Association of Cigarette Smoking With Superoxide Dismutase Enzyme Levels in Subjects With Chronic Periodontitis

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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). In the present study, the influence of smoking on the periodontium was determined by estimating the levels of SOD in light and heavy smokers with periodontitis.

Methods: Seventy subjects in the age range of 20 to 55 years, including 60 smokers and 10 non-smokers (controls), were selected. Clinical parameters recorded were plaque index (PI), probing depth (PD), and attachment loss (AL). 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. Gingival crevicular fluid (GCF) and saliva samples were collected. 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 intergroup 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 non-smokers to light smokers to heavy smokers. These findings highlight the need to augment the efforts of smoking-cessation programs. The benefits of reduced smoking and improved antioxidant levels may motivate smoking cessation. *J Periodontol 2009;80:657-662.*

KEY WORDS

Antioxidants; chronic periodontitis; cigarette smoking; gingival crevicular fluid; saliva; superoxide dismutase.

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Periodontitis is an inflammation of the periodontium that extends beyond the gingiva and produces destruction of the connective tissue attachment of the teeth.[1] The severity of the disease process can be modified by a variety of factors. Recently, the role of reactive oxygen species (ROS) has been established in the pathogenesis of periodontitis.

In healthy individuals, ROS are produced during various physiologic processes. Normally there is a balance between ROS and antioxidants that may be disturbed by a variety of factors, including smoking. This dysregulation may damage the cells by variant mechanisms, such as peroxidation of lipid membranes, protein inactivation, and induction of DNA damage.[2]

There is enhanced production of ROS, such as superoxide (O_2^-), hydroxyl, nitric oxide, hydrogen peroxide, and hypochlorous acid, by polymorphonuclear leukocytes (PMNs) in periodontitis. Stimulation by bacterial antigen results in production of O_2^- by PMNs via the metabolic pathway of the "respiratory burst" during phagocytosis.[2-4] Inflammatory cells, such as fibroblasts, vascular endothelial cells, and osteoclasts, also produce ROS.[2,5-7] They are highly toxic to the ingested microorganisms as well as to the host cells. Excess production of ROS results in tissue damage.

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Smoking also increases ROS production and is a significant source of oxidative stress.[8] It causes depletion of systemic endogenous antioxidant capacity, resulting in increased pro-oxidant burden.[9] Protection against these species is provided by antioxidants that may be in the form of enzymes (e.g., superoxide dismutase [SOD], catalase, and glutathione peroxidase) or low molecular weight free-radical scavengers (e.g., vitamin E, total thiol, and glutathione).

SOD is an antioxidant enzyme that acts against superoxide, an oxygen radical that is released in inflammatory pathways and causes connective tissue breakdown. This enzyme is released as a homeostatic mechanism to protect the tissues, and it can be detected in extra- and intracellular compartments. It is of various types: type 1: Cu^{2+}/Zn^{2+}-dependent enzyme found within cytosol, type 2: Mn^{2+}-dependent enzyme located within the mitochondria, and type 3: extracellular enzyme found in low levels.[10]

SOD has also been localized within human periodontal ligament, and it may represent an important defense mechanism within gingival fibroblasts against superoxide release.

Biochemical analysis of periodontal soft tissues cleaved from freshly extracted human teeth showed that the human periodontal ligament contained about twice as much SOD activity as human skin, but less than red blood cells. The concentrations were positively or negatively related to probing depth (PD) and had a definite correlation with the progression of disease.[11]

Studies[9,12] comparing antioxidant levels in smokers and non-smokers with periodontitis and gingivitis have shown decreased levels of antioxidants in smokers compared to non-smokers.

In the present study, the influence of smoking on periodontal health was evaluated by estimating the levels of SOD enzyme in light and heavy smokers with no attachment loss (AL) (healthy), 0 > AL <4 mm (mild periodontitis), and AL ≥4 mm (moderate periodontitis). The levels of SOD in light and heavy smokers were also compared.

MATERIALS AND METHODS

A convenience sampling method was followed using patients who were referred to the Department of Periodontics, Manipal College of Dental Sciences, between October 2007 and March 2008. Initially, 100 subjects who fulfilled the inclusion criteria were selected; 70 subjects participated in the study. Ethical clearance for the study was obtained from the Kasturba Hospital, Manipal Ethical committee prior to the commencement of the study. A written informed consent was obtained from the subjects who participated in the study.

