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Salivary oxidative stress level among tobacco chewers and smokers- A comparative Study

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ABSTRACT

Background: Tobacco in both smoke and smokeless forms contains various toxic contents which produce oxygen free radicals causing damage to the oral tissues. Since saliva encounters tobacco in both the forms it has antioxidant defence system and also can serve as a biomarker for oral diseases. Thus, this study aims to evaluate and compare the salivary SOD, GSH-Px, CAT and MDA levels among smokers and smokeless tobacco users.

Materials and Methods: Unstimulated saliva from 240 males who visited tobacco cessation clinics for the first time was collected. Standard protocol was followed to collect saliva and assess salivary antioxidants levels from each 80 participants with habit of smoking, smokeless tobacco uses and both. The collected data was statistically analysed.

Results: The mean salivary SOD and MDA levels were significantly high among the participants with the habit of smoking and chewing, followed by chewing and smoking respectively ($p<0.05$). There was significant high reduction in the GSH-Px and CAT in participants with both the habits compared to chewing and smoking alone ($p<0.05$). Pairwise comparisons also showed a significant difference in the mean salivary oxidative stress.

Conclusion: Both smoking and smokeless tobacco modifies salivary antioxidant activity. The estimation of salivary oxidative stress can serve as a diagnostic and prognostic biomarker for oral tissue damage and dysplasia. However, an awareness of an increase in the salivary oxidative stress needs to be directed to reduce the disease burden in near future.

Keywords: *saliva, antioxidant stress, CAT, MDA, smokers, tobacco chewers*

INTRODUCTION

Tobacco is being obtained from 65 known species of tobacco plant. Of which Nicotiana tobaccum is the one which is grown widely and commercially. About 24.3% and 25.9% of the adults are current smokers and tobacco chewers in India.[1] This increased use of tobacco has led to an increase in the prevalence of non-communicable diseases such as ischemic heart disease, cancers, diabetes and chronic respiratory diseases.

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India spends nearly $27.5 billion for treating smoking and smokeless tobacco related diseases among people aged ≥35years.[2] Thus, encountering a massive economic burden. Both smoking and smokeless tobacco use attributed to 3500 death every day in India.[3]

Tobacco products either as smoke or smokeless form contain 5000 toxic substances. There is a structural analogy between nicotine and the neurotransmitter acetylcholine (Ach). Thus, nicotine combines with receptors of acetylcholine easily and endeavours actions similar to Ach, which uptights mental and physical arousal, several emotional aspects, learning and memory. This mechanism of action of nicotine makes one addicted to tobacco.[6] Evidence reports that the absorption of nicotine into the bloodstream is twice in oral smokeless forms of tobacco compared to smoke forms of tobacco. Also, nicotine absorbed from smokeless tobacco stays for a longer time in bloodstream.[7]

These toxic substances in both smoke and smokeless forms of tobacco apart from addictive nature, also produce oxidative stress causing tissue damage and apoptosis (programmed cell death).[8] Oxidative stress is the body's inability to counteract the harmful effects of excessive production of reactive oxygen species like superoxide and hydrogen peroxide.[9] Imbalance in the rate of production of reactive oxygen species (ROS) and rate of clearance by endogenous antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione, glutathione peroxidase (GSH-Px), glutathione redox enzymes, glutathione reductase (GRd) is the reason for cellular and extracellular damage.[9] O2- is a highly cytotoxic principle reactive oxygen species produce by all aerobic organisms. This production of O2- and exposure to tobacco use increases the levels of antioxidant enzymes mentioned earlier.[10] The highly reactive O2 is then converted to H2O2 by SOD which in turn is converted into molecular oxygen and water by CAT and GSH-Px as shown in figure 1.[11] The reactive oxygen species cause plasma membrane injury and cell death by oxidation and formation of lipid peroxidase on reaction with fatty acids.[11] On reaction with proteins and DNA, ROS by oxidation causes loss of enzyme activity and abnormal folding of proteins and mutation of DNA respectively.[12]

