Estimation of serum superoxide dismutase and glutathione peroxidase levels in tobacco chewers and smokers: A comparative study

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Abstract

Introduction: Habitual tobacco use is known to cause various health hazards by initiating oxidative stress through the release of free radicals. The free radicals induce cellular damage that is reflected through alterations in antioxidant enzymes levels like superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) within circulating erythrocytes. Therefore, it is important to estimate the erythrocytic SOD and GPx levels to assess tobacco induced oxidative stress.

Aim: The aim of the present study is to assess the effect of tobacco chewing and smoking on serum antioxidant enzymes superoxide dismutase and glutathione peroxidase levels.

Materials and Methods: The present study comprised of 4 study groups including healthy control (n=25), tobacco smokers (n=25), tobacco chewers (n=25) and combination habit group (n=25). The case subjects were further sub-divided depending upon the frequency of habits i.e. > 10 times/day and <10 times/day and duration of habits i.e. > 10 years and <10 years. Erythrocytic SOD and GPx levels were then estimated using the Randox and Ransel kits respectively.

Result: Mean erythrocytic SOD levels were significantly decreased in cases compared to control group whereas mean erythrocytic GPx level was significantly increased in cases compared to controls. When SOD and GPx were compared for the frequency and duration of habits, GPx levels were significantly increased among smokers who smoked <10smokes/. Whereas, there was a significant difference in SOD levels amongst tobacco chewers with < 10 years of habit.

Conclusions: The present study confirmed the relationship between antioxidative enzymes activity, oxidative stress and tobacco. Frequency and duration of tobacco consumption were shown to alter antioxidant enzymes levels. Therefore, estimation of these enzyme levels will help in early intervention of tobacco habit by patient education and motivation and can minimize the risk of tobacco induced lesions.

Keywords: Tobacco, Erythrocytic superoxide dismutase (SOD), Glutathione peroxidase (GPx), Oxidative stress.

Introduction

Tobacco use is the single extremely important preventable cause of illness and death.[1] The tobacco epidemic is one of the biggest public health threat the world has ever faced, killing more than 7 million people every year.[2] As a consequence, there is an increasing incidence of tobacco related mortality as compared to other causes of death like alcohol and addictive drugs.[3]

Tobacco is considered as one of the major risk factor for a number of chronic diseases including cancer, lung diseases and cardiovascular diseases. [4] Usually, commercially available tobacco is made entirely or partly of tobacco leaf as the raw material, which is intended to be smoked, sucked, chewed or snuffed. Tobacco constituents are known to exacerbate aspects of the respiratory burst and enhance reactive oxygen species (ROS) production.[5] Cigarette smoke is known to contain 10^7 oxidant molecules per puff of which 10^{14} are oxygen free radicals.8. The heat (generated during smoking) as well as pH (changes during chewing) of body fluids due to tobacco consumption affect the formation and stabilization of free radicals.[6]

On the other hand, smokeless tobacco (SLT), available as snuff or chewing tobacco causes approximately twice the amount of nicotine to be absorbed per dose when compared to cigarettes.

Nicotine when orally absorbed stays longer in the bloodstream.[7] Furthermore, smokeless tobacco extract is more toxic than nicotine in terms of their respective oxidative stress actions and produces oxidative tissue damage and apoptosis.[8] Also, the alkaline conditions observed in betel nut chewing is reported to favor the formation of free radicals.[9]

Free radicals and oxidants are important both as toxic and beneficial compounds, as they can be either harmful or helpful to the body.[10] At low or moderate concentrations, ROS and reactive nitrogen species (RNS) are necessary for the maturation process of cellular structures and act as weapons for the host defense system.[11,12] When formed in excess, free radicals and oxidants generate a phenomenon called oxidative stress, a deleterious process that can seriously alter the cell membranes and other structures such as proteins, lipids, lipoproteins, and deoxyribonucleic acid (DNA).[11-15] Free radical associated damage leads to an imbalance between pro-oxidant and anti-oxidant states. This imbalance plays an important causative role in carcinogenesis.[16]

The human body has several mechanisms to counteract oxidative stress by producing antioxidants that have a shielding role by scavenging the free radicals.[10] The most important enzymatic antioxidants is SOD, which catalyzes dismutation of the superoxide

anion (O_2^-) into hydrogen peroxide (H_2O_2), which is then deactivated to H_2O by CAT and GPx enzymes.[17] Therefore, antioxidants can enhance the immune defense and lower the risk of cancer and degenerative diseases.

