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ASSOCIATION OF SMOKELESS TOBACCO AND SMOKING WITH SALIVARY CATALASE AND GLUTATHIONE PEROXIDASE LEVELS IN SUBJECTS WITH CHRONIC PERIODONTITIS: A CROSS-SECTIONAL BIOCHEMICAL STUDY

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Abstract:

Purpose: To estimate and correlate salivary catalase and glutathione-peroxidase levels in Chronic Periodontitis (CP) patients who are non-tobacco users, smokers and smokeless tobacco (gutkha) users & periodontally healthy subjects.

Methods: The study included 120 subjects (age range: 18 to 60 years). Participants were divided into four groups: 30 healthy subjects (Group I), 30 subjects with CP (Group II), 30 smokeless tobacco (gutkha) users with CP (Group III) and 30 smokers with CP (Group IV). Clinical parameters included probing depth (PD), clinical attachment level (CAL), and gingival index (GI). Following this, salivary catalase (CAT) and glutathione peroxidase (GPx) levels were estimated using UV-spectrophotometry, and the data were analyzed using SPSS software. Mann-Whitney U and Pearson correlation coefficient tests were used for analysis.

Results: The periodontally healthy subjects demonstrated significantly higher salivary levels of CAT and GPx (an antioxidant parameter) compared to periodontitis subjects who were gutkha chewers and smokers. Pairwise comparison by the Mann-Whitney U test showed a significant difference in the mean levels of GPx among all groups ($p < 0.05$). The mean salivary levels of CAT were significantly lower in group II as compared to other groups (II<IV<III<I). The clinical parameters GI, PD and CAL among the four groups were also statistically significant ($p < 0.05$).

Conclusion: The study results suggested that salivary levels of both catalase and glutathione peroxidase were found to be decreased in chronic periodontitis patients who had oral abusive habits such as smoking and gutkha chewing

Keywords: Antioxidants, Biological Markers, Catalase, Glutathione Peroxidase, Chronic Periodontitis, Saliva, Smokeless tobacco, Smoker.

Introduction:

Periodontitis is an inflammatory disease whose pathophysiology is related to the accumulation of microbial plaque on teeth and the excessive host response to periodontal pathogens, destroying periodontal tissue [1,2]. Various factors can influence the severity of the disease process. Recently, the role of reactive oxygen species (ROS) in the pathogenesis of periodontitis has been established. In normal physiology, a dynamic equilibrium exists between ROS activity and antioxidant defense capacity [3]. Oxidative stress arises within tissues when there is an imbalance, caused by a reduction in antioxidant defense and/or an increase in ROS production or activity [4,5].

The polymorphonuclear leucocyte (PMN) constitutes the first line of cellular host defenses against bacteria in the gingival sulcus. The antimicrobial activities of PMNs and monocytes encompass both oxygen-dependent and oxygen-independent mechanisms. The oxygen-dependent pathway involves the production of ROS molecules which are capable of initiating periodontal tissue destruction [6,7]. The production of ROS by PMNs is primarily focused on bacterial killing, but the extracellular release of ROS results in the destruction of surrounding healthy tissues. ROS can cause tissue damage via multiple mechanisms, including DNA damage, lipid peroxidation, protein damage, enzyme oxidation, and the stimulation of pro-inflammatory cytokine release by monocytes and macrophages [8, 9].

Antioxidants are substances that delay or prevent the oxidation of cellular oxidizable substrates. Catalase (CAT) protects the cell from hydrogen peroxide generated within them. CAT plays a crucial role in the acquisition of tolerance to oxidative stress during the adaptive response of cells. Glutathione is essential to the glutathione peroxidase (GPx) antioxidants enzyme system which also removes hydrogen peroxide [10,11].

Antioxidant defense systems comprise three main components: (1) preventive antioxidants, which suppress the formation of free radicals (e.g. superoxide dismutase, catalase, glutathione peroxidase), (2) radical-scavenging antioxidants, which scavenge radicals to inhibit chain reactions (e.g. vitamins A, C and E) and (3) repair and de novo enzymes, which repair, damage and reconstitute membranes (e.g. DNA repair enzymes) [12,13].

