

## Assessment of Serum Cotinine and Oxidative Stress Markers in Tobacco Users

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Augmented production of free radicals associated with smoking with decrease in antioxidant levels and increase in peroxidation of biomolecules have been shown in various studies. The studies with smokeless tobacco use having depictive data were lacking. To estimate serum cotinine (CTN) levels and oxidative stress markers in tobacco smokers and smokeless tobacco users and to compare them with tobacco nonusers. This cross sectional study was performed in 180 study subjects divided into 6 groups - tobacco smokers, tobacco chewers, tobacco mishri users, dual tobacco chewers and mishri users, dual smokers and smokeless tobacco users and tobacco nonusers. Serum CTN levels were estimated. Oxidative stress was estimated by Malondialdehyde (MDA) and enzymatic antioxidant Superoxide Dismutase (SOD). Serum MDA levels were significantly high and erythrocytic SOD levels were significantly low in tobacco users than tobacco nonusers ( $P < 0.001$ ). There was a significant negative relationship between cotinine and SOD, a significant positive relation between cotinine and MDA in tobacco users. There was a significant negative relationship between duration of tobacco use and SOD, a significant positive relation between duration of tobacco use and MDA. There was a significant negative relationship between amount of tobacco use and SOD. Tobacco users with longer duration of tobacco use with increased levels of serum cotinine have increased oxidative stress which can be a risk factor for many diseases. Tobacco chewing equally increases oxidative stress as that of smoking.

**Keywords:** Serum cotinine levels, Malondialdehyde, Superoxide Dismutase, tobacco smokers, smokeless tobacco users.

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According to WHO report, tobacco is responsible for an extensive amount of morbidity and mortality among middle-aged adults worldwide and for causing more than 5 million deaths every year.<sup>1</sup> Smoking and smokeless tobacco products are the two main forms of tobacco use.<sup>2</sup> India is one of the largest producer and second highest consumer of tobacco in the world with 229 million tobacco

users, mainly in the form of smokeless tobacco (SLT).<sup>2,3</sup>

Global Adult Tobacco Survey India (GATS-India) 2009-2010 observed that prevalence of smokeless tobacco use (26%) is significantly more than that of smoking (14%).<sup>4</sup> Smoking is available as cigarettes, beedis, cigars, cheroots etc. SLT is available as gutkha, khaini, pan masala,

mawa or snuff or mishri, gul, bajjar, gudakhu (used for application to the teeth and gums).<sup>2</sup> The use of SLT in India is escalating as they are cheap, socially acceptable and easily available with addictive habit.<sup>2</sup>

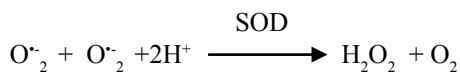
After absorption of tobacco constituents in the blood circulation, it affects almost all organs causing increased risk of atherosclerosis, hypertension, chronic obstructive pulmonary disease, cancers etc.<sup>5</sup> Adverse effects of tobacco use have been associated to the different effects of chemical constituents of it on biological systems. Although the underlying mechanisms involved in the pathogenesis of diseases associated with tobacco use are not exactly known, free radical induced damage has been suggested to play a major role.<sup>6</sup>

Tobacco contains 5,000 compounds, most of which are well known sources of free radicals,<sup>7</sup> when they get absorbed in blood circulation, causes changes in cells resulting in increased production of free radicals. These free radicals in turn are capable of initiating and promoting oxidative damage by inactivating endogenous antioxidant defense systems consisting of enzymes such as superoxide dismutase, catalase and glutathione peroxidase and nonenzyme molecules (uric acid, ascorbic acid, reduced glutathione etc.) which keep the free radical levels within necessary limit. Increased production of free radicals associated with smoking may exceed the capacity of defense system or in case of insufficiency or depletion of antioxidants it may leads to the oxidant vs. antioxidant imbalance and hence oxidative stress.<sup>8,9</sup>

Oxidative stress results in damage to macromolecules such as lipids, proteins and DNA. This leads to enhancement of pro-inflammatory reactions, lipid peroxidation, induction of DNA strand break, inactivation of certain proteins, accelerated aging, immunity disturbances etc. which has been implicated as the major pathologic mechanisms of all smoking related disease.<sup>10</sup> Several studies have reported depletion of antioxidants as a result of cigarette smoking and tobacco use.<sup>12,13</sup>