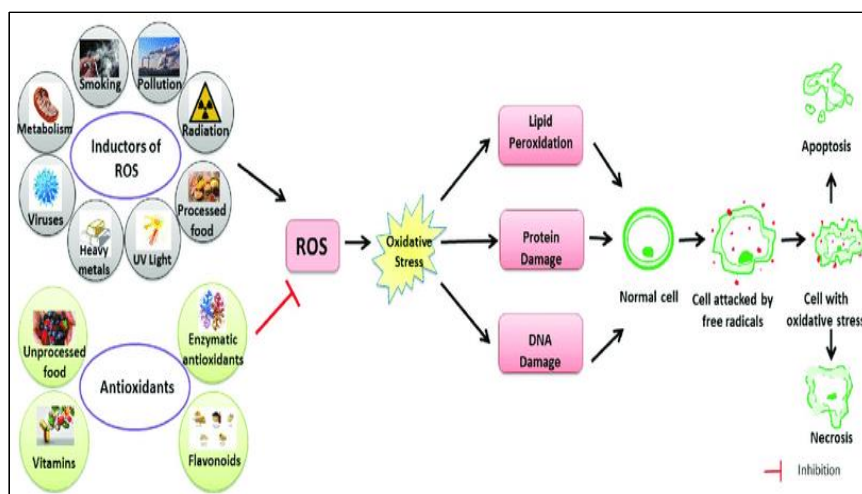

FIGURE 1: Production of reactive oxygen species and its effect

The first non-invasive diagnostic body fluid to rendezvous smoke or smokeless tobacco is saliva. Various enzymes and molecules such as uric acid and peroxidase system in saliva helps in fighting against the reactive oxygen species, there by presenting saliva as diagnostic and preventive factor.[13] About three different types of superoxide dismutase (SOD) (Fe-SOD; Mn-SOD; Cu-Zn- SOD) is found in all body tissues and few body fluids which includes saliva.[14] These SOD's fight against the ROS produced on tobacco use. Our research and knowledge have

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resulted in high-quality publications from our team. [15-29]

Thus, the present study aims to estimate and compare antioxidants such as SOD, GSH-Px, malonyl-dialdehyde (MDA) -levels among smokers and smokeless tobacco users in saliva.

MATERIALS AND METHODS

Two hundred and forty male patients with age ranging from 25-55 years were selected at convenience from the tobacco cessation counselling (TCC) clinic of Department of Public Health Dentistry, Saveetha Dental College and Hospitals (Chennai, India). Of the 240 patients, Group 1, 80 patients having the smoking habit in any form (cigarette, beedi); Group 2, 80 patients having the habit of use of smokeless tobacco (gutka, pan masala and oral snuff);

Group 3, 80 patients having the habit of use of both smoke and smokeless tobacco. All current tobacco users who were systemically healthy with no history of use of antibiotic, anti-inflammatory and/or anti-oxidant drugs at least for the past 6 months. Informed consent was obtained from the potential patients after explaining the purpose of the study. Cognitive behavioural therapy was given to all the patients recruited for the study. Ethical clearance to conduct the study was obtained from the scientific review board (SRB) of Saveetha Dental College and Hospitals, Chennai.

Salivary sample collection

Unstimulated salivary samples from all the participants were collected. Patients visiting TCC clinics were asked not to eat or drink anything before sample collection. An effort was taken such that no participants smoked or used tobacco one hour prior to saliva collection. The samples were collected only in the morning from 9am to 11 am in order to prevent variations due to circadian rhythm. The participants were asked to rinse their mouth with 15ml of distilled water to clear away exfoliated cells and debris. Then, they were asked to let saliva pool in the bottom of the mouth and drool it into sterile containers. About 1 ml of the unstimulated saliva was collected and centrifuged in the biochemistry department lab immediately at 3000 rpm for 5 minutes and stored at 4oC.[30] Then, the supernatant saliva was aspirated and used for biochemical assay.

