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Original Article

Biochemistry Section

Tobacco Usage and Serum Cotinine: A Hospital Based Study

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ABSTRACT

Introduction: Tobacco usage is mainly in the form of smoking and smokeless tobacco. Some studies commented that smokeless tobacco has high nicotine and toxic effect, but the studies with depictive data have been limited. Cotinine is one of the nicotine metabolite, which is used as a marker to quantify tobacco exposure.

Aim: To estimate serum cotinine (CTN) levels in tobacco smokers and smokeless tobacco users and to compare them with tobacco non-users.

Materials and Methods: This cross-sectional study was performed in 240 study subjects divided into four groups- only tobacco smokers (G1), only smokeless tobacco users (G2), dual tobacco smokers and smokeless tobacco users (G3), and tobacco non-users (G4). G2 was divided into G2a, G2b, and G2c. Serum CTN levels were estimated using enzyme linked immunosorbant assay (ELISA) kit. Chi-square test, unpaired t-test, ANOVA and regression analysis were used for statistical analysis.

Results: The mean serum CTN levels in groups G1, G2a, G2b, G2c, G3 and G4 were found to be 117.45 ng/mL, 138.09 ng/mL, 72.35 ng/mL, 100.34 ng/mL, 145.21 ng/mL and 5.28 ng/mL respectively. When the mean values were compared between the groups the values were found to be statistically highly significant. The finding suggests significantly higher CTN levels in tobacco users compared with tobacco non-users, also in tobacco chewers compared with tobacco smokers, as well as for dual tobacco smokers and smokeless tobacco users compared with smokers.

Conclusion: Dual tobacco smokers and smokeless tobacco users tend to have relatively high level of serum CTN, which suggests tobacco dependence.

Keywords: Serum cotinine (CTN) levels, Smokeless tobacco users, Tobacco chewers, Tobacco smokers

INTRODUCTION

Worldwide tobacco use is a major public health problem and is a preventable cause of many diseases like cardiovascular diseases, chronic obstructive pulmonary disease, cancers, reproductive outcomes, oral diseases etc., [1]. India is the second largest producer and consumer of tobacco in the world [2]. In India, scenario of tobacco consumption is far worse, as it is prevailing in all socioeconomic and ethnic groups in urban as well as remote villages.

In recent years, public health associations have given more attention to smokeless tobacco products. According to Global Adult Tobacco Survey India (GATS-India) 2009-2010, prevalence of smokeless tobacco use (26%) is significantly more than that of smoking (14%). Amongst the tobacco users, 8.7% only smoked, 20.6% consumed only smokeless tobacco and 5.3% both smoked as well as consumed smokeless tobacco. In females, the prevalence of smokeless tobacco use was much higher than smoking. The study also reported that overall tobacco use in India is 34.6%; males (47.9%) and females (20.7%) with comparatively higher prevalence rate in the rural areas than urban area [3]. The use of smokeless products in India is on rise as they are cheap and easily available e.g., gutkha, khaini, pan masala, mawa or snuff or mishri, gul, bajjar, gudakhu used for application to the teeth and gums [1,4].

Tobacco contains more than 4,200 chemicals [5]. Nicotine is an important alkaloid and major addicting substance in tobacco. On an average, about 70 to 80% of nicotine is rapidly metabolised to CTN in the liver. CTN has a longer half-life of 18-20 hours and can be reliably estimated in blood, saliva and urine, so it is most commonly measured [6]. Blood CTN is regarded as most direct, acceptable, and sensitive and best marker for monitoring tobacco exposure in actively exposed individuals [7].

Due to addiction of nicotine, its use is increasing among population mainly in younger generation [8]. Usually it is thought that harmful effects of tobacco are associated with smoking, but effects of smokeless tobacco are neglected. Also, biomarkers of tobacco exposure have earlier been analysed for cigarette smokers [9,10] and for cigar smokers [11], but less is known about biomarkers among smokeless tobacco users. Very few studies have been done in India regarding smokeless tobacco use, dual use of smokeless tobacco and smoked tobacco, smoking and nontobacco users. In this study we estimated serum CTN as marker of tobacco exposure in tobacco smokers and smokeless tobacco users and compared them with tobacco non-users.

MATERIALS AND METHODS

Study Design

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

Ethics

Ethical clearance was taken from Institutional Ethics Committee before the start of the research. (ECR/307/Inst/MH/2013). The nature and purpose of the study was explained and informed written consent was taken from every subject.

Methodology

Sample size was calculated based on the previous study done by Srivastava A et al., as follows: to obtain mean difference in serum CTN level of 108.23 nanogram per mililitre (ng/mL) (146.89±33.21 ng/mL vs 38.66±10.66 ng/mL) among smokeless tobacco users and tobacco non-users with permissible error

10%, confidence interval 95%, power 80%, it come around minimum 10 in each group [12]. Open Epi, version 3, open source calculator was used.

