Influence of interventional nonsurgical periodontal treatment on levels of salivary and serum nitric oxide in smokers and nonsmokers with chronic periodontitis

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Abstract:
Background and Objective: Nitric oxide (NO) is a widespread signaling molecule which is known to influence varied biological processes. However, an uncontrolled high level of NO accelerates tissue destruction. The pathogenesis of periodontal disease is also affected by smoking which alters the inflammatory response. The present study was thus performed to assess the effect of nonsurgical periodontal treatment on salivary and serum NO levels in smokers and nonsmokers with chronic periodontitis. Materials and Methods: Forty patients with chronic periodontitis, including 20 nonsmokers and equal number of smokers participated in the present study. Probing depth, clinical attachment level, plaque index, gingival index were assessed, serum and saliva samples were obtained from the patients at baseline and after Phase I therapy at 6 weeks to estimate NO by Griess colorimetric reaction. Results: Smokers showed higher serum and saliva NO levels 30.3 ± 3.28 and 50.4 ± 4.07 µM as compared to nonsmokers 20.05 ± 2.42 µM and 37.5 ± 2.95 µM, respectively, at baseline. After Phase I therapy, both the groups exhibited significant improvement in clinical parameters and reduction in serum and saliva NO levels; however, reduction was higher in nonsmokers. Conclusion: More destructive expression of periodontal disease in smokers causes an increase in the concentrations of NO and less reduction after Phase I therapy as compared to nonsmokers with chronic periodontitis. Hence, NO levels in saliva and serum could be used as indicators of periodontal inflammatory condition.

Key words:
Chronic periodontitis, colorimetry, nitric oxide, smoking

INTRODUCTION

Periodontitis is one of the most prevalent chronic infections of the periodontium. Although bacteria are the primary etiologic agent in the pathogenesis of the periodontal disease, host-bacterial interaction also plays a crucial role in defining the outcome of the disease. However, simultaneous presence of both factors at the same time complicates the clarification of the underlying immunopathogenic mechanism. It is postulated that the inflammation and infection within periodontal tissues lead to production of nitric oxide (NO) in high quantities, which have been linked to etiopathogenesis of periodontal diseases. NO is a prominent signaling molecule that participates in essentially every cellular and organ function in body. NO is thought to influence variety of biological processes by virtue of its antibacterial, antiviral, antitumoricidal, and antiparasital properties. However, it possesses some undesirable effects when present in uncontrolled and high levels. It interacts with superoxide anions leading to formation of highly toxic peroxynitrite. In the presence of infection and inflammation, significant quantities of both superoxide and NO can be produced by cells of the immune system which rapidly react to form peroxynitrite by a nonenzymatic reaction. Peroxynitrite causes lipid peroxidation.

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damage to proteins, and nucleic acids, and results in DNA strand breaks.[3] A group of isoenzymes that produce NO in mammalian cells named NO synthases (NOS) exists as three distinct isoforms, namely, endothelial NOS (eNOS), neural NOS (nNOS), inducible NOS (iNOS). iNOS is expressed in response to proinflammatory stimuli and produces large amounts of NO for sustained time periods.[3]

Substantial evidence exists implicating NO in the pathogenesis of periodontitis. Lipopolysaccharide, bacterial toxins, and antigenic cell components from periodontopathogenes have been shown to induce and accelerate periodontal tissue destruction by increasing iNOS levels and NO synthesis in murine macrophages.[5,6] The inflammatory status within periodontal diseases leads to expression of iNOS which produces increased concentration of NO. The locally produced NO when in higher quantities acts as a cytotoxic molecule against periodontal pathogens and surrounding cells possibly leading to tissue destruction.[7]

Smoking is the most common and significant local factor associated with periodontal disease which leads to increased oxidative stress in the lungs and further may enhance the susceptibility to the periodontal pathogen.[9] Consecutively, excessive production of reactive oxygen species by activation of immune response leads to a hyperinflammatory state in periodontitis patients. Therefore, cigarette smoke intensifies the severity of periodontal destruction.