SOD analysis of salivary samples

A standard procedure was followed to measure superoxide dismutase (SOD). It was assayed using the method of Misra and Fridovich.[31] The stored saliva was diluted with water and about 0.25 volume and 0.15 volume of chilled ethanol and ice-cold chloroform was added to it. This mixture was flustered well for one minute at 4oC and centrifuged. Then, this supernatant saliva was added into the tube containing 0.5 ml of 0.1M sodium carbonate buffer (pH of 10.2, HiMedia Laboratories Pvt. Ltd. Mumbai, India), Ethylenediaminetetraacetic Acid (EDTA) (1M, HiMedia Laboratories Pvt, Ltd, Mumbai, India) and 0.5 ml of enzyme. A final volume of 2.5 ml was obtained by adding distilled water to the mixture. About 0.2 ml of epinephrine (1mM, Sigma-Aldrich, China) was added to initiate the reaction and the change in absorbance on inhibition of conversion of epinephrine to adenochrome was measured at 480 nm using a spectrophotometer. The inhibition in terms of percentage on concentration of SOD was plotted. Percentage of inhibition was calculated using the formula:

% inhibition = 100 – (Asample/min/Ablank/min) x 100

The amount of enzyme needed to inhibit self-oxidation of epinephrine by 50% is the one unit of SOD. The units of SOD in salivary samples were expressed as units/ml. The principle of this assay is that, SOD inhibits the self-oxidation of adrenaline to adenochrome at pH 10.2. On oxidation of epinephrine at alkaline pH, SOD enzyme indirectly catalyses to produce superoxide (O2-) anion.

The adenochrome formation amount and rate was slowed down on reaction of SOD enzyme with O2-

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GSH-Px analysis of salivary samples

Salivary glutathione peroxidase (GSH-Px) level was calculated using the method describe by Rotruck et al (1973).[32] About 0.2ml of EDTA (50mM sodium phosphate buffer with 0.40mM EDTA at PH 7.0), 1mM of sodium azide, 200mM of glutathione and 0.042% (w/w) hydrogen peroxide with glutathione reductase enzyme, 10.0mM sodium phosphate buffer and ß-Nicotinamide Adenine Dinucleotide Phosphate (ß-NADPH) were mixed to obtain a final solution of 0.08mM, 1.0mM, 0.4mM, 0.25mM concentration in an incubation volume of 2 ml. The incubation was executed at 37oC and reaction was stopped at one minute intervals by adding 5% (tricyclic antidepressant) TCA. The residual glutathione content was determined by centrifugation of the contents with addition of 2ml of supernatant added 8ml of phosphate solution tailed by 1ml of 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) in a spectrophotometer at 412nm immediately.

Catalase analysis of salivary samples

The catalase (CAT) assay of salivary samples was carried out using the ELISA kit instructions (SINNOWA Medical Science & Technology, Jiangsu, China). CAT levels were measured by an affinity tag-labeled capture antibody and a reporter conjugated detection antibody that immunocaptures the sample analyte in solution. A blue colration is generated by adding TMB substrate which is catalysed by HRP. A stop solution was added to terminate the reaction with a colour change from blue to yellow. A signal generated proportionally to the amount of bound analyte and intensity at 450nm was measured and expressed in units of U/ml.

MDA analysis of salivary samples

MDA analysis of salivary samples was measured using the method describe by Stalnaya and Garishvili (1973).[33] To the 0.3ml of supernatant saliva, 3ml of 0.025 M Tris-HCl and 0.175 M KCl (pH 7.4) was mixed. Then to 2.5ml of diluted saliva, 1ml of 17%(w/v) TCA was mixed and centrifuged at 4000 x g for 10 minutes. The obtained precipitate was dislodged by centrifugation which was then reacted with 1ml of 0.8% (w/v) of 2-thiobarbituric acid (TBA) in a boiling water bath for 10 minutes. On cooling to room temperature, the absorption of supernatant was recorded by UV-visible spectrophotometer at 532nm (Thermo Fisher Scientific, UK). The obtained values were then compared with a series of standard solutions of 1,1,3,3 tetraethoxypropane (TMP) and expressed as micromoles per millilitre (mcmol/L).

