Original Article

Oxidant and antioxidant status among tobacco users: A cross-sectional study

ABSTRACT

Background: Smokeless and smoking tobacco use results in increased oxidative stress and lipid peroxidation, which play a major role in the causation of cancer in tobacco habituates. Malondialdehyde (MDA) is a product of lipid peroxidation, and glutathione peroxidase (GPx) and superoxide dismutase (SOD), the main enzymes in the antioxidant defense system, are assessed among tobacco users. This study gave insight into the relationship between tobacco use, oxidative stress, and antioxidant enzyme activity.

Aims and Objectives: This study aimed to estimate the levels of lipid peroxidation product MDA and antioxidant enzymes SOD and GPx among tobacco users and compare them with controls.

Method: A case–control study comprising 30 smokeless tobacco users, 30 smokers, and 30 controls was enrolled for the study. Serum MDA was assayed by the thiobarbituric acid method; serum SOD and GPx were assayed using Ransel antioxidant kits. The results were statistically analyzed using descriptive and inferential statistical analysis.

Results: Serum MDA levels, which indicate oxidative stress, were increased among all tobacco users and significantly increased among smokeless tobacco users as compared to smokers. Serum SOD and GPx levels were decreased among both forms of tobacco users compared with controls. With an increase in duration and frequency of tobacco use, there was a significant increase in serum MDA levels among both smokers and chewers and a decrease in serum SOD and GPx levels.

Conclusion: In the present day, the tobacco epidemic has attained enormous proportions with the tobacco habit starting as early as 13–14 years and leading to serious conditions with high morbidity and mortality. These biochemical parameters such as MDA, SOD, and GPx, which act as marker of oxidant and antioxidant system, can constitute important tools for evidence-based medicine for educating patients and motivating interventions in tobacco cessation therapy.

Keywords: Antioxidants, glutathione peroxidase (GPx), malondialdehyde (MDA), superoxide dismutase (SOD), tobacco users

INTRODUCTION

The global epidemic of tobacco use—both smokeless and smoking forms—continues more than a half-century after its use was causally linked to lung cancer and other diseases.[1] According to the World Health Organization (WHO), India is home to 12% of the world's smokers. An estimated one million Indians die annually from tobacco-induced diseases, and projections forecast that by 2020, tobacco will account for 13 percent of deaths in India. India also has one of the highest rates of oral cancer in the world, partly attributed to the high prevalence of tobacco chewing.[2]

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Literature research has shown an effect on oxidant and antioxidant defense system among tobacco users, especially in smoking form.[3-5] Environmental stressors including ultraviolet (UV) radiation and various pollutants such as tobacco and polycyclic aromatic hydrocarbons generate reactive oxygen species (ROS) in the body, which results in increased oxidative stress and lipid peroxidation.[6] Lipid peroxidation is a chain reaction providing a continuous supply of free radicals that initiate further peroxidation unless checked by antioxidants. Under physiological conditions, the human antioxidant defense system including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione (GSH) allows the elimination of excess ROS.[7] In healthy cells, an intricate balance between prooxidant and antioxidant states is maintained, but in oxidative stress there is an imbalance between prooxidants and antioxidants due to decreased antioxidant capacity and increased free radical generation rendering tobacco users to peroxidative stress. Moreover, the heat generated during smoking and the pH changes during tobacco chewing results in certain changes in the body fluids, which affects the formation and stabilization of free radicals.[8] These free radicals cause changes in antioxidant levels, and the free radical-associated damages are reflected through antioxidant enzyme activities in the blood.[8] Chronic use of tobacco also results in quantitative and qualitative changes in saliva. Change in the resting whole-mouth salivary flow rate (SFR) plays a significant role in the pathogenesis of various oral conditions.[9] Increased blood levels of malondialdehyde (MDA) and decreased blood levels of antioxidants such as CAT, SOD, and GPx have been reported in oral cancer patients.[10]

In view of the possible oxidative stress involved with tobacco consumption, this study was undertaken to estimate MDA levels and the first-line defense antioxidants such as SOD and GPx among tobacco users and compare them with controls. These biological parameters would be of importance in evaluating the role of tobacco on antioxidant status among tobacco users.