All the evidence suggests the role of NO and smoking in pathogenesis of periodontal destruction. Literature search reveals insufficient understanding about the influence of nonsurgical periodontal treatment on levels of NO in smokers with chronic periodontitis. The present study was thus performed to assess the levels of NO in smokers and nonsmokers with chronic periodontitis and the influence of Phase I periodontal treatment on these levels.

**MATERIALS AND METHODS**

Patients with moderate chronic periodontitis aged 30–55 years were selected from those visiting the Department of Periodontics and Implantology of VSPM Dental College and Research Centre, Nagpur. These patients were assessed clinically and biochemically. The study was conducted following the clearance from the VSPM’s Institutional Ethics Committee. A special pro forma was designed so as to have a systematic recording of information and observations which included detailed case history, clinical examination, and periodontal indices and written consent of the patient. The study protocol was in accordance to the Helsinki Declaration, as revised in 2013.

Salivary nitrite level was considered as the outcome variable to calculate the sample size. The sample size was calculated as 20 patients per group, based on the previous study,[10] where percentage increase in salivary NO in smokers as compared to non-smokers was 48.3%, with the absolute precision, Δ = 20%, α = 10%, power = 80%.

The study comprised of two groups: Group I, nonsmokers with chronic periodontitis and Group II, smokers with chronic periodontitis. Each group comprised of 20 patients. Patients having chronic periodontitis with more than 30% of sites exhibiting pocket probing depth (PPD) ≥5 mm, clinical attachment level (CAL) ≥5 mm, and with at least minimum of 20 teeth present were included in the study. Patients with history of smoking ten or more cigarettes per day for the last 3 years were included in Group II while those with no history of smoking were placed in Group I.

Patients exhibiting the following conditions were excluded from the study: (a) pregnant women or lactating mothers, (b) patients with history of any periodontal treatment in the past 6 months, (c) patients with systemic diseases such as hypertension, diabetes mellitus, renal disease, rheumatoid arthritis, or any other form of inflammatory involvement, and (d) patients on antibiotics and anti-inflammatory therapy.

**Clinical parameters**

PPD and CAL were measured using William’s graduated periodontal probe (Hu Friedy, Chicago, IL, USA) on four sites from the cementoenamel junction to the base of the pocket of all the present teeth. Plaque index (PI)[11] and gingival index (GI)[12] were also recorded. The periodontal specialist was calibrated before the beginning of the clinical trial in a pilot study.

**Collection of samples**

For serum sample collection, the area over the antecubital fossa was disinfected and two mL of blood was withdrawn. The blood samples were allowed to settle down and centrifuged at 1500 rpm for 10 min. For saliva sample collection, patients were told not to drink or eat anything from morning until sampling. Unstimulated whole saliva samples (2 mL) were obtained over 5 min and placed in sterile Eppendorf tubes and centrifuged at 2500 rpm for 10 min. The supernatant of both the samples were collected and stored until biochemical analysis at −80°C.

**Estimation of NO levels using colorimetric detection kit (DetectX®, Arbor Assays, Inc., USA)**

For quantitative estimation of NO, all the reagents, standards, and samples were prepared according to manufacturer’s instruction (DetectX®, Arbor Assays, Inc., USA). Due to the unstable nature and physical characteristics of NO, assessment by direct detection methods is difficult. However, colorimetric methods can be applied to estimate its stable breakdown products nitrate (-NO$_3$) and nitrite (-NO$_2$) as described by Green et al. The NO colorimetric detection kit (DetectX®, Arbor Assays, Inc., USA) was used for quantitative measurement of nitrate and nitrite in serum and saliva. For estimation of total NO content, samples were incubated with diluted nitrate reductase enzyme and nicotinamide adenine dinucleotide. After incubating at room temperature for 20 min, color reagent A followed by color reagent B were added and incubated for 5 min. The absorbance of the color product was read on microplate reader at 550–570 nm. The standard curve was plotted with known concentration of the NO, and corresponding values of absorbance by linear regression equation were used for determination of nitrite levels in the samples.