Since free radicals have been implicated in the pathogenesis of various human diseases, this has prompted interest in evaluating cellular levels of antioxidant agents. CAT is an antioxidant enzyme with high specific activity present in all aerobic cells. In erythrocytes, catalase and GPx jointly protect hemoglobin from oxidative damage [14,15].

Smoking, being a risk factor for periodontitis, is associated with an increased risk for periodontal attachment loss and bone loss [16]. The biological occurrence of the increased periodontal disease severity and rate of progression associated with smoking has been hypothesized to be due to interactions among smoking, bacterial periodontal pathogens and the host [17]. Exposure to environmental cigarette smoke is associated with increased leukocyte counts, chemotaxis, and an increased release of reactive oxidants from stimulated neutrophils. Smokers demonstrate 2.6 – 6 times increased prevalence of periodontal diseases compared to non-smokers [4].

Smokeless tobacco has also been shown to affect the immune response both in vitro and in vivo [18]. Smokeless tobacco contains chemical carcinogens that include polynuclear aromatic hydrocarbons

(usually benzo-a-pyrene), polonium-210, and N-nitrosamines [10]. Although available in various forms, smokeless tobacco is primarily used in two main forms: chewing tobacco (loose leaf, plug, or twist) and snuff (either moist or dry) [19]. Clinical attachment level (CAL), gingival recession, mobility, furcation and lesions like leukoplakia, periodontal disease and delayed wound healing were significantly higher amongst smokeless tobacco users [20].

During gingival inflammation, gingival crevicular fluid (GCF) flow increases, and components of the inflammatory response are detectable in saliva, including lipid peroxidation products [21]. Saliva can be easily collected and hence may offer a basis for specific diagnostic tests for periodontitis.

Although there is ample documentation supporting the untoward effect of smoking on periodontal health, little is known about the probable effects of non smoked tobacco products. Given the importance of antioxidants in periodontal pathogenesis, this study aimed to estimate and correlate catalase and glutathione peroxidase levels in saliva (i.e., measure local effects) in subjects with chronic periodontitis compared to healthy participants. Further, the influence of smoking and use of smokeless tobacco on these parameters were observed.

Materials and Methods:

Study Design: In the present cross-sectional study, 120 systemically healthy subjects aged between 18 and 60 years were randomly selected from the outpatient department, Department of Oral Medicine, Diagnosis, and Radiology, P.M.N.M. Dental College and Hospital, Bagalkot. Further, all the participants were clearly explained the need and design of the study. Written informed consent was obtained from all recruits. The Institutional Review Board approved the research project.

The selection of patients was made according to the criteria approved by the 1999. International Workshop on the Classification of Periodontal Diseases and Conditions. Furthermore, in the Department of Periodontics, 120 patients were divided into four groups: clinically healthy periodontium (Group I), chronic periodontitis (Group II), gutkha chewers with chronic periodontitis (Group III), and smokers with chronic periodontitis (Group IV).

All study participants with a minimum of 20 teeth and no history of acute or chronic systemic disorders were included. Gutkha chewers with chronic periodontitis (Group III) were enrolled if they regularly chewed smokeless tobacco at least 1 sachet daily for at least 12 months . Subjects belonging to the group IV were enrolled if they had smoked ≥100 cigarettes in their lifetime and currently smoked . Pregnant and lactating females, individuals with trauma or who underwent recent tooth extraction or who had received any periodontal, antimicrobial and anti-inflammatory therapy or antioxidants supplements in last 3 months before sampling or diagnosed with oral submucous fibrosis were excluded from the study.

Periodontal Parameters: Depending upon the gingival index (GI), probing depth (PD) and CAL measurements, study subjects were divided into 4 groups:

Group I (n= 30): Periodontally healthy subjects characterized by GI=0 (absence of clinical inflammation), PD ≤ 3 mm and CAL=0,

Group II (n= 30): Subjects with CP characterized by at least 30% sites with PD ≥ 5 mm, GI >1 and CAL ≥4 mm,

Group III (n=30): Gutkha chewers with CP and

Group IV (n=30): Smokers with CP

One calibrated examiner obtained all the measurements to reduce intra-examiner variability for GI, PD, and CAL [16]. Both PD and CAL were recorded using the Williams graduated periodontal probe at four sites around all present teeth, excluding the third molars.