Consequently, monitoring biological parameters like antioxidant enzyme system would be of fundamental importance in evaluating the role of tobacco on antioxidant status in tobacco users.[9] Particularly, as the plasma level of antioxidant enzymes are known to be negatively correlated with cancer mortality rates.[18]

Nearly 80% of the more than 1 million smokers worldwide live in low- and middle-income countries where the burden of tobacco related illness and death is heaviest.[19] Considering the prevalence of tobacco use in India which was estimated at 37% among the population of 15 years and above, there is a significant mortality burden.[20] And India is one of the few countries where prevalence of smoking and smokeless tobacco use is high and is characterized by dual use of tobacco (use of both smoking and SLT products) thus making India one of the highest tobacco consumers in the world.[21]

The objectives of this study were to estimate erythrocytic SOD and GPx enzyme levels in tobacco chewers, smokers and those without the habit of chewing and smoking tobacco, as well as to compare the antioxidant levels within the study and control group.

Materials and Methods
Study Design: This study was conducted among 100 age matched healthy individuals at the Department of Oral Medicine and Radiology at VIDS and RC Bengaluru.

This study included 100 participants of whom 25 were healthy controls (without any tobacco habits), 25 were tobacco smokers, 25 were tobacco chewers and 25 were in the combination habit group. The age group ranged from 18-75 years. The study was approved by the Ethical and Research Committee of VIDS and RC Bengaluru, to use human participants in the research study.
Group I- Tobacco smokers
Group II- Tobacco chewers
Group III- Combination habit
Group IV- Controls

All the subjects were personally questioned through a questionnaire. It included details of tobacco consumption (form, duration, type & frequency), dietary supplements such as vitamins, drug intake, alcohol, medical history etc.
Inclusion Criteria:
1. Subjects of age group 18-75 years with tobacco chewing and smoking habits not less than a year and with no tobacco related lesions.
2. Control group consisted of age and sex matched

healthy individuals with no tobacco habits.
Exclusion Criteria:
1. Patients with habit of alcohol consumption.
2. Patients with any underlying systemic disease which may also influence the antioxidant level.
3. Patients using dietary supplements.

Exclusively healthy subjects with history of tobacco habit but lacking any tobacco related lesions were chosen to see if habit alone was accountable for the alteration of antioxidant enzyme levels much before the commencement of any tobacco related lesions. These findings can have diverse influence over the outcome of the study.

Sample Preparation
About 2ml of venous blood was collected from all study subjects. Then the collected blood sample was transferred to vacutainer container with EDTA as anticoagulant, plasma was separated, and erythrocytes were washed with 0.9% normal saline. Centrifugation was done for 10 min at 3000 rpm after each wash. These erythrocytes were made up to 2ml with cold re-distilled water and mixed and left to stand at 4°C for 15 min. This hemolysate was then used to estimate the SOD and GPx level.

SOD and GPx were assayed using RANSEL antioxidant enzyme kit (RANDOX laboratories).

Statistical Analysis
The collected data were statistically analyzed with the use of following tests:
1. ANOVA.
2. Mann-Whitney Test.
3. Pearsons correlation coefficient.

Results
There was no statistical difference observed for age between all the groups (Table 1). However, in the age group of 20- 35 years a general tendency towards smoking was seen whereas in the age group of 36-50 years this trend changed into combination of tobacco smoking and chewing. In the later age group of 51-70 years tobacco chewing was more common. (Table 1 & Fig.1).

Gender distribution showed that most of the participants of the control and case groups were male with female participants present only in the tobacco chewers group (24%) (Table 2).

The values of mean with standard deviation, of erythrocytic SOD were calculated for all the 4 groups and given in Table 3a & Fig. 2. When compared to the control group the mean value of erythrocytic SOD was significantly (P<0.05) decreased in all the three case groups. The highest mean erythrocytic SOD level was 411.438 ± 81.277 in the control group and it was least in the smokers group (250.718±76.548). ANOVA test showed a highly statistical significant difference in the level of erythrocytic SOD among all the four groups

(p<0.001). When Tukeys post hoc test (Table 3b) was applied to see the difference between each group, it was found that there was no significant difference between the (I) smokers group and (III) tobacco Chewers + Smokers group (P=0.102) as well as (II) tobacco chewers and (IV) control group i.e. (P=0.783), whereas highly significant (**P<0.001) difference between the other groups.