The age of the subjects ranged between 20 and 55 years; all were males.

The patients were questioned regarding their smoking status and systemic diseases, such as diabetes, rheumatoid arthritis, and cardiovascular problems. Their medical records were checked to validate information provided about systemic illnesses that might influence the outcome of the study. The presence of any of these systemic diseases was an exclusion criterion. Patients who had been prescribed anti-inflammatory or antimicrobial therapy within the past 3 months, regular users of mouthwash or vitamin supplements, or those who had special dietary requirements were also excluded from the study.

Clinical examination included the recording of full-mouth PDs and AL, except for third molars. The mean number of teeth remaining was >24. Plaque index[13] was recorded for all teeth. Only the subjects with mean plaque index ≤1 were included in the study.

The subjects were divided into two major groups based on smoking status: 30 light smokers (<10 cigarettes/day) and 30 heavy smokers (≥10 cigarettes/day).[14] Each smoking group was divided into three subgroups based on PD and AL: healthy (no AL; n = 10), mild periodontitis (PD ≥4 but <6 mm and AL >0 and <4 mm; n = 10), and moderate periodontitis (PD ≥6 mm and AL ≥4 mm; n = 10).

The control group consisted of 10 non-smoking subjects who were periodontally healthy. These subjects exhibited sulcus depths of 2 to 3 mm with no AL.

Collection of Saliva Samples

Whole unstimulated saliva was collected in glass beakers; 2 ml was transferred into Eppendorf tubes. This standardized the volume collected from each patient. The samples were centrifuged at 3,000 revolutions per minute (rpm) at 4°C for 5 minutes; the supernatant was stored at –80°C until analysis.

Collection of Gingival Crevicular Fluid (GCF) Samples

GCF sampling was performed between 8:00 am and 10:00 am. 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 × 8 mm) were used for collecting the samples by the intracrevicular method.[15] A total of six strips per person were placed for 1 minute at the entrance of the sulcus or pocket, and the fluid seeping out was collected. Reduced production of GCF has been reported in smokers.[16] Therefore, a time period of 1 minute was chosen to ensure the collection of sufficient quantity of GCF for SOD estimation. The same procedure was followed for all subjects. Any paper contaminated with blood was discarded, and the collection was repeated after

30 minutes. The volume of GCF was estimated by pre- and postweighing the filter paper strips.[17] All six GCF strips were pooled with 1 ml Tris-HCl buffer (pH 6.5), eluted for 30 minutes, and stored until the SOD assay.

Assay for SOD Enzyme

One-half milliliter of water, 0.25 ml ethanol, and 0.15 ml chloroform were added to 0.5 ml GCF eluate or saliva, mixed in a vortex machine, and placed in an ice chest for 15 minutes; thereafter, 0.1 ml water was added. The solution was centrifuged at 2,000 rpm for 15 minutes. The supernatant was mixed with SOD substrate to estimate the levels of SOD. The substrate was prepared fresh every time by mixing 23.6 ml of 0.05 *M* phosphate buffer with 37.2 mg methionine and 0.16 ml riboflavin.

The SOD level was analyzed by the reduction of nitroblue tetrazolium by free radicals generated with the riboflavin system.[18] The formazan formed by this reaction was detected spectrophotometrically at 560 nm and compared to the standard. Illumination of riboflavin in the presence of O_2 and an electron donor, such as methionine on EDTA, generated superoxide ions that were used as the basis of assay for SOD. The samples were illuminated for 10 minutes before readings were taken. The SOD levels were calculated as units per milliliter.

Statistical Analysis

Inter- and intragroup comparisons of the levels of SOD in the GCF and saliva of light and heavy smokers were done using multivariate analysis of variance (ANOVA) followed by multiple comparisons with the Bonferroni correction. The difference between two values was considered significant if the *P* value was <0.05 and highly significant at $P<0.01$.

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 non-smokers (controls) and light and heavy smokers with mild or moderate periodontitis. The mean values of various characteristics are given in Table 1.

The mean levels of SOD in the GCF and saliva of light and heavy smokers were decreased compared to the control group (Table 2).

