Estimation of Levels of Non-Enzymatic Antioxidants In Saliva of Chronic Periodontitis Patients with Type 2 Diabetes Mellitus Before And After Phase 1 Periodontal Therapy- A Prospective Follow Up Study

Dr Seba Abraham (Professor and HOD), Dr Aparna M Nair (Final Year MDS)  
Dept Of Periodontics, PMS College Of Dental Science Trivandrum

I. Introduction

Periodontitis is one of the most common oral infections in human beings and it is also cited as one of the most significant reasons for tooth loss in adults. Investigations have displayed an increased risk of periodontitis in diabetic patients and this is because of the sheer number of cellular and molecular adaptations taking place in the periodontium as a sequel to prolonged hyperglycemia. The imbalance between periodontal tissue destruction and repair concerning the host response to bacterial challenge results in periodontitis. Polymorphonuclear Neutrophils (PMNs) functions as the primary host defense against periodontal pathogens. They produce and intensify the concentration of Reactive oxygen species (ROS) molecules which are adept in instigating periodontal tissue destruction. Reactive oxygen species (ROS), including hydrogen peroxide, superoxide anion, and the hydroxyl radical, and reactive nitrogen species (RNS), including peroxynitrite, nitric oxide(NO), are common offshoots formed by the typical aerobic metabolism of oral cavity cells or by the inhalation of oxidizing agents in tobacco smoke and additional air pollutants.

Diabetes mellitus (DM) includes a varied group of conditions characterized by altered glucose tolerance and damaged lipid and carbohydrate metabolism. Hyperglycemia is often referred to as a trademark of Diabetes mellitus. Hyperglycemia prompts oxidative stress, which functions as an imbalance between the creation of reactive oxygen species or free radicals and the antioxidant defense mechanisms existing in biological systems. Oxidative stress is validated by an escalation in the reactive oxygen species levels and a fall in antioxidant defenses. Several researchers, in their epidemiological, experimental, and clinical examination have testified that the pervasiveness and severity of periodontal diseases is considerably greater among diabetics than non-diabetics and their bidirectional relationship. High glucose (HG) can induce the production of superoxide and hydrogen peroxide, the predecessors of reactive free radicals, which are competent enough to kindle the deterioration of antioxidant systems, by directly damaging many biomolecules, intensify lipid peroxidation and advance the insulin resistance in diabetes. These free radicals are generated in organelles like cell membrane mitochondria, nucleus, lysosomes, peroxisomes, endoplasmic reticulum and cytoplasm. Reactive free radicals are highly potent enough to create chemical modifications and thereby damage proteins, lipids, carbohydrates and nucleotides in the tissues. The most common method for measuring free radical activity is to analyze the level of the end products of lipid peroxidation such as hydrogen peroxide, peroxyxinitrite, singlet oxygen peroxynitrosic acid and peroxyl nitrite. Periodontitis has been acknowledged as the sixth complication of diabetes and its occurrence in type 2 diabetic patients is more than twice often been remarked that oxidative stress is a shared factor in periodontal disease and type 2 diabetes, and that the disparity in redox control resulting independently that of non-diabetic patients. Diabetic patients demonstrate an increased severity of disease. It has from these disease states acts synergistically and augments the biochemical and clinical sequence of these diseases in a bidirectional manner.

All organisms retain an antioxidant defense mechanism against the detrimental effects of ROS. Antioxidants are classified as “those substances that are present at low concentrations, when related with those of an oxidisable substrate, which will considerably suspend or hinder oxidation of that substrate.” In normal physiology there is a vibrant balance between ROS activity and antioxidant defense capacity and when this balance changes in favor of ROS, either by a fall in antioxidant defense or a surge in ROS production or activity, it ensues in oxidative stress. Oxidative stress was described as ‘a disruption in the pro-oxidant antioxidant equilibrium in favor of the former, leading to probable impairment.’ The fluctuations in the host response as a result of several diseases can affect the supposed severity and prognosis of periodontitis.