The values of mean with standard deviation, median and range of erythrocytic GPx were calculated for all the 4 groups and are given in Table 4a & Fig 3. When compared to the control group the mean value of erythrocytic GPx was significantly (P<0.05) increased in all the three case groups. The highest mean erythrocytic GPx value was seen in tobacco chewers group, 25511 ± 2149.80 and was the least in control group i.e. 12000 ± 4320.04. ANOVA test showed a statistically highly significant difference in the level of erythrocytic GPx among all the four groups (p<0.001). (Table 4b)

Depending on the frequency of smoking the groups were further divided into two subgroups, those consuming tobacco in any form <10 times/day and >10 times/ day. (Table 5). Student t test showed that there is no significant difference (p=0.524) in the mean erythrocytic SOD level in both of these subgroups. With regards to mean erythrocytic GPx level, a significant difference (P=0.05) was noticed in the subgroup of <10 smokes /day showing an increased value (17219±5071). On evaluation of relationship between duration of smoking and SOD and GPx values among different groups (Table 6). Student t test showed that there was significant difference (p=0.04) only in the mean erythrocytic SOD level of tobacco chewers group. Whereas there was no significant difference in the mean GPx level among all groups.

On evaluation of relationship between age and erythrocytic SOD, GPx was shown in (Table 7). Pearson's correlation coefficient showed no significant correlation between the age and erythrocytic SOD and GPx among all the 4 groups. The correlation was negative indicating that as the age increases erythrocytic SOD level decreases. Whereas with respect to the erythrocytic GPx in all the habit groups, Pearson's correlation showed no significant correlation between the age and erythrocytic GPx in all the four groups.
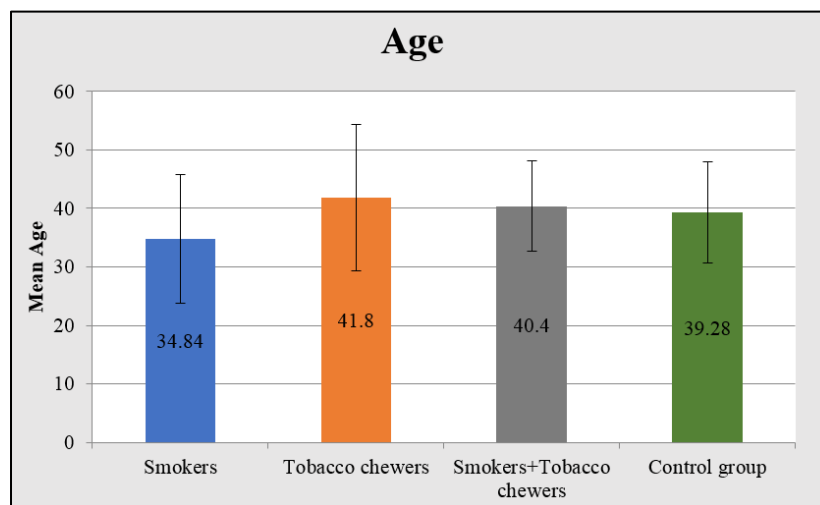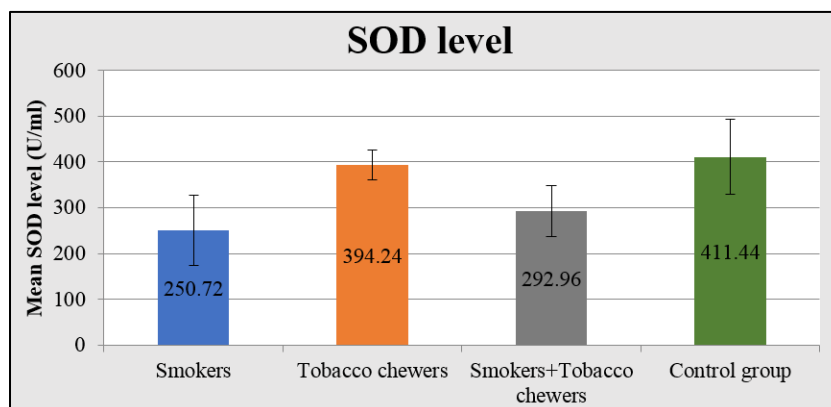

Fig. 1: Comparison of mean age among the study groups
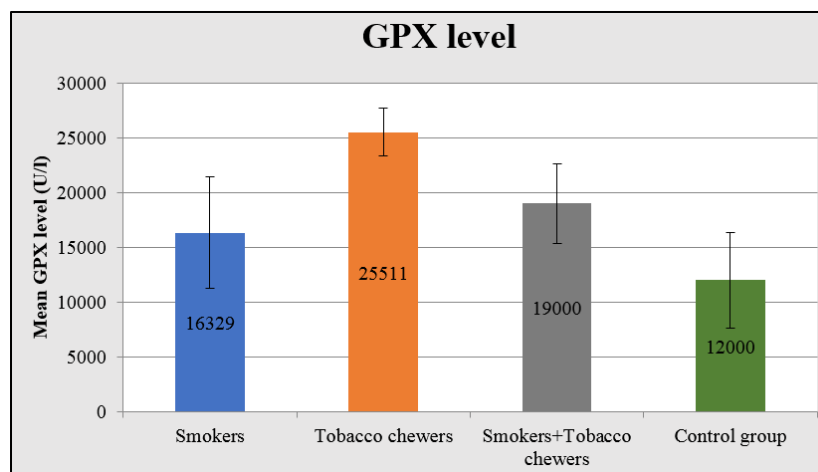

