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Estimation Of Tissue Destruction In Smokers & Non-Smokers With Chronic Periodontitis Through Salivary Biomarkers – A Spectrophotometric Analysis

Indranil Sarkar[1], Sreelakshmi Sekhar[2], Annaji Sreedhara[3]
[1]Assistant Professor, [2]Private Practitioner. [3]Prof. & HOD
[1]Dept. of Dentistry, Institute of Post Graduate Medical Education & Research, Kolkata
[2]D706 Antarctica, Casa Rio, Lodha Palava City, Dombivli, Thane, Maharashtra.
[3]Dept. of Dentistry, PES Institute of Medical Science and Research, Kuppam, Andhra Pradesh.

***Corresponding Author:**
Dr. Indranil Sarkar
Assistant Professor, Dept. of Dentistry, Institute of Post Graduate Medical Education & Research, Kolkata

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Abstract

Background

Periodontal disease is initiated by colonization of specific bacteria, causing abnormal host response through reactive oxygen species (ROS) and excess proteolytic enzymes leading to host tissue destruction. Smoking is responsible for increased prevalence of periodontal diseases which enhance the damage to lipids through lipid peroxidation, contributing to carcinogenesis.

Objectives

To evaluate the increased tissue destruction in smokers with chronic periodontitis through estimation of biomarkers, malondialdehyde (MDA) and salivary total sialic acid (TSA) as an indicator of oxidative stress.

Materials and methods

50 subjects with chronic periodontitis were recruited. The subjects were divided into 2 groups: (i) Control group (non-smokers) 25 subjects (ii) Case group (smokers) 25 subjects. The demographic characteristics of the patients, plaque index, gingival index, pocket depth, clinical attachment level was initially recorded. Saliva samples were collected & analysed for salivary lipid peroxidation & salivary sialic acid levels

Results

Amongst the control group, the mean salivary MDA level was 0.65± 0.14µM/L and TSA level was 15.615± 3.74 mg/dl. Among the case group, the same values were 1.20± 0.27µM/L and 26.59± 3.82 mg/dl respectively. On comparison it was found that increased salivary TSA is associated with Lipid peroxidation in smokers.

Conclusion

Free sialic acid an end product of hydrolysis of mucin by ROS in smokers causes lipid peroxidation and destruction of periodontal tissues. Increased salivary TSA and MDA in smokers with periodontitis might be related to ROS associated periodontal tissue destruction and can be considered an important salivary biomarker for periodontal diseases.

Keywords: Bio-markers, Lipid peroxidation, Malondialdehyde, Periodontitis, Smokers, Total sialic acid

Introduction

Periodontal diseases are one of the most common chronic multi factorial diseases affecting the population world-wide. Several contributory factors include, microbial challenge from *Porphyromonas gingivalis* (Pg) and *Aggregatibacter actinomycetemcomitans* (Aa), genetic factors, host immune-inflammatory factors, environmental and

acquired factors; all of which mediate the periodontal tissue destruction.

Periodontal disease is initiated by the colonization of specific bacteria and their products on the gingiva which causes abnormal host response, involving the release of reactive oxygen species (ROS) and excess proteolytic enzymes which lead to increased release of biomarkers and damage to host tissue [1]. Periodontal tissue damage from free radical production is related to low antioxidant (AO) capacity caused by several factors such as smoking and/or poor nutritional status [2]. Low antioxidant (AO) capacity leads to oxidative stress induced salivary lipid peroxidation and can be evaluated via malondialdehyde (MDA) levels. Total sialic acid (TSA) levels have also been used as a biomarker for human cancer of lung, prostrate, brain, gastrointestinal system and cardiovascular diseases [3] which mainly indicate the host tissue destruction.

Smoking is one of the hazardous environmental risk factor for the increased prevalence of periodontal diseases in the population [4]. It causes chronic reduction of blood flow, altered neutrophil function, increased production of cytokines and growth factors, inhibits fibroblastic growth and attachment leading to decreased collagen production and vascularity [5]. Smoking magnifies the effects of lipid peroxidation and free radicals in periodontal tissues. In addition, gingivitis and periodontitis in smokers leads to consequent decreased AO levels in serum, saliva, gingival tissue and gingival crevicular fluid (GCF) [6].

The study aims to measure and compare the salivary lipid peroxidation (as an end product of oxidative stress) and salivary sialic acid levels in smokers and non-smokers with chronic periodontitis as there is dearth of reports on salivary total sialic acid (TSA) and Malondialdehyde (MDA) levels in smokers and non-smokers and their influence on periodontitis.

Materials And Methods

The study design selected was of a case control study. Out of total 50 subjects recruited from the out-patient department of Periodontics, Coorg Institute of Dental Sciences, Virajpet. 25 subjects were non-smokers & 25 subjects as current smokers diagnosed with chronic periodontitis.