Saliva sampling: Saliva samples were collected from the selected subjects for analysis. The participants were asked not to eat or drink one hour before saliva collection. The smokers/tobacco chewers were prohibited from smoking/tobacco chewing one hour prior to saliva collection. Sampling was performed in a quiet room between 9 am to 11:30 am to prevent any circadian variation. The participants were instructed to rinse the mouth using distilled water. The stimulated saliva from subjects chewing on paraffin was collected for at least 5 minutes in eppendorf tube and the salivary samples were centrifuged for 5 minutes at 3000 rpm to remove cell debris . The supernatant was stored at -80°C, until tests were performed. Salivary catalase and glutathione peroxidise enzymes were measured through UV-spectrophotometer (Shimadzu Model).

Assay of salivary catalase and glutathione peroxidase levels: For catalase estimation, 50 mM PBS was prepared by adding 1.71 gram of KH_2PO_4 and 2.5 grams of NaH_2PO_4 in 250 ml of distilled water. Also, 0.7 mM of H_2O_2 was also prepared. Later on, 1.95 ml of PBS and 1 ml of H_2O_2 was added to 50 microlitre of saliva and readings of absorbance were recorded at 240 nm at 0 and 1 minute. One unit (U) is equal to 1 mmol of H_2O_2 decomposed/min .

For glutathione peroxidase analysis, the main reagent was made by mixing 8.00 mL of KH_2 PO_4 buffer (100 mmol/L; 1 mmol EDTA/L; pH 7.4), 4.00 mL of glutathione reducatse (5000 U/L,), 2.00 mL of reduced glutathione (2.5 mmol/L), and 2.00 mL of NADPH (2.5 mmol/L). The main reagent (200 mL) and the sample (25 µL of 1:200 saliva plus 10 µL of H_2O) were added to the cuvette and the absorbance at 340 nm was monitored for 200 s (step A). Then 10 mL of *tert*-butyl hydroperoxide (25 mmol/L) were added as start reagent. The absorbance was monitored for another 225 s (step B). The final reaction volume was 250 mL. The difference in absorbance per minute between steps B and A was used to calculate the enzyme activity by using a molar absorptivity of NADPH at 6.22 x10^3 L mol^{-1} cm^{-1}.The unit is mmol of NADPH oxidized/min .

Statistical Analysis*:* Kruskal Wallis ANOVA, Mann-whitney U and Wilcoxon matched pairs test were used to evaluate the mean GI, PD and CAL scores among all the four groups. Statistical analysis for CAT was done by ANOVA and Tukeys multiple post hoc procedures. For GPx , Kruskal Wallis, Wilcoxon matched pair test and Mann-Whitney U tests were used. SPSS software (version 19) was used for analysis.

Results: The present cross-sectional study was carried out among 120 subjects and categorized into four groups based on their history and clinical presentation. Pairwise comparison of all the subjects revealed the absence of female subjects in Group III and Group IV and comparatively younger subjects in Group III [Table 1].

Table 1: Study population: Periodontal and salivary parameters (Mean ± SD)**:** Gingival Index (GI); Probing Depth (PD); Clinical Attachment Level (CAL); Catalase (CAT)(U); Gutathione peroxidase (GPX) (μ mol)

Characteristics	**Group I**	**Group II**	**Group III**	**Group IV**	**P Value**
Age (yrs)	37 ± (5.1)	41 ± (4.9)	32.5 ± (5.6)	54 ± (4.2)	
Male/Female	80/20	70/30	100/0	100/0	
GI Score	0.93 ± (0.44)	1.86 ± (0.50)	1.83 ± (.53)	1.40 ± (0.56)	<0.001*
PD (mm)	1.63 ± (0.41)	5.53 ± (0.57)	5.2 ± (0.40)	5.4 ± (0.56)	<0.001*
CAL (mm)	0	6.13 ± (0.86)	6.03 ± (0.76)	5.9 ± (0.84)	<0.001*
CAT (U)	36.62 ± (6.2)	18.86 ± (3.56)	27.17 ± (4.83)	23.06 ± (4.64)	
GPX (mg/ml)	1901.7 ± (340.6)	1220 ± (215.03)	724.44 ± (125.6)	434.2 ± (70.27)	

