI, GBTI, PI scores for the study groups are given at Table 2. The differences in PD, CAL and GI between all groups were significant (P < 0.001). GBTI and PI scores were significantly higher in periodontitis (2.69 [0.53] and 1.19 [0.4]) and gingivitis (2.33 [0.7] and 0.92 [0.39]) groups than the control group (0.14 [0.13] and 0.1 [0.05]) (P < 0.001). Difference for these parameters did not reach to a significant level between periodontitis and gingivitis groups (P > 0.05). Table 3 provides information on the descriptive data for clinical parameters, GCF volume and nitrite/nitrate levels regarding individual evaluation of the sampled teeth. The differences regarding all clinical measurements and GCF volume differed significantly between all groups (P < 0.001). Clinical parameters, including the GCF volumes, demonstrated gradual increases with the presence of gingival inflammation.

**Table 1. Data regarding age, gender, number of teeth present and smoking habits**

|                  | Healthy | Gingivitis | Periodontitis | Test statistics | P     | Pairwise comparisons |
|------------------|---------|------------|---------------|-----------------|-------|----------------------|
| **Gender**       |         |            |               |                 |       |                      |
| (male - female)  | 10 - 10 | 8 - 12     | 9 - 11        | χ² = 0.4        | 0.817 | -                    |
| **Smokers**      |         |            |               |                 |       |                      |
| (n)              | 4       | 3          | 8             | χ² = 6.43       | 0.106 | -                    |
| **Age**          |         |            |               |                 |       |                      |
| Mean (SD) (years)| 29.9 (2.17) | 33.9 (8.38) | 42.65 (8.04) | KW = 31.68     | 0.001 | 1 vs 3 P < 0.001*    |
| **Teeth count**  |         |            |               |                 |       |                      |
| Mean (SD) (n)    | 27.9 (0.3) | 26.95 (1.35) | 26.35 (1.98) | KW = 12.24     | 0.002 | 1 vs 2 P > 0.05      |

*Statistically significant, Dunn test.
SD = standard deviation.
Table 2. Data regarding full-mouth probing depth (PD), clinical attachment level (CAL) measurements (mm), gingival index (GI), gingival bleeding time index (GBTI), plaque index (PI) scores, and saliva nitrite and nitrate level (µM) for healthy (1), gingivitis (2) and periodontitis (3) group

| Healthy (n = 147) | Gingivitis (n = 183) | Periodontitis (n = 150) | Difference | Pairwise Comparisons |
|-------------------|----------------------|-------------------------|------------|----------------------|
| **Full-mouth**    |                      |                         |            |                      |
| PD                | 1.35 (0.19)          | 1.86 (0.25)             | 3.26 (0.7) | 2.96 2.13 - 5 49.86 | P < 0.001² |
| Cal              | 1.35 (0.19)          | 1.89 (0.26)             | 4.12 (0.93)| 4.06 2.8 - 5.69 49.86| P < 0.001² |
| GI                | 0.16 (0.17)          | 1.2 (0.35)              | 1.57 (0.3) | 1.56 1.02 - 2 43.14 | P < 0.001² |
| GBTI             | 0.14 (0.13)          | 2.33 (0.7)              | 2.69 (0.53)| 3 1.21 - 3 42.51 | P < 0.001² |
| PI                | 0.1 (0.05)           | 0.92 (0.39)             | 1.19 (0.4) | 1.18 0.49 - 1.98 41.48| P < 0.001² |
| Saliva nitrite level (µM) | 8.67 (8.68)      | 5.5 0.27 - 33.14        | 5.56 (4.53)| 4.12 0.91 - 16.64 5.55 (5.39) | 4.12 0 - 18.28 1.34 | P > 0.05 |
| Saliva nitrate level (µM) | 20.65 (12.2)    | 21.68 0 - 38.07         | 18.5 (16.7) | 9.23 0 - 46.49 16.19 (15.49) | 15.98 0 - 46.49 1.178 | P > 0.05 |

SD = standard deviation; N/A = not available; KW = Kruskal-Wallis test.

*Statistically significant, Dunn test.

