Original Article

Estimation of superoxide dismutase levels in saliva and gingival crevicular fluid among smokers and non-smokers in periodontitis patients - An Observational Study

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Abstract

Background: Smoking, which is an important risk factor for periodontitis, induces oxidative stress in the body and causes an imbalance between reactive oxygen species (ROS) and antioxidants, such as superoxide dismutase (SOD). The present study was done to quantify and compare the level of SOD enzyme levels in gingival crevicular fluid (GCF) and saliva among smokers and nonsmokers. **Methodology:** One hundred and thirty-five individuals in the age range of 20–55 years, including 45 light smokers, 45 heavy smokers, and 45 nonsmokers (controls), were selected and the clinical parameters recorded were plaque index, probing depth, and attachment loss. Smokers were divided into light smokers (<10 cigarettes/day) and heavy smokers (>10 cigarettes/day) and into three subgroups: healthy, mild periodontitis, and moderate periodontitis. GCF and saliva samples were collected then SOD levels were analyzed using spectrophotometric assay. **Results:** The mean levels of SOD in the GCF and saliva of smokers were decreased compared to controls. Intra- and inter-group analyses showed a significant reduction in the levels of SOD in the GCF and saliva of heavy smokers compared to light smokers and the control group. **Conclusions:** There was a progressive reduction in SOD levels from healthy nonsmokers to light smokers to heavy smokers.

Keywords: Antioxidants, gingival crevicular fluid, reactive oxygen species, spectrophotometric assay, superoxide dismutase

Introduction

In recent years, the term "reactive oxygen species" (ROS) has been adopted to include molecules such as hydrogen peroxide, hypochlorous acid, and singlet oxygen,[1] which though, not radical in nature, and are capable of radical transformation in the extra- and intra-cellular environments.[1] There is at present ample evidence that proves the role of ROS in the destruction of the periodontal tissues.[2] ROS can cause tissue damage by a variety of different mechanisms which include DNA damage, lipid peroxidation, protein damage, oxidation of important enzymes (e.g., anti-proteases), and stimulation of pro-inflammatory cytokines release.[2]

To counteract the detrimental effects of ROS *in vivo*, a variety of antioxidants (AOs) defense mechanisms exist within the body. Various AOs include glutathione, Vitamin C, and Vitamin E as well as enzymes such as catalase, superoxide dismutase (SOD), and various peroxidases.[3] In normal physiology, there is a dynamic equilibrium between ROS activity and AO defense capacity and when that equilibrium shifts in favor of ROS, either by the reduction in AO defense or an increase in ROS production or activity, oxidative stress results. This imbalance between the ROS-AO has been implicated as one of the progressive or pathogenic factors for periodontal disease.[4]

SOD is an AO enzyme that acts against superoxide anion. It is localized within human periodontal ligament and is an important defense within gingival fibroblasts against superoxides.[5] Smoking, which is an important risk factor for periodontitis, induces oxidative stress in the body and causes an imbalance between ROS and AOs, such as SOD.

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A progressive reduction in SOD levels has been seen from healthy nonsmokers to light smokers to heavy smokers, thus highlighting the role of oxidative stress in causing periodontal disease in smokers.[6]

Since saliva constitute as the first line of defense against the free radical-mediated oxidative stress during the process of mastication and digestion promoting a variety of reactions including lipid peroxidation, and gingival crevicular fluid (GCF) reflecting the gingival tissue change, both qualitatively and quantitatively distinct from that of saliva, plasma, and serum, both were chosen as samples to assess the SOD enzyme level in chronic periodontitis of smokers and nonsmokers.[7,8]

Hence, the objective of the study was to quantify the level of SOD enzyme in GCF and saliva in smokers and nonsmokers and also to compare the SOD enzyme levels in light and heavy smokers with nonsmokers.

Methodology

The present study was an observational, descriptive study carried out in the Department of Periodontics, Vishnu Dental College, Bhimavaram, Andhra Pradesh, India. The study protocol was approved by the institutional ethics committee. Before enrolment in the study, informed written consent was obtained from each patient. Male individuals of age range between 20 and 55 years were included in the study from the outpatient department of the institution.