Statistical Analysis

The obtained data were analysed using Statistical Package for Social Sciences (SPSS) software version 23.0. Normal distribution of the data was assessed using Kolmogrov-smirnov numerical test and data were found to be normally distributed. Mean comparison of SOD, GSH-Px and MDA levels among smokers, smokeless tobacco users and both users were carried out using One-Way ANOVA. Pairwise comparison was carried out with Tukey's post-hoc test. Significant p-value <0.05 was considered.

RESULTS

The mean age of participants in Group 1 was 37.58 ± 6.79; Group 2 was 39.29 ± 7.45 and Group 3 was 38.13 ± 7.91 with no significant difference between the groups (p=0.776). The mean SOD among participants of Group 1 was found to be 28.78 ± 9.184; Group 2 was 39.67 ± 11.892 and Group 3 was 45.29 ± 12.131. There was a significant difference in the mean SOD among the groups (p=0.000) (Table 1). Also, there was a significant difference in mean GSH-Px among the participants in the groups (p=0.000) as shown in Table 2. Similarly, the mean catalase (CAT) among the participants in Group 1 was 7.51 ± 1.26; Group 2 was 6.12 ± 0.94; Group 3 was 5.03 ± 0.64 U/ml with a significant difference (p=0.028) as shown in Table 3. There is a significant difference in MDA levels among the groups (p=0.017) shown in Table 4. Pair-wise comparison using Tukey's HSD test showed a significant mean difference in SOD, GSH-Px, CAT and MDA between the groups (p<0.05). Mean levels of SOD, GSH-Px, catalase and MDA levels of all three groups were shown in Figure 2 and 3.

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TABLE 1: Mean comparison of SOD units/ml among the study participants

Group	Mean ± SD	F value	p value
1	28.78 ± 9.184	72.546	0.000
2	39.67 ± 11.89		
3	45.29 ± 12.13		
Pair-wise comparison by Tukey's HSD test			
Comparison	Mean difference	p value	95% CI
Group 1 vs Group 2	-10.89	0.018	-14.52 to -4.268
Group 2 vs Group 3	- 5.62	0.007	-9.467 to -1.277
Group 3 vs Group 1	16.51	0.001	9.583 to 19.48

TABLE 2: Mean comparison of salivary GSH-Px among the study participants

Group	Mean ± SD	F value	p value
1	1.78 ± 0.042	81.752	0.000
2	0.56 ± 0.006		
3	0.07 ± 0.001		
Pair-wise comparison by Tukey's HSD test			
Comparison	Mean difference	p value	95% CI
Group 1 vs Group 2	1.22	0.004	0.56 to 2.89
Group 2 vs Group 3	0.49	0.035	0.08 to 1.64
Group 1 vs Group 3	1.71	0.000	0.91 to 2.59

TABLE 3: Mean comparison of salivary catalase U/ml among the study participants

Group	Mean ± SD	F value	p value
1	6.93 ± 0.531	83.167	0.000
2	5.85 ± 0.473		
3	3.82 ± 0.432		
Pairwise comparison by Tukey's HSD test			
Comparison	Mean difference	p value	95% CI
Group 1 vs Group 2	1.08	0.001	0.09 to 2.47
Group 2 vs Group 3	2.03	0.028	1.27 to 3.52
Group 1 vs Group 3	3.13	0.000	1.58 to 4.26

TABLE 4: Mean comparison of MDA mcmol/ml among the study participants

Group	Mean ± SD	F value	p value
1	17.56 ± 4.125	85.249	0.000
2	28.94 ± 5.278		
3	34.21 ± 5.648		
Pair-wise comparison by Tukey's HSD test			
Comparison	Mean difference	p value	95% CI
Group 1 vs Group 2	-11.38	0.006	-8.62 to -2.89
Group 2 vs Group 3	-5.27	0.012	- 9.34 to -3.49
Group 3 vs Group 1	16.65	0.000	11.48 to 22.23