Table 3. Data regarding individual probing depth (PD), clinical attachment level (CAL) measurements (mm), gingival index (GI), gingival bleeding time index (GBTI), plaque index (PI) scores, GCF volume (µl) and total nitrate and nitrate level (nmol) of the sampled teeth

| Sampled teeth | Healthy (n = 147) | Gingivitis (n = 183) | Periodontitis (n = 150) | Difference | Pairwise comparisons |
|---------------|-------------------|----------------------|-------------------------|------------|----------------------|
| PD            | 1.26 (0.37)       | 1 - 2.33             | 1.84 (0.41)             | 3.68 (0.97)| 1.66 - 6.5 49.59    | P < 0.001² |
| CAL           | 1.26 (0.37)       | 1 - 2.33             | 1.89 (0.48)             | 4.47 (1.33)| 1.66 - 8.5 49.87    | P < 0.001² |
| GI            | 0.01 (0.06)       | 0 - 0.25             | 1.07 (0.49)             | 0.25 - 2.5 | 1.59 (0.36)| 0.75 - 2.25 44.97    | P < 0.001² |
| GBTI          | 0.01 (0.11)       | 0 - 1                | 2.10 (1.07)             | 1 - 3      | 2.78 (0.57)| 1 - 3 44.97 | P < 0.001² |
| PI            | 0.03 (0.08)       | 0 - 0.25             | 0.81 (0.54)             | 0 - 2.5    | 1.14 (0.49)| 0.25 - 2.25 40.80    | P < 0.001² |
| GCF volume (µl) | 0.32 (0.17) | 0.05 - 0.98          | 0.64 (0.43)             | 0.13 - 2.57| 1.23 (0.6)| 0.25 - 2.89 45.56    | P < 0.001² |
| Total nitrite level (nmol) | 0.83 (0.31) | 0.26 - 1.68          | 1.07 (0.62)             | 0.14 - 4.93| 1.08 (0.59)| 0 - 3.72 7.39 | P < 0.05² |
| Total nitrate level (nmol) | 7.7 (2.71) | 0 - 22.88           | 7.51 (4.16)             | 0 - 50.4   | 7.38 (1.91) | 0.02 - 15.7 0.43 | P > 0.05 |

SD = standard deviation; N/A = not available.

*Statistically significant, Dunn test.

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Nitrite/nitrate levels in saliva and GCF

Salivary nitrite/nitrate levels (µM) for the study groups are given at Table 2. Salivary nitrite/nitrate levels did not differ significantly among the three study groups. Total nitrite levels in GCF were higher in periodontitis and gingivitis (1.08 [0.59] nmol and 1.07 [0.62]) groups than the control group (0.83 [0.31] nmol) (P < 0.001). The difference in GCF nitrite level did not reach to a significant level between gingivitis and periodontitis groups (P > 0.05). GCF nitrate level did not differ significantly among the control, gingivitis and periodontitis groups (7.7 [2.71] nmol, 7.51 [4.16] nmol and 7.38 [1.91] nmol) (P > 0.05).

Analysis of nitrate/nitrite ratio in saliva and GCF

In the present study, nitrate/nitrite ratios in saliva and GCF were also evaluated; the ratio was calculated with the division of nitrate by nitrite. Saliva nitrate/nitrite ratios were higher in periodontitis and gingivitis groups (2.91 and 3.32) than the control group. On the other hand, GCF nitrate/nitrite ratio demonstrated a gradual decrease with the presence of inflammation.

DISCUSSION

NO is an important inflammatory mediator which shows both direct and indirect effects. Direct effects include the chemical reactions on its biological target. The indirect effects involve the actions of NO metabolites; reactive nitrogen species (RNS). The indirect effects can then subdivide to nitrosative stress and oxidative stress [28,37]. An excess of reactive oxygen species (ROS) and depletion of anti-oxidant levels in tissues result with oxidative stress [28,39]. The indirect effects need NO, activated by superoxide (O₂⁻) or oxygen to form RNS, then undergo consecutive reactions with their related biological targets [28,37]. Nitrosative stress also involves intermediates, produced from nitrosated thiol, hydroxy, and amine groups [37]. Oxidative and nitrosative stress are involved in many diseases and medical conditions, such as cancer, diabetes, atherosclerosis, congestive heart failure, myocardial infarction, metabolic syndrome and periodontitis [39-41]. A recent study has demonstrated the involvement of nitrosative stress in the aggravation of periodontitis under a diabetic condition, suggesting that nitrosative stress plays a crucial role in the development of diabetic-associated periodontitis [17]. In another recent study, Subantimicrobial Dose Doxycycline (SDD) therapy was used as an adjunct to SRP treatment against nitrosative stress in chronic periodontitis. Adjunctive SDD therapy was useful against inflammatory response by reducing the generation of nitrosative stress via inhibiting iNOS. Nitrosative stress reduction was correlated with improvements in periodontal status. The results suggested the utility of nitrosative markers in chronic periodontitis diagnosis and prognosis [28].