Oxidizing free radicals produced by cigarette smoke attack on the polyunsaturated fatty acids of plasma membranes causing lipid peroxidation (LPO), is particularly damaging because it proceeds as a self perpetuating chain

reaction. Malondialdehyde (MDA) is major end product of LPO.<sup>14</sup> Superoxide dismutase (SOD) is one important antioxidant enzyme present in nearly all cells exposed to oxygen, with the main function to catalyze the dismutation of two superoxide molecules into oxygen and hydrogen peroxide.<sup>15</sup>



SOD detoxifies superoxide to less toxic  $\text{H}_2\text{O}_2$ , which is then completely detoxified by other antioxidant enzymes like glutathione peroxidase and catalase.

Nicotine is an important alkaloid and major addicting substance in tobacco.<sup>16</sup> Near about 70 to 80 % of nicotine is rapidly metabolized to cotinine (CTN) in the liver. CTN has a longer half-life of 18-20 hours and can be reliably estimated in blood, saliva and urine. Blood CTN is regarded as sensitive, direct and specific marker of tobacco exposure.<sup>17</sup>

As it has been difficult to measure the true oxidative stress levels because of the reactivity of free radicals, malondialdehyde (MDA) and superoxide dismutase (SOD) used as biomarker of oxidative stress.

Usually it is thought that harmful effects of tobacco are only associated with smoking and effects of smokeless tobacco are neglected. The most widely prevalent and most studied form of tobacco use globally is cigarette smoking. Very few studies were done for smokeless tobacco use regarding comparison of oxidative stress especially in developing countries. Therefore, the purpose of this study was to explore the levels of serum cotinine (CTN) as marker of tobacco exposure, status of antioxidant enzyme superoxide dismutase (SOD) in erythrocyte and malondialdehyde (MDA) in serum of tobacco smokers and smokeless tobacco users and compared them with tobacco nonusers.

## MATERIAL AND METHODS

### Study design

This was a cross sectional study, conducted on subjects attending OPD, Krishna Hospital, Karad, Maharashtra from January 2016 to December 2017.

### Ethics

Ethical clearance was taken from Institutional Ethics Committee. Informed written consent was taken from every subject after explaining about the study.

### Methodology

Based on the previous study done by Arora KS,<sup>12</sup> sample size was calculated as follows: to obtain mean difference in plasma MDA level of 1.49 nmol/ml ( $3.10 \pm 0.41$  nmol/ml vs  $1.61 \pm 0.66$  nmol/ml) among tobacco smokers and tobacco nonusers with permissible error 10%, confidence interval 95%, power 80% it come around minimum 10 in each group by using formula  $n = (SD_1^2 + SD_2^2) (Z_{1-\alpha/2} + Z_{1-\beta})^2 / d^2$ . Open Epi, version 3, open source calculator was used.

Hence, in each group 30 subjects were included and the study consisted of total 180 subjects between age group 35-60 years with the following six groups.

The group comprised of-

Group 1(G1) - 30 tobacco smokers

Group 2(G2) - 30 tobacco chewers

Group 3(G3) - 30 tobacco mishri users

Group 4(G4) - 30 dual, tobacco chewers and tobacco mishri users

Group 5(G5) - 30 dual, tobacco smokers and smokeless tobacco users

Group 6(G6) - 30 tobacco nonusers

Inclusion and Exclusion criteria

**Inclusion criteria:** age between 35-60 years

Group 1 included smokers (an adult who has smoked 100 cigarettes or beedis in his or her lifetime and who currently smokes cigarettes or beedis)

Group 2 included tobacco chewers (ever using chewing tobacco or using it at least once within the past 30 days)

Group 3 included tobacco mishri users (ever using tobacco mishri or using it at least once within the past 30 days)

Group 4 included dual, tobacco chewers and tobacco mishri users

Group 5 included dual, tobacco smokers and smokeless tobacco users

Group 6 included subjects who never used tobacco, either smoking or smokeless form in their lifetime.

