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RESEARCH ARTICLE

PERIODONTAL THERAPY AND SALIVARY NITRIC OXIDE METABOLITES

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ABSTRACT

Background: Nitric oxide (NO) being a free radical has been evidenced to play a controversial role in the etiopathogenesis of periodontal disease. The present longitudinal study aims to investigate the levels of salivary NO metabolites in healthy as well as in chronic periodontitis patients, its correlation with the periodontal clinical parameters and the effects of periodontal treatment on salivary NO metabolites in CP patients.

Methods: A case-control interventional study was conducted. A total of 54 subjects -36 CP patients and 18 healthy controls were enrolled for this study. The controls were allotted as Group I whereas the CP patients were divided based on the treatment delivered as Group II (scaling and root planing [SRP]) or Group III (SRP with periodontal flap surgery). Clinical parameters, such as probing depth (PD), clinical attachment level (CAL), sulcus bleeding index (SBI), and plaque index (PI) were recorded at baseline and at 12 weeks post- operative. The salivary NO metabolite levels were determined using a spectrophotometric technique.

Results: At baseline, the salivary Nitrate, Nitrite and NO levels for Group II and Group III were higher than Group I controls. At 12 weeks post-operative the salivary Nitrate, Nitrite and NO levels for Group II and Group III were significantly reduced when compared with the baseline values. It was also evident that there was a more significant reduction in salivary NO metabolite levels in Group III which underwent surgical periodontal therapy as compared to Group II which underwent only non-surgical therapy ($p< 0.05$).

Conclusions: Within the limits of the present study, salivary NO metabolites were significantly higher in CP patients when compared to controls. Its levels were positively correlated with the periodontal clinical parameters. The study also concludes that surgical periodontal treatment carried out in Group III patients showed an effective reduction in the salivary NO metabolite levels as compared to the non-surgical periodontal treatment performed in Group II patients. Therefore, surgical periodontal treatment offers added advantage in reducing the damaging effects of NO free radical.

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INTRODUCTION

Periodontitis is a complex disease mediated by multiple molecular mechanisms. Although the principal causative agent is chiefly gram negative anaerobic or facultative bacteria within the subgingival biofilm, majority of the periodontal tissue destruction is believed to be caused by an inappropriate host response to these microorganisms and their products.

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Several inflammatory mediators are known for their timely and unperturbed diagnosis of periodontal disease and one of the most important biochemical marker is nitric oxide (NO) (Poorsattar Bejeh-Mir, 2014). NO is a free radical and its production necessitates a complex reaction that entails five electron transfers which requires the presence of several cofactors and substrates (Rosselli, 1998). NO is synthesized from L-arginine by the action of NO synthase (NOS), an enzyme existing in three isoforms namely Brain NOS (bNOS) or neuronal NOS (nNOS or NOS1), an inducible (iNOS or NOS2) and endothelial NOS (eNOS or NOS3), (Han, 2013).

Various studies have been performed on NO after its discovery as the endothelium derived relaxing factor (EDRF) by Furchgott and Zawadzki in both the medical and dental specialties (Furchgott, 1980). NO is now known to play important functional roles in both physiological as well as pathological processes of the human body. The major pathway for NO metabolism is the stepwise oxidation to nitrite (NO_2) and nitrate (NO_3), (Yoshida, 2010). The nitrite reductase activity in mammalian tissues has been linked to the mitochondrial electron transport system. So not only can nitrite and nitrate determination reflect NO production but may also serve as an alternative source of NO. Thus accurate detection of both anions becomes crucial in NO biology (Weitzberg, 2010). Oxidative and nitrosative stresses are involved in various diseases and medical conditions, such as cancer, diabetes, atherosclerosis, congestive heart failure, myocardial infarction, metabolic syndrome and periodontitis.