Saliva contains a wide range of antioxidants including urate, ascorbate, reduced glutathione, oxidized glutathione and others. These antioxidants work in unison, and the over-all antioxidant capacity may be the most pertinent factor for evaluating the defense ability. It has been acknowledged that saliva functions as a faithful mirror of the body’s health, as it consists of proteins, hormones, antibodies, and other molecules that are regularly analyzed in the regular blood tests to effectively screen health and disease.
The complex and multifaceted relationship between DM, periodontal disease, and salivary antioxidant status has not been effectively elucidated. Oxidative stress can be closely examined by inflammatory indicators like salivary antioxidants, reactive nitrogen species and reactive oxygen species. They play a very important role in the pathogenesis of periodontitis\textsuperscript{29}. However, this mechanism is not coherently explicated in the case of diabetic patients with protracted periodontitis. We have theorized that the diabetic state would lessen the salivary antioxidants. There will also be an increase in lipid peroxidation products. Furthermore, this antioxidant deficiency may aid in analysing the crucial link between Type 2 Diabetes Mellitus and inflammatory periodontal disease. There is sufficient confirmation to hint that there is indeed a change in the levels of salivary antioxidants, ROS, RNS molecules in non-diabetics and chronic periodontitis. Nevertheless, there is a lack of coherent proof regarding the effect of SRP on salivary antioxidants (urate, ascorbate) and reactive nitrogen species (peroxynitrate) in Chronic periodontitis (CP) patients with DM. To the best of our knowledge there is no exhaustive study till date that compares the salivary levels of antioxidant and reactive nitrogen species (peroxynitrate) in CP patients with Type 2DM before and after Non-surgical periodontal therapy. Therefore, the present study is projected to consider the salivary antioxidant (urate, ascorbate) level and peroxynitrite level in Chronic periodontitis patients with Type 2 DM before and after Scaling And Root planning.

II. Material And Methods

Study Population:
Subjects taken for the study comprised both male and female Type2 controlled Diabetic patients who reported to the Department of periodontics, PMS College Of Dental Science. The subjects were drawn from a wide age spectrum of 30 to 60 years. Subjects were carefully chosen according to the specific criteria given by AAP and ADA. Patients were excluded if they suffered from any other systemic disease other than diabetes mellitus, uncontrolled diabetes mellitus, or had endured oral prophylaxis in the last 6 months, and those with aggressive periodontitis. Similarly, subjects with a history of antibiotic treatment within the time frame of the preceding three months or those with smoking history were also excluded. Subjects were enrolled from May 2014 to May 2015. The study was piloted in full agreement with the ethical principles, of the World Medical Association Declaration of Helsinki. The patients were given concise and brief information about the procedure before the initiation of the procedure and their written consent was obtained. Prior to treatment, patients were given guidelines on oral hygiene. The study was done in the morning between 9am to 11:00 am. Salivary, Ascorbate, peroxynitrite, and urate were analyzed spectrophotometrically before scaling & root planning, 1 month, 3 months after scaling & root planning.

III. Results

Table 1: Comparison of ascorbate in saliva of chronic periodontitis patients with Type 2 DM before & after phase 1 therapy

<table>
<thead>
<tr>
<th>Ascorbate</th>
<th>Mean</th>
<th>SD</th>
<th>N</th>
<th>F#</th>
<th>p</th>
<th>Pair</th>
<th>Mean Diff.</th>
<th>p $</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline (A)</td>
<td>0.20</td>
<td>0.05</td>
<td>30</td>
<td></td>
<td>0.000</td>
<td>A &amp; B</td>
<td>0.07*</td>
<td>0.000</td>
</tr>
<tr>
<td>1st month (B)</td>
<td>0.27</td>
<td>0.04</td>
<td>30</td>
<td>553.07**</td>
<td>0.000</td>
<td>A &amp; C</td>
<td>0.12*</td>
<td>0.000</td>
</tr>
<tr>
<td>3rd month (C)</td>
<td>0.33</td>
<td>0.04</td>
<td>30</td>
<td></td>
<td>0.000</td>
<td>B &amp; C</td>
<td>0.06*</td>
<td>0.000</td>
</tr>
</tbody>
</table>