* Statistically significant ***P< 0.001, **p<0.01, *p<0.05

The differences between the four groups were significant (P< 0.001) in terms of GI, PD and CAL scores (Table 1). The mean scores of all the parameters (GI, PD, CAL) were significantly higher among groups II, III and IV compared to Group I (p < 0.001). Likewise, pairwise comparison of GI, PD and CAL between group I and other groups (II/II/IV) by Mann-Whitney U test were highly significant (p = 0.000) [Table 2]. With respect to GI, pairwise comparison between group IV and groups (II, III) showed significant difference (p < 0.001 and p < 0.003 respectively). While considering PD, significant difference was seen between groups II and III (p < 0.014) [Table 2].

Table 2: Pair wise comparison of GI, PPD and CAL among four groups by Mann-Whitney U test.

Parameters	Group Comparison	P Value
Gingival Index	Group I vs II/III/IV	P = 0.000***
	Group II vs III/IV	P = 0.793 / 0.001***
	Group III vs IV	P = 0.003**
Pocket Depth	Group I vs II/III/IV	P = 0.000***
	Group II vs III/IV	P = 0.014** / 0.323

	Group III vs IV	P = 0.141
Clinical Attachment Level	Group I vs II/III/IV	P = 0.000***
	Group II vs III/IV	P = 0.758 / 0.288
	Group III vs IV	P = 0.440

*Statistically Significant *** P< 0.001, **p<0.01, *p<0.05

The results of our study showed that the mean salivary levels of CAT were significantly lower in group II as compared to other groups (II<IV<III<I). Mean salivary GPx levels were found to be decreasing in sequential order from group I to group IV (Table 1). Upon pairwise Tukey's post hoc comparison after applying ANOVA test, significant difference was seen in the mean values of CAT among all groups ($p < 0.05$).Similarly, significant difference in the mean levels of GPx was found among all groups ($p < 0.05$) except between group III and IV ($p = 0.198$) [Table 3].

Table 3: Pair wise comparison of four groups with CAT and GPx levels by ANOVA and Tukey's Post–hoc multiple comparisons.

Groups		Other groups	CAT	GPx
Group I	v/s	Group II	p=0.000***	p=0.000**
		Group III	p=0.000***	p=0. 000***
		Group IV	p=0.000***	p=0. 000***
Group II	v/s	Group III	p=0. 000***	p=0.005***
		Group IV	p=0.007**	p=0.000**
Group III	v/s	Group IV	p=0.008	p=0.198

P value is significant in all the groups except for GPx in Group III v/s IV
Statistically Significant when ***p≤ 0.001, **p<0.01, *p<0.05

According to Pearson correlation test, salivary CAT and GPx levels are directly proportional to each other in groups I and II but inversely proportional in groups III and IV [Table 4].

Table 4: Pearson co-relation between CAT and GPx groups

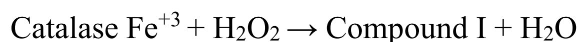
	R value	P value
Group I	0.039	0.838
Group II	0.098	0.607
Group III	-0.117	0.538
Group IV	-0.049	0.799

Discussion: Antioxidants are present in all body fluids and tissues, and protect against endogenously-formed free radicals, usually produced by leakage of the electron transport system [22]. The strongest indication implicating ROS in periodontal destruction of the connective tissues during periodontal diseases arises in considering PMN infiltration as a key event of host response against bacterial invasion [23].