The individuals were divided into three major groups based on smoking status: 45 nonsmokers (control group), 45 light smokers (<10 cigarettes/day), and 45 heavy smokers (>10 cigarettes/day).[9] Each smoking group was divided into three subgroups based on probing depth (PD) and attachment loss (AL): healthy (no AL; $n = 15$), mild periodontitis (PD + 4 but < 6 mm and AL > 0 and < 4 mm; $n = 15$), and moderate periodontitis (PD + 6 mm and AL + 4 mm; $n = 15$). The control group consisted of 45 nonsmoking individuals who were periodontally healthy. These individuals exhibited sulcus depths of 2–3 mm with no AL.

Clinical examination included the recording of full mouth PDs and AL, except for third molars and the mean number of teeth remaining was >24. Plaque index (PI) was recorded for all teeth. Only the individuals with a mean PI <1 were included in the study.

Individuals with good systemic health and should not have received periodontal treatment for at least the past 6 months prior to the study were included in the study. Patients who had received periodontal treatment during the previous 6 months, who had been prescribed anti-inflammatory/antimicrobial therapy within the past 3 months, patients on regular use of vitamin supplements, or those who had special dietary requirements and the presence of systemic disease that might influence their periodontal condition were excluded from the study.

Biochemical analysis

Collection of saliva samples

Two milliliter of whole un-stimulated saliva were collected in glass beakers and were transferred into Eppendorf tubes for standardization of the volume collected. Then, the samples were centrifuged at 3000 revolutions/min (rpm) at 4°C for 5 min; the supernatant was stored at −80°C.

Collection of gingival crevicular fluid

Sampling was done from a single site of the tooth with maximum PD and AL. The area was isolated with cotton rolls and gently air dried. Care was taken to eliminate salivary contamination. Preweighed number 1 filter paper strips (2 mm × 8 mm) were used for collecting the samples by the intracrevicular method.[10] A total of six strips per person were placed for 1 min to ensure the collection of sufficient quantity of GCF at the entrance of the sulcus or pocket, and the fluid seeping out was collected.[11] There was a reduced production of GCF has been reported in smokers for SOD estimation, and the same procedure was followed for all individuals. Any paper contamination with blood was discarded, and the collection was repeated after 30 min. The volume of GCF was estimated by pre- and post-weighing the filter paper strips.[12] All the six strips were pooled with 1 ml Tris-hydrochloride buffer (Ph 6.5) eluted for 30 min and stored until the SOD enzyme assay.

Assay for superoxide dismutase enzyme

The activity of SOD was assayed by the method of Kakkar *et al*. (1984) based on the formation of nicotinamide adenine dinucleotide (NADH)-phenazine methosulphate-nitroblue tetrazolium formazan.

Reagents used

1. Sodium pyrophosphate buffer 0.052M (Ph 8.3)
2. Phenazine methosulfate 186 μmol/L
3. Nitroblue tetrazolium (NBT) 300 μmol/L
4. NADH 789 μmol/L.

The assay mixture consisted of 1.2 ml of sodium pyrophosphate buffer, 0.1 ml of phenazine methosulfate, 0.3 ml of NBT, 1 ml of appropriately diluted enzyme preparation, and water in a total volume of 3 ml. Then, the reaction was started by the addition of 0.2 ml of NADH.[13] After incubation at 30°C for 90 s, the reaction was arrested by the addition of 1 ml of glacial acetic acid. Subsequently, the reaction mixture was shaken with 4–0 ml n-butanol and allowed to stand for 10 min. Finally, the butanol layer was separated after centrifugation and measured at 560 nm. Instead of enzyme preparation, water was used to serve as control and processed similar to the test assay. Enzyme activity was expressed as units/ml.

Statistical analysis

Inter- and intra-group comparison of SOD levels in the saliva and GCF of light and heavy smokers were done using multivariate analysis of variance following by multiple comparisons with the Bonferroni correction. The difference between the two values was considered statistically significant if the $P < 0.05$.

Results

The present study was done to evaluate the influence of smoking on periodontitis by estimating the levels of SOD in the GCF and saliva of healthy nonsmokers (controls) and light and heavy smokers with mild or moderate periodontitis. The mean levels of SOD in the GCF and saliva of light and heavy smokers were decreased compared to the control group. Intragroup analysis of light and heavy smokers showed a highly statistically significant decrease ($P < 0.05$) in the levels of SOD in GCF and saliva compared to nonsmokers [Tables 1 and 2].