* - Significant at 0.01 level, * - Significant at 0.05 level
#One-Way Repeated Measures ANOVA (Sphericity Assumed)
$ - Pair wise multiple comparison with Bonferroni Correction

Graph 1: Comparison of ascorbate in saliva of chronic periodontitis patients with Type 2 DM before & after phase 1 therapy

Mean ascorbate levels showed statistically significant (P < 0.001) difference between before and after treatment. Average ascorbate was increased from 0.20 mg/dl to 0.27mg/dl after treatment in first month follow up which was again increased to 0.33 mg/dl in the 3 month follow up.

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**Table 2:** Comparison of urate in saliva of chronic periodontitis patients with Type 2 DM before & after phase 1 therapy

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SD</th>
<th>N</th>
<th>F#</th>
<th>P</th>
<th>Pair</th>
<th>Mean Diff.</th>
<th>p $</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline (A)</td>
<td>0.19</td>
<td>0.12</td>
<td>30</td>
<td></td>
<td>0.000</td>
<td>A &amp; B</td>
<td>0.07*</td>
<td>0.000</td>
</tr>
<tr>
<td>1st month (B)</td>
<td>0.27</td>
<td>0.13</td>
<td>30</td>
<td>169.45**</td>
<td>0.000</td>
<td>A &amp; C</td>
<td>0.17*</td>
<td>0.000</td>
</tr>
<tr>
<td>3rd month (C)</td>
<td>0.36</td>
<td>0.11</td>
<td>30</td>
<td></td>
<td></td>
<td>B &amp; C</td>
<td>0.09*</td>
<td>0.000</td>
</tr>
</tbody>
</table>

**: - Significant at 0.01 level, *: - Significant at 0.05 level
#One-Way Repeated Measures ANOVA (Sphericity Assumed)
$: Pair wise multiple comparison with Bonferroni. Correction

**Graph 2:** Comparison of urate in saliva of chronic periodontitis patients with Type 2 DM before & after phase 1 therapy

Mean urate showed significant (P < 0.001) difference between before and after treatment. Average urate was increased from 0.19 mg/dl to 0.27 mg/dl after treatment in first month follow up which was again increased to 0.36 mg/dl in the 3 month follow up.

**Table 3:** Comparison of peroxynitrite in saliva of chronic periodontitis patients with Type 2 DM before & after phase 1 therapy

<table>
<thead>
<tr>
<th>Peroxynitrite</th>
<th>Mean</th>
<th>SD</th>
<th>N</th>
<th>F#</th>
<th>p</th>
<th>Pair</th>
<th>Mean Diff.</th>
<th>p $</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline (A)</td>
<td>1019.6</td>
<td>161.9</td>
<td>30</td>
<td></td>
<td>0.000</td>
<td>A &amp; B</td>
<td>52.07*</td>
<td>0.001</td>
</tr>
<tr>
<td>1st month (B)</td>
<td>967.6</td>
<td>130.1</td>
<td>30</td>
<td>31.22**</td>
<td>0.000</td>
<td>A &amp; C</td>
<td>95.33*</td>
<td>0.000</td>
</tr>
<tr>
<td>3rd month (C)</td>
<td>924.3</td>
<td>120.4</td>
<td>30</td>
<td></td>
<td></td>
<td>B &amp; C</td>
<td>43.27*</td>
<td>0.000</td>
</tr>
</tbody>
</table>

**: - Significant at 0.01 level, *: - Significant at 0.05 level
#One-Way Repeated Measures ANOVA (Sphericity Assumed)
$: Pair wise multiple comparison with Bonferroni. Correction

**Graph 3:** Comparison of peroxynitrite in saliva of chronic periodontitis patients with Type 2 DM before & after phase 1 therapy
Mean peroxynitrite showed statistically significant (P < 0.001) difference between before and after treatment. Average peroxynitrite was reduced from 1019.6 µg/ml to 967.6 µg/ml after treatment in first month follow up which was again reduced to 924.3 µg/ml in the 3 month follow up.