METHODOLOGY

The study was conducted at a dental and medical college in Bangalore, India, between December 2011 and July 2013. The non-probabilistic sampling method was used for sample size estimation, and the sample size was estimated from the cited study.[11] The sample size was 30 each for smokeless tobacco users, smokers, and controls. Ethical clearance from the institution's review board was obtained, and the patient's informed consent was taken.

Protocol for patient selection: Individuals in the age group of 25–50 years, using tobacco in any form, for more than 2 years with a frequency of 3 or more than three times a day, were included in the study. Individuals receiving nutritional supplements—vitamins A, C, E, and vitamin B complex—with a history of cardiovascular, endocrine, hepatic, and gastrointestinal disorders and having any potentially malignant disorders and malignancy, were excluded.

Details of tobacco consumption (duration, type, and frequency), dietary supplements, medical history, etc., were recorded in a questionnaire. Under aseptic conditions, 5 ml of venous blood from the median cubital vein was drawn by vein puncture.

Preparation of blood sample for MDA: 3 ml of the 5 ml blood sample collection was transferred to a clean test tube and allowed to clot. Serum was separated by centrifugation at 3000 rpm and stored at -20°C until the estimation of MDA levels.

Processing of sample for GPx: 1 ml of the 5 ml blood sample was transferred to a heparinized vacutainer and stored at -20°C until analysis.

Preparation of lysate for SOD estimation: 1 ml of the 5 ml blood sample was collected in an ethylenediaminetetraacetic acid (EDTA) vacutainer and centrifuged at 3000 rpm to separate the plasma, which was then removed. The red blood cell (RBC) pellet was then washed three times with sterile saline to ensure the complete removal of plasma, leucocytes, and platelets. The washed RBCs were hemolyzed by the addition of sterile distilled water (1:5). Then, the lysate was centrifuged at 3000 rpm for 15 mins to make the lysate ghost free. The supernatant was stored at -20°C until analysis. The estimation of both enzymes, that is, SOD and GPx, was analyzed using the Ransel antioxidant enzyme kit (manufactured by Randox). Thiobarbituric acid reactive substances (TBARS) were estimated by the method described by Wilbur *et al*.[11]

Statistical method: Descriptive and inferential statistical analysis was performed in this study. The sample size was estimated using nMaster software (developed by the Department of Biostatistics, CMC, Vellore) from the cited study.[11]

Standard deviation of MDA levels in smokeless tobacco users: 0.97.

Standard deviation of MDA levels in controls: 1.22.

Estimated difference between the means: desired confidence interval of -95.

The sampling method used was non-probabilistic sampling wherein all the patients meeting the inclusion and exclusion criteria were recruited after informed consent during the study period.

RESULTS

The mean age was 33.10 ± 4.96 years among controls, 39.26 ± 7.78 years among smokeless tobacco users, and 34.93 ± 8.44 years among smokers. The gender distribution was statistically similar in the three groups with $P = 0.206$. The duration of tobacco use was made into three subgroups: <5 yrs, 6–10 years, and >10 yrs. The duration of tobacco use was statistically similar in the two groups studied with $P = 0.978$. The frequency of habit use was divided into three subgroups: <5, 6–10, and >10 times per day. The frequency of the habit was statistically similar among the two groups with $P = 0.889$.

Serum MDA levels were divided into five subgroups, SOD levels into three subgroups, and GPx levels into six subgroups, respectively [Tables 1-3, respectively]. The mean MDA levels were found to be statistically significant among the controls and smokeless tobacco users, among the controls and smokers, and among the smokeless and smoking forms of tobacco users with *P* value <0.001 for each group. Mean serum SOD levels were found to be statistically significant among the controls and smoking and smokeless forms of tobacco users. Mean serum GPx levels were found to be statistically significant among the controls and smokeless tobacco users and among the controls and smokers with a *P* value of <0.001 for each group [Graph 1a-c].