NO plays an important role in the progression of periodontal disease (Kendall, 2001). Locally produced NO is cytotoxic against periodontal pathogens as well as the tooth surrounding tissue (Matejka, 1998). These molecules mainly affect the DNA causing strand breaks, base-pair mutations, additions or deletions which ultimately leads to cell death (Bogdan, 2001).
It has been evidenced that dental plaque formation increases NO production, by the up regulation of iNOS expression in gingival cells, which may be induced not only directly by bacterial proliferation but also indirectly through cytokine production stimulated by bacterial plaque. More specifically, bacterial lipopolysaccharides stimulate NO expression in bone, as well as in other tissues (Kendall, 2001 and Matejka, 1998). In addition to this, it was also found out that during periodontitis an increase in NO synthesis was evident within the gingival tissues which further substantiates its role in periodontitis (Matejka, 1998). Salivary NO metabolites are considered as the inflammatory hallmark of periodontal disease. NO was found to act synergistically with prostaglandin E_2 in osteoclastic resorption and vasodilation which is indispensable in the initiation and progression of periodontitis (Han, 2013).

Assessment of NO metabolites which are formed during the host-microbe interaction plays a vital role in comprehending the etiopathogenesis of periodontitis and also serves as an important diagnostic tool to monitor the disease. Reduction of this nitrosative stress was correlated with improvements in periodontal status (Matejka, 1998) However the effects of non-surgical and surgical periodontal therapy, which are considered the basic periodontal treatment modalities on these metabolites are not very much evident in the literature. Though literature regarding NO and NO synthase enzyme is vast, the importance of its stable metabolites being involved in the pathogenesis and progression of periodontitis and the effects of periodontal therapy on them seems to be underestimated. To the best of our knowledge no study has reported the effects of non-surgical and surgical periodontal therapy on the individual metabolites of salivary NO (total NO, Nitrate, Nitrite) in patients with chronic periodontitis. Hence the present study was purposed to determine the levels of salivary NO metabolites in controls and chronic periodontitis patients and also to evaluate the correlation between the levels of salivary NO metabolites and periodontal clinical parameters. The study also aims to compare the salivary levels of NO metabolites in chronic periodontitis patients before and after scaling, root planing and surgical periodontal therapy.

MATERIALS AND METHODS

The study design used was a case control interventional study. The study was carried out in the Department of Periodontics, Bapuji Dental College and Hospital, Davangere. A total of 54 subjects which included (27 males and 27 females; between the age group of 25-50 years, average age: 37.23 years) were selected for the study. The research protocol was initially submitted to the Institutional Ethical Committee and Review Board. After the ethical approval, all the participants were explained about the study and a written consent was obtained from each patient before enrolling them in the study. The subjects recruited in the present study were divided into 3 groups comprising 18 patients each. The inclusion criteria included; subjects having at least 15 natural teeth who were systemically healthy.

Control group: Individuals having a plaque score of ≤ 0.9, SBI score of ≤ 1, PD ≤ 3 in $\geq 90\%$ of the measured sites, and CAL ≤ 2 were included in the group. There was no radiographic sign of alveolar bone loss (i.e., a distance of < 3mm between the cemento- enamel junction and bone crest at > 95% of the proximal tooth sites).

Periodontitis group: Individuals having a plaque score between 2 to 3, SBI score between 3 to 5, PD between 4 to 7 mm and CAL ≤ 8 mm with radiographic evidence of more than 50% of alveolar bone loss in ≥ 2 quadrant.

The following were the exclusion criteria: 1) Aggressive periodontitis; 2) subjects with known systemic illness; 3) pregnancy or lactation; 4) subjects under medications known to affect the outcome of periodontal therapy; 5) insufficient platelet count (<200,000/mm3); 6) subjects with history of tobacco consumption. The clinical parameters assessed were PI (Silness and Loe, 1964), SBI (Muhlemann and Son, 1971), PD and CAL. In all the 3 groups clinical parameters were recorded and saliva samples were collected at baseline. In Group I patients, no periodontal treatment was done. Each subject was given careful instructions on oral hygiene maintenance. In Group II patients, only scaling and root planing (SRP) was performed. In Group III patients, Phase I periodontal therapy was performed. After 1 month, the periodontal condition was revaluated by re-probing the entire mouth and checking for the presence of calculus and signs of persistent inflammation. Patients with persistent inflammation in areas of moderate-to-deep pockets were treated with flap surgery. Intraoral antisepsis was performed with 0.12% chlorhexidine digluconate rinse, and iodine solution was used to perform extraoral antisepsis. After administration of local anesthesia, incisions were placed according to the flap operation performed i.e. Kirkland flap, papilla preservation flap or modified Widman flap depending on anatomical and pocket morphology. Mucoperiosteal flaps were reflected and meticulous defect debridement and root planing were performed. Osseous re-contouring was performed wherever required. Mucoperiosteal flaps were repositioned and secured in place using 4-0 non-absorbable silk surgical suture.* The surgical area was protected and covered with periodontal dressing. Saliva samples were collected and clinical parameters were recorded after 12 weeks in all the 3 groups.

