ORIGINAL ARTICLE

Assessment of Some Biochemical Oxidative Stress Markers in Male Smokers with Chronic Periodontitis

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Abstract The present study aims to assess and compare the biochemical oxidative stress markers in male smokers and non-smokers with chronic periodontitis. One hundred thirty-four male chronic periodontitis patients and 64 apparently healthy male volunteers were recruited for the study. The periodontal status was evaluated by measuring gingival index, plaque index, papillary bleeding index and clinical attachment loss using UNC-15 probe. The biochemical markers estimated were total antioxidant capacity, RBC-superoxide dismutase, glutathione peroxidase, vitamin C, malondialdehyde and C-reactive protein. The obtained results indicate higher oxidative stress in chronic periodontitis. Smokers with chronic periodontitis show significantly higher periodontal clinical parameters and relatively higher systemic oxidative stress. Vitamin C estimation may be an important biochemical parameter in conjunction with clinical parameters for diagnosis of chronic periodontitis in smokers.

Keywords Chronic periodontitis · Smoking · Oxidative stress · Biochemical oxidative stress markers

Introduction

Extensive experimental evidence indicates that cigarette smoking represents a major preventable cause of human disease. Besides the well recognized systemic effects of cigarette smoke, a variety of oral manifestations have been associated with this habit. Increased odds to develop oral cancer, caries, and periodontal disease and tooth loss have been reported [1]. Tobacco smoking is one of the main risk factor associated with chronic destructive periodontal disease and there is an emerging evidence to suggest that sub-gingival calculus formation is more prevalent and severe in smokers compared to the non-smokers [2]. Smokers are almost three times as likely to have severe periodontitis compared to the non-smokers [3]. Smoking is associated with oxidative stress. Smoke derived oxidants are a major factor in inflammatory reactions to cigarette smoke. These oxidants can alter antioxidant defenses, and can up regulate inflammation by a number of mechanisms leading to extensive oxidative damage [4–6], and therefore a cumulative effect of smoking and chronic periodontitis on oxidative stress markers needs to be addressed. We hypothesize that smoking aggravates the intensity of Periodontitis and exacerbate the oxidative stress in chronic periodontitis compared to the non-smokers. Therefore the present study has been undertaken to

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comparatively assess the oxidative stress markers in chronic periodontitis patients with and without smoking habits.

Materials and Methods

The study was undertaken as per the approval of the Institutional Ethics Committee (Registration number: 391/CPCSEA) of Grant Medical College and Sir J. J. Group of Hospitals, Mumbai, following norms of the World Medical Association of Helsinki; ethical principles for medical research involving human subjects (amended by 55th WMA General Assembly, 2004). A written informed consent was obtained from all the subjects enrolled in the study. The subjects had the right to refuse to participate in the study or to withdraw consent to participate at any time without reprisal.

Study Groups

A total of 134 male patients with chronic periodontitis, 85 of them with smoking habits, who visited the Department of Dentistry, Grant Medical College, Mumbai, and 64 apparently healthy non smoker male volunteers were recruited for the study and divided into the following groups:

Group I   Non-smoker healthy controls; $n = 64$ (mean age 39.64 ± 5.04)
Group II   Non-smokers with chronic periodontitis; $n = 49$ (mean age 41.72 ± 4.36)
Group III Smokers with chronic periodontitis; $n = 85$ (mean age 44.2 ± 5.88)

The patients in the study groups were clinically evaluated for chronic periodontitis according to the criteria of the American Academy of Periodontology (1999) [7]. The patients had minimum of 20 teeth present of which at least 30 % had a site with probing depth 5 mm and clinical attachment level ≥ 3 mm. The patients had poor oral hygiene and the gingiva showed bleeding on probing and had the characteristics of chronic inflammation. The patients were otherwise healthy, with no history of major illness and consumption of antioxidants, antibiotics, anti inflammatory or any other drugs and had not received any periodontal therapy for at least 6 months prior to the inception of the study. Individuals having past illness and undergoing any treatment, diabetics and alcoholics were excluded from the study. The patients in the smoker group were active smokers (predominantly cigarette smokers) with smoking habit for over 3 years and frequency of smoking ≥ 5 cigarettes/day; however smokers with any systemic illness were excluded from the study. The individuals in the control group were from the same

geographical location with apparently good oral and systemic health and without smoking habits.

Clinical Measurements

The periodontal status of all individuals was evaluated by measurement of gingival index (GI) as developed by Loe and Silness [8], plaque index (PI) as described by Silness and Loe [9], papillary bleeding index (PBI) developed by Muhlemann [10] and clinical attachment loss (CAL). CAL is measured on six sites of each tooth (i.e. mesiobuccal, buccal, distobuccal, mesiolingual, lingual and distolingual) and the individual scores were compared on a scale for characterization of periodontitis as slight, moderate or severe [11]. All clinical measurements were evaluated by a single dental professional using University of North Carolina (UNC-15) probe (Hu-Friedy, Chicago).

