Quantification and Comparison of the Impact of the Smoking Status on Oral Polymorphonuclear Leukocyte and Malondialdehyde Levels in Individuals with Chronic Periodontitis: A Double-Blinded Longitudinal Interventional Study

Abstract

Background: The formation of reactive oxygen species by oral polymorphonuclear leukocytes (oPMNs) is amplified in smokers with chronic periodontitis (CP) causing tissue damage which can be measured by quantifying levels of malondialdehyde (MDA). Objective: To quantify and compare the impact of smoking status on oPMN and MDA in individuals with CP before and after scaling and root planing (SRP). Materials and Methods: Sixty individuals were divided into four groups, namely, periodontally healthy (Group A), current smokers with CP (Group B), former smokers with CP (Group C), and nonsmokers with CP (Group D). Parameters assessed were bleeding on probing (BOP), gingival index (GI), probing pocket depth (PPD), clinical attachment level, gingival recession, periodontal inflamed surface area, salivary MDA, and oPMN at baseline and 6 and 12 weeks after SRP. Results: Increased PPD ($P = 0.01$) and decreased GI ($P = 0.021$) was noted in Group B as compared to C and D at baseline. Periodontal intervention caused a greater resolution of inflammation in Groups C and D as compared to B as noted from the GI and BOP. A reduction in MDA ($P = 0.074$) was noted in Groups C and D as compared to B, and oPMN levels were higher ($P = 0.009$) in Group C and D as compared to B. Conclusion: Greater periodontal destruction is seen in current smokers than former and nonsmokers with CP. MDA can be considered as a reliable biomarker for oxidative stress as it directly correlates with the oPMN levels.

Keywords: Chronic periodontitis, cigarette smoking, malondialdehyde, oxidative stress, saliva

Introduction

Periodontitis is an inflammatory disease of the tooth supporting structures which is characterized by breakdown of the hard and soft tissues.\(^1\) Smoking, a well-established risk factor for periodontitis, has several detrimental effects on periodontal tissues including chronic reduction of blood flow, inhibition of fibroblast growth and altered neutrophil function.\(^6\) It also stimulates the oxidative burst of polymorphonuclear leukocytes (PMN), increases, in excess, reactive oxygen species (ROS) production, and leads to lipid peroxidation (LPO).\(^7\)\(^8\)

PMN serve as sentinels of innate immunity. An efflux of PMNs into the gingival crevice, which is a critical local host defense event in periodontal infections that effectively mitigates microorganisms by means of generation of ROS and phagocytosis.\(^10\)\(^12\)

While healthy norms have been determined for circulating blood neutrophil counts in order to identify patients with suspected systemic infections, the levels of oral polymorphonuclear leukocytes (oPMN) in oral health and periodontal diseases confounded by smoking has not been described.

ROS are short lived derivatives of oxygen metabolism, causing DNA and protein damage, with a shelf life of $10^{-6}$–$10^{-8}$ s.\(^5\)\(^13\)–\(^15\) Saliva is a true indicator of ongoing destruction occurring in the oral cavity as a whole, and thus can be of diagnostic use as a source of biomarkers for determination of oxidative stress levels.\(^16\) The ROS mediated tissue breakdown can be measured by quantification of malondialdehyde (MDA), which is one of the final decomposition products of LPO.\(^17\)

oPMN recruitment to the gingival sites with release of proteolytic enzymes and ROS

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are considered as important indicators of severe periodontal breakdown in susceptible individuals. However, there exist lacunae in literature regarding the impact of smoking on oPMN levels and also their correlation to MDA levels in saliva. The quantification of oPMNs and the final products of LPO for analysis of oxidative stress can serve as important tools in the study of periodontal disease severity. Furthermore, the role of periodontal interventional therapy on levels of oPMNs and MDA in saliva needs to be explored. Thus, the aim of this study was to quantify and compare the impact of the smoking status on oPMN and MDA in individuals with chronic periodontitis (CP) before and after scaling and root planing (SRP). The role of MDA as a potential biomarker to assess tissue destruction was also assessed.

