**A multifaceted molecule, Nitric oxide – its possible role in periodontitis**

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**Abstract**

**Aim:** To assess and compare the levels of Nitric Oxide (NO) in Gingival Crevicular Fluid (GCF) and serum of chronic periodontitis (mild to moderate and severe) patients and healthy controls.  

**Methods:** The present comparative study comprised three groups, Group I: 30 healthy subjects without periodontitis as control group, Group II: 30 patients affected with mild to moderate periodontitis and Group III: 30 patients affected with advanced periodontitis. Thus, a total of 90 subjects in the age group 25-60 years participated. Patients with clinical attachment loss (CAL) of 2-4 mm and >5mm in at least 10 sites were classified as mild to moderate periodontitis and severe periodontitis respectively. NO levels in GCF and serum were assayed by measuring the accumulation of stable oxidative metabolite, nitrite with Griess reagent.

**Results:** The mean value of NO levels in GCF among subjects with periodontal disease (Group 2 & Group 3) was significantly higher compared to control group i.e. (6.68 ± 0.75µM/l and 9.45± 0.94µM/l respectively compared to 3.75±0.73µM/l/l). Similarly the mean value of NO levels in serum among subjects with periodontal disease (Group 2 and Group3) was significantly higher compared to control group (Group1) i.e. (40.96 ± 6.58µM/l and 65.69 ± 6.00µM/l resp. compared to 22.98 ± 2.73µM/l/l).  

**Conclusions:** The findings from the study suggest that NO levels are increased in GCF and serum in subjects with periodontitis compared to healthy controls. The increase in NO levels was directly proportional to the severity of periodontal disease.  

**Key words:** Biological fluids; Inflammatory diseases; Nitric oxide; Periodontitis.

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Nitric oxide as a biomarker in periodontal disease

Introduction

Periodontal disease is a chronic inflammatory reaction of periodontal tissues in response to infection caused by a group of bacteria. The toxins, enzymes and metabolites of the bacteria (predominantly Gram-negative anaerobic) present in the dental plaque play a key role in the initiation of the inflammatory process. It is conceivable that endotoxins and/or pro-inflammatory cytokines produced by inflammatory cells in response to bacteria trigger resident and/or immigrant cell populations for the expression of various biological markers such as proteins, enzymes, host cells, hormones, bacterial products, volatile compounds and ions (1). Reactive intermediate oxygen species are formed continuously as a consequence of metabolic and other biochemical reactions as well as external factors like infection. Nitric oxide (NO) is a simple free radical synthesized in mammalian cells by family of enzymes, the nitric oxide synthases acting both as a physiological messenger and cytotoxic molecule (2). NO is not only important in host defense and homeostasis but it is also regarded as harmful and has been implicated in the pathogenesis of a wide variety of inflammatory and autoimmune diseases (3).

NO has been intensively studied in various inflammatory conditions related to medical health. In addition NO has been linked in etiopathogenesis of periodontal disease. NO is a short living product of nitrogen metabolism. NO is generated by oxidative deamination of L-arginine by a group of isoenzymes collectively termed NO synthases (NOS) (4). NOS exists as three distinct isoforms, namely endothelial NOS, neural NOS and inducible NOS (iNOS). The first two are constitutively produced (cNOS). In contrast iNOS is expressed in response to proinflammatory stimuli and produces large amount of NO for sustained time periods. Free NO has many characteristics of free toxic radicals, and therefore it can affect various cell reactions and biological functions, including inhibition of neutrophil chemotaxis, adhesion to endothelium, and up regulation of tumor necrosis factor alpha (5). Periodontal diseases are chronic inflammatory infections accompanied by destruction of surrounding connective tissue and alveolar bone. Cytokines and other bacterial products stimulate the expression of iNOS and interfere with periodontal disease progression (6-8). The primary causative agents are gram-negative bacteria, which stimulate cells like macrophages, fibroblasts to generate NO. More specifically, bacterial lipopolysaccharides stimulate NO expression in bone as well as other tissues (9, 10). The presence of NO in periodontal disease may reflect the participation of an additional mediator contributing to periodontal tissue damage and bone resorption responsible for progression of periodontitis.

In vivo, NO quickly combines with oxygen to produce stable compounds nitrite and nitrate abbreviated as (NOx), which are commonly used as measure of NO production in biological fluids. NO produced in high concentrations proves to be crucial in non-specific host defense and is cytotoxic (10). The evaluation of involvement of nitric oxide in the periodontal disease will enable us to understand the complexity of periodontal disease progression. Hence this study was designed with aim to measure NO levels in gingival crevicular fluid (GCF) and serum in patients with mild to moderate and severe chronic generalized periodontitis and to compare these levels in individuals without periodontitis.