Sample Collection

A total of 4 ml venous blood was collected in disposable syringe from all the subjects following standard precautionary measures. Of this, 1 ml blood was transferred to sodium heparin vacutianer (VACUETTE) and was used for analysis of total antioxidant capacity (TAOC) RBC-superoxide dismutase (RBC-SOD) and glutathione peroxidase (GPx). The remaining 3 ml of blood was transferred to Serum Sep Clot Activator vacutianer (VACUETTE) and allowed to stand at room temperature for 30 min and centrifuged at 3,000 rpm for 20 min to obtain serum, which was stored at -4 °C until further analysis. The serum was analyzed of Vitamin C, malondialdehyde (MDA) and C-reactive protein (CRP). All the biochemical markers were measured on calibrated semi auto analyzer BIOTRON BTR-830 (Ranbaxy laboratories, India).

Biochemical Studies

The plasma TAOC was measured by the Ferric Reducing Ability of Plasma (FRAP) assay according to the method of Benzie FFI and Stain JJ [12]. The reaction measures antioxidant reduction of Fe^{3+}TPTZ (tripyridyl triazine) to blue colored Fe^{2+}TPTZ. FRAP values were obtained by comparing the absorbance change at 593 nm in test reaction mixture with those containing ferrous ions in known concentration.

The RBC-SOD was measured using the RANSOD kit (Randox Laboratories, UK). The assay principle employs xanthine and xanthine oxidase to generate superoxide radicals which react with 2, 4-idophenyl 3–4 nitrophenol 5-phenyl tetrazolium chloride (INT) to form a red formazan dye. The superoxide activity was then measured by the

degree of this reaction. 1 unit of enzyme activity is that which causes 50 % inhibition of rate of reduction of INT under assay condition. Change in absorbance was recorded at 505 nm [13].

Blood GPx was measured using RANSEL kit (Randox Laboratories, U. K.). GPx catalyses the oxidation of glutathione by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, the oxidized glutathione is immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP$^+$. The decrease in absorbance at 340 nm was measured [14].

The serum vitamin C (ascorbic acid) content was measured using the dinitro-phenyl hydrazine (DNPH) method [15]. In strong acidic medium, ascorbic acid is oxidized to diketogluonic acid which reacts with 2,4 DNPH to form diphenyl hydrazone which dissolves in strong sulphuric acid solution to produce a red colored complex which was measured at 500 nm.

The serum MDA was estimated according to the method of Kei [16]. Lipoproteins were precipitated from the serum by adding trichloro acetic acid. 0.05 M sulphuric acid and 0.67 % thiobarbituric acid (TBA) in 2 M sodium sulphate were added and the coupling of lipid peroxide with TBA was carried out by heating. The resulting chromogen was extracted in *n*-butanol whose absorbance was measured at 530 nm.

The serum CRP was measured by the quantitative latex turbidity method using the CRP-TURBI (Spinreact, Spain). The latex particles coated with specific anti-human CRP was agglutinated when mixed with samples containing CRP. The agglutination causes absorbance change (measured at 540 nm), dependent upon the CRP content of the test sample that can be quantified by comparison from a calibrator of known CRP concentration [17].

Statistical Analysis

The measured values for clinical parameters and biochemical markers were subjected to statistical analysis using Statistical Package for Social Sciences (SPSS version 11.5) for MS Windows. The values on clinical parameters and biochemical markers were expressed as mean ± SD across three study groups. The underlying normality assumption was tested for each clinical and biochemical parameter using percentile to percentile (PP) plot technique before applying any statistical test. Comparison of significance of difference of average of clinical parameters and biochemical markers across the three study groups was done using analysis of variance (ANOVA) technique with Tukey's correction for multiple group comparison. P value < 0.05 is considered to be statistically significant.

Observations and Results

Clinical Measurements

The average clinical measurements listed in Table 1 demonstrates significantly higher levels of clinical parameters ($P < 0.001$) among group II compared to group I. Further the group III showed significantly deranged clinical parameters ($P < 0.001$) compared to group I and group II.

Biochemical Studies

The statistical comparison of biochemical markers estimated between the study groups are shown in Table 2. The markers were found to be significantly different ($P < 0.001$) in group II as compared group I. The average TAOC, GPx and vitamin C were significantly lower ($P < 0.001$) and RBC-SOD, MDA and CRP were significantly higher ($P < 0.001$). Further the group III has shown significantly deranged biochemical markers compared to group I but when compared with group II the extent of derangement did not reach statistical significance (except vitamin C).