NO has been shown to have a paradoxical relationship with bone [42,43]. Although excessive production of NO may be associated with bone loss in some inflammatory conditions, NO also mediates beneficial effects of estrogen on bone by the NO/cyclic guanosine monophosphate pathway [44]. Knockout mice deficient in endothelial NOS exhibit osteoporosis as a result of a defect in bone formation [15]. Similarly, mice that are deficient in iNOS exhibit altered bone healing [45]. Increased bone mineral density has been reported in mice deficient in all three isoforms of NOS [46]. On the other hand, in response to inflammation, iNOS is expressed, resulting in higher amounts of NO production [14,15]. Increased numbers of iNOS positive cells have been demonstrated in periodontally diseased tissues [47-49]. Another previous study has demonstrated that enhanced formation of NO, increased iNOS production and consequent nitrosative stress play a significant role in the pathogenesis of periodontitis [16].

In the present study, we evaluated nitrate and nitrate levels in GCF and saliva from patients with generalized gingivitis and chronic periodontitis. The level of nitrite in GCF of 147 healthy, 183 gingivitis and 150 chronic periodontitis sites were analyzed. In accordance with the literature [50-52], the findings of this study demonstrated that GCF volumes exhibited clear increases at diseased sites compared to healthy sites. Also the present study shows that inflammatory conditions increases GCF nitrite level significantly in comparison with control subjects. Comparable findings have been reported previously [53]. They studied nitrite and myeloperoxidase metabolism in both GCF and peri-implant sulcular fluid, and demonstrated a tendency to increase in these ingredients with the presence of gingival/peri-implant inflammation. On the other hand, in the present study, nitrate levels did not differ significantly among study groups. In order to evaluate the change in levels of nitrite and nitrate according to each other, nitrate/nitrite ratio was calculated. The gradual decrease in nitrate/
nitrite ratio with the presence of inflammation and higher nitrite level in inflamed groups emphasizes that role of the nitrite in periodontal disease pathogenesis is more pronounced than nitrate. It might be speculated that nitrite may be a more beneficial indicator than nitrate.

NO production in saliva was also measured using the level of nitrite/nitrate in saliva of 20 gingivitis and 20 chronic periodontitis and 20 control subjects. Analyses demonstrated no significant difference among the study groups regarding saliva nitrite level. Almost equal nitrate levels in inflammatory groups may be due to quite similar values for the presence/extent of gingival inflammation and plaque accumulation detected in full mouth recording of gingivitis and chronic periodontitis groups or large overlap between study groups.

Based on the previously demonstrated discrepancy between “concentration” and “total activity” modes of data presentation for GCF, were not completely correlated [54,55]. With respect to total activity level, nitrite and nitrate concentrations in gingivitis or periodontitis were lower than healthy sites. This contrast between 2 modes of data presentation suggests the volume-dependent nature of the concentration expression. As the available GCF volume in a given site affects concentration expression, it may be suggested that GCF share similar volumetric features with respect to the appropriate mode of data presentation.

In the present study, two biological fluids, GCF and saliva, were analyzed. Although the results may shed light on the diagnostic potential of GCF, the results regarding saliva NO level still remain controversial. Nitrite and nitrate levels did not demonstrate any significant correlation between these two biological fluids. Saliva can be affected qualitatively and quantitatively by a variety of factors. The broad similarity of clinical parameters, in particular with regard to PD, could explain the non-significant results between gingivitis and periodontitis sites concerning nitrite/nitrate levels. On the other hand, qualitative and quantitative evaluation of GCF is more specific for periodontal disease pathogenesis than saliva. It can be suggested that certain amount of NO is derived from GCF to the saliva. Further studies on this to evaluate and compare the components of saliva and GCF, especially with respect to the inflammatory process, NO and nitrosative stress, are needed to increase our understanding of the role of each component and the diagnostic potential of these parameters and pathogenesis of periodontal disease.

CONCLUSIONS

In the limits of the present study it can be suggested that gingival crevicular fluid seems to have a better diagnostic potential than saliva. Nitrite may be a better marker for gingival inflammation rather than periodontitis.

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