Materials and Methods

The study was conducted in the Department of Periodontics, Mahatma Gandhi Mission’s Dental College and Hospital. The study protocol was carried out in accordance with the ethical standards outlined in the 1964 Declaration of Helsinki, as revised in 2008, and the protocol was approved by the Institutional Review Board. The duration of the study was from June 2015 to October 2016. The study, along with the risks and benefits, was explained, and a written informed consent was obtained from each patient agreeing to participate in the study.

Individuals meeting the following criteria were recruited for the study: (1) Individuals aged 18–55 years. (2) Individuals diagnosed as having CP with probing pocket depth (PPD) ≥5 mm, clinical attachment level (CAL) ≥4 mm.[18] (3) Individuals who were nonsmokers and periodontally healthy with PPD ≤3 mm and gingival index (GI) ≤1 to serve as controls. (4) Presence of minimum 20 natural teeth excluding third molars. The exclusion criteria for this study were: (1) Individuals who were immunologically and systemically compromised (history of diabetes mellitus, bacterial, viral and fungal infections, psychiatric disease, malignancies, hematologic disorders, etc.). (2) Individuals who had received any form of periodontal therapy, surgical, or nonsurgical within past 6 months. (3) Individuals with aggressive periodontitis. (4) Individuals who had received antibiotic therapy within past 6 months. (5) Individuals on anti-inflammatory drug regimen for the past 6 months. (6) Individuals on supplementary multivitamins, antioxidants, and sympathomimetics for the past 6 months. (7) Individuals wearing orthodontic or prosthetic appliances. (8) Pregnant and lactating women. (9) Individuals with untreated grossly carious teeth, oral ulceration, and other oral pathosis. (10) Individuals using mouth rinses for the past 6 months. (11) Individuals consuming alcohol or any form of smokeless tobacco for the past 6 months. (12) Individuals who were physically and/or mentally challenged.

Seventy-three individuals were recruited for the study among which 18 individuals were periodontal healthy who served as controls (Group A), 20 individuals were current smokers smoking ≤10 cigarettes/day for a minimum of 2 years diagnosed with CP (Group B), 15 individuals were former smokers who had quit smoking for ≥6 months diagnosed with CP (Group C), and 20 individuals were nonsmokers diagnosed with CP (Group D) (aged 21–53 years; mean age: 36.7 years). A flowchart of the methodology is shown in Figure 1.

Periodontal examination

A detailed history was attained from the individuals by the first examiner. Clinical periodontal examination was carried out during the first visit (which served as a baseline) by a second and third examiner who were blinded to the history and smoking habit of the individuals. The level of agreement between the examiners was determined using kappa statistics which showed a high level of agreement (K >0.80). Bleeding on probing (BOP),[19] GI,[20] PPD, CAL, and gingival recession (GR) were recorded using a University of North Carolina (UNC) -15 probe (Hu-Friedy, Chicago, IL, USA). PPD was grouped into full mouth (FM-PPD) for all four groups and diseased site (D-PPD) for Groups B, C, and D. Periodontal inflamed surface area (PISA)[21] was calculated using PPD, BOP, and GR using a spreadsheet by the formula given by Huijol et al.[22] These parameters were assessed at baseline and 6 and 12 weeks after SRP.

Figure 1: Flowchart of methodology
Sample collection

To avoid contamination of the samples with blood, all collections were performed before any dental procedures. Unstimulated whole saliva samples were obtained with the patient seated upright, with instructions to allow the saliva to pool at the bottom of the mouth and 1 ml was drained into an Eppendorf tube. The collected sample was then centrifuged Z323K, (Hermle Labortechnik, Wehingen, Germany) at 3000 g for 10 min. One hundred microliter of the supernatant fraction was stored at −20°C until further analysis of MDA. Individuals were then asked to rinse 10 ml of Hank’s Balanced Salt Solution for 30 s and expectorate the contents in a 50 ml falcon tube which was refrigerated at 4°C until further analysis of oPMNs.[23] This was repeated at 6 and 12 weeks after SRP.