IV. Discussion

Periodontal diseases can be cited as inflammatory diseases which possess multifactorial etiology. The inflammatory and immune reactions to the bacteria and viruses that affect the periodontal and associated tissues invariably influence the systemic circulation and eventually the peripheral systems of the body. This ensues in an intricate bi-directional succession of host - microbial interactions encompassing cellular and humoral aspects and networks of cytokines, chemokines, and growth factors. According to Haffajee et al the principal etiological cause for periodontal disease is specific which consists primarily of gram-negative anaerobic or facultative bacteria. However, the majority of periodontal tissue damage is instigated by an incongruous host reaction to those microorganisms and their products. The timely detection of indicators of tissue destruction can thus be assimilated as a beneficial aid in the diagnosis of periodontal diseases.

Imbalance between reactive oxygen species and antioxidant defense has also been implicated as an etiologic factor for periodontal diseases. It may present itself as an escalation in oxidative stress, a reduction of total antioxidant capacity, or a fall in individual antioxidant level. Chapple and Matthews implicated oxidative stress in the pathogenesis of periodontitis.

Diabetes associated oxidative stress is often perceived a consequence in the production of free radicals or a reduced antioxidative defence capacity. A few studies have assessed the salivary antioxidants in patients with diabetes before nonsurgical therapy, and contradictory reports were stated. So far, no studies have testified the effects of scaling and root planning on salivary levels of ascorbate, urate and peroxynitrite before and after phase 1 therapy in type 2 diabetic chronic periodontitis patients. Imbalances between the levels of free radicals, antioxidants and reactive oxygen species were proposed to play an imperative part in the inception and advance of various inflammatory oral diseases.

The present study was implemented to evaluate the salivary levels of urate, ascorbate and peroxynitrite at baseline, 1 month and 3 months after scaling and root planning. Thirty subjects between the age group of 30 and 60 were carefully chosen according to inclusion criteria. Salivary antioxidants (urate, ascorbate), reactive nitrogen species (peroxynitrite) and periodontal status were evaluated at baseline, 1 month and 3 month after Non-surgical periodontal therapy.

In the present study, there was a significant (P < 0.001) reduction in PI and OHI score from baseline to 1 and 3 months. The mean OHI score at base line was 2.79 which were improved to 1.30 at 1 month and to 1 at 3 months after SRP. The mean PI at base line was 1.94 which reduced to 0.78 at 1 month and 0.62 at 3 months. This is in accordance with the study done by Nada Novokovic et al who testified a reduction in mean PI from 1.39 to 0.36 at 2 months after SPR in chronic periodontitis patients. A significant improvement in OHI score and PI in our study could be ascribed to the level of oral hygiene maintained by the patients knowing that they are involved in a study and might have maintained good oral hygiene (Hawthorne effect) which could have affected the score of the indices.

There is also a significant (P < 0.001) decrease in GI and BOP at 1 month and 3 month when compared to baseline. The mean GI at base line was 2, which was reduced to 0.68 at 1 month and 0.03 at 3 month. BOP was obtained as 1.93 at baseline, which was reduced to 1.08 at 1 month and 0.33 at 3 months. This is comparable to study done by Nada Novokovic et al where he reported a decrease in GI in CP patients from 1.96 to 0.55 and reduction in BP from 1.81 to 0.43 at 2 months after SRP. Non-surgical therapy led to a significant reduction in periodontal tissue inflammation which was identified by alterations in clinical factors in our study. SRP therapy is intended to reduce the disease triggering microbes allied with oral plaque. This may
aid in diminishing the inflammatory indicators. Full mouth SRP might also remove the bulk of calculus and the related microbial flora.