Discussion

Increasing evidence points to smoking as a major risk factor for periodontitis, affecting the prevalence, extent and severity of disease [18]. Tobacco smoking has widespread systemic effects many of which may provide mechanism for the increased susceptibility to periodontal diseases. Various factors contribute to the deleterious periodontal effects of smoking, including alterations in both microbial and host response factors. The proposed mechanisms for the negative periodontal effects of smoking may include vascular alterations, altered neutrophil function, decreased IgG production, decreased lymphocyte proliferation, increased prevalence of perio-pathogens, altered fibroblast attachment and functions, difficulty in eliminating pathogens by mechanical therapy and negative local effects on cytokinesis and growth factor production. The balance between destructive cytokines such as IL-1α, IL-1β, TNF-α, IL6, IL8 and prostaglandins and protective cytokines such as TGF-β, IGF and interferons shifts more towards destructive profile in smokers. Also it has been speculated that the interference of smoking with inflammatory signs and symptoms is mediated by vasoconstrictive effects of nicotine [2, 3, 19–21]. The present study has observed that smokers with chronic periodontitis exhibit more clinical periodontal damage and relatively higher oxidative stress compared to non-smokers with chronic periodontitis.

Table 1 Inter group comparison of the clinical parameters

Clinical parameters	Mean ± SD			P* value		
	Group I (n = 64)	Group II (n = 49)	Group III (n = 85)	Group I versus group II	Group I versus group III	Group II versus group III
GI	0.7 ± 0.08	2.4 ± 0.5	1.9 ± 0.5	0.001	0.001	0.001
PI	0.5 ± 0.2	2.3 ± 0.6	3.0 ± 0.5	0.001	0.001	0.001
PBI	0.8 ± 0.5	2.6 ± 0.4	1.7 ± 0.4	0.001	0.001	0.001
CAL (mm)	1.9 ± 0.3	7.7 ± 0.9	8.5 ± 1.0	0.001	0.001	0.021

Values are mean ± SD

* P values are obtained using one way analysis of variance (ANOVA) with Tukey's correction for multiple group comparisons. P value < 0.05 is considered to be statistically significant

Table 2 Inter group comparison of the biochemical markers

Biochemical markers	Mean ± SD			P* value		
	Group I (n = 64)	Group II (n = 49)	Group III (n = 85)	Group I versus group II	Group I versus group III	Group II versus group III
TAOC (μM/L)	925.2 ± 68.4	859.2 ± 78.5	804.3 ± 77.8	0.054	0.001	0.079
RBC-SOD (U/g Hb)	309.9 ± 37.2	530.8 ± 86.5	547.7 ± 69.3	0.001	0.001	0.992
GPX (U/g Hb)	14.3 ± 1.2	8.8 ± 1.4	8.1 ± 1.0	0.001	0.001	0.311
Vit C (μM/l)	39.7 ± 0.06	28.3 ± 0.05	22.7 ± 0.05	0.001	0.001	0.001
MDA (nM/ml)	2.1 ± 0.2	4.3 ± 0.4	4.5 ± 0.5	0.001	0.001	0.536
CRP (mg/l)	1.9 ± 0.3	3.4 ± 0.4	3.5 ± 0.5	0.001	0.001	0.999

Values are mean ± SD

* P values are obtained using one way analysis of variance (ANOVA) with Tukey's correction for multiple group comparisons. P value < 0.05 is considered to be statistically significant

Tymkiw [21] has revealed that smokers exhibited a decrease in several proinflammatory cytokines and chemokines and certain regulations of T cells and NK cells. This reflects the immunosuppressant effects of smoking which may contribute to an enhanced susceptibility to periodontitis [21]. As smoking is associated with vasculature alterations, altered fibroblast attachment and function, an altered clinical observation may be expected. The present study has observed that smokers with chronic periodontitis showed lower GI and PBI and higher PI and CAL as compared to non-smokers with chronic periodontitis. In accordance with our study, various studies [22–24] have observed altered clinical parameters in smokers with chronic periodontitis compared to non-smokers.

Various studies [4, 5, 25] in literature have associated cigarette smoking and oxidative stress, and most of them have observed higher incidences of oxidative stress in smokers. Cigarette smoke contains number of free radicals which are capable of causing an increase in generation of various reactive oxygen species (ROS). These ROS in turn are capable of initiating and promoting oxidative damage. Oxidative stress is also implicated in the pathogenesis of periodontitis. Various studies have associated periodontitis

with compromised antioxidant activities. Studies [26, 27] have reported lower TAOC in saliva, gingival blood and peripheral blood of subjects with chronic periodontitis as compared to healthy control group as observed in our study. Al Bayaty et al. [28] has observed lowered antioxidant in smokers as compared to non-smokers. A greater reduction of total antioxidant activity in smokers may be due to increased oxidants induced by smoking [29]. The above findings support our observations.