Treatment

All the individuals then received FM SRP in one sitting using ultrasonic scaler Suprasson P5 Booster, Satelec, France and/or Gracey curettes (Hu-Friedy, Chicago, IL, USA) performed by the first examiner. Home care instructions of using Modified Stillman’s technique of toothbrushing, with a soft bristle toothbrush and fluoridated dentifrice were given to the individuals. Individuals were asked to refrain from other unassigned forms of oral hygiene practices like mouth rinses and interdental cleaning aids during the study period.

Analysis of malondialdehyde [Figure 2]

According the method given by Nourooz-Zadeh et al.,[24] 1000 µl of 0.67% thiobarbituric acid and 500 µl of 20% trichloroacetic acid was added to the 100 µl of supernatant of unstimulated whole saliva [Figure 2]. The solution was incubated at 100°C for 20 min and then centrifuged at 2000 g for 5 min. The supernatant then underwent measurement of absorbance at 532 nm in a colorimeter Equiptronics EQ-650, India. MDA level was calculated by dividing Molar Extinction Coefficient (C) by the measurement of Absorbance value.

\[ C = 1.56 \times 10^5 \text{ M}^{-1}\text{L.Cm}^{-1} \]

Analysis of oral polymorphonuclear leukocyte [Figure 3]

The oral rinse samples were centrifuged at 2800 g for 5 min, following which, the supernatant was discarded using a pipette. One milliliter of the remnant pellet was used to produce a smear on the histology slide which was stained with acridine orange dye. The number of oPMNs in the samples were measured by counting 40 randomly selected squares of 0.5 cm² under fluorescence microscope (Nikon, Eclipse, Melville, NY, USA) and scored [Figure 3].[23]

Statistical analysis

Data analysis was done using Windows PC-based software “MedCalc Statistical Software MedCalc Statistical Software version 13.3.1 (MedCalc Software BVBA, Mariakerke, Belgium)” version 13.3.1 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2014) by a blinded statistician. The data for oPMNs count and MDA levels were expressed as means with standard deviation. As data were normal in distribution, the differences between the four groups were analysed for variability in the mean values for the oPMNs count and MDA levels at baseline, 6, and 12 weeks, using one-way analysis of variance followed by post hoc Bonferroni’s multiple comparison method for pair-wise comparisons.

Results

The study was a double-blinded longitudinal interventional study. Of the 73 participants, 13 did not adhere to the oral hygiene instructions or failed to come for a follow-up
visit or were excluded to maintain uniformity among the four groups. Group A consisted of 15 individuals who were periodontally healthy, Group B consisted of 15 individuals who were current smokers smoking ≤10 cigarettes per day for ≥2 years with CP, Group C consisted of former smokers who had quit smoking for ≥6 months with CP, and Group D consisted of individuals with CP who were nonsmokers. A mean of the descriptive statistics of the study are displayed in Table 1. A sharp decrease in the GI, BOP, FM-PPD, D-PPD, PISA, and MDA levels were seen in Groups C and D as compared to Group B [Figure 4]. Results of post hoc Bonferroni’s multiple comparison test [Tables 2 and 3] showed a statistically significant difference in GI between Groups B and C (P = 0.021), B, and D (P = 0.018) at baseline. A statistically significant difference in BOP was noted between Groups B and C (P = 0.013), B and D (P = 0.024) at baseline. When the D-PPD was evaluated, a statistically significant difference was seen between Groups B and C, B and D at baseline (P = 0.01), 6 weeks (P = 0.001), and 12 weeks (P = 0.001), but not statistically significant between Groups C and D at baseline (P = 0.067), 6 weeks (P = 0.088), and 12 weeks (P = 0.151). There was no statistically significant difference in GR between Groups B and C, C and D, B and D at baseline, 6 and 12 weeks. A statistically significant difference in CAL between Groups A and B, A and C, A and D was seen at baseline, 6, and 12 weeks. PISA showed a statistically significant difference between Groups A and C (P = 0.029), A and D (P = 0.045) at baseline. At 12 weeks, a statistically significant difference in PISA was seen between Groups A and B (P = 0.001), A and C (P = 0.001), A and D (P = 0.001). When MDA was compared, a statistically significant difference was seen between Groups A and B (P = 0.001), A and C (P = 0.005), A and D (P = 0.001) at baseline.