Inclusion Criteria:

1. Subject's age ranging from 35-55 yrs.
2. Subject with a minimum complement of 20 teeth.
3. Subjects who are diagnosed with chronic periodontitis, with at least two non-adjacent sites with probing pocket depths or clinical attachment level of ≥ 5 mm.
4. Case group (smokers with chronic periodontitis. Smokers were defined as who smoked more than 100 cigarettes in life time and are currently smoking since last two years) -25 subjects.
5. Control group (non-smokers with chronic periodontitis, who never smoked more than 100 cigarettes in life time and are not smoking now) - 25 subjects.

Exclusion Criteria:

1. Patients with any other known systemic diseases.
2. Subjects who had undergone any periodontal treatment in the past 6 months.
3. Subjects who have received antibiotics/ anti-inflammatory drugs/ steroids in the past 6 months.
4. Pregnant or lactating women.
5. Subjects who are on anti-oxidant therapy.
6. Subjects who are using any other forms of tobacco other than cigarette form and alcohol users.

The purpose of the study and its benefits were explained to every selected patient, and written informed consent was obtained from those who agreed to participate voluntarily and ethical clearance was obtained from institution's ethical committee (IRB/CIDS/173/2018 dated on 09-08-2018). The procedures followed were in accordance with the prescribed standards of the institutional ethical committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008.

Method Of Collection Of Data

The purpose of the study was explained to every selected patient and written consent obtained. Demographic data like age, sex, history of smoking, address and contact details recorded, the plaque index (Silness and Loe 1964) [7], gingival index (Loe H and Silness J 1963) [8], pocket depth (measured with

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UNC 15 probe, Hu-Friedy) and clinical attachment level (CAL) were recorded initially.

Procedure Of Collection Of Saliva Sample

Unstimulated saliva samples were collected into a sterile plastic container from each subject before breakfast after brushing between 8-9am by rinsing the mouth with distilled water in restful and quiet circumstances. Saliva samples were stored at -20^{0}C until analysis.

Measurement Of Lipid Peroxidation Levels In Saliva

The Lipid peroxidation levels were determined by thiobarbituric acid reactive substances (TBARS) by spectrophotometric assay. Thiobarbituric acid interacts with malondialdehyde and yields a fluorescent product [9]. The amount of fluorescent light is directly proportional to lipid peroxidation levels in the sample. This can be measured by using a light of wavelength ranging from 400-700nm through colorimetric assays. All patients were asked to spit in a sterile test tube and 5 ml of unstimulated saliva were collected [10]. Saliva was centrifuged at 12,000 rpm at a temperature of 4°C for 24 min to obtain a supernatant. TBA was used to assess the MDA content of the saliva by Buege and Aust method [11]. MDA interacts with TBA to form a yellow fluorescent product. A spectrophotometer was used to measure the absorbance of this product at 335 nm.

Measurement Of Sialic Acid Levels In Saliva

The sialic acid levels in saliva were determined using Winzler's Diphenyl amine technique [12]. The technique is based on difference in solubility between proteins and glycoproteins. Proteins were precipitated using chloric acid. Precipitation of Glycoproteins in supernatant were done with phosphorovolframic acid. 0.5 ml of whole saliva sample was taken and 4.5 ml of 0.15 mol/L Sodium Chloride (NaCl) solution and 2.5 ml of 1.8 mol/L Perchloric acid ($HClO_4$) was added. The samples were incubated for 10 min at room temperature and centrifuged at 3000 rpm for 10 minutes at a temperature of 4°C. Supernatant was collected for glycoproteins measurement. Supernatant was dissolved in 1 ml of 10% Sodium Carbonate (Na_2CO_3), 3.5 ml water (H_2O) and 0.5 ml Folin-Ciocalteau reagent. Samples were dyed for a period of 30 min. The absorbance was assessed at the wave length of 750 nm. Protein concentration (C) was calculated according to the following equation C=A*F (A - absorbance; F - calibration coefficient =4.62). To 5 ml of supernatant, 1 ml of 5% Phosphorovolframic acid was added. Samples were centrifuged after 10 minutes of precipitation at 3000 rpm for 10 min at 4°C. The supernatant was discarded at completion of centrifugation. The sediment was dissolved in 1 ml of 10% Sodium carbonate (Na_2CO_3), 3.5 ml of water (H_2O) and 0.5 ml of Folin-Ciocalteau reagent. Samples were dyed after 30 min. The absorbance was assessed at wave length of 750 nm. The absorbance was measured at wave length of 750 nm. Glycoprotein concentration (C) was calculated using the formula; C=Tyr*F. Where "Tyr" is the tyrosine concentration in mg/ml; Tyr in nmol/ml = absorbance (A) × 800, F is calibration coefficient = 23.8.

Statistical Analysis

The continuous variables are described through mean and standard deviation. Unpaired T-test was used to compare the statistically significant difference between two groups with a 95% confidence interval. A "P" value less than 0.05 is considered as statistically significant.

Results:

On comparison between cases and controls, plaque index & gingival index showed statistically significant differences. The gingival inflammation was less in current smokers (cases) compared to non-smokers (controls). (Table -1)

The mean MDA scores were 1.20±0.27 in current smokers and 0.65±0.14 in non-smokers with statistically highly significant difference. Similarly, the mean TSA was 26.59±3.82 and 15.61±3.74 in smokers and non-smokers respectively. The difference in means was statistically highly significant (Table - 2).