Among smokeless tobacco users, serum MDA (nmol/ml) vs SOD (U/ml) was found to be statistically significant with a *P* value of 0.002. Among smokers, serum MDA (nmol/ml) vs SOD (U/ml) was also found to be statistically significant with a *P* value of <0.001. Among smokers, serum MDA (nmol/ml) vs GPx (U/gmhb was found to be statistically significant with a *P* value of 0.009. Among controls, serum SOD (U/ml) vs GPx (U/gmhb was found to be statistically significant with a *P* value of 0.032 [Table 4].

Among smokeless tobacco users, only serum MDA and SOD levels were found to be statistically significant [Table 5], whereas among smokers all three values, serum MDA, SOD, and GPx levels, were found to be statistically significant with the duration of the tobacco use [Table 6].

Table 1: Serum MDA levels in five subgroups among the study groups

MDA (nmol/ml)	Controls		Smokeless tobacco users		Smokers	
	No	%	No	%	No	%
<1	6	20.0	0	0.0	0	0.0
1–2	10	33.3	2	6.7	5	16.7
2–5	13	43.3	6	20.0	12	40.0
5–10	1	3.3	16	53.3	12	40.0
>10	0	0.0	6	20.0	1	3.3
Total	30	100.0	30	100.0	30	100.0

Table 2: Serum SOD levels in three subgroups among the study groups

SOD (U/ml)	Controls		Smokeless tobacco users		Smokers	
	No	%	No	%	No	%
100–150	0	0.0	17	56.7	10	33.3
151–200	22	73.3	13	43.3	20	66.7
>200	8	26.7	0	0.0	0	0.0
Total	30	100.0	30	100.0	30	100.0

Table 3: Serum GPx levels in six subgroups among the study groups

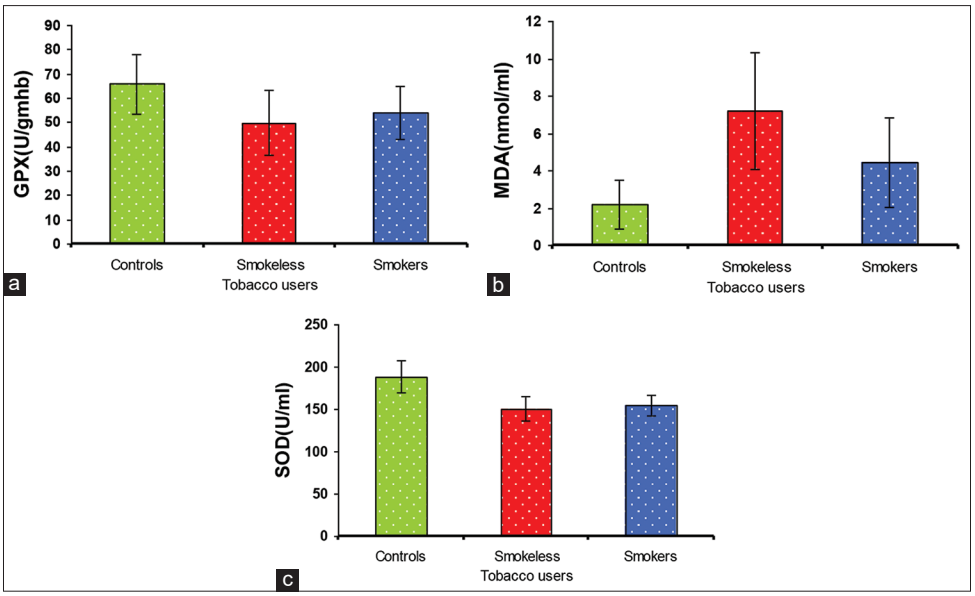
GPx (U/gmhb)	Controls		Smokeless tobacco users		Smokers	
	No	%	No	%	No	%
<30	0	0.0	2	6.7	1	3.3
31–40	0	0.0	7	23.3	2	6.7
41–50	5	16.7	6	20.0	9	30.0
51–60	6	20.0	10	33.3	10	33.3
61–70	8	26.7	4	13.3	7	23.3
>70	11	36.7	1	3.3	1	3.3
Total	30	100.0	30	100.0	30	100.0