Material and methods

Sample collection

Patients attending the outpatient Department of Periodontics, A.B. Shetty Memorial Institute of Dental Sciences, Mangalore from Jan 2008-AUG 2009 who were in the age group of 35-45yrs were the target population of the study. Inclusion
criteria included patients with age group between 35-45 years without any systemic disease and patients who have not undergone any periodontal therapy in the previous year while exclusion criterion constituted patients with history of systemic antibiotic therapy 2 months prior to study, smoking, pregnancy and lactation. The present comparative study comprised three groups, Group I: 30 healthy subjects without periodontitis as control group, Group II: 30 patients affected with mild to moderate periodontitis and Group III: 30 patients affected with advanced periodontitis. Thus, a total of 90 subjects were recruited from the target population. Patients with clinical attachment loss (CAL) of 2-4 mm and >5mm in at least 10 sites were classified as mild to moderate periodontitis and severe periodontitis respectively. All the measurements and samples were collected before start of any periodontal therapy. The study was independently reviewed and approved by the Research Ethical Committee of A.B Shetty Memorial Institute of Dental Sciences, Mangalore. Informed consent was obtained from all the individuals prior to their participation in the study.

Clinical examination

The following measures of oral health were collected to assess the severity of periodontal destruction: probing pocket depth and clinical attachment level. Pocket depth was measured in (mm) using Williams graduated periodontal probe as the distance from the gingival margin to the base of the pocket. Clinical attachment loss was calculated from recession and pocket depth measures and represented as the distance in (mm) from the CEJ to the base of the pocket. All the measurements were recorded by a single examiner before start of any periodontal therapy. Clinical attachment loss was examined at six sites per tooth excluding third molars.

Gingival crevicular fluid and serum collection

Sample of GCF were obtained before probing the site by placing white color-coded 1-5 µL calibrated volumetric microcapillary pipettes which were obtained from Sigma Aldrich Chemicals Company, limited, USA. One microliter of GCF was collected by placing a microcapillary pipette extracrevicularly. The collected GCF samples were transferred to a vial that contained 100µl of phosphate buffer with pH-7.1, centrifuged, followed by NO estimation. Two ml of blood was collected from the ante-cubital fossa by venipuncture using 20-gauge needle with 2 ml syringes and immediately transferred to laboratory. Blood sample was allowed to clot at room temperature and after one hour, serum was extracted from blood by centrifuging at 3000g for 5 min, followed by biochemical estimation of NO.

NO quantification

GCF and serum nitrite levels were measured using the Griess colorimetric reaction (11). 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 570nm. Samples of GCF and serum were transferred to a 96-well ELISA plate, and an equal volume of Griess reagent was added to each well. In order to obtain standard curves, serial dilutions of sodium nitrite in PBS (pH 7.2) were also included. After 10minutes, the optical density was measured using an ELISA plate reader (SpectraMax 340, Molecular devices, USA) with a 570 nm filter.

Statistical analysis

The results of measurement were described using means and standard deviations for each measured parameter. NO among the
Nitric oxide as a biomarker in periodontal disease

three groups were compared for statistical significance by one-way ANOVA (and post hoc analysis – Tukey was used for multiple comparisons) at a probability level of < 0.05.

Results

Table 1 shows the mean value of nitric oxide levels in GCF among subjects with periodontal disease (Group2 and Group3) compared to control group (Group1) i.e. (6.68 ± 0.75µM/l and 9.45± 0.94µM/l resp. compared to 3.75 ± 0.73µM/l). The NO levels in severe periodontitis patients was approximately thrice in comparison to healthy controls.