Smoking and smokeless tobacco have shown to impair various aspects of innate and acquired host immune responses. There has been an increased prevalence of chewing tobacco owing to its free accessibility, lower price and also rising education concerning well-known hazards of smoking in India. Adults currently using smokeless tobacco are twice more likely to have severe active periodontal disease than adults who never used smokeless tobacco [9]. Saliva is the first biological fluid that inhaled cigarette smoke (CS) and smokeless tobacco encounters. Stimulated saliva contains a lower concentration of antioxidants but when flow rates are taken into account, antioxidant capacity is higher than in unstimulated saliva [24].

In consideration of the strong evidence in favor of smoking as a major risk factor for destructive periodontal disease, the possible risk associated with the use of smokeless tobacco products is worthy of attention. In this backdrop, an attempt has been made in the present study to investigate the possible association between smokeless tobacco and smoking with the salivary levels of enzymatic antioxidants such as catalase and glutathione peroxidase in chronic periodontitis subjects [25].

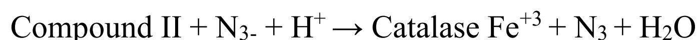
Catalase catalyzes the rapid decomposition of hydrogen peroxide by two types of reactions. Both types include a first step formation of compound I, which consists of the enzyme and hydrogen peroxide. The catalytic activity catalyzes a reaction with a second molecule of hydrogen peroxide producing water and oxygen. The reaction can be described in most general terms by the following equations [26]:



Catalase $Fe^{+3} + H_2O_2 \rightarrow$ Compound I + H_2O

Compound I + $H_2O_2 \rightarrow$ Catalase $Fe^{+3} + H_2O_2 + O_2$

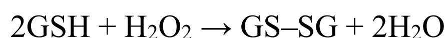
Compound I + $N_{3-} + H^+$ H2O2 $\rightarrow$ Compound II + N_3



Compound II + $N_{3-} + H^+ \rightarrow$ Catalase $Fe^{+3} + N_3 + H_2O$

N= Azide

Glutathione peroxidase, catalyze the reduction of a variety of hydroperoxides (ROOH and H_2O_2) using reduced glutathione (GSH), thereby protecting mammalian cells against oxidative damage as shown in following equation [27].



$2GSH + H_2O_2 \rightarrow GS\text{–}SG + 2H_2O$

Although glutathione peroxidase (GPX) shares the substrate, H_2O_2, with catalase, it alone can react effectively with lipid and other organic hydroperoxides. The glutathione redox cycle is major source of protection against low levels of oxidant stress, whereas catalase becomes more significant in protecting against severe oxidant stress [28].

It has been reported that the gingival blood flow is increased in smokeless tobacco users [29]. Exposure

of keratinocytes and monocytes to aqueous extracts of smokeless tobacco increases production of inflammatory mediators, such as prostaglandin E2 (PGE2) and interleukin–1 (IL-1) and increases keratinocyte proliferation. This may be an explanation for the more severe periodontal conditions and disturbance in antioxidant levels in gutkha chewers. The primary periodontal alteration in smokeless tobacco users is localized gingival recession (25-30 %) [30]. The tobacco specific nitrosamines (TSNAs) are metabolites of nicotine and are major carcinogens. Chronic inflammation may promote the carcinogenic effect of these nitrosamines through the generation of ROS [31].

Smoking exerts a major effect on protective elements of immune response, resulting in an increase in the extent and severity of periodontal destruction [25]. Smoking may have an adverse effect on fibroblast function, chemotaxis of neutrophils, immunoglobulin, production and induction of peripheral vasoconstriction [26]. Nicotine metabolites concentrate in the periodontium and cause functional alterations in phagocytosis and the oxidative burst [27]. The direct exposure to cigarette smoke represent only a portion of total oxidative stress and contributes to additional endogenous oxidant formation through effects on inflammatory immune response [28].

The results obtained in the current study demonstrate that the highest value of salivary CAT and GPx is found in healthy subjects. The obligatory use of body-reserve of antioxidants to detoxify the excess of free radicals in smokers and smokeless tobacco users therefore results in alteration in antioxidant levels. The antioxidant disturbance may be further enhanced by their lower intake of both supplemental and dietary antioxidants [29].

It was observed that there was no significant correlation between CAT and GPx with age, in any of the groups when seen group wise. Although, there exists positive correlation between CAT and GPx (Pearson co-relation) (Table 4).