**Exclusion criteria**

Individuals with systemic illness like hypertension, diabetes mellitus, liver, cardiac or

renal diseases, pregnancy and history of any other substance abuse (alcohol, drugs).

Systematic sampling method was used for selection of subjects. According to inclusion & exclusion criteria and six groups, subjects were selected from the patients coming to OPD. According to the group, every third patient was selected in the study after informed consent till completion of required sample size.

A proforma containing demographic data, past history of tobacco use and medical history was filled for every subject. Questionnaire was used to collect details of tobacco use which included type, approximate amount and duration of consumption of tobacco.

In tobacco users, amount of tobacco use in grams per day calculated as follows: Average number of packets of biddies / cigarettes/ chewing tobacco / mishri used per day was inquired. To calculate tobacco use by each subject, tobacco content<sup>18</sup> in each tobacco products was multiplied by number of packets used per day. Subjects were divided into those using above or below 5 grams per day.

### Biochemical investigation

After overnight fasting, 10 ml of venous blood sample was collected in plain bulb with aseptic precautions from all the subjects. Blood was processed in Biochemistry laboratory of KIMSU, Karad. Serum was separated by centrifugation. Serum CTN level was measured by cotinine enzyme linked immunosorbant assay kit (Calbiotech).<sup>19</sup> It is a solid phase competitive ELISA. The CTN levels were measured in nanogram per millilitre (ng/ml). Serum Malondialdehyde [as Lipid Peroxide (LPO)] estimated by Kei Satoh method.<sup>20</sup> Erythrocytic Superoxide Dismutase estimated by I.N.T. [2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride] method by using RANSOD kit supplied by RANDOX Laboratory USA.<sup>21</sup>

### Statistical analysis

Chi-square test, unpaired t test and ANOVA were used to find the significance of study parameters between different groups. The data analyzed using IBM SPSS Statistics, version 20. P value <0.05 was considered as statistically significant.

## RESULTS

On comparison of the mean values of erythrocytic SOD and serum MDA between the groups using ANOVA test, the values were found statistically significant ( $P < 0.001$ ). Serum MDA levels were significantly high and erythrocytic SOD levels were significantly low in tobacco users (G1, G2, G3, G4 & G5) than tobacco nonusers (G6) ( $P < 0.001$ ). Serum MDA levels were significantly high and erythrocytic SOD levels were significantly low in tobacco smokers (G1) than tobacco mishri

users (G3) ( $P = 0.021$  and  $p = 0.022$  respectively). In other tobacco users, no significant difference found between serum MDA levels and erythrocytic SOD levels [Table 1].

On using unpaired t test, mean serum cotinine and serum MDA were significantly high, while mean erythrocytic SOD was significantly low in subjects using  $> 5$  grams of tobacco per day than those using  $\leq 5$  grams of tobacco per day ( $P < 0.001$ ) [Table 2].

On using unpaired t test, mean serum MDA levels were significantly high and mean

**Table 1.** Comparison of SOD & MDA between the study groups

Study groups	E.SOD (U/ml of whole blood) Mean $\pm$ SD	S. MDA (nmol/ml) Mean $\pm$ SD
G1(n=30)	296.50 $\pm$ 20.67	2.69 $\pm$ 0.34
G2(n=30)	306.76 $\pm$ 24.09	2.50 $\pm$ 0.40
G3(n=30)	311.63 $\pm$ 34.29	2.46 $\pm$ 0.32
G4(n=30)	313.13 $\pm$ 24.34	2.41 $\pm$ 0.40
G4(n=30)	302.40 $\pm$ 24.33	2.52 $\pm$ 0.36
G5(n=30)	362.30 $\pm$ 20.68	1.51 $\pm$ 0.41
ANOVA F	26.73	36.76
P Value	$< 0.001$	$< 0.001$

**Table 2.** Effect of amount of tobacco use on cotinine, SOD and MDA in tobacco users

Biochemical parameter	Amount of tobacco user per day		t value	P value
	$\leq 5$ grams	$> 5$ grams		
Serum cotinine (ng/ml)	88.29 $\pm$ 29.44	159.01 $\pm$ 48.10	-11.127	$< 0.001$
E.SOD (U/ml of whole blood)	313.07 $\pm$ 25.60	292.12 $\pm$ 22.02	-6.424	$< 0.001$
S. MDA (nmol/ml)	2.39 $\pm$ 0.30	2.76 $\pm$ 0.38	4.942	$< 0.001$