Hence, 60 subjects per group were included and the study consisted of total 240 subjects between age group 35-60 years with the following four groups.

The group comprised of:

Group 1 (G1)-60 subject who were tobacco smokers

Group 2 (G2)-60 subject who were smokeless tobacco users

G2a-20 subjects who were tobacco chewers

G2b-20 subjects who were tobacco mishri users

G2c-20 subjects who were dual, tobacco chewers and tobacco mishri users

Group 3 (G3)-60 subject who were dual, tobacco smokers and smokeless tobacco users

Group 4 (G4)-60 subject who were tobacco non-users

Inclusion and Exclusion Criteria

Inclusion criteria: Age between 35-60 years.

Group 1 included subjects who were smokers (an adult who has smoked 100 cigarettes or bidis in his or her lifetime and who currently smokes cigarettes or bidis) [13] based on data from the January-September 2014 National Health Interview Survey.

Group 2 included subjects who were smokeless tobacco users (smokeless tobacco use was defined as ever using such products and using a smokeless tobacco product such as chewing tobacco, using mishri or both at least once within the past 30 days) [14].

Group 3 included subjects who were dual, tobacco smokers and smokeless tobacco users.

Group 4 included subjects who never used tobacco, either smoking or smokeless form in their lifetime.

Exclusion criteria: Individuals with history of any other substance abuse (alcohol, drugs), associated co-morbid systemic illness, pregnancy, diabetes mellitus and subjects taking regular medications.

Systematic sampling method was used for selection of the subjects. According to four groups and inclusion and exclusion criteria, subjects were selected from the patients coming to OPD. Every third patient was selected according to the group who was willing to participate in the study till completion of required sample size. A proforma was filled for every subject containing demographic data, past history of tobacco use, medical history and family history. Details of tobacco use was collected which included type, approximate amount and duration of consumption of tobacco through questionnaire.

Amount of tobacco use in grams per day calculated as follows: Tobacco user subjects were asked on an average how much packets of bidis or cigarettes or chewing tobacco or mishri they used per day. To evaluate how much tobacco each subject consumed, tobacco content [15] in each tobacco products was multiplied by number of packets consumed per day. Thus, subjects were divided into those using above or below 5 grams per day [16]. According to Society for Research on Nicotine and Tobacco Subcommittee (SRNT) on biochemical verification, non-smokers are the people who have never smoked or who are ex-smokers with serum CTN concentration <15.0 nanogram per mililitre (ng/mL). It states that current smokers as people who have a self-reported habit with serum CTN concentration >15 ng/mL [17]. Similar values are taken as reference in the study.

After overnight fasting, 5 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 CTN ELISA kit (Calbiotech) [18]. It is a solid phase competitive ELISA. The CTN levels were measured in ng/mL. Standardisation of method of estimation of serum CTN was done. The sample and CTN enzyme conjugate was added to the wells coated with anti-cotinine antibody. CTN in the sample competes with a cotinine enzyme Horse Radish Peroxidase conjugate for binding sites. Unbound CTN and CTN enzyme conjugate was washed off by distilled water. After addition of the substrate, the intensity of colour was inversely proportional to the concentration of CTN in the sample and concentration of serum CTN calculated. A standard curve was prepared relating colour intensity to the concentration of the CTN [19].

STATISTICAL ANALYSIS

Chi-square test and unpaired t-test was used to find the association. ANOVA has been used to find the significance of study parameters between different groups. Post-hoc Tukey test was used to find the pairwise significance. Regression analysis was also used. The data analysed using IBM SPSS Statistics, version 20. The p-value<0.05 was considered as statistically significant.

RESULTS

Dual, tobacco chewers and tobacco mishri users were younger than members of other tobacco user groups with mean age of 43.55 years. When mean values of age in years were compared between the groups, the values were statistically significant p<0.001. In the study groups G1, G2a, G2c and G3 all the subjects were males [Table/Fig-1].

Group		N	Age in years			ANOVA		Gender	
			Min	Max	Mean±SD	F	p-value	Male	Female
G1		60	37	60	52.98±5.71	10.59	<0.001	60	0
G2	G2a	20	42	58	51.50±3.81			20	0
	G2b	20	40	58	49.35±4.33			4	16
	G2c	20	36	55	43.55±5.75			20	0
G3		60	37	60	52.20±5.68			60	0
G4		60	36	60	48.85±6.50			35	25
Total		240						199	41

[Table/Fig-1]: Demographic characteristics of subjects in study groups.