Saliva collection & biochemical analysis of no metabolites

A total of 108 samples of 2mL of whole unstimulated saliva was collected from both the control and chronic periodontitis

patients after 2 hours of refraining from eating or drinking, before and after treatment in sterile plastic tubes. The collected saliva samples were centrifuged at 12,000 rpm at 4^0 C for 10 minutes and the supernatants were frozen at -80^0 C until used. Salivary NO metabolites levels were measured applying the Griess calorimetric reaction (Figure: 1) using a NO calorimetric assay kit§ and measuring under a UV spectrophotometer (Figure: 2).

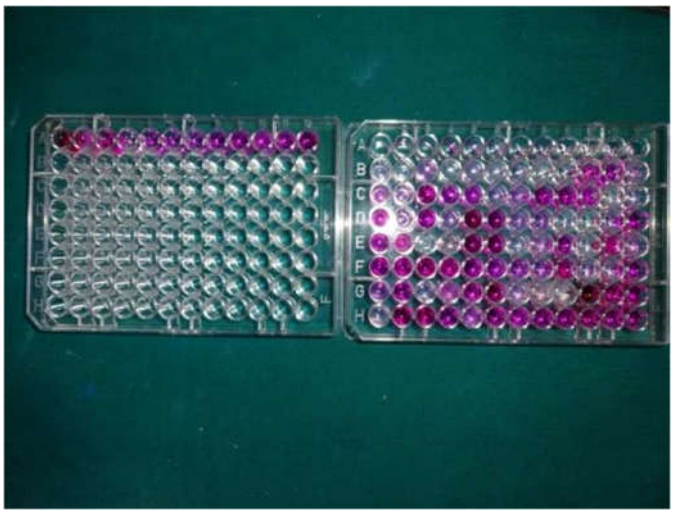


Figure 1. Development of purple colour after addition of Griess reagent R1&R2

Figure 2. Samples analysed in Spectrophotometer for estimation of NO metabolites

Sample Analysis

Measurement of Total NO

200µl of the Nitrate/Nitrite assay buffer was added to the wells. To this 80 µl of the sample was added followed by addition of 10 µl of Nitrate reductase enzyme cofactor preparation. Then 10 µl of the Nitrate reductase preparation was added to each of the wells. The plate was then covered and incubated at room temperature for one hour. After the required incubation time, 50 µl of Griess reagent R1 was added to all the wells. Immediately 50 µl of Griess reagent R2 was added to each of the wells. It was then incubated for 10 minutes at room temperature. The absorbance and the corresponding concentrations of NO were read at 540 nm using a spectrophotometer.

Measurement of Nitrite

200 µl of the Nitrate/Nitrite assay buffer was added to the wells. No other reagents were added. To this 100 µl of the sample was added. The plate was then covered and incubated at room temperature for one hour. After the required incubation time, 50 µl of Griess reagent R1 was added to all the wells. Immediately 50 µl of Griess reagent R2 was added to each of the wells. It was then incubated for 10 minutes at room temperature. The absorbance and the corresponding concentrations of Nitrite were read at 540 nm using a spectrophotometer.

Measurement of Nitrate

The values of Nitrate were obtained by subtracting the values of Nitrite from total NO.