The success of any periodontal treatment is determined by pocket depth reduction and gain in CAL. In the current study a statistically (P < 0.001) substantial reduction in PD and gain in CAL was perceived when compared to the baseline at all-time intervals. However, an increase in PD was detected at 3 month when compared to 1 month in – patients; and this could be attributed to the improper oral hygiene maintenance. The mean pocket depth at base line was 6.1mm and was lowered to 4.9 mm at 1 month and again increased to 5.1 mm at 3 month. The mean CAL at baseline was 6.2mm and 5.9mm and 5.7mm at 1 month and 3 month consequently. This is in concordance with another study conducted by Nada Novokovic et al where he described 1.38mm reduction in mean PD from base line and 0.02 mm gain in mean CAL compared to base line.

Thorough SRP has removed the bacterial burden which resulted in a discernible reduction in inflammation. Complete re-epithelization and healing by long junctional epithelium might have resulted in the reduction in PD and gain in CAL.

A decrease in the concentration of salivary ascorbate levels with an increase in severity of periodontal disease was detected through various investigative studies. The study done by Aziz A S et al reported an increase in plasma ascorbate level from (base line) 27.06 µM/L to 30.99 µM/L at 3 month after SRP. In our study, a dedicated effort was undertaken to compare the salivary ascorbate and urate and peroxynitrite levels in diabetic CP patients before and after SRP was done. Since there is no other study till date, that compares the salivary ascorbate and urate levels in Diabetic CP patients before and after non surgical periodontal therapy, we will be comparing our results with non diabetic CP patients.

In the present research, the mean salivary ascorbate levels at baseline (0.20mg/dl) was increased to 0.27mg/dl at 1 month and to 0.36mg/dl at 3 months after SRP. Thus, it demonstrates a statistically significant (P < 0.001) difference between mean salivary ascorbate levels before and after treatment. The mean difference in the ascorbate levels between baseline and 1 month is 0.07 and between baseline and 3 month is 0.12 and between 1 month and 3 month is 0.06 which indicates a statistically significant (P < 0.001) increase in mean ascorbate level from base line to 1 month, base line to 3 month and 1month to 3 month.

Our research augments the hypothesis that low ascorbate status in diabetes may be due to a higher turnover rate of ascorbic acid, with increased oxidation to its oxidized form dehydroascorbate. Another conceivable mechanism for the impaired ascorbate status is a competitive inhibition between glucose and ascorbic acid, which both share a close structural homology and possibly occupy common membrane transport sites. Yet another possible reason for the decreased ascorbate levels could be ascribed to lower consumption of fruits and vegetables rich in antioxidants or increased usage of antioxidants to counter oxidative stress. Post-SRP therapy had a substantial progress in salivary ascorbate levels signifying lowered oxidative stress and improved antioxidant status. In our study, salivary ascorbate level was lower in Diabetic chronic periodontitis at baseline as compared to 1 month and 3rd month post operatively which agrees with other similar studies carried out in related area.

Vitamin C through its antioxidant action neutralizes oxidative stress and this may result in its reduction in plasma and saliva. It is therefore quite feasible that periodontitis causes lower salivary vitamin C through this mechanism. Human beings lack the ability to synthesise vitamin C due to mutation in the gene coding for L – gluconolactone oxidase, an enzyme that is mandatory for the biosynthesis of VitaminC via the glucuronic acid pathway. Thus, vitamin C is acquired through the diet. This vitamin is especially abundant in fresh fruit and in particular, citrus fruit and vegetables.

In the present study, a significant reduction in concentration of salivary urate was observed at base line and a subsequent increase in urate levels at 1 and 3 months was noticed.