The mean serum CTN levels in groups G1, G2a, G2b, G2c, G3 and G4 were found to be 117.45 ng/mL, 138.09 ng/mL, 72.35 ng/mL, 100.34 ng/mL, 145.21 ng/mL and 5.28 ng/mL respectively. When the mean values were compared between the groups, the values were statistically highly significant p<0.001 [Table/Fig-2].

Group		N	Min	Max	Mean±SD	ANOVA	
						F	p-value
G1		60	56.00	170.00	117.45±30.15	91.04	<0.001
G2	G2a	20	76.00	250.00	138.09±61.11		
	G2b	20	37.46	110.00	72.35±24.34		
	G2c	20	69.00	169.00	100.34±30.30		
G3		60	90.00	290.00	145.21±59.98		
G4		60	4.00	9.00	5.28±1.03		
Total		240					

[Table/Fig-2]: Comparison of serum CTN levels between the study groups.

The mean serum CTN levels were significantly higher in tobacco users than tobacco non-users and in tobacco chewers than tobacco smokers. The mean serum CTN levels were significantly higher in dual tobacco smokers with smokeless tobacco users than tobacco smokers and dual tobacco chewers with tobacco mishri users. The mean serum CTN levels in tobacco mishri users were significantly lower than tobacco smokers, tobacco chewers, and dual tobacco chewers with tobacco mishri users and dual tobacco smokers with smokeless tobacco users. No significant

difference was found between serum CTN levels of tobacco chewers and dual tobacco smokers with smokeless tobacco users [Table/Fig-3].

(I) Group	(J) Group	Mean difference (I-J)	Std. error	p-value	95% CI	
					Lower Bound	Upper Bound
G1	G2a	-20.64350*	10.20718	0.044	-40.7532	-0.5338
	G2b	45.10000*	10.20718	<0.001	24.9903	65.2097
	G2c	17.10650	10.20718	0.095	-3.0032	37.2162
	G3	-27.76667*	7.21757	<0.001	-41.9864	-13.5470
	G4	112.16717*	7.21757	<0.001	97.9475	126.3869
G2a	G2b	65.74350*	12.50119	<0.001	41.1142	90.3728
	G2c	37.75000*	12.50119	0.003	13.1207	62.3793
	G3	-7.12317	10.20718	0.486	-27.2329	12.9865
	G4	132.81067*	10.20718	<0.001	112.7010	152.9204
G2b	G2c	-27.99350*	12.50119	0.026	-52.6228	-3.3642
	G3	-72.86667*	10.20718	<0.001	-92.9764	-52.7570
	G4	67.06717*	10.20718	<0.001	46.9575	87.1769
G2c	G3	-44.87317*	10.20718	<0.001	-64.9829	-24.7635
	G4	95.06067*	10.20718	<0.001	74.9510	115.1704
G3	G4	139.93383*	7.21757	<0.001	125.7141	154.1535

[Table/Fig-3]: Pairwise comparison of serum CTN levels between the study groups. *(I) and (J) represent the pair wise comparison between groups that has been symbolically represented in the first 2 columns

In three groups, 60% in group (G1), 26.7% in group (G2) and 41.7% in group (G3) were using tobacco since 21-30 years [Table/Fig-4].

Mean serum CTN was least (88.61 ng/mL) in subjects using tobacco since 1-10 years. There was no significant difference in mean serum CTN levels after 11 to more than 30 years [Table/Fig-5].

Duration of tobacco use in years	Types of tobacco users			Total
	G1	G2	G3	
1-10	2 (3.3%)	14 (23.3%)	3 (5.0%)	19 (10.6%)
11-20	20 (33.3%)	19 (31.7%)	20 (33.3%)	59 (32.8%)
21-30	36 (60.0%)	16 (26.7%)	25 (41.7%)	77 (42.8%)
>30	2 (3.3%)	11 (18.3%)	12 (20.0%)	25 (13.9%)
Total	60 (100%)	60 (100%)	60 (100%)	180 (100%)

[Table/Fig-4]: Duration of tobacco use in years in the study groups.

Duration of tobacco use in years	N	Serum CTN (ng/mL) Mean±SD	ANOVA	
			F	p-value
1-10	19	88.61±25.43	3.68	0.013
11-20	59	128.86±58.95		
21-30	77	121.40±41.08		
>30	25	133.62±62.64		

[Table/Fig-5]: Duration of tobacco use in years and mean serum CTN levels in the study groups.

In three groups, 66.7% in group (G1), 88.3% in group (G2) and 66.7% in group (G3) were using ≤5 grams of tobacco per day [Table/Fig-6].

Mean serum CTN was significantly high in subjects using >5 grams of tobacco per day than those using ≤5 grams of tobacco per day [Table/Fig-7].