Calculation of Results

A standard curve was drawn by plotting the NO metabolite concentration of the standards on the horizontal axis and the corresponding absorbance on the vertical axis. The absorbance for each sample was located on the vertical axis and read off the corresponding NO metabolite concentration on the horizontal axis. The results were subjected to statistical analysis.

Statistical Analysis

The obtained values were tabulated and subjected to statistical analysis. The results were given as mean & standard deviation values. One way ANOVA (Analysis of variance) test was done for multiple group comparisons followed by post-hoc Tukey's test for group-wise comparison. Changes in clinical and salivary NO parameters for each group were tested by paired't' test. Unpaired-'t' test was done to compare post-operative changes in Group II and group III. The statistical analysis was performed by using STATISTICA software. $p \leq 0.05$ was considered statistically significant, $p < 0.001$ was considered statistically highly significant and $p > 0.05$ was considered statistically non-significant.

RESULTS

The PI, SBI, PD and CAL values between Group I, II and III were statistically significant at baseline as well as at 12 weeks post-operative. Post-hoc Tukey's test comparing the mean difference in the clinical parameters at baseline was significant between Group I & II and I & III, but not significant between Group II & III as the inclusion criteria is the same for both the groups. The Post-hoc Tukey's test at 12 weeks post-operative showed statistically significant mean difference between Groups I, II & III. The results of paired 't'-test comparing the values of clinical parameters at baseline and 12 weeks post-operative for group II and III showed statistically significant results (p value <0.05). Results of independent sample 't'-Test (Unpaired 't'-test) comparing the mean values of clinical parameters for Group II and Group III at the end of 12 weeks was statistically significant ($p<0.01$).

NO Metabolite Values

There was a statistically significant Post-hoc Tukey's test comparing the mean difference in NO metabolite values at

Table 1. Correlation between nitrate levels and clinical parameters of study groups at baseline

Clinical Parameters	Group I		Group II		Group III	
	'r'	p Value	'r'	p Value	'r'	p Value
PI	0.1261	0.6180	0.6091	0.0070	0.1521	0.5470
SBI	0.3327	0.1770	0.5796	0.0120	0.2213	0.3770
PD	0.0297	0.9070	0.7099	0.0010	0.1805	0.4740
CAL	0.1706	0.4990	0.6760	0.0020	0.2501	0.3170

Pearson's coefficient of correlation. p value: p>0.05= NS (Not Significant) p≤0.05= S (Significant), p<0.001= HS (Highly Significant)

Table 2. Correlation between nitrate levels and clinical parameters of study groups at 12 weeks time intervals

Clinical Parameters	Group I		Group II		Group III	
	'r'	p Value	'r'	p Value	'r'	p Value
PI	0.1158	0.6470	0.3071	0.2150	0.0672	0.7910
SBI	0.2851	0.2510	0.5747	0.0130	0.2168	0.3880
PD	0.1269	0.6160	0.4202	0.0830	0.1409	0.5770
CAL	0.0932	0.7130	0.6945	0.0010	0.2339	0.3500

Pearson's coefficient of correlation. p value: p>0.05= NS (Not Significant) p≤0.05= S (Significant), p<0.001= HS (Highly Significant)

Table 3. Correlation between nitrite levels and clinical parameters of study groups at baseline

Clinical Parameters	Group I		Group II		Group III	
	'r'	p Value	'r'	p Value	'r'	p Value
PI	0.2046	0.4150	0.1290	0.6100	0.3513	0.1530
SBI	0.2450	0.3270	0.0415	0.8700	0.0709	0.7800
PD	0.1216	0.6310	0.1814	0.4710	0.4791	0.0440
CAL	0.1494	0.5540	0.0977	0.7000	0.6154	0.0070

Pearson's coefficient of correlation. p value: p>0.05= NS (Not Significant) p≤0.05= S (Significant), p<0.001= HS (Highly Significant)

Table 4. Correlation between nitrite levels and clinical parameters of study groups at 12 weeks time intervals