**Table 3.** Effect of duration of tobacco use on cotinine, SOD and MDA in tobacco users

Biochemical parameter	Duration of tobacco use		t value	P value
	$\leq 15$ grams	$> 15$ grams		
Serum cotinine (ng/ml)	114.92 $\pm$ 34.46	120.53 $\pm$ 49.57	1.48	0.20
E.SOD (U/ml of whole blood)	318.71 $\pm$ 33.28	302.66 $\pm$ 23.10	3.150	0.002
S. MDA (nmol/ml)	2.38 $\pm$ 0.40	2.55 $\pm$ 0.36	-2.381	0.019

**Table 4.** Correlation of cotinine with SOD and MDA

Cotinine	Tobacco users		Tobacco nonusers	
	®	P value	®	P value
SOD	-0.696	$< 0.001$	-0.069	0.719
MDA	0.779	$< 0.001$	-0.123	0.487

erythrocytic SOD was significantly low in subjects using tobacco for  $> 15$  years than those using for  $\leq 15$  years ( $P = 0.019$ ,  $P = 0.002$  respectively). No significant difference was found in mean serum cotinine level [Table 3].

Correlation analysis showed that there was a significant negative relationship between

cotinine and SOD ( $\text{R} = -0.696$ ,  $P < 0.001$ ), a significant positive relation between cotinine and MDA ( $\text{R} = 0.779$ ,  $P < 0.001$ ) in tobacco users. No significant correlation was found between cotinine and SOD or MDA in tobacco nonusers [Table 4].

Correlation analysis showed that there was a significant negative relationship between duration of tobacco use and SOD ( $\text{R} = -0.291$ ,  $P < 0.001$ ), a significant positive relation between duration of tobacco use and MDA ( $\text{R} = 0.184$ ,  $P = 0.024$ ). No significant correlation was found between cotinine and duration of tobacco use [Table 5].

Correlation analysis showed that there was a significant negative relationship between amount of tobacco use and SOD ( $\text{R} = -0.348$ ,  $P < 0.001$ ), a significant positive relation between amount of tobacco use and MDA & cotinine ( $\text{R} = 0.458$ ,  $P < 0.001$ ;  $\text{R} = 0.689$ ,  $P < 0.001$  respectively) [Table 6].

## DISCUSSION

Adverse health effects of tobacco use have been attributed to chemical constituents in tobacco leading to generation of free radicals and oxidative stress and their deleterious effects on biomolecules such as lipids, membrane proteins and nucleic acids. MDA has long life-time ranging from hours to weeks, so used as biomarker of LPO<sup>22</sup> and oxidative stress along with estimation of SOD in erythrocytes in vitro and in vivo for various diseases. The levels of biomarkers of oxidative

stress in relation to duration of tobacco use and amount of tobacco use were estimated in tobacco users and tobacco nonusers.

Present study showed that serum MDA levels were significantly high and erythrocytic SOD levels were significantly low in tobacco users than tobacco nonusers. The study by Begum SF *et al.*<sup>23</sup> showed that MDA levels were significantly increased in the saliva of gutkha group than nontobacco users. The study by Arora KS *et al.*<sup>12</sup> and OBI Ugochukwu *et al.*<sup>24</sup> showed that plasma SOD was significantly decreased whereas plasma MDA levels were significantly increased in smokers as compared to non-smokers. Similarly, in the study by Supriya K *et al.*<sup>13</sup> cigarette smoker, cigarette smoke exposure and tobacco user showed increased MDA and decreased SOD, and catalase. In the studies undertaken by Altuntas *et al.*<sup>25</sup> and Nagaraj *et al.*<sup>26</sup> serum MDA were significantly higher in smokers than controls.