Fig. 2: Comparison of mean SOD level among the study group

Fig. 3: Comparison of mean GPX level among the study groups

Table 1: Age distribution among the groups

Age group	Smokers		Tobacco chewers		Tobacco chewers + smokers		Control	
	N	%	N	%	N	%	N	%
20 -35	16	64	9	36	6	24	8	32
36 – 50	6	24	9	36	16	64	15	60
51 - 70	3	12	7	28	3	12	2	8
Total	25	100	25	100	25	100	25	100

Table 2: Gender distribution among the groups

	Male		Female	
Group	N	%	N	%
Smoker	25	100	0	0
Tobacco chewers	19	76	6	24
Tobacco chewers + smokers	25	100	0	0
Control	25	100	0	0

Table 3a: Comparison of mean erythrocytic SOD levels in various groups

Study Group	Mean	Std Dev	F value	P value	Multiple comparison
Smokers	250.72	76.54	36.364	<0.001**	I vs II, IV (P<0.001**) I vs III (p =0.102)
Tobacco chewers	394.24	33.00			II vs I, III (P<0.001**) II vs IV (p =0.783)
Tobacco chewers + smokers	292.96	55.94			III vs II, IV (P<0.001**) III vs I (p =0.102)
Control	411.44	81.27			IV vs I, III (P<0.001**) IV vs II (p =0.783)

Table 3b: Post hoc tuckey test for erythrocytic superoxide dismutase in various groups

Study Group	Tobacco Smoker	Tobacco Chewer	Tobacco Chewer+ Smoker	Control
Smokers	_	(P<0.001**)	P=0.102	(P<0.001**)
Tobacco chewers	(P<0.001**)	_	(P<0.001**)	P=0.783
Tobacco chewers + smokers	P=0.102	(P<0.001**)	-	(P<0.001**)
Control	(P<0.001**)	P=0.783	(P<0.001**)	-

ANOVA followed by tukeys post hoc test
**P<0.001 highly significant

Table 4a: Comparison of mean erythrocytic GPX levels in various groups

Study Group	Mean	Std Dev	F value	P value	Multiple comparison
Smokers	16329	5078.12	51.47	<0.001**	I vs II, IV (P<0.001**) I vs III (p =0.085)
Tobacco chewers	25511	2149.80			II vs I, III,IV (P<0.001**)
Tobacco chewers + smokers	19000	3621.52			III vs II, IV (P<0.001**) III vs I (p =0.085)
Control	12000	4320.04			IV vs I, II, III (P<0.001**)

**P<0.001 highly significant

Table 4b: Post hoc tuckey test for erythrocytic GPx in various groups

Study Group	Tobacco Smoker	Tobacco Chewer	Tobacco Chewer+ Smoker	Control
Smokers	_	(P<0.001**)	P=0.085	(P<0.001**)
Tobacco chewers	(P<0.001**)	_	(P<0.001**)	(P<0.001**)
Tobacco chewers + smokers	P=0.085	(P<0.001**)	-	(P<0.001**)
Control	(P<0.001**)	(P<0.001**)	(P<0.001**)	-

ANOVA followed by tukeys post hoc test
**P<0.001 highly significant

Table 5: Relationship between frequency of smoking and Erythrocytic SOD, GPx levels among different groups

Group	Freq/day	N	Particulars	SOD (U/ml)	GPx(U/l)
Tobacco smokers	<10 $\geq$ 10	20 05	Mean ± SD	245.62 ± 77.94 271.13 ± 75.14 P=0.524 (NS)	17219 ± 5071 12770± 3575 P=0.05 (S)
Tobacco chewing	<10 $\geq$ 10	19 06	Mean ± SD	389.67 ±26.11 408.74 ± 49.43 P=0.4(NS)	25686 ±2210.82 24956±2025.37 P=0.47 (NS)
Tobacco chewing + smoking	<10 $\geq$ 10	16 9	Mean ± SD	291.67±57.08 295.25±57.16 P=0.88 (NS)	18127±3577.57 20553±3333.25 P=0.10 (NS)