Intragroup analysis of light and heavy smokers showed a highly statistically significant decrease ($P<0.01$) in the levels of SOD in GCF and saliva compared to non-smokers. Further comparisons using the Bonferroni correction showed a statistically significant decrease in the levels when healthy smokers were compared to smokers having mild or moderate periodontitis ($P<0.05$). However, there was no statistically significant difference ($P>0.05$) in the levels between the groups with mild and moderate periodontitis.

A highly statistically significant reduction ($P<0.01$) in the levels of SOD in the GCF and saliva was present when controls and light and heavy smokers were compared (Tables 3 and 4). Multiple comparisons using the Bonferroni correction showed 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 except in healthy light smokers. A comparison of subgroups of light with heavy smokers showed a statistically significant reduction in the levels of SOD in GCF ($P<0.05$) but not in saliva of healthy smokers (Tables 5 and 6).

DISCUSSION

There is an abundance of literature supporting the relationship between smoking and periodontal

Table 1.

Values (mean ± SD) of Various Parameters Recorded in Controls and Subgroups of Light and Heavy Smokers

Group		Age (years)	Cigarettes Smoked/Day (n)	Smoking Duration (years)	Plaque Score	PD (mm)	AL (mm)
Controls*		28.4 ± 6.80	0.00 ± 0.00	0.00 ± 0.00	0.46 ± 0.27	2.3 ± 0.48	0.00 ± 0.00
Light smokers	Healthy	29.7 ± 8.79	3.0 ± 1.82	6.16 ± 4.78	0.36 ± 0.30	2.8 ± 0.52	0.00 ± 0.00
	Mild periodontitis	33.4 ± 9.18	5.6 ± 1.84	7.0 ± 5.16	0.47 ± 0.36	4.8 ± 0.71	2.49 ± 0.66
	Moderate periodontitis	30.4 ± 7.54	6.1 ± 1.79	12.8 ± 8.65	0.60 ± 0.33	6.8 ± 0.78	4.88 ± 0.78
Heavy smokers	Healthy	34.08 ± 10.34	13.3 ± 2.05	7.7 ± 6.29	0.39 ± 0.49	2.5 ± 0.53	0.00 ± 0.00
	Mild periodontitis	34.5 ± 11.23	15.94 ± 2.05	11.85 ± 6.47	0.52 ± 0.46	4.8 ± 0.97	2.4 ± 0.94
	Moderate periodontitis	38.5 ± 9.76	16.54 ± 2.67	13.83 ± 6.22	0.63 ± 0.38	8.6 ± 1.33	5.78 ± 1.34

* Healthy non-smokers.

Table 2.

SOD (mean ± SD) in GCF and Saliva of Controls and Light and Heavy Smokers

Category	SOD in GCF (U/ml)	SOD in Saliva (U/ml)
Controls*	68.09 ± 17.22	69.89 ± 13.96
Light smokers		
Healthy	52.88 ± 14.25	56.69 ± 13.62
Mild periodontitis	35.10 ± 3.51	40.59 ± 12.64
Moderate periodontitis	34.43 ± 9.38	35.51 ± 15.93
Heavy smokers		
Healthy	31.04 ± 7.00	41.86 ± 10.94
Mild periodontitis	19.33 ± 6.88	23.53 ± 11.58
Moderate periodontitis	16.07 ± 10.72	20.21 ± 14.93

* Healthy non-smokers.

Table 3.

Intergroup Comparison of SOD in GCF Using ANOVA

	ANOVA	
Category	F Value	*P* Value
Controls*		
Light smokers	38.99	0.00
Heavy smokers		

P <0.01 = highly significant.
* Healthy non-smokers.

Table 4.

Intergroup Comparison of SOD in Saliva Using ANOVA

	ANOVA	
Category	F Value	*P* Value
Controls*		
Light smokers	22.689	0.00
Heavy smokers		

P <0.01 = highly significant.
* Healthy non-smokers.

Table 5.