Discussion

Periodontitis is a multifactorial complex disease that results in an up regulated immune–inflammatory response to bacterial plaque which leads to periodontal breakdown. Although bacteria are the primary etiologic factor in periodontal disease, the patients host response is a determinant of disease susceptibility.1,2 Both the incidence and severity of periodontitis is greater in smokers than in nonsmokers,23 as was seen in our study also. Smokers seem to have less gingival inflammation than nonsmokers because of the masking effect of nicotine on inflammation due to vasoconstriction of capillaries.26 Likewise, in this study, higher GI and BOP scores were noted in nonsmokers and former smokers with CP than current smokers with CP.

It has also been noted that smokers do not respond to periodontal treatment as well as nonsmokers do, reflecting the impairment of the tissue repair process.27,28 The effect of smoking seems reversible and tissue healing is hastened upon cessation of smoking.29,30 The periodontal parameters of PPD, CAL and PISA showed a greater resolution at the 12 weeks period in the former smoker and nonsmoker individuals than the current smoker individuals with CP. Furthermore, the PPD at diseased sites showed a statistically significant reduction in nonsmokers and former smokers as compared to the current smokers. Thus, the findings of the present study are in agreement with the conjecture that smoking cessation produces favorable effects on periodontal health.

MDA is one of the stable end product formed as a result of peroxidation of lipids by ROS, which has mutagenic and cytotoxic effects.31 Increased salivary MDA levels are seen in patients with periodontitis32 and it is an oxidative damage indicator. Due to the presence of free radicals

| Table 1: Descriptive statistics showing the mean values of gingival index, bleeding on probing, full-mouth probing pocket depth, diseased sites probing pocket depth, gingival recession, clinical attachment loss, periodontal inflamed surface area, malondialdehyde levels, and oral polymorphonuclear neutrophil for all four groups | Group | Time | GI (%) | BOP (%) | FM-PPD (mm) | D-PPD (mm) | GR (mm) | CAL (mm) | PISA (sq. mm) | MDA (n.moles/ml) | oPMN level |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Group A | Baseline | 21.3 | 0 | 1.232 | 0 | 0 | 0 | 103.81 | 2.34 | 97.2 |
| (n=15) | 6 weeks | 13.1 | 0 | 1.134 | 0 | 0 | 0 | 78.69 | 2.75 | 82.4 |
| | 12 weeks | 12.68 | 0 | 1.079 | 0 | 0 | 0 | 67.24 | 2.12 | 75.4 |
| Group B | Baseline | 37.52 | 0 | 4.44 | 0 | 6.07 | 1.6 | 118.78 | 6.19 | 91.66 |
| (n=15) | 6 weeks | 26.39 | 0 | 3.05 | 0 | 5.54 | 1.56 | 36.62 | 5.61 | 79.2 |
| | 12 weeks | 20.71 | 0 | 2.56 | 0 | 5.96 | 1.67 | 25.6 | 3.55 | 74.46 |
| Group C | Baseline | 40.18 | 0 | 3.37 | 0 | 5.33 | 2.75 | 200.65 | 4.02 | 115.33 |
| (n=15) | 6 weeks | 23.18 | 0 | 2.59 | 0 | 4.65 | 2.64 | 64.48 | 3.43 | 91.86 |
| | 12 weeks | 19.39 | 0 | 2.51 | 0 | 4.35 | 2.59 | 40.14 | 2.78 | 84.53 |
| Group D | Baseline | 44.44 | 0 | 4.65 | 0 | 5.67 | 1.39 | 148.45 | 4.57 | 114.66 |
| (n=15) | 6 weeks | 23.08 | 0 | 3.1 | 0 | 4.98 | 1.36 | 50.49 | 4.07 | 96.46 |
| | 12 weeks | 18.44 | 0 | 2.92 | 0 | 4.65 | 1.34 | 35.47 | 2.49 | 87.13 |
in cigarette smoke, increased levels of salivary MDA are seen in smokers.\textsuperscript{[33]} In this study, the salivary MDA levels at baseline were significantly higher in individuals with CP as compared to the controls. Current smokers had the highest level of salivary MDA as compared to former and nonsmokers at baseline. However, after intervening nonsurgically, the MDA values showed a consistent reduction in all the four groups but remained higher in current smokers at the 12 weeks period. These findings of our study, similar to a study conducted by Guentsch et al.\textsuperscript{[16]} suggested that both periodontitis and smoking causes a substantial change in LPO in saliva. The combination of periodontitis and smoking tended to result in significant increase in MDA compared to that recorded in periodontally healthy control patients. Thus, MDA could prove to be a reliable biomarker in pathologies associated with oxidative stress and production of free radical damage, that is, CP.