Student t test
NS- not significant
S – significant p<0.05
Mean SOD: I < III < II, **Mean GPx:** I < III < II

Table 6: Relationship between duration of smoking and Erythrocytic SOD, GPx levels among different groups

Group	Duration of habit(years)	N	Particulars	SOD (U/ml)	GPx(U/l)
Tobacco smokers	<10 $\geq$ 10	15 10	Mean ± SD	230.04±86.40 281.73±47.22 P=0.06 (NS)	15561 ±5343.5 17482 ±4678.57 P=0.35 (NS)
Tobacco chewing	<10 $\geq$ 10	15 10	Mean ± SD	404.29±35.88 379.17±21.94 P=0.04 (S)	25352±2287.56 25749±2019.73 P=0.35 (NS)
Tobacco chewing + smoking	<10 $\geq$ 10	16 9	Mean ± SD	291.48±55.88 295.59±59.32 P=0.86 (NS)	18614±4206.40 19687±2310.99 P=0.41 (NS)

Mean SOD: I < III < II, **Mean GPx:** I < III < II
Table 7: Correlation between age and Erythrocytic SOD, GPx levels among different groups

Group	SOD		GPx	
	Age r value	p value	Age r value	p value
Tobacco smokers	-0.061	0.774 (NS)	0.222	0.287 (NS)
Tobacco chewing	-0.162	0.439 (NS)	0.161	0.443 (NS)
Tobacco chewing + smoking	-0.101	0.632 (NS)	0.009	0.966 (NS)
Control	-0.214	0.304 (NS)	0.094	0.653 (NS)

Discussion

India is the third largest producer and consumer of tobacco in the world with a high prevalence of smoking tendency in youngsters within the age range of 5-20 years. A similar tendency has been reflected in our study wherein in the age group of 20-35 years smoking was more prevalent (64%). Unlike the West, tobacco use, especially smoking is a male-dominated phenomenon among children and adolescents in India. Smoking by women in India is still socially unacceptable but SLT use is common due to its easy availability and low cost. Similarly, in our study women participants were absent in both tobacco smokers and the combination habit groups and formed only 24% of the total group in the tobacco chewers group.

The continuous release of reactive free radicals from different forms of tobacco inflicts an oxidative stress on the circulating erythrocytes. The human erythrocytes are essential for the biological oxidation of free radicals as they are rich in polyunsaturated fatty acids, contain hemoglobin which can function as an oxidase as well as a peroxidase and are one of the most potent catalysts of lipid peroxidation. The erythrocyte antioxidant defense system consists of SOD, GPX and CAT. SOD converts superoxide radicals into hydrogen peroxide, which is degraded to water and oxygen by GPX at physiological concentrations. When hydrogen peroxide concentration increases to toxic levels, CAT also contributes to degradation metabolism of H_2O_2.

Due to limited biosynthetic capacity of erythrocytes, they are completely dependent on antioxidant defensive components when faced with per-oxidant challenge, concluding in hemolysis. Thus, the present study was undertaken to assess the effect of tobacco on these erythrocyte antioxidant enzymes.

The mean SOD levels among all the four groups were shown to be statistically significant. The highest SOD levels were found in the control group (411.44) with the least value in the tobacco smoker group (250.72). The results of our study with respect to the erythrocyte SOD levels agreed with that of Naga Sirisha CV et al.[9] In another study, Patel BP et al. analyzed the erythrocytic and tissue antioxidant enzyme activities in terms of glutathione S transferase (GST), GRx, SOD, CAT, and GPx and plasma thiol levels.[6] Erythrocytic SOD was significantly lower in cases with tobacco habit as compared to those without tobacco habit. This slight decrease in SOD observed in cases compared to controls suggested SOD's utilization in scavenging the free radicals as it catalyzes the dismutation of superoxide to hydrogen peroxide, which is then removed by either GPx or CAT.[9] Similar results were shown in a study done by Supriya K et al. SOD was highly significantly reduced in combination habit group and active smokers; significantly reduced in tobacco chewers when compared to control.[22]

In contrast, few studies have shown an increase in the erythrocyte SOD levels in tobacco consumers especially smokers. Abou-Seif estimated erythrocyte and plasma antioxidant enzyme activities and antioxidants as well as concentrations of total sulfate and thiocyanate in a group of healthy subjects and three groups of smokers. Erythrocyte SOD and CAT activities as well as plasma SOD were elevated all the three groups of smokers when compared with the corresponding activity in the control group.[23] Similarly, Jenifer et al. observed significantly increased erythrocytic and salivary SOD enzyme levels in smokers with periodontitis, compared to nonsmokers. This was primarily attributed to the fact that erythrocyte antioxidants have protective effects against oxidative damage induced by smoking. Thereby explaining the hypothesis that an exaggerated response by erythrocytes occurs in response to increased oxidative stress in smokers.[5]