Amount of tobacco use in grams per day	Types of tobacco users			Total
	G1	G2	G3	
≤5	40 (66.7%)	53 (88.3%)	40 (66.7%)	133 (73.9%)
>5	20 (33.3%)	7 (11.7%)	20 (33.3%)	47 (26.1%)
Total	60 (100%)	60 (100%)	60 (100%)	180 (100%)

[Table/Fig-6]: Amount of tobacco use in grams per day in the study groups.

Amount of tobacco use in grams per day	N	Serum CTN (ng/mL) Mean±SD	t	p-value
≤5	133	98.81±23.31	16.20	<0.001
>5	47	187.95±50.07		
Total	180			

[Table/Fig-7]: Amount of tobacco use in grams per day and mean serum CTN levels in the study groups.

In the study groups G1, G2a, G2b, G2c and G3 the difference was statistically significant. There was excellent positive correlation between serum CTN levels and amount of tobacco use in grams per day (p<0.001). There was no significant correlation between serum CTN levels and duration of tobacco use in years [Table/Fig-8].

Groups		Serum CTN level	p-value
		Pearson correlation coefficient®	
G1	Amount of tobacco use in grams per day	0.893	<0.001
	Duration of tobacco use in years	0.006	0.977
G2a	Amount of tobacco use in grams per day	0.914	<0.001
	Duration of tobacco use in years	-0.135	0.570
G2b	Amount of tobacco use in grams per day	0.960	<0.001
	Duration of tobacco use in years	0.154	0.516
G2c	Amount of tobacco use in grams per day	0.938	<0.001
	Duration of tobacco use in years	0.061	0.350
G3	Amount of tobacco use in grams per day	0.789	<0.001
	Duration of tobacco use in years	0.004	0.977

[Table/Fig-8]: Correlation between serum CTN levels and tobacco use status in each study group.

Linear regression analysis [Table/Fig-9-11] revealed that serum CTN levels were significantly associated (p<0.001) with amount of tobacco use in grams per day in tobacco smokers, smokeless tobacco users and dual tobacco smokers with smokeless tobacco users. But serum CTN levels were not significantly associated with age and duration of tobacco use in years. It shows that, the amount of tobacco use in grams per day was significant predictor of serum CTN levels in all tobacco user groups. The duration of tobacco use in years was not found to be significant predictor of serum CTN levels in all tobacco user groups.

	Unstandardised coefficients		Standardised coefficients	t	p-value
	B	Std. error	beta		
Constant	44.132	18.760	-	2.352	0.022
Age	-0.198	0.385	-0.038	-0.516	0.608
Amount of tobacco use in grams per day	18.912	1.285	0.889	14.713	<0.001
Duration of tobacco use in years	0.101	0.312	0.024	0.325	0.747

[Table/Fig-9]: Linear regression analysis showing association of CTN level in tobacco smokers group.
F=73.76, p<0.001, R^2=0.79

	Unstandardised coefficients		Standardised coefficients	t	p-value
	B	Std. error	Beta		
Constant	-20.58	26.35	-	-0.781	0.438
Age	0.469	0.636	0.055	0.737	0.464
Amount of tobacco use in grams per day	31.786	2.193	0.900	14.493	<0.001
Duration of tobacco use in years	-0.477	0.365	-0.095	-1.307	0.197

[Table/Fig-10]: Linear regression analysis showing association of CTN level in smokeless tobacco users group.
F=76.55, p<0.001, R^2=0.80

	Unstandardised coefficients		Standardised coefficients	t	p-value
	B	Std. error	Beta		
Constant	1.444	46.958	-	0.031	0.976
Age	0.116	0.901	0.011	0.129	0.898
Amount of tobacco use in grams per day	33.373	3.484	0.789	9.578	<0.001
Duration of tobacco use in years	-0.230	0.644	-0.030	-0.357	0.722

[Table/Fig-11]: Linear regression analysis showing association of CTN level in dual tobacco smokers with smokeless tobacco users group.
$F=30.84$, $p<0.001$, $R^2=0.62$

DISCUSSION

CTN has long half-life than nicotine, more stable and can be easily measured in the body fluid. So it has been approved as a short term indicator of recent active nicotine exposure. CTN measurement in body fluids provides an estimation of recent exposure to tobacco products but does not indicate the duration of exposure to tobacco [20].

Present study shows that tobacco use as chewing tobacco with tobacco mishri use was more common in younger age group than other tobacco user groups with mean age of 43.55 years. This shows that smokeless tobacco users get addicted to tobacco use at an earlier age. Males use tobacco in smoking as well as smokeless form; however females use tobacco exclusively in smokeless form as mishri. In India, even though socially women are not allowed to smoke, use of smokeless tobacco is culturally acceptable. In Maharashtra smokeless tobacco in the form of mishri is abused to a greater extend for application on teeth and gums with a false belief that it is germicidal and helps in cleaning teeth and curing toothache [1].

A significantly higher CTN levels in tobacco