Multiple comparisons using the Bonferroni correction exhibited a highly statistically significant reduction in the levels in GCF when the control group was compared to all of the subgroups of light and heavy smokers. This reduction in levels of SOD was also evident in saliva. A comparison of subgroups of light with heavy smokers showed a statistically significant reduction in the levels of SOD in GCF and saliva ($P < 0.05$) [Tables 3 and 4].

Discussion

A number of AO mechanisms exist in the human body whose primary role is to eliminate or inactivate free radicals/ROS as soon as they are formed or to aid repair the damage caused by free radicals. Recently, the AO status of serum, saliva, and GCF in patients with periodontitis has been widely investigated. For the purpose of analyzing salivary AO Status, the whole saliva is the most relevant, as it contains GCF, immune cells, and tissue metabolites and also reflects most closely the predominant intraoral condition. Stimulation, on the other hand, may increase the flow of GCF and this may result in a false increase in the concentration of AOs in the saliva.[9]

In the present study, a reduction in the levels of SOD was more evident in GCF than in saliva. This could be attributed to the dilution of the ROS in saliva that resulted in decreased consumption of its AO capacity. Because the volume of GCF is low, ROS are more concentrated in it, resulting in an increased reaction with AOs and hence, their reduced levels.[6] According to some researchers, GCF is a serum transudate, so it may also be hypothesized that a reduction in the levels of AOs in the blood may be reflected in GCF.[14-16] It is known that the SOD is mainly found in cells and tissues and only a minor activity is seen in extracellular fluids which might be one of the reasons for low levels of SOD activity in GCF when compared to saliva.[5]

In this study, the oxidative stress induced by smoking was reflected by the reduced GCF and salivary SOD concentrations in smokers. The mean values were lowest in heavy smokers with moderate periodontitis. Similar results were reported in earlier studies.[14,17-19] This study also compared the levels of SOD between light and heavy smokers with no, mild, and moderate periodontitis. There was a decrease in the levels of SOD as AL and PD increased. These findings are in accordance with a previous study in which there was a significant reduction

Table 1: Superoxide dismutase levels (mean±standard deviation) in saliva of nonsmokers, light, and heavy smokers

Groups	Mean±SD	*F*-ratio	*P*
Nonsmokers			
Healthy	63.56±1.62	68.331	0.000*
Light smokers			
Healthy	54.32±5.48		
Mild periodontitis	43.74±2.59		
Moderate periodontitis	38.16±5.749		
Heavy smokers			
Healthy	43.24±1.101		
Mild periodontitis	31.11±1.550		
Moderate periodontitis	28.8±1.463		

*ANOVA the mean difference is significant at the 0.05 level.
SD – Standard deviation; ANOVA – Analysis of variance

Table 2: Superoxide dismutase levels (mean±standard deviation) in the gingival crevicular fluid of nonsmokers and light and heavy smokers

Groups	Mean±SD	*F*-ratio	*P*
Nonsmokers			
Healthy	61.40±1.26	122.78	0.000*
Light smoker			
Healthy	46.18±2.54		
Mild periodontitis	35.60±1.26		
Moderate periodontitis	34.34±5.79		
Heavy smoker			
Healthy	35.14±2.89		
Mild periodontitis	22.04±1.20		
Moderate periodontitis	21.88±1.20		

*ANOVA the mean difference is significant at the 0.05 level.
SD – Standard deviation; ANOVA – Analysis of variance

Table 3: Multiple intergroup comparisons of superoxide dismutase in gingival crevicular fluid using Bonferroni correction

Group 1	Group 2	*P*
Nonsmokers (control group)	Light smokers (healthy)	0.01*
Nonsmokers (control group)	Light smokers (mild periodontitis)	0.00*
Nonsmokers (control group)	Light smokers (moderate periodontitis)	0.00*
Nonsmokers (control group)	Heavy smokers (healthy)	0.00*
Nonsmokers (control group)	Heavy smokers (mild periodontitis)	0.00*
Nonsmokers (control group)	Heavy smokers (moderate periodontitis)	0.00*
Light smokers (healthy)	Heavy smokers (healthy)	0.04*
Light smokers (mild periodontitis)	Heavy smokers (mild periodontitis)	0.00*
Light smokers (moderate periodontitis)	Heavy smokers (moderate periodontitis)	0.00*