When erythrocytic GPx levels were assessed among various groups our study revealed GPx levels were the highest among the tobacco chewers (25511± 2149.80) followed by those with the combination habit (19000 ± 3621.52) and later by tobacco smokers (16329 ± 5078.12). It was the least in the control group (12000± 4320.04). Our results were comparable to that shown by Naga Sirisha CV et al, Patel B et al., where mean GPx levels were found to be slightly increased in cases compared to controls as it acts after SOD and this increase could be co-related to an adaptive phenomenon when free radical generation exceeds the reducing capacity of SOD.[9,6] On the other hand in a study by Volkovova et al, Metta S et al. Bolzan et al. and Orhan et al.[24-27] found a significant decrease of GPx activity in smokers in comparison with non-smokers. According to Metta S et al. erythrocytic activity of GPX is the most significant indicator of oxidative stress in smokers in association with acute myocardial infraction (AMI) as smoking increases erythropoiesis by producing more carboxyhemoglobin and making hemoglobin inaccessible for oxygen transport, leading to altered erythrocyte indices.[25] Alternatively, Bogdansky et al. and Leonard M et al found that smoking didn't have any significant effect on the activity of any of the erythrocytic enzyme levels.[28]

When frequency of the habit and the erythrocytic SOD and GPx levels were assessed, our study revealed significant difference only with respect to erythrocytic GPx level and the frequency of habit of tobacco smoking. Whereas there was no significant difference observed between the frequency of tobacco smoking with that of erythrocytic SOD levels; and tobacco chewing or combination habits with both erythrocytic SOD and GPx levels. Therefore, GPx levels were decreased with the increase of frequency in tobacco smoking group. Comparable results were seen with studies done by Pannuru et al., Hemalatha et al. and Kim et al[29-31] where it was observed that heavy and chronic smoking decreased the red cell antioxidant status. This could be due to the down regulation of the gene responsible for encoding the enzyme GPx1 as quoted by Hansen et al, who further concluded in their study that erythrocytic GPx activity could be influenced by several factors including the intensity of smoking. Burlakova et al, observed that in high intensity smokers, the number of DNA double strands breaks were significantly higher than in non-smokers. This could explain the down regulation of gene expression of antioxidant enzymes.[32]

A further evaluation of relationship between duration of tobacco habit with that of erythrocytic SOD and GPx levels revealed that GPx levels did not have any significant association with the duration in any of the habit groups. However, erythrocytic SOD levels showed some variations within the habit groups. Though there was no significant association between the duration of the habit and the SOD levels in the tobacco smoking group, the tobacco chewers group showed the significant decrease in SOD levels with increase in the habit duration and vice versa in the combination habit group i.e. SOD levels increased with an increase in the duration of habit. Correspondingly, Samal IR et al. on evaluation of erythrocyte malondialdehyde (MDA) levels and activities of SOD and GRx in tobacco chewers, established a significant duration dependent decrease in erythrocyte SOD and GR activity.[33] This could be explained by a study done by Russo M et al. In this study, analysis of the time-course data revealed an increase of intracellular content of free radicals induced by cigarette smoking condensate (CSC), that was not immediate but occurred after a latency period of 6h. These results suggest that acute exposure to excessive levels of free radicals may down-regulate the gene expression of antioxidant enzymes, whereas chronic continuous exposure may increase the gene expression of these enzymes.[34] In another study by Pryor et al. when oxidative stress-induced toxic effects of cigarette smoking were evaluated, there was a probability that those which had a rapid onset could be mainly attributed to gas phase, that contains short-lived oxidants and not to CSC that mostly contains the long-lived radical semiquinone and

causes an increase of free radical content in exposed cells after 6h.[35]

Although few of the observations in our study agree with that of the studies mentioned above, there are other observations where there is a disagreement. These contradictory findings may be attributed to a number of factors including the age, sex and nutrition status of the individual, the type of tobacco used, exposure condition and specific tissue.