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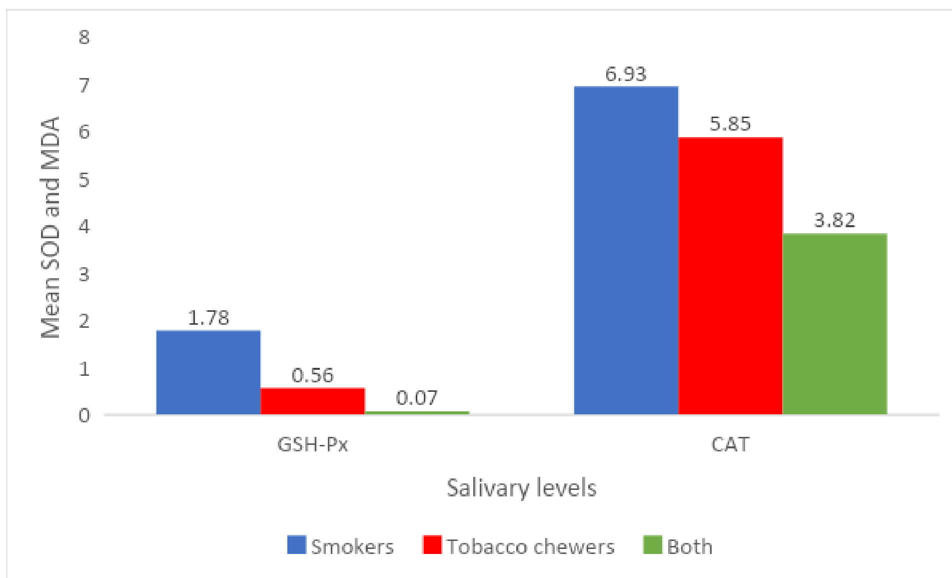

FIGURE 2: Mean level of SOD and MDA levels among the study participants

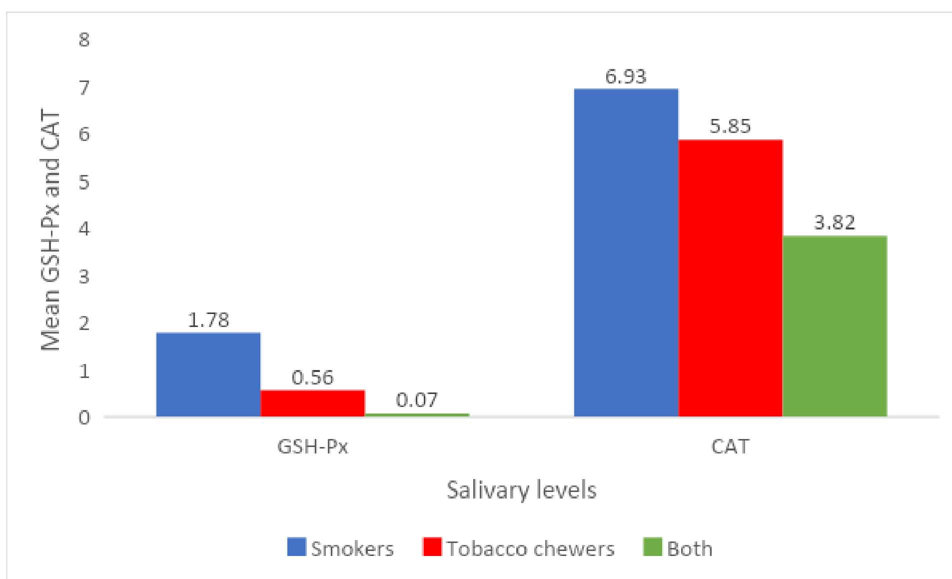

FIGURE 3: Mean level of GSH-Px and CAT levels among the study participants

DISCUSSION

An imbalance in the rate of production of reactive oxygen species (ROS) and rate of clearance by endogenous antioxidants produce oxidative stress. This oxidative stress and free radical reactions contribute as aetiology for numerous systemic diseases such as cardiovascular disease, vascular ageing, precancerous and cancerous lesions etc. [34-36] Reactive oxygen species react with lipids, proteins and DNA/RNA by oxidation to cause protein damage and mutation of DNA.[37] The first biological fluid to come to encounter tobacco in the form of smoke and smokeless is saliva.