Table 4: Pearson correlation of MDA, SOD, and GPx in three groups studied

Pair	Controls		Smokeless tobacco users		Smokers	
	r	*P*	*r*	*P*	*r*	*P*
MDA (nmol/ml) vs SOD (U/ml)	0.146	0.441	-0.533	0.002**	-0.708	<0.001**
MDA (nmol/ml) vs GPx (U/gmhb	0.005	0.978	-0.098	0.605	-0.470	0.009**
SOD (U/ml) vs GPx (U/gmhb	0.392	0.032*	0.184	0.330	0.286	0.125

DISCUSSION

Tobacco use is considered the major etiological factor for oral cancer development, accounting for 30–40% of all cancer cases in India. Tobacco consumption generates ROS such as superoxide anion (O_2), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH), and MDA and nitric oxide (NO) are



Graph 1: Comparison of study variables in three groups studied. (a) GPx (b) MDA (c) SOD

Table 5: Correlation of MDA, SOD, and GPx according to duration among smokeless tobacco users

Duration of habit in smokeless tobacco users	MDA (nmol/ml)	SOD (U/ml)	GPx (U/gmhb)
<5	3.45±1.7	160.83±10.23	56.02±9.78
6–10	7.02±2.85	155.55±17.17	51.35±12.41
>10	9.11±2.1	141.31±6.87	45.58±15.02
P	<0.001**	0.004**	0.264

Table 6: Correlation of MDA, SOD, and GPx according to duration among smokers

Duration of habit in smokers	MDA (nmol/ml)	SOD (U/ml)	GPx (U/gmhb)
<5	2.16±0.5	162.09±7.18	60.99±7.32
6–10	5.06±2.16	152.5±14.38	54.88±9.7
>10	6.18±1.95	148.18±11.49	47±10.75
P	<0.001**	0.020*	0.006**

directly involved in the multistage process of carcinogenesis by bringing out continuous endogenous damage to cellular deoxyribonucleic acid (DNA).[8] An antioxidant defense system is essential for the deactivation and removal of these ROS.

MDA is a highly reactive three-carbon dialdehyde produced as a byproduct of polyunsaturated fatty acid peroxidation and arachidonic acid metabolism. MDA readily combines with several functional groups of molecules including proteins, lipoprotein, and DNA. It is formed during oxidative degeneration as a byproduct of ROS and is accepted as an indicator of lipid peroxidation.[12] In our study, the mean MDA level for smokeless tobacco users was much higher (7.21 ± 3.11) when compared to controls (2.17 ± 1.32 nmol/ml). This finding is in accordance with the study of Samal I R *et al.* where the mean serum MDA level for controls was 3.871±0.22 nmol/ml and for smokeless tobacco users was 20.75 ± 0.97 nmol/ml. This can be due to the different durations for which smokeless and smoking tobacco products are used. Among smokeless tobacco user subgroups, serum MDA levels were significantly increased with the duration of tobacco use [Table 5]. This finding is similar to the study conducted by I R Samal.[12] So, the longer the duration of usage, the higher the oxidative stress. Among smokers, the MDA level was more than double that of controls as compared to smokeless tobacco users where it was found to be more than three times [Table 7]. This is in accordance with the study conducted by Lykkesfeld J *et al.*[3] among the Danish population. In addition to 4000 odd hazardous chemicals, cigarette smoke contains free radicals that can cause cellular damage. It was demonstrated that one cigarette puff contains 1014 free radicals in the tar phase and 1015 radicals in the gas phase, and the tar and gas phases are two major phases in cigarette smoke. Tar-phase free radicals are mostly quinine–hydroquinone, and they are not highly reactive; however, gas-phase free radicals are generally more reactive.[13] Hence, smokers are more prone to oxidation from the inhalation of large numbers of gas phase and other radicals giving rise to increased oxidative damage.[14] Moreover, depletion of plasma antioxidants otherwise protecting against oxidative damage such as lipid peroxidation has consistently been observed among smokers leading to increased stress.[3]