Although, our study showed no significant correlation between the age and erythrocytic SOD levels among all the 4 groups, a negative correlation between SOD and age was observed indicating that as the age increased erythrocytic SOD levels decreased. Whereas, according to Pearson's correlation there was no significant correlation between the age and erythrocytic GPx levels in all the habit groups. Our results are in partial agreement with studies done by Anderson et al.[36] and Bolzan et al.[37] Anderson et al. where age-related decrease in erythrocytic SOD but no age-related change for GPx and CAT activity was observed. Conversely, Volkovova K et al. reported no significant correction of SOD and GPx activity with age.[24] Maurya PK et al. observed reduced antioxidants enzymes activity in the older age groups when compared to the young. They suggested this was caused by increased free radicals damage that contributes to aging.[38] This could be attributable to a shift towards increased lipid peroxidation with advancing age leading to an imbalance between antioxidant & pro-oxidant factors in free radical metabolism.[39]

Conclusion

Consequent to the disruption in the equilibrium between pro-oxidant and antioxidant systems and a decreased antioxidative defense system make the mucosa more vulnerable to toxic effect of ROS. Based on previous literature, such changes over time cause oral potential malignant disorders. Increased oxidative stress in tobacco users cause significant alterations in the erythrocytic antioxidant enzymes levels like superoxide dismutase and glutathione peroxidase. The resulting excessive free radicals play critical role in causing tissue damage including carcinogenesis. The current study establishes a possible relationship between the erythrocytic antioxidant levels, oxidative stress and tobacco consumption that may be compensatory regulation in response to oxidative stress. However, further studies with a larger sample size are needed to better understand the role of oxidative damage and enzymatic antioxidants levels in tobacco users.

References

1. World Health Organization. WHO Report on the Global Tobacco Epidemic, 2008: The M Power Package. Geneva: World Health Organization; 2008.
2. World Health Organisation. (2017). Tobacco. [online]Available at

http://www.who.int/mediacentre/factsheets/fs339/en/ [Accessed 1 Nov 2017].

3. Benowitz NL. Nicotine Addiction. *N Engl J Med.* 2010;362(24):2295–303.

4. Ambrose JA, Barua RS. The pathophysiology of cigarette smoking and cardiovascular disease: An update. *J Am Coll Cardiol.* 2004;43:1731-37.

5. Jenifer HD, Bhola S, Kalburgi V, Warad S, Kokatnur VM. The influence of cigarette smoking on blood and salivary super oxide dismutase enzyme levels among smokers and nonsmokers—A cross sectional study. *J Tradit Complement Med.* 2015;5(2):100–5.

6. Patel BP, Rawal UM, Shah PM, Prajapati JA, Rawal RM, Dave TK, et al. Study of tobacco habits and alterations in enzymatic antioxidant system in oral cancer. *Oncology.* 2005;68:511-9.

7. Tilashalski K, Rodu B, Mayfield C. Assessing the nicotine content of smokeless tobacco products. *J Am Dent Assoc.* 1994;125:590–94.

8. Tilashalski K, Rodu B, Cole P. A pilot study of smokeless tobacco in smoking cessation. *Am J Med.* 1998;104:456–58.

9. Naga Sirisha CV, Manohar RM. Study of antioxidant enzymes superoxide dismutase and glutathione peroxidase levels in tobacco chewers and smokers: a pilot study. *J Cancer Res Ther.* 2013;9(2):210–14.

10. Pham-Huy LA, He H, Pham-Huy C. Free radicals, antioxidants in disease and health. *Int J Biomed Sci.* 2008;4(2):89–96.

11. Droge W. Free radicals in the physiological control of cell function. Review. *Physiol Rev.* 2002;82:47–95.

12. Young I, Woodside J. Antioxidants in health and disease. *J Clin Pathol.* 2001;54:176–86.

13. Willcox JK, Ash SL, Catignani GL. Antioxidants and prevention of chronic disease. Review. *Crit Rev Food Sci Nutr.* 2004;44:275–95.

14. Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. *Physiol Rev.* 2007;87:315–424.

15. Genestra M. Oxyl radicals, redox-sensitive signalling cascades and antioxidants. Review. *Cell Signal.* 2007;19:1807–19.

16. Beevi SS, Rasheed AM, Geetha A. Evaluation of oxidative stress and nitric oxide levels in patients with oral cavity cancer. *Jpn J Clin Oncol.* 2004;34:379-85.

17. Grazioli V, Schiavo R, Casari E. Antioxidant enzymaticactivities and lipid peroxidation in cultured human chondrocytesv from vertebral plate cartilage. *FEBS Lett.* 1998;431:149–53.

18. Chen J, Geissler C, Parpia B, Li J, Campbell TC. Antioxidant status and cancer mortality in China. *International Journal of Epidemiology.* 1992;21(4):625-35.

19. World Health Organisation (WHO).Tobacco fact sheet 339. http://www.who.int/mediacentre/factsheets/fs339/en .Accessed on 20 July 2017.