Multiple Intergroup Comparisons of SOD in GCF Using Bonferroni Correction

Category	Category	*P* Value
Controls*	Light smokers	
	Healthy	0.02
	Mild periodontitis	0.00
	Moderate periodontitis	0.00
	Heavy smokers	
	Healthy	0.00
	Mild periodontitis	0.00
	Moderate periodontitis	0.00
Light smokers	Heavy smokers	
Healthy	Healthy	0.00
Mild periodontitis	Mild periodontitis	0.005
Moderate periodontitis	Moderate periodontitis	0.001

P <0.01 = highly significant; *P* <0.05 = significant.
* Healthy non-smokers.

diseases; however, the exact mechanism by which smoking exerts its deleterious effects on the periodontium is still unclear. The induction of oxidative stress in the body by nicotine and the subsequent depletion of antioxidants may be one of the mechanisms for the tissue damage.

The ROS and their corresponding antioxidants may be identified in the GCF and saliva.[2] 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 antioxidant capacity. Because the volume of GCF is low, ROS are more concentrated in it, resulting in an increased reaction with antioxidants and, hence, their reduced levels.

According to some researchers,[19-21] GCF is a serum transudate, so it may also be hypothesized that a reduction in the levels of antioxidants in blood may be reflected in GCF.

ROS are also generated in many physiologic processes, such as mitochondrial oxidation, oxygen transportation by hemoglobin, and cytochrome P_{450} activity. The delicate balance between the ROS and tissue concentrations of antioxidants may be disturbed by various factors, including smoking.[12] Elevated levels of ROS stimulate the neutrophils to upregulate the adhesion integrins, leading to their increased accumulation in tissues and a local sealing off of antioxidant enzymes, such as SOD, catalase, and protease inhibitors.[8] Consequent to this, there is degradation and collagenolysis of ground substance or increased stimulation of excessive proinflammatory cytokines through nuclear transcription factor-kappa B activation or an increased production of prostaglandin E_2 via lipid peroxidation and superoxide release; all are linked to bone resorption.[21] Cigarette smoking may also result in the reduction of SOD due to inactivation by increased production of hydrogen peroxide.[22]

Table 6.

Multiple Intergroup Comparisons of SOD in Saliva Using Bonferroni Correction

Category	Category	*P* Value
Controls*	Light smokers	
	Healthy	0.42
	Mild periodontitis	0.00
	Moderate periodontitis	0.00
	Heavy smokers	
	Healthy	0.00
	Mild periodontitis	0.00
	Moderate periodontitis	0.00
Light smokers	Heavy smokers	
Healthy	Healthy	0.18
Mild periodontitis	Mild periodontitis	0.03
Moderate periodontitis	Moderate periodontitis	0.08

$P<0.01$ = highly significant; $P<0.05$ = significant.
* Healthy non-smokers.

In the present 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.[9,12,19,23]

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[24] in which there was a significant reduction in the levels of SOD within gingival tissues adjacent to deep pockets. 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. There was also a significant reduction in the levels of SOD in the GCF 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[12] performed on the gingival tissue samples of light and heavy smokers.

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. An increased accumulation of cadmium in blood and a decrease in the levels of SOD enhance the destructive process, which was reported earlier.[20] 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.[25]

A study[26] reported increased SOD levels in the saliva of elderly male smokers (≥65 years). This contradictory finding could be related to the age of the subjects who were evaluated for antioxidant status. It has been suggested that antioxidant defenses and DNA repair may be induced in old age. This may be a response to a higher level of oxidative damage, as mitochondria become leakier and release more reactive oxygen.[27] However, in the present study, the subjects were of a comparatively younger age group; the chances for age-related free-radical damage, and, hence, an increased production of antioxidants, were low.

In the present study, female smokers were not evaluated because of their low prevalence in India (≤4%).[28,29] Another limitation of the study was that the smoking status was recorded based on self-reporting by the subjects. It has been suggested that the estimation of serum cotinine assays is more reliable for the evaluation of smoking status. Therefore, further studies incorporating a larger sample size, including female smokers, coupled with the estimation of serum cotinine assays are warranted. The inclusion of non-smokers with mild or moderate periodontitis and smokers with severe periodontitis may also be considered in future studies.

CONCLUSIONS

The results of the present study suggest that cigarette smoking is associated with a significant decrease in the levels of SOD. Because the SOD levels were lower in heavy smokers compared to light smokers and controls, a reduction in smoking exposure might be helpful in improving the antioxidant levels. Therefore, the information derived from the present study may be used in smoking-cessation programs.

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