*The mean difference is significant at the 0.05 level

Table 4: Multiple intergroup comparisons of superoxide dismutase in saliva using Bonferroni correction

Group 1	Group 2	*P*
Nonsmokers (control group)	Light smokers (healthy)	0.02*
Nonsmokers (control group)	Light smokers (mild periodontitis)	0.00*
Nonsmokers (control group)	Light smokers (moderate periodontitis)	0.00*
Nonsmokers (control group)	Heavy smokers (healthy)	0.00*
Nonsmokers (control group)	Heavy smokers (mild periodontitis)	0.00*
Nonsmokers (control group)	Heavy smokers (moderate periodontitis)	0.00*
Light smokers (healthy)	Heavy smokers (healthy)	0.02*
Light smokers (mild periodontitis)	Heavy smokers (mild periodontitis)	0.00*
Light smokers (moderate periodontitis)	Heavy smokers (moderate periodontitis)	0.00*

*The mean difference is significant at the 0.05 level

in the levels of SOD within gingival tissues adjacent to deep pockets.[20] A comparison of controls to smokers with mild and moderate periodontitis showed a substantial depletion of SOD levels in the GCF and saliva. However, when healthy smokers were compared to the control group, the difference in SOD levels was less, although it was still significant for GCF and saliva. There was also a significant reduction in the levels of SOD in the GCF and saliva of heavy smokers compared to light smokers. This shows that levels of SOD decrease with an increase in smoking status, which may result in the worsening of already existing periodontal disease. Similar results were reported in a study performed on the gingival tissue samples of light and heavy smokers.[18]

This reduction in the levels of SOD may be related to an increased concentration of cadmium in cigarette smoke. Cadmium replaces the bivalent metals in SOD, such as zinc, copper, and manganese, resulting in its inactivation. Increased accumulation of cadmium in blood and a decrease in the levels of SOD enhance the destructive process, which was reported earlier.[15] The saturation of already present SOD by the increased concentration of free radicals in cigarette smoke is another possible mechanism for the increased destruction of the periodontium, especially in heavy smokers.[21]

Brock *et al*. in his study on local and systemic AO capacity in periodontitis and health with saliva, GCF and blood as samples concluded that GCF total AO concentration was significantly lower ($P < 0.001$) in periodontitis individuals compared to healthy controls. In the present study, the level of SOD was assessed in saliva and GCF of smokers and nonsmokers of different grades of periodontitis. The result showed that mean level of superoxide of saliva and GCF was higher in controls whereas there was a gradual reduction in the level of SOD in both saliva and GCF of healthy light smokers and healthy heavy smokers. When compared between groups there was a mean difference in the level of SOD both in saliva and GCF which was found to be higher in healthy light smokers when compared to healthy heavy smokers probably related to oxidative burst influenced by smoking.[7]

A study by Garg *et al*. on levels of lipid peroxides and AOs in smokers and nonsmokers have revealed that SOD levels were higher in nonsmokers, both in tissue (2.406 ± 0.477) and blood (2.611 ± 0.578) than heavy smokers. Similar results were obtained in the present study. The levels of SOD were less in both GCF and saliva of smokers with chronic periodontitis when compared to the controls.[18] In the present study, female smokers were not evaluated due to their low prevalence in India (<4%).[6] Another limitation of the study was that the smoking status was recorded based on self-reporting by the individuals. It has been suggested that the estimation of serum cotinine assays is more reliable for the evaluation of smoking status. Therefore, further studies, including female smokers, coupled with the estimation of serum cotinine assays, are warranted. The inclusion of nonsmokers with mild or moderate periodontitis and smokers with severe periodontitis may also be considered in future studies.

Conclusions

There was a progressive reduction in SOD levels from healthy nonsmokers to light smokers to heavy smokers in both saliva and GCF suggestive of a reduction in smoking exposure might be helpful in improving the AO levels. Thus, by making an effort to incorporate smoking cessation programs at different levels would be advantageous. Ultimately, this study would help in motivating the individuals in knowing the importance of AOs in maintaining the good oral health.

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Conflicts of interest

There are no conflicts of interest.

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