Table 1: Comparison of mean scores of nitric oxide levels (µg/dl) in GCF in different groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean± Standard deviation</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>3.75±0.73</td>
<td>2.09</td>
<td>5.01</td>
</tr>
<tr>
<td>Group 2</td>
<td>6.68±0.75</td>
<td>5.32</td>
<td>8.12</td>
</tr>
<tr>
<td>Group 3</td>
<td>9.45±0.94</td>
<td>7.66</td>
<td>12.02</td>
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</tbody>
</table>

One way ANOVA, p = 0.0005

Table 2: Mean Scores of nitric oxide levels (µg/dl) in serum in different groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean± Standard deviation</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>22.98±2.73</td>
<td>18.29</td>
<td>28.56</td>
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<tr>
<td>Group 2</td>
<td>40.96±6.58</td>
<td>26.66</td>
<td>50.32</td>
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<tr>
<td>Group 3</td>
<td>65.69±6.00</td>
<td>54.43</td>
<td>75.09</td>
</tr>
</tbody>
</table>

One way ANOVA, p = 0.0005

Discussion

The production of NO has been recognized as an inflammatory marker in a variety of human diseases associated with inflammation. Infection and inflammation are the hallmarks of periodontal disease. NO synthesis is increased in periodontal disease, as a result of inflammatory cell infiltration in the periodontal tissues. Macrophages are known to be the main source of iNOS in periodontal tissues (12). Periodontal diseases are initiated and sustained by factors produced or released by the subgingival microflora. These substances can directly damage host cells and tissues while other activate endogenous cellular and humoral inflammatory systems which secondarily affect the integrity of the periodontium (13).

The present study was conducted in an attempt to evaluate the NO levels in GCF and serum of periodontally healthy group and compare it with subjects who had mild to moderate and severe periodontal disease. A significant trend was observed where the NO levels increased with increase in severity of the disease. NO levels in the advanced periodontitis group were significantly higher than in individuals without periodontitis in addition to those with milder and moderate forms of disease. This is similar to that noted in other studies where a ligature induced periodontitis showed a significant increase in NO levels (14,15). Inter comparison of nitrite levels in serum and GCF between the groups showed more increased levels of nitrite in group 2 and group 3 compared to group 1.
Nitric oxide as a biomarker in periodontal disease

small sample study was conducted to assess the serum NO levels in our previous article (17) which included healthy controls without periodontitis and patients with generalized chronic periodontitis. The results of our past study were consistent with present results; however no attempt was made in our previous study to compare between the subgroups of periodontitis.

The significant higher level of NO in group 2 and group 3 may contribute to the development of the frequently found clinical symptoms of periodontitis. The increased alveolar bone resorption may be due to the stimulatory effect of NO on the activity of the osteoclasts. Besides the cytotoxic and tissue damaging effect of NO itself, the increased prostaglandin E production due to stimulating effect of NO on cyclooxygenase activity, might play synergistic role in osteoclastic bone resorption and vasodilation (16,17).

In addition, studies have shown that rapid serum diffusion of NO could contribute to increased aqueous nitrite and nitrate, implicating NO in pathophysiology and progression of diabetic retinopathy as well as in periodontal disease (18).

The present study results are also in agreement with a previous study findings where salivary nitrite was detected which stood for the content of NO, and it was found that NO in saliva of patients with chronic periodontitis were significantly higher than that in saliva of healthy individuals. Also in the same study a significant relationship was found between attachment level and salivary NO level and between probing depth and salivary NO level (6). However, no attempt was made in the present study assess salivary NO levels. The results of present study are also consistent with various other reports of increased NO in periodontitis (19-21).

Recently it has been proposed that selective inhibition may be a promising novel approach for the treatment of periodontitis. Mercaptoethylguanidine (MEG) which is a selective inhibitor of iNOS and a scavenger of peroxynitrite significantly reduced the plasma extravasation in gingivomucosal tissue and decreased the degree of alveolar bone destruction in rat model of periodontitis (22). Bone resorption in rats with induced periodontal disease could be prevented with isosorbid, an iNOS inhibitor (23,24). Other inhibitors used include aminoguanidine and the enzyme arginase, which competes with the substrate (L-arginine), thus reducing NO production (25). These previous reports further support the influence of NO on periodontal disease.

Conclusions
The findings from the study suggest that NO levels are increased in GCF and serum in subjects with periodontitis compared to healthy controls. The increase in NO levels was directly proportional to the severity of periodontal disease. The role of NO in inflammation represents one of the most studied yet a controversial subject. However, the precise role of NO in tissue damage in periodontal disease still remains unclear. Further studies must be undertaken to know the multiple signaling pathways that are involved in production of NO that may help to design new therapeutic strategies for the treatment of inflammatory tissue injury. It is also important to assess nitrite levels in other forms of periodontitis and gingivitis and compared within subgroups to reveal expression of NO during different stages of periodontal disease progression.

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Nitric oxide as a biomarker in periodontal disease

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Nitric oxide as a biomarker in periodontal disease