This could be explained by significantly higher CTN levels in tobacco users compared with tobacco nonusers as found in our previous study.<sup>27</sup> Lowered antioxidant enzyme SOD found in this study suggests that chronic tobacco use reduce the antioxidant pool of tobacco user which is used to neutralize increased levels of free radicals leading to oxidative stress.<sup>28</sup> Study by Hazieli *et al.*<sup>29</sup> showed contrasting results with significantly higher SOD enzyme activity in the blood (i.e., in erythrocytes) and saliva of smokers with periodontitis, compared to nonsmokers with periodontitis and healthy controls.

**Table 5.** Correlation between duration of tobacco use and biochemical parameters

Biochemical parameter	$\text{R}$	P value
Duration of tobacco use with cotinine	0.129	0.116
Duration of tobacco use with SOD	-0.291	<0.001
Duration of tobacco use with MDA	0.184	0.024

**Table 6.** Correlation between amount of tobacco use and biochemical parameters

Biochemical parameter	$\text{R}$	P value
Amount of tobacco use with cotinine	0.689	<0.001
Amount of tobacco use with SOD	-0.348	<0.001
Amount of tobacco use with MDA	0.458	<0.001

Serum MDA levels were significantly high and erythrocytic SOD levels were significantly low in tobacco smokers than tobacco mishri users. This could be due to low serum CTN level in tobacco mishri users as proved by our previous study.<sup>27</sup> In other tobacco users, no significant difference was found between serum MDA levels and erythrocytic SOD levels. This suggests that smokers as well as tobacco chewers have same potential to cause increase oxidative stress.

Our study showed that as amount of tobacco use increased, cotinine and MDA were increased, while SOD was decreased. Similarly Nagaraj *et al.*<sup>26</sup> found that oxidative stress level was elevated in accordance with the intensity of smoking. But Altuntas *et al.*<sup>25</sup> found no relationship between lipid peroxidation and the number of cigarettes smoked by an individual.

Present study showed that as duration of tobacco use increased, MDA was increased, while SOD was decreased. OBI Ugochukwu *et al.*<sup>24</sup> showed that as the duration of smoking increased, total antioxidant capacity and SOD were decreased and MDA was increased. Also Altuntas *et al.*<sup>25</sup> noted that MDA levels were significantly higher and total antioxidant status were significantly lower in current smokers compared with nonsmokers and it correlated with duration of smoking.

In the present study a significant negative relationship was found between cotinine and SOD, a significant positive relation between cotinine and MDA in tobacco users. Similarly the study by Begum SF<sup>23</sup> showed significant positive correlation between cotinine and MDA in gutkha and khaini users.

In the present study a significant negative relationship was found between duration of tobacco use and SOD with a significant positive relation between duration of tobacco use and MDA. No significant correlation was found between cotinine and duration of tobacco use. Also there was a significant negative relationship between amount of tobacco use and SOD, a significant positive relation between amount of tobacco use and MDA & cotinine. Similarly OBI Ugochukwu *et al.*<sup>24</sup> showed that SOD was negatively correlated and the MDA level positively correlated in the current cigarette smokers with duration of smoking.

The antioxidant enzyme superoxide dismutase (SOD) serves as primary line of defense

in destroying free radicals which requires Zinc (Zn) for its activity. In tobacco users, there is decrease in Zn as cadmium (Cd) in tobacco has affinity to replace the bivalent metals like (Zn) from SOD and inactivate it. So depletion of SOD activity in tobacco users may be primarily due to inadequate availability of Zn in blood.

Due to high nicotine release and addiction potential of SLT, it is equivalent to smoking with most rapid absorption of nicotine.<sup>30</sup> Similarly our finding suggests that smoking as well as tobacco chewing increases oxidative stress due to increased levels of serum cotinine.

#### Limitations

We did not have exact information on amount of tobacco used per day, which is approximately calculated from number of packets used per day. Further research is recommended to include other smokeless products.

#### CONCLUSION

Tobacco users with increased levels of serum cotinine have increased oxidative stress. This suggests that tobacco use could weaken the body's antioxidant enzymes defense mechanism and increase lipid peroxidation, which can be a risk factor for many diseases. It was also observed that oxidative stress tends to increase as duration and amount of tobacco use increases. Tobacco chewing equally increases oxidative stress as that of smoking. Therefore, stress should be given to inform tobacco users regarding health hazards due to tobacco use in every form.

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