Clinical Parameters	Group I		Group II		Group III	
	'r'	p Value	'r'	p Value	'r'	p Value
PI	0.0988	0.6960	0.2658	0.2860	0.2898	0.2430
SBI	0.1566	0.5350	0.0387	0.8790	0.1414	0.5760
PD	0.0220	0.9310	0.2023	0.4210	0.6920	0.0010
CAL	0.1316	0.6030	0.0591	0.8160	0.6591	0.0030

Pearson's coefficient of correlation. p value: p>0.05= NS (Not Significant) p≤0.05= S (Significant), p<0.001= HS (Highly Significant)

Table 5. Correlation between nitric oxide levels and clinical parameters of study groups at baseline

Clinical Parameters	Group I		Group II		Group III	
	'r'	p Value	'r'	p Value	'r'	p Value
PI	0.1754	0.4860	0.0714	0.7780	0.1948	0.4390
SBI	0.3415	0.1660	0.0735	0.7720	0.1139	0.6530
PD	0.0256	0.9200	0.3353	0.1740	0.3006	0.2250
CAL	0.1693	0.5020	0.1212	0.6320	0.5154	0.0290

Pearson's coefficient of correlation. p value: p>0.05= NS (Not Significant) p≤0.05= S (Significant), p<0.001= HS (Highly Significant)

Table 6. Correlation between nitric oxide levels and clinical parameters of study groups at 12 weeks time intervals

Clinical Parameters	Group I		Group II		Group III	
	'r'	p Value	'r'	p Value	'r'	p Value
PI	0.1282	0.6120	0.0846	0.7390	0.2492	0.3190
SBI	0.2823	0.2560	0.2205	0.3790	0.0179	0.9440
PD	0.0905	0.7210	0.3517	0.1520	0.5869	0.0100
CAL	0.1233	0.6260	0.2563	0.3050	0.6005	0.0080

Pearson's coefficient of correlation. p value: p>0.05= NS (Not Significant) p≤0.05= S (Significant), p<0.001= HS (Highly Significant)

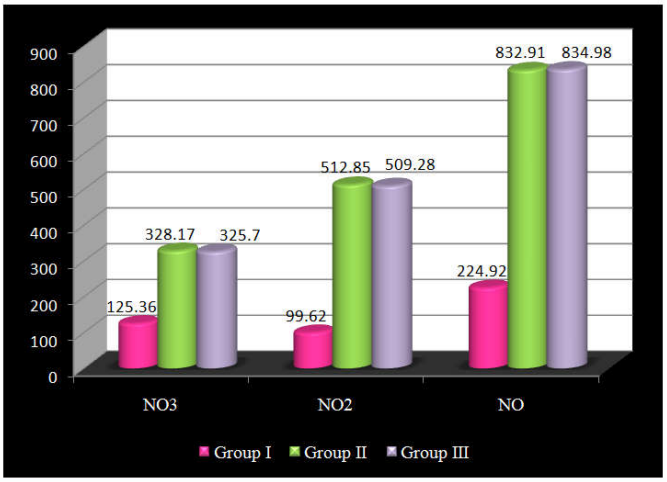
baseline between Group I & II and I & III were statistically highly significant (p value = 0.001) but between group II & III it was not significant (p value = 0.9894). At 12 weeks post-operative, there was significant difference between the 3 groups. The correlation between NO, NO_2 and NO_3 levels and clinical parameters was analyzed at baseline and at 12 weeks after the periodontal therapy. There was a positive correlation between the periodontal clinical parameters and Nitrate (Table: 1 & 2), Nitrite (Table: 3 & 4) and NO levels (Table: 5 & 6) at baseline and at 12 weeks post-operative for Group II & III.