All the clinical parameters were measured and the serum and saliva samples were collected at baseline and at 6 weeks after Phase I periodontal treatment was initiated after recording
the baseline periodontal parameters and collecting serum and saliva samples. All patients underwent therapy including oral hygiene instructions, scaling, and root planing.

Statistical analysis
Statistical software STATA version 13.0 (StataCorp. 2015. Stata Statistical Software: Release 14. College Station, TX: StataCorp LP) was used for data analysis. Clinical parameters (PPD, CAL, PI and GI) were presented as mean ± standard deviation. Mean PPD, CAL, PI and GI were compared before and after 6 weeks of therapy in Group I and Group II by performing paired t-test for normalized data. Mean change in clinical parameters after Phase I therapy was compared between smokers and nonsmoker group by performing independent t-test. Pearson’s correlation coefficient (r) was calculated to assess the direction and strength of correlation between serum and salivary NO level and clinical parameters. Baseline parameters were compared between Group I and Group II by applying independent t-test. P < 0.05 was considered as statistically significant.

RESULTS
Mean age of patients in Group I was 45.9 ± 12.43 years with a range between 35 and 63 years, and in Group II, it was 43.4 ± 9.30 years with range of 35–60 years. Out of 20 nonsmokers with chronic periodontitis, sixteen were male and four were female. Considering the geographical location of the study and also social background where females do not tend to smoke or reveal smoking history, Group II, i.e., smoker with chronic periodontitis comprised only of male patients.

Clinical parameters at baseline and after Phase I therapy
At baseline, clinical periodontal parameters were higher for Group II as compared to Group I but the difference was nonsignificant except for the GI, which was higher for Group I as compared to Group II (P < 0.001) [Table 1].

All clinical parameters showed a reduction from baseline to posttherapy visit, and the differences were statistically significant for both the groups (P < 0.001) [Table 1]. When comparing the mean change between baseline and after treatment for both the groups, statistically significant difference was found, however Group I responded better in terms of clinical parameters after Phase I therapy than Group II (P < 0.001) [Table 2].

Serum and saliva nitric oxide levels at baseline and after Phase I therapy
Although NO levels in both serum and saliva were significantly higher at baseline compared to after 6 weeks for both the groups, Group II showed statistically higher levels at baseline as compared to Group I and the difference was statistically significant. The differences between baseline and after 6 weeks serum and salivary NO levels among the groups were statistically significant (P < 0.0001) [Table 3].

On comparison the serum and saliva NO levels at baseline among the groups showed significant difference (P<0.0001). Salivary NO levels were higher than serum NO levels in both the groups. The levels of serum NO levels showed highly significant correlation with saliva levels of NO in Group I (r = 0.8607, P < 0.0001) as well as in Group II (r = 0.9224, P < 0.0001) [Table 4].

The results showed that there was a positive significant relationship between the values of both the serum and the salivary NO levels with respect to their clinical parameters (PPD, CAL, GI, PI) [Table 5].

DISCUSSION
Over the past decade, many studies have been conducted to elucidate the role of NO in the immunoinflammatory response and characterize its potential involvement in the pathogenesis of periodontal disease. The levels of NO may indicate the status and the severity of underlying disease processes. It could be utilized as an inflammatory biomarker that may enable the clinicians to direct environmentally based prevention or treatment programs.