In our study, we have assessed SOD and GPx as they form the first-line endogenous antioxidant defense system in the body. The mean level of serum SOD among smokeless tobacco users

Table 7: Comparison of study variables in the three groups studied

Variables	Controls	Smokeless tobacco users	Smokers	Controls vs smokeless tobacco users	Controls vs smokers	Smokeless tobacco users vs smokers
MDA (nmol/ml)	2.17±1.32	7.21±3.11	4.41±2.40	<0.001**	0.001**	<0.001**
SOD (U/ml)	188.40±18.67	150.43±14.45	154.43±12.30	<0.001**	<0.001**	0.574
GPX (U/gmhb)	65.79±12.19	49.79±13.41	54.23±10.88	<0.001**	0.001**	0.340

was 150.43 ± 14.45 U/ml, which was much lower compared with the control group, that is, 188.40 ± 18.67 U/ml, which is in accordance with the previous study. SOD level was significantly decreased ($P = 0.004$) with an increase in the duration of habit, which is in accordance with the study by Samal *et al*.[12]

This can be attributed to the production of free radicals by smokeless tobacco products, which are highly reactive, act as initiators and/or promoters of carcinogenesis, and alter the cellular antioxidant defense system. An effective detoxification mechanism comprising SOD and CAT works in a sequential manner in the disposal of superoxide radical and the conversion of H_2O_2 to water.[15]

In our study, the mean serum SOD level among smokers was 154.43 ± 12.30 U/ml, much lower than the control group, that is, 188.40 ± 18.67 U/ml, which is in accordance with the finding of Bolzan *et al*.[16] but was contrary to that of Durak I *et al.* who suggested that smoking caused no impairment in the enzymatic antioxidant defense system and did not lead to oxidant stress in the erythrocytic activities of SOD.[16,17] Serum SOD levels were significantly decreased with an increase in the duration of tobacco use, which is in accordance with the findings of Garg N Singh *et al*.[18] and Zhou JF *et al*.[19] This decrease in SOD level among smokers could be due to the inactivation of SOD by H_2O_2. The variability of the effects of smoking on antioxidant enzyme activities may be due to multiple reasons, such as interaction between direct and passive smoke exposures, different smoking patterns, and differences in the compositions and brands of smoking tobacco used.[20,21]

The mean level of serum GPx in smokeless tobacco users was 49.79 ± 13.41 U/gmhb (unit per gram hemoglobin), which was significantly lower than the control group (65.79 ± 12.19 U/gmhb) with a *P* value <0.001.

This result is contrary to the study findings, which reported an increase in the mean serum GPx level in smokeless tobacco users as compared to controls. This can be explained by the fact that GPx acts after SOD in antioxidant defense and this could be an adaptive phenomenon when free radical generation exceeds the quenching capacity of SOD.[8,22] Various smokeless tobacco forms used in India have additives, which by themselves have certain antioxidant properties. Serum GPx levels were not significantly altered in the subgroups of tobacco smokers with an increase in duration of habit. The mean level of serum GPx among tobacco smokers was lower than the control group similar to other studies.[4,23]

Strength: Elevated MDA levels and diminished antioxidant can be used as predictors of premalignant lesions resulting from tobacco use.

Limitation: Small sample size.

CONCLUSION

This study gives an insight into the relationship between tobacco use, oxidative stress, and antioxidant enzyme activity. The elevated MDA levels and diminished antioxidant enzyme levels observed in this study can act as a predictor for pre-potentially malignant lesions. These biochemical parameters can constitute important tools for evidence-based medicine for educating patients and motivational intervention in tobacco cessation therapy and would prevent the development of tobacco-induced lesions.

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Conflicts of interest

There are no conflicts of interest.

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