The human mouth has a constant bacterial presence that is kept under control, in part, by a continual influx of oPMN from the surrounding periodontal tissues that form a protective barrier between bacteria and the periodontal supporting tissues.\textsuperscript{[34]} The rate at which oPMN migrate through the gingival sulcus into the oral cavity, i.e., the orogranulocytic migratory rate, directly correlates with the PPD and GI and is not sensitive to the presence of plaque or calculus.\textsuperscript{[11,35]} Thus, in accordance with this finding, the oPMN counts were found to be elevated in former smokers and nonsmokers with CP. It is hypothesized that in smokers, rather than migrating via the gingival crevice, the oPMNs accumulate in periodontal tissue where they release their constituents leading to increased degradation of connective tissue components.\textsuperscript{[36]} Accordingly, in this study, the number of oPMN migrating through the gingival crevice into the oral cavity were found to be depleted in the oral rinse samples of current smoker with CP.

Figure 4: Comparison between (a) gingival index, (b) bleeding on probing, (c) diseased site probing pocket depth, (d) periodontal inflamed surface area, (e) oral polymorphonuclear leukocytes, (f) malondialdehyde levels between the groups at baseline, 6, and 12 weeks
These findings, along with the fact that cigarette smoke is known to increase production of oxygen free radicals by PMNs and to decrease activities of some free radical scavengers,[37] together results in a synergistic destructive effect on the periodontium. Furthermore, there seems to be an early benefit of smoking cessation in terms of periodontal treatment outcome. This finding is especially relevant in clinical practice, where we may infer that smokers do not need to have stopped smoking for a long time to increase their chances of improved response to therapy. Our study even demonstrated that the quantification of oPMN with high sensitivity was possible using a single, rapid and noninvasive oral rinse assay.