In the literature, there is a paucity of studies which focus on the effect of smoking on NO levels, and also, its role in complex pathogenesis of periodontal disease is not well understood. Furthermore, whether nonsurgical periodontal therapy has any effect on the levels of NO in chronic periodontitis patient with history of smoking have only been preliminarily investigated, but the effects have not been unequivocally established. In the present study, we attempted to evaluate the levels of both serum and salivary NO in chronic periodontitis patients and

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Table 1: Comparison of clinical parameters among study groups at baseline and after Phase I therapy

| Parameter     | Group I         | Group II        | P     | Group I         | Group II        | P     |
|---------------|----------------|----------------|-------|----------------|----------------|-------|
| PPD (mm)      | 4.77±0.89 (31.7) | 5.08±0.32 (31.7) | <0.0001 | 5.1±0.31 (31.7) | 5.63±0.94 (31.7) | <0.0001 |
| CAL (mm)      | 5.41±0.94 (31.7) | 5.53±0.51 (31.7) | <0.0001 | 5.39±0.26 (31.7) | 5.91±0.67 (31.7) | <0.0001 |
| GI            | 2.02±0.35       | 2.17±0.24       | 0.97±0.34 (37.6) | 2.17±0.24       | 2.17±0.24       | <0.0001 |
| PI            | 2.15±0.38       | 2.17±0.24       | 0.97±0.34 (37.6) | 1.24±0.30       | 1.24±0.30       | 0.97±0.34 (37.6) |

Data is expressed as mean±SD. PPD – Probing pocket depth; CAL – Clinical attachment level; GI – Gingival index; PI – Plaque index; SD – Standard deviation

Table 2: Comparison of mean change in study variables before and after Phase I therapy in both the groups

| Study variables | Mean±SD (%) | P     |
|----------------|-------------|-------|
| Group I        | Group II    |       |
| PPD (µM)       | 0.97±0.67 (20.50) | 0.45±0.30 (9.05) | 0.0005 |
| CAL (µM)       | 0.78±0.46 (14.4) | 0.42±0.37 (7.6) | 0.0100 |
| GI             | 0.75±0.34 (37.6) | 0.28±0.13 (17.7) | <0.0001 |
| PI             | 0.91±0.31 (42.3) | 0.48±0.19 (22.5) | <0.0001 |
| Serum NO (µM)  | 6.35±2.36 (31.7) | 3.07±1.9 (10.1) | <0.0001 |
| Saliva NO (µM) | 8.72±2.20 (31.7) | 4.32±1.9 (8.6) | <0.0001 |

Data is expressed as mean±SD. PPD – Probing pocket depth; CAL – Clinical attachment level; GI – Gingival index; PI – Plaque index; NO – Nitric oxide; SD – Standard deviation
Table 3: Level of nitric oxide (μM) in serum and saliva in both groups

| Parameter | Group I |         |         |         |         | Group II |         |         |         |
|-----------|---------|---------|---------|---------|---------|----------|---------|---------|---------|
|           | At baseline | After Phase I therapy | Mean change | P       | At baseline | After Phase I therapy | Mean change | P       |
| Serum NO  | 20.05±2.42 | 13.7±0.04 | 6.35±2.36 | <0.0001 | 30.3±3.28 | 27.27±3.79 | 3.07±1.90 | <0.0001 |
| Saliva NO | 37.5±2.95 | 28.8±2.60 | 8.72±2.20 | <0.0001 | 50.4±4.07 | 46.1±4.67 | 4.32±1.90 | <0.0001 |

NO – Nitric oxide

Table 4: Correlation of levels of nitric oxide in serum and saliva among study groups

| Group          | r     | P     |
|----------------|-------|-------|
| Group I        | 0.8607 | <0.0001 |
| Group II       | 0.9224 | <0.0001 |

Table 5: Correlation of serum nitric oxide, saliva nitric oxide and study parameters in both the groups

| Clinical parameters | Serum NO | Saliva |
|---------------------|----------|--------|
|                     | Group I  | Group II | Group I  | Group II |
| PPD                 | 0.5387   | 0.5289  | 0.5261   | 0.5466   |
| r                   | 0.0143, S | <0.0001 | 0.0172, S | <0.0001 |
| P                  | 0.8280  | 0.6276  | 0.5367   | 0.7334   |
| G1                  | 0.0374, S | <0.0001 | 0.0165   | 0.0053   |
| CAL                 | 0.8644  | 0.7417  | 0.4929   | 0.6293   |
| P                  | 0.0001  | 0.0027  | 0.0001   | 0.0030   |

Data is expressed as mean ± SD. PPD – Probing pocket depth; CAL – Clinical attachment level; GI – Gingival index; PI – Plaque index; S – Significant; SD – Standard deviation

Also to assess the effect of smoking on the inflammatory marker and reassess these levels after nonsurgical periodontal therapy with a view to determine its diagnostic and prognostic values.