20. Household Consumer Expenditure, Socio-economic survey.55th Round. July 1999-June 2000. NSSO.Government of India; New Delhi;2012.

21. Singh A, Ladusingh L. Prevalence and determinants of tobacco use in India: evidence from recent Global Adult Tobacco Survey data. PloS One.

22. Supriya K, Chaudhary Janardan, Shamal S.N. Oxidative stress in cigarette smoker and smokeless tobacco user among ethnic group north-eastern population of Uttar Pradesh, India. *Int J Res Med Sci.* 2017;5(4):1439-44

23. Abou-Seif MA. (1996). Blood antioxidant status and urine sulfate and thiocyanate levels in smokers. *J Biochem Toxicol.* 11:133–8.

24. Volkovova K, Beno I, Staruchova M, Bobek P, Mekinova D, Tatara M. Antioxidative enzyme activity in the blood of healthy persons. *Bratis l Lek Listy.* 1996;97(3):134–8.

25. Metta S, Basalingappa DR, Uppala S, Mitta G. Erythrocyte Antioxidant Defenses Against Cigarette Smoking in Ischemic Heart Disease. *J Clin Diagn Res.* 2015;9(6):BC08–11.

26. Bolzan AD, Bianchi MS, Bianchi NO. Superoxide dismutase, catalase and glutathione peroxidase activities in human blood: influence of sex, age and cigarette smoking. *Clin Biochem.* 1997;30(6):449–54.

27. Orhan H, Evelo CTA, Sahin G. Erythrocyte antioxidant defense response against cigarette smoking in humans – the glutathione S-transferase vulnerability. *J Biochem Mol Toxicol.* 2005;19:226–33.

28. Bogdanska, J.J.; Korneti, P.; Todorova, B. Erythrocyte superoxide dismutase, glutathione peroxidase and catalase activities in healthy male subjects in republic of macedonia. *Bratisl Lek Listy.* 2003;104:108–114.

29. Durak I, Yalcin S, M. Y. Burak Çimen M Y B, Büyükkoçak S, Kaçmaz M, Öztürk S H, Effect of smoking on plasma and erythrocyte antioxidant defense systems. *J Toxicol Environ Hlt Part A.* 1999;56:373-8.

30. Pannuru P, Vaddi DR, Kindinti RR, Varadacharyulu N. Increased erythrocyte antioxidant status protects against smoking induced hemolysis in moderate smokers. *Hum Exp Toxicol.* 2011;30(10):1475–81.

31. Kim SH, Kim JS, Shin HS, and Keen CL. Influence of smoking on markers of oxidative stress and serum mineral concentrations in teenage girls in Korea. *Nutrition.* 2003;19:240-243.)

32. Burlakova EB, Zhizhina GP, Gurevich SM, Fatkullina LD, Kozachenko AI, Nagler LG, et al. Biomarkers of oxidative stress and smoking in cancer patients. *J Cancer Res Ther.* 2010;6(1):47–53.

33. Samal, I.R., Maneesh, M., Chakrabarti, A., 2006. Evidence for systemic oxidative stress in tobacco chewers. *Scand J Clin Lab. Invest.* 66, 517–522.

34. Russo M., Cocco S., Secondo A. Cigarette smoking condensate causes a decrease of the gene expression of Cu-Zn superoxide dismutase, Mn superoxide dismutase, glutathione peroxidase, catalase, and free radical induced cell injury in SH-SY5Y human neuroblastoma cells. *Neurotox Res.* 2011;19:49–5

35. Pryor WA, Hales BJ, Premovic PI, Church DF (1983) The radicals in cigarette tar: their nature and suggested physiological implications. *Science.* 220:425–427.

36. Andersen HR, Nielsen YB, Nielsen F, Grandjean P. Antioxidative enzyme activities in human erythrocytes. *Clin Chem.* 1997;43(4):562–8.

37. Bolzan AD, Bianchi MS, Bianchi NO. Superoxide dismutase, catalase and glutathione peroxidase activities in human blood: influence of sex, age and cigarette smoking. *Clin Biochem.* 1997;30(6):449–54.

38. Maurya PK, Kumar P, Siddiqui N, Tripathi P, Rizvi SI. Age-associated changes in erythrocyte glutathione peroxidase activity: correlation with total antioxidant potential. *Indian J Biochem Biophys.* 2010;47(5):319–21.

39. Guemouri, L. Artur, Y. Herbeth, B. Jeandel, C. Cuny, G., Siest, G. Biological variability of superoxide dismutase, glutathione peroxidase, and catalase in blood. *Clin Chem.* 1991;37(11):1932−37.