Discussion

Tissue damage in periodontal disease results from bacterial toxins. Inflammation is directly related to production of free radicals and inversely to low antioxidant (AO) capacity which is modified by a number of other factors such as smoking and poor nutritional status. Since no previous study was done on salivary MDA and TSA together in smokers and non-smokers with chronic periodontitis, the present

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study was undertaken to measure & compare the salivary lipid peroxidation and salivary sialic acid levels (as an end product of oxidative stress) in smokers & non-smokers with chronic periodontitis.

The present study showed that salivary MDA and TSA levels were increased significantly in current smokers compared to non-smokers. Tobacco smoke contains nearly about four thousand constituents. Nicotine is considered the most pharmacologically active tobacco component responsible for wide variety of systemic diseases.

Studies have reported that nicotine is one of the risk factors in the development of atherosclerosis [13] and periodontitis. In this study, MDA and TSA levels in saliva of smokers were higher than those of non-smokers. These elevated levels of salivary biomarkers are reflected as risk factors for periodontitis in smokers. There are few studies conducted on levels of serum TSA in smokers which reported that serum TSA is generally elevated in smokers [14, 15, 16]. However, reports of previous studies concerning high serum TSA levels in smokers are somewhat controversial. Patel et al. reported that TSA levels were not affected by smoking. [16] whereas, it has been reported that TSA levels are elevated in serum of smokers [17] compared to that of non-smokers. The evidence collected so far demonstrates that smoking increases the formation of endogenous N-nitroso compounds thereby magnifying the risk of exposure by preformed cancerous tobacco products [18]. Serum TSA is used as a bio-marker for various cancers including prostate, colorectal and breast cancers[19, 20].

This study found that there was a significant increase in salivary MDA levels in tobacco smoking group than non-smoking controls. In a study conducted by Guentsch et al. it was noted that lipid peroxidation in the saliva was enhanced in smokers with periodontitis [21-24]. Also, saliva is a suitable source to detect the body's oxidative stress (OS) level [25]. Nicotine along with other compounds in tobacco smoke bring intracellular oxidative stress induced damage to several biologic molecules. Many ROS interceded cellular processes are activated by these compounds [26-28].

In this study, the salivary TSA levels were parallel to MDA levels. Reactive Oxygen species are one of the important coordinators of many inflammatory response [29]. Tobacco smoke induces neutrophils to amplify oxygen free radicals generation [30]. Constant local irritation from tobacco smoke activates chronic inflammation and OS. Moreover, tissue injury can itself generate more oxidants which may worsen the tissue destruction over time [31]. Additionally increase in salivary gland OS causes alteration in salivary secretion leading to qualitative changes in salivary proteins.

Saliva is the first body fluid to come in contact with inhaled cigarette smoke and also is closely related to the gastrointestinal tract. Also, salivary ROS which is increased due to use of tobacco products and mucosal inflammation cause oxidative damage in various cells and organs through systemic circulation.

Increased salivary TSA and MDA levels in smokers might be related to cancers of lung, brain and cardio-vascular diseases. Our findings suggest that there is a closer interaction between inflammatory events and smoking which lead to destruction of periodontal tissues. It has been reported that several systemic diseases, medications as well as mucosal inflammations [32] cause changes in salivary components - which confirms our hypothesis.

Conclusion:

From the results, we can conclude that increased salivary TSA is associated with lipid peroxidation in smokers. Besides inflammatory markers, salivary TSA may also be considered as a substitute for the oxidative stress in tobacco exposure which is found to be in well harmony with MDA.

Therefore, a saliva-based test could be helpful for early detection and monitoring of effects of these habits since saliva is most readily and easily collectable sample. This study suggests that salivary MDA and TSA may be used as useful bio-markers for oxidative stress induced tissue destruction. This study in addition is important for the reason that the findings draw consideration to a significant potential public health hazard. The outcomes of this study should be investigated with further studies in large populations by randomized clinical trials.

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TABLE 1: Comparison of clinical parameters among smokers (case) and non-smokers (control) with chronic periodontitis [HS – Highly Significant, S – Significant, NS – Non-Significant]

	Groups	Mean	Std. Deviation	t value	p value
Plaque index	Case	1.67	0.36	2.507	0.016 S
	Control	1.96	0.46		
Gingival index	Case	1.24	0.65	4.496	<0.001 HS
	Control	1.91	0.35		
Probing pocket	Case	6.52	0.65	0.484	0.631 NS

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depth	Control	6.44	0.50		
Clinical attachment level	Case	6.52	0.65	1.930	0.060 NS
	Control	6.88	0.66		

TABLE 2: Comparison of Malondialdehyde (MDA) and Total Sialic Acid (TSA) levels among smokers (case) and non-smokers (control) with chronic periodontitis [HS – Highly Significant]

	Groups	Mean	Std. Deviation	t value	p value
MDA in µM/L	Case	1.20	0.27	8.887	<0.001 HS
	Control	0.65	0.14		
Sialic Acid in mg/dl	Case	26.59	3.82	10.258	<0.001 HS
	Control	15.61	3.74		

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