A previous study done by Scully et al also noted a reduction in mean salivary urate levels with increase in severity of periodontitis. In our present study, there is a statistically significant increase in salivary urate level at all time intervals when compared to the base line. The mean urate level at baseline was (0.19mg/dl) which increased to 0.27 mg/dl at 1 month and again increased to 0.36 mg/dl at 3 month after SRP showing statistically significant difference between before and after treatment. The mean difference in the urate levels between baseline and 1 month is 0.07 mg/dl and between baseline and 3 month is 0.17 mg/dl and between 1 month and 3 month is 0.09 mg/dl which indicates there is a statistically significant (P < 0.001) increase in mean urate level from base line to 1 month, base line to 3 month and 1month to 3 month subsequently.

This is in agreement with previous studies done by NadNovokovic et al, Pinar Gumus et al, AditiMaduret et al. SRP is aimed at reducing inflammation. The decreased pathogen load and local inflammation may help in upregulating salivary antioxidants and down regulating reactive nitrogen species as observed in our study. After scaling, when the inflammation had reduced, there was a subsequent increase in uric acid levels.
This is the first study done to compare the levels of salivary peroxynitrite before and after scaling and root planing in diabetic chronic periodontitis patients. There is a statistically significant decrease in salivary peroxynitrite level before and after SRP at 1 month and 3 month. Mean salivary peroxynitrite level at baseline is 1019.6µg/ml which is reduced to 967.6µg/ml at 1 month and again reduced to 924.3µg/ml at 3 month after SRP. The mean difference in the peroxynitrite levels between baseline and 1 month , base line and 3 month and 1 month and 3 month is 52.07µg/ml , 95.33µg/ml and 43.27µg/ml respectively indicating a statistically significant (P < 0.001) reduction in mean peroxynitrite level from base line to 1 month, base line to 3 month and 1 month to 3 month subsequently after phase 1 therapy. To the best of our knowledge no studies have evaluated the salivary peroxynitrite levels in chronic periodontitis patients with or without Diabetes. Hence we compare the results of our study to chronic obstructive pulmonary disease (COPD) and Rheumatoid arthritis (RH) which are inflammatory conditions similar to periodontitis. Kanasawa et al in 2003 assessed the imbalance between levels of nitrogen oxides and peroxynitrite inhibitory activity in chronic obstructive pulmonary disease in which they found increased peroxynitrite activity and increased levels of nitrogen oxides in induced sputum from patients with COPD.  

The study done by Kaur and Halliwell described an elevated level of nitrotyrosine in serum , which is a marker of peroxynitrate production, from patients with Rheumatoid Arthritis. When compared with other similar studies, our research also noticed an elevated level of salivary peroxynitrite – a marker of oxidative stress in diabetic patients with periodontitis. There will be reduction in antioxidants and increase in Reactive nitrogen species resulting in chronic periodontitis. Phase 1 periodontal therapy causes reduction in periodontal pathogens and inflammation and intents to improve host defence by reinstating disturbed balance between antioxidant and reactive oxygen species. This points to an increase in the antioxidants and a decrease in reactive nitrogen species which are clearly evident from our study. Levels of peroxynitrite directly correlate with disease activity and hence can be considered as a marker for diabetic patients with periodontitis.

The collection of salivary components is a simple, non-invasive procedure that can be executed without a high level of technical proficiency. In addition, salivary samples can be effortlessly frozen and directed to laboratories for the examination of biomarkers. The analysis of saliva can also offer a cost-efficient method for the screening of large populations, and may denote asubstitutetechneque for patients in whom blood drawing is difficult, or when compliance is an issue. Saliva is predominantly useful for qualitative rather than quantitative diagnosis, which makes it essential for evaluating the efficiency during the maintenance phase. Stimulation of saliva results in elevated flow of GCF, thereby increasing salivary antioxidants level. Therefore, unstimulated whole-saliva samples were used for the present study.

One of the main drawbacks of our study is the lack of a definitive control group that would have helped us to conclude whether the levels of salivary antioxidants are categorically interrelated to the diabetic status, independently of the clinical periodontal situation. This was a short term study and we recommend further longitudinal studies with large sample size to establish the findings of the present study and to appraise the efficaciousness of periodontal therapy in various systemic diseases associated with periodontitis.

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