DISCUSSION

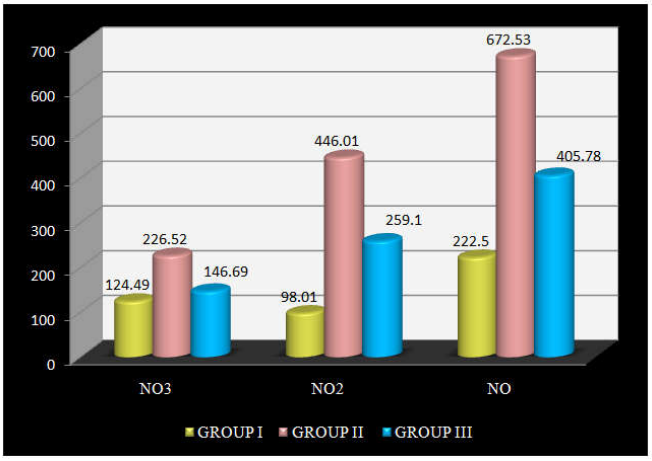
Periodontitis is a chronic inflammatory reaction of periodontal tissues which occurs in response to infection caused by a

specific group of bacteria causing destruction of the connective tissue and alveolar bone bringing about premature loss of tooth consequently leading to a great socio- economic burden on individuals. Recent studies have suggested that NO has both direct and indirect effects on biological tissues. Direct effects include the chemical reactions on its biological target whereas the indirect effects involve the actions of their metabolites and the reactive nitrogen species (RNS).

Graph 1. Comparison of no metabolites between different groups at baseline



Graph 2. Comparison of no metabolites between different groups at 12 weeks post-op



Graph 2. Comparison of no metabolites between different groups at 12 weeks post-op

Several literatures have been documented regarding the relationship of NO with periodontitis (Han, 2013; Matejka, 1998; Parwani, 2012; Batista, 2002; Lappin, 2000; Reher, 2007; Miozza, 2010; Shaker, 2013 and Jagadish, 2014), but the effect of periodontal treatment on the stable metabolites of NO are very few. Hence this study was purposed to determine the levels of salivary NO metabolites in controls and chronic periodontitis patients its correlation with the periodontal clinical parameters and the effects of periodontal treatment on salivary NO metabolites in CP patients. Saliva was used as a tool to measure NO metabolites as it is a simple and non-invasive procedure which gives the levels of NO produced in the local environment. Also in a study done by Bejeh-Mir *et al*[1] saliva was found to be a more sensitive diagnostic tool to determine the NO metabolites when compared to GCF. The periodontal clinical parameters for Group II and III decreased significantly after SRP and flap surgery respectively when compared to the baseline values. It was also found out that there was a significant reduction in the clinical parameters at the end of 12 weeks in Group III as compared to Group II ($p<0.05$). This suggests that patients who underwent open flap debridement resulted in a harmonious relationship between the hard and soft tissues due to the reduction of the inflammation which created an oral environment conducive for plaque control. An important clinical outcome of periodontal treatment is gain in CAL. In a study done by Kendall *et al*[7] it was proved that iNOS was strongly expressed in periodontal sites with CAL of $\geq$ 6 mm and that enhancement of NO production via the iNOS pathway in periodontal lesions resulted in the progress of periodontitis. There are various studies that support the fact that NO is increased in diseased periodontal tissues (Kendall, 2011; Batista *et al*., 2002; Lappin, 2000 and Garrett, 1990).

Another study by Reher (Reher, 2007) revealed that periodontal disease and its severity are related to salivary nitrite concentration. Recently, a study showed that subjects with periodontitis had significantly higher nitrite in serum than healthy subjects (Menaka, 2009). In the present study we found out that salivary Nitrate, Nitrite and NO values were significantly higher in Group II and Group III patients who had compromised periodontal conditions when compared to Group I. This is in contrast with the study done by Topcu *et al* (Topcu Ali, 2004), who described that salivary NO metabolite levels did not differ significantly among healthy, gingivitis and periodontitis groups. The NO metabolite values of both Group II and III were reduced after periodontal therapy on analysis after 12 weeks. On comparing the NO metabolite levels between Group II and III, it was inferred that the values of Group III were reduced to a greater extent when compared to its pre-test levels, which in turn was a significant decrease when compared to that of Group II. The adjusted mean scores of NO metabolites at the end of 12 weeks taking the pre-test values as a covariate was significantly reduced in Group III when compared to Group II $p<0.01$.