This was the first interventional longitudinal study that evaluated the effects of nonsurgical periodontal therapy in current smokers, former smokers and nonsmokers with CP correlating levels of MDA, along with oPMN count. However, the current smokers were not sub classified according to the number of cigarettes smoked per day. Second, estimation of salivary or serum cotinine levels would have accurately measured the exposure of individuals to cigarette smoke rather than an interview.
Table 3: Post hoc Bonferroni’s multiple comparison test comparing mean diseased sites probing pocket depth, gingival recession and clinical attachment loss between Groups B, C and D at baseline, 6, and 12 weeks

| Timeline | Group | Group | MD   | P    | MD   | P    | MD   | P    |
|----------|-------|-------|------|------|------|------|------|------|
| Baseline | B     | C     | 0.74 | 0.001* | −1.14 | 0.192 | 0.64 | 0.567 |
|          | D     | C     | 0.41 | 0.010* | 0.2   | 1.00  | −0.71 | 0.401 |
|          | C     | B     | −0.74 | 0.001* | 1.14  | 0.192 | −0.64 | 0.567 |
|          | D     | C     | −0.32 | 0.067 | 1.35  | 0.73  | −1.35 | 0.4   |
|          | D     | B     | −0.41 | 0.010* | −0.2  | 1.00  | 0.71  | 0.401 |
|          | C     | B     | 0.32  | 0.067 | −1.35 | 0.73  | 1.35  | 0.4   |
| 6 weeks  | B     | C     | 0.88  | 0.001* | −1.08 | 0.226 | 0.05  | 1.00  |
|          | D     | C     | 0.55  | 0.001* | 0.19  | 1.00  | −0.28 | 1.00  |
|          | C     | B     | −0.88 | 0.001* | 1.08  | 0.226 | −0.05 | 1.00  |
|          | D     | C     | −0.33 | 0.088 | 1.28  | 0.089 | −0.33 | 1.00  |
|          | D     | B     | −0.55 | 0.001* | −0.19 | 1.00  | 0.28  | 1.00  |
|          | C     | B     | 0.33  | 0.088 | −1.28 | 0.089 | 0.33  | 1.00  |
| 12 weeks | B     | C     | 0.92  | 0.001* | −1.05 | 0.241 | −0.01 | 1.00  |
|          | D     | C     | 0.62  | 0.001* | 0.19  | 1.00  | −0.26 | 1.00  |
|          | C     | B     | −0.92 | 0.001* | 1.05  | 0.241 | 0.01  | 1.00  |
|          | D     | C     | −0.3  | 0.151 | 1.25  | 0.094 | −0.25 | 1.00  |
|          | D     | B     | −0.62 | 0.001* | −0.19 | 1.00  | 0.26  | 1.00  |
|          | C     | B     | 0.3   | 0.151 | −1.25 | 0.094 | 0.25  | 1.00  |

*P<0.05-Statistically significant. MD: Mean difference; GI: Gingival index; BOP: Bleeding on probing; FM-PPD: Full-mouth probing pocket depth; D-PPD: Diseased sites probing pocket depth; GR: Gingival recession; CAL: Clinical attachment loss; PISA: Periodontal inflamed surface area; MDA: Malondialdehyde levels; oPMN: Oral polymorphonuclear neutrophil; MD: Mean difference

Thirdly, no microbiological evaluation was done in this study. Studies with a larger sample size are therefore needed to assess the effects of nonsurgical periodontal therapy in individuals who are smokers, nonsmokers or former smoker with CP and periodontally healthy controls. Further research in development of chair side tests for rapid detection of MDA and oPMN levels in saliva, to assess oxidative stress in periodontally compromised patients is warranted.

Conclusion

Within the scope of this study, it can be concluded that the clinical periodontal parameters of inflammation (GI and BOP) are higher in former smokers and nonsmokers with CP as compared to current smokers and controls, whereas periodontal destruction (PPD and CAL) is higher in current smokers as compared to former smokers and nonsmokers with CP. A better resolution of the diseased site is seen in former and nonsmokers than current smokers with CP.

The orogranulocyte migratory rate is higher in former and nonsmokers than in current smokers with CP which shows the impact of cigarette smoking on the oral cavity. Furthermore, the reduction in salivary MDA directly correlates with reduction in oPMN and periodontal inflammation, which indicates that MDA may be considered as a consistent biomarker for oxidative stress in CP.

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Conflicts of interest

There are no conflicts of interest.

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