Group I showed better response to nonsurgical periodontal therapy as compared to Group II in terms of PPD and CAL. These results are in conformance with the studies by Ah et al.,[14] and Preber H, Bergström.[15] The severity of periodontal destruction and inferior clinical response could be attributed to the smoking-induced host response alteration and healing capacity.16-18

In our study at baseline, the levels of serum and saliva NO levels were significantly higher in Group II as compared to Group I. Similar results were reported by Wadhwa et al.[10]

Possible reasons for this could be that the detrimental effects of smoking manifested by vasoconstrictive action of nicotine results in compromised vasodilation. The presence of large number of reactive oxygen species and free radicals in cigarette smoke enhances the susceptibility to the periodontal pathogens which ultimately leads to increase in oxidative stress in smokers.[19]

Biochemical and immunological markers exist within saliva or serum that can relatively ascertain the severity of periodontal disease and even may predict its progression. In the present study, we have measured NO in serum as well as in saliva and found a positive correlation between them. Collection of salivary constituents is a simple, noninvasive procedure. In addition, samples can be easily transferred in the laboratories in frozen state. The Griess reaction can be potentially used as a tool for future epidemiological research as it provides a simple, highly sensitive method for estimating micromolar concentration of NO.

Both the groups showed statistically significant improvement in both serum and saliva NO levels after nonsurgical periodontal therapy but Group I exhibited more improvement as compared to Group II. Furthermore, significant correlation has been found between the levels of NO in serum and saliva in both the groups. Similar findings were obtained in the study by Parwani et al.[9] who reported significantly increased saliva NO levels in gingivitis and periodontitis individuals and its correlation with clinical parameters. The possible reason could be due to increased localized production of NO by various inflammatory mediators in the cascade of inflammation. The activation of pro-inflammatory mediators in periodontal disease because of the host bacterial interaction enables them to travel through blood to other organs and tissues, and initiate systemic inflammatory response. This mechanism would facilitate the understanding on the capabilities of periodontal blood markers in inducing systemic consequences.

After Phase I therapy, both the groups showed a significant reduction in serum and saliva NO levels. More importantly, serum NO levels were correlated with saliva NO levels along with the clinical parameters. Consequently, serum NO can be utilized as systemic oxidative stress marker as it may reflect release of periodontal NO and can be utilized as systemic nitrosative stress markers.

The results in the present study were evaluated at time interval of 6 weeks posttherapy. However, it is desirable to evaluate the results on a long term basis which enable more definitive and consistent conclusions to be made. Assessor for the assessment of all the clinical parameters and estimation of NO was the same, and there were no blinded examinations. Therefore, possibility of operator bias to some extent cannot be ruled out.

**CONCLUSION**

Within the limits of our study, it can be concluded that serum and salivary NO concentrations can be used as a suitable marker of the inflammatory condition of the periodontium. More destructive expression of periodontal disease in smokers leads to an increase in the concentrations of NO in serum and saliva as compared to nonsmokers with chronic periodontitis. After Phase I therapy, the mean serum and saliva NO concentrations reduced significantly, but the reduction was
greater in nonsmokers with chronic periodontitis as compared to smokers with chronic periodontitis.

However, further longitudinal studies are needed to estimate the effect of nonsurgical periodontal treatment on NO levels and underlying mechanism. It will be beneficial in clarifying their role in the pathogenesis of periodontitis and to validate NO as “Novel Biomarker” of periodontal disease.

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**Conflicts of interest**
There are no conflicts of interest.

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