It has been shown that in the oral cavity, salivary nitrate will come in contact with bacteria that are capable of rapidly reducing nitrate to nitrite as part of their respiration. Areas of low oxygen tension encourage the reduction of nitrate. The ability of the oral cavity to reduce nitrate is known to vary widely among individuals. The product of nitrate respiration by bacteria is nitrite.[12] It was observed that the levels of Nitrite when compared to the Nitrate values in group I was found to be less which explains the fact that salivary Nitrite is produced as result of action of bacterial reductase on salivary Nitrate. This is in accordance with the study performed by Bejeh-Mir (Poorsattar Bejeh-Mir, 2014). Recently a study has employed ozone-based reductive chemiluminescence to compare nitrite concentration in the saliva of periodontal disease (PD) and healthy individuals or in the various blood compartments of the same individuals before and after periodontal treatment. They concluded that patients with periodontitis had lower nitrite concentration in whole saliva, and this situation remained unchanged after periodontal treatment. Nevertheless, erythrocytes and whole blood nitrite levels diminished after periodontal treatment (Menaka, 2009). In the present study, there was a positive correlation between levels of NO metabolites and the clinical parameters namely PI, SBI, PD and CAL. Similar findings were seen in study done by

Andrukhov *et al* (Meschiari, 2015) and Han *et al*. (Han, 2013). This study offers validation that NO synthesis is increased in periodontal disease. It has also been substantiated that NO metabolites plays an imperative role in the pathogenesis of periodontitis, either directly or indirectly by modulating the production of other pro-inflammatory cytokines. There is a linear increase in the levels of NO metabolites with periodontal clinical parameters. However, we have detected that surgical periodontal therapy has reduced the levels of NO metabolites to a greater level when compared to non-surgical therapy. The reason for the significant decrease after 12 weeks in patients who underwent only non- surgical periodontal therapy can also be attributed to various unexplained host interactions along with the removal of the etiologic factors which had eliminated the inflammation.

As this study was only conducted for a time period of 12 weeks, further studies should be done with longer time intervals to observe if the levels of NO metabolites are changing with the non-surgical periodontal therapy alone. Also during the experimental period, changes in salivary NO levels associated with generic factors such as stress, diet, life-style, hygiene or exercise, were assumed to be equivalent between the treatment and control groups, a limitation of the study. The present study strongly suggests that though both non-surgical and surgical periodontal therapy were effective in arresting the progression of periodontal disease, surgical periodontal treatment was more proficient in reducing the overall inflammation and inclined to bring about more attachment gain and pocket reduction as compared to non-surgical periodontal treatment alone. Further prospective clinical trials should be conducted with larger sample size to confirm our findings, as well as to better understand the mechanisms by which NO can modulate periodontal disease. Despite various published papers till date on NO, this minuscule molecule still holds many mysteries yet to be discovered.

Conclusion

Data from this study connotes the following:

- Salivary NO Metabolite levels were significantly higher in chronic periodontitis patients (Group II and Group III) as compared to periodontally healthy controls (Group I) ($p<0.001$). These results are consistent with existing literature.
- Salivary NO Metabolite levels in chronic periodontitis patients were positively correlated to the periodontal disease severity and activity.
- Both non-surgical and surgical periodontal treatment were effective in improving the clinical parameters and Salivary NO Metabolite levels significantly ($p<0.001$).
- In chronic periodontitis patients, surgical periodontal treatment led to significantly greater reduction in Salivary NO Metabolite levels as compared to when only non-surgical periodontal treatment was performed ($p<0.001$).

To conclude, our study shows that surgical periodontal therapy was more effective in reducing periodontal inflammation than non-surgical periodontal treatment alone. Further longitudinal studies including larger sample size should be carried out to validate the impact of periodontal disease and various treatment options either locally or systemically to reduce the production of these NO metabolites.

**- Mersilk (4-0), Ethicon, Johnson & Johnson Ltd. India.*
†- Coe-Pak, GC America, U.S.A.

§- Nitrate/Nitrite Colorimetric Assay Kit, Cayman Chemicals Ellsworth Ann Arbor, MI, U.S.A.

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