Antioxidant enzymes SOD and GPx provide protection within the cell against ROS. SOD removes damaging ROS from cellular environment by catalyzing dismutation of two superoxide radicals to H_2O_2 and GPx reduces H_2O_2 by oxidation of reduced glutathione. Various studies [30, 31] have observed higher SOD activity in chronic periodontitis group than in controls which is in accordance with our study. Smoking is associated with altered expression and activity of antioxidant enzymes. According to the studies [32, 33] cigarette smoking is associated with a significant decrease in the level of SOD, however some other studies [34, 35] have documented that compared to non smoker controls the *bidi* smokers have shown increased erythrocyte SOD activity. We have observed a relatively higher SOD

activity in smokers compared to non-smokers. In the present study, lower GPx activity in chronic periodontitis group is observed than in controls. Similar findings were reported by studies [26, 32]. Guentsch et al. [36] have showed that periodontally healthy subjects demonstrated significantly lower levels of GP_x than the periodontitis group including smokers. However studies [34, 37] have revealed that compared to non smoker controls the *bidi* smokers have shown decreased GPx activity, which is in accordance with our study.

Vitamin C is a low molecular weight, water soluble antioxidant. It has protective effect on maintaining tissue homeostasis by playing important role in collagen synthesis and therefore helps in maintenance of structural integrity of the connective tissue. Vitamin C also has a beneficial role as radical scavenger [38]. Vitamin C, through its antioxidant action, neutralizes oxidative stress, and in doing so may be depleted in plasma. It is therefore quite possible that periodontitis causes lower plasma vitamin C through this mechanism [39]. In this study serum vitamin C level was lower in chronic periodontitis as compared to healthy controls, which was also documented by [40, 41].Smokers are constantly over exposed to free radicals through inhalation of long lived carbon and oxygen centered radicals that subsequently deplete the plasma antioxidant vitamin C. Similar to our study, studies [42, 43] have reported lower vitamin C levels in smokers than in non-smokers.

MDA is one of the most frequently used indicator of lipid peroxidation and may be a potential biomarker indicating oxidative stress [44]. Our finding indicates higher serum MDA level in chronic periodontitis as compared to the healthy controls. Many researchers have also reported higher MDA levels in chronic periodontitis [45, 46]. Smoking may enhance the effect of ROS in periodontitis, thereby increasing the tissue destruction resulting from oxidative stress, which may lead to higher lipid peroxidation. Increased circulating products of lipid peroxidation (F_2-isoprostanes) in smokers have been documented and observed that smoking causes oxidative modification of biologic compounds in humans [43]. Similar observations of higher MDA among the smokers are also documented by studies [36, 42], which is in accordance to our finding.

CRP is an acute phase protein, which is used as an inflammatory marker. It has been demonstrated by various studies [47–49] that CRP levels are higher in periodontitis patients than in healthy subjects, which support our findings. Mandraffino [37] have observed that smokers show higher levels of CRP, however a non-significant slightly higher value for CRP is observed in smokers than non-smokers [28]. We have observed a non significant difference in the levels of CRP in smokers compared to the non-smokers.

In this study, only male patients were recruited, considering urban Indian social customs with respect to smoking habits. Also their smoking status was based on the verbal autopsy. The non-recruitment of healthy smokers (smokers without periodontitis) puts a limitation for better inter and intra group comparison. Albeit limitations the study demonstrate that smokers with chronic periodontitis showed altered clinical parameters, a relatively higher oxidant and low antioxidant defense compared to non-smokers with chronic periodontitis. Among the biochemical parameters estimated in the study, mean values of all of them were different in smokers than non-smoker group and only vitamin C levels could reach statistical significance, which may be linked to smoking habit. In keeping with the current emerging findings vitamin C may be considered as an important biochemical marker in diagnosis of periodontitis in high risk group like smokers. Clinical causality of smoking as a risk factor cannot be established by this study, being a cross sectional study. We recommend further longitudinal studies to investigate the causality role of smoking for inflammatory damages to periodontium and its effect on systemic biochemical oxidative stress markers.

Conclusion

Patients with chronic periodontitis show higher tendency of oxidative stress which may be due to oxidant: antioxidant imbalance, reflecting higher ROS activity which leads to periodontal inflammation and its pathogenesis. The study also demonstrates that the smokers are at a higher risk of inflammatory damages in periodontium and systemic oxidative stress. Vitamin C estimation may be an important biochemical parameter for diagnosis of chronic periodontitis in smokers. The findings of the study may serve to be a potential clinico-biochemical tool in the better management of the underlying disease and may help in understanding the deleterious effects of smoking with respect to oral health.

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