Studies have shown that nicotine increases ROS in a time and concentration-dependent manner. Barr and co workers have reported that as low as 0.1 μM concentration of nicotine induces ROS by approximately 35%. Bagchi et al. reported that ST extract produces oxidative tissue damage [30]. 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, including periodontium .

Hamid-reza et al showed that salivary GPx was significantly lower in smoking group than non-smoking group [31], which indicates compatibility of salivary levels of antioxidants, suggesting that saliva may be an accurate biofluid for evaluation of antioxidants enzymes. Saggu T K al also evaluated salivary antioxidant enzymes through spectrophotometery method and concluded that exposure to cigarette smoke caused a statistically significant decrease in the levels of GPx in the saliva of smokers [32]. This explains that cigarette smoke may alter the detoxification of hydrogen peroxide through a decrease of GPx activity. However, our results are not in accordance with the study by Rai B et al who reported total glutathione levels in saliva to be higher in subjects with periodontitis in smokers as compared to non-smokers. Thus, smoking and periodontitis compromised the antioxidant capacity of saliva in systemically healthy patients [33].

The current study results were found consistent with the findings as reported by M. K Reejamol who showed the effect of cigarette smoking on periodontal damage in terms of antioxidants in gingival tissue [21]. Thus, it reveals that smoking increases the level of free radicals in periodontal tissues, which in turn may augment the level of periodontal destruction. Ellis and collaborators analyzed gingival tissues

from patients with severe periodontal disease and showed that the activity of catalase was decreased [34]. In contrast, the catalase activity was assayed in erythrocytes by Al-Abrash et al, who reported an increase in catalase activity in patients who suffered from diseases associated with oxidative stress [7].

Recently, Biju Thomas et al, found that levels of glutathione, catalase and selenium are significantly lower in serum of diabetic patients with periodontitis and also in healthy individuals with periodontitis but are highest in healthy controls showing that the serum levels are inversely proportional to inflammation and tissue destruction [35]. Likewise, we obtained results in saliva that are compatible with those reported for serum, which suggests that our protocol, based on spectrophotometry, is sensitive enough to provide accurate results on periodontal tissue status.

The most salient finding of our study is that salivary CAT and GPx levels were higher in gutkha chewers as compared to smokers group. This observation may be related to the actual physical location of the ST on the external tissue surface, the differences in tissue characteristics, or the greater surface area of alveolar mucosa in contact with the tobacco. These findings suggest that smokeless tobacco induces oxidative stress leading to significant changes in salivary CAT and GPx levels.

Oral conditions, such as periodontal infections, may be risk factors or indicators for important medical outcomes represents a paradigm shift in thinking about causality and the directionality of oral and systemic associations [33-36]. Determination of reliable periodontal molecular markers measurable in saliva bares precious significance regarding numerous specific pathophysiologic conditions present in periodontal pockets. Furthermore, GCF sampling requires specific sensitive techniques and equipment for quantification of biomarkers. In the clinical sense, this could present difficulty as the mandatory requirements are a complete absence of saliva and blood, which is often difficult to achieve with active periodontal pockets [37].

Although promising results have been obtained in a relatively small sample, further investigations are strongly encouraged to obtain and confirm compatibility among GCF and salivary levels under the same homogeneous protocol and increased sample size for more precise characterization of investigated antioxidants as biomarkers.

Conclusion: The present analysis may well be of great importance for further understanding the relationship between saliva and anti-oxidants. Regarding the use of the measured biochemical parameters as biomarkers, results suggest that measured salivary antioxidants can be accurate for evaluation of periodontal status. It becomes imperative to consider smokeless tobacco as harmful as smoking for periodontitis.

Ethical Approval: Ethical approval is obtained from Institute Ethical Committee and Review Boards.

Conflict of Interest: I confirm that the manuscript is original and has not been published elsewhere. Nor is it sent to any other journals for consideration. The manuscript in its submitted form has been read and approved by all authors. There is no financial relationship between any author and any commercial firm(s), which may pose a potential, perceived, or real conflict of interest.

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