The prime objective of the present study was to estimate and compare the salivary SOD, GSH-Px, catalase and MDA levels among the smokers, smokeless tobacco users and both users. To the best of our knowledge, this is the first study to evaluate the anti-oxidants level among tobacco users in both forms.

The present study results showed a significant increase in the mean levels of salivary SOD

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among participants who smoke and also use smokeless tobacco (45.29 ± 12.13) compared to smokers and smokeless tobacco users. On comparison of mean GSH-Px with smokers and chewers, it was found that there is a significant decrease among the participants who have both the habits of smoking and chewing (0.07 ± 0.001). Similarly, there is a significant decrease in the catalase enzyme among participants who use smokeless tobacco (5.85 ± 0.473) and both (3.82 ± 0.432) compared to smokers. It was also found a significant increase in MDA levels among participants having the habit of smoking and chewing (34.21 ± 5.648).

A previous study among the people of similar geographic location showed a significant increase in salivary SOD among smokers compared to non-smokers which in consistency with the present study results.[38,39] In contrast to the results of present study, a case-control study among khat chewers showed no significant change in the SOD and GSH-Px levels between chewers and non-chewers.[40] The reason behind this difference could be attributed to the concentration of toxic constituents in different forms of smokeless tobacco.

A case-control study among tobacco chewers showed a significant increase and decrease in the salivary MDA and CAT levels compared to controls.[41] These results are in consistency with results of our study which also showed a significant increase and decrease in the salivary MDA and CAT levels among tobacco chewers.

A previous study among 200 smokers and 200 non-smokers of people belonging to the same geographic location of the present study showed no significant difference in salivary CAT levels.[42] These results were in contrast to the results of the present study which can attributed to the fact of difference in the constituents of indigenous tobacco products.

One of the biomarkers for lipid peroxidation is MDA which is found to be higher in both forms of tobacco users. Also, salivary MDA levels are found to be increased in other pathological conditions such as periodontitis, cardiovascular disease, oral potentially malignant disorders and oral cancer. [43-45] Lipid peroxidation by constituents of tobacco in the form of smoking and chewing has contributed to an increase in the salivary MDA levels than smoking and tobacco chewing alone. The increase in the salivary CAT among participants who have the habit of both smoking and chewing can be due to peroxidation of proteins and oxidation of DNA.[46] Thus, oxidative stress in saliva among users of both the forms of tobacco can be attributed to lipid and protein peroxidation. Increased oxidative stress by ROS and improper functioning of antioxidant defence might be the contributing aetiology for cancer related oral diseases.

Though an effort was taken to consider the eligibility criteria in the present study, the finding of our study could be limited, since we failed to control the confounding factors that can impact salivary oxidative stress such as gingival, periodontal disease and diet. Also, we failed to consider the rate, frequency, amount and years of habit (smoking and chewing) which might be directly proportional to salivary oxidative stress. Further longitudinal studies with control of confounding factors are needed to assess the clinical effects of increased salivary oxidative stress in function as a diagnostic and prognostic biomarker.

CONCLUSION

There is a marked increase in the salivary SOD, GSH-Px, CAT and MDA levels among tobacco chewers and both smokers and chewers contributing to increased salivary oxidative stress. The people with habit of both smoking and chewing are at high risk for various oral diseases such as potentially malignant disorders, cancer, periodontitis and dental caries compared to smokers and tobacco chewers alone. Awareness programs need to be targeted in this context to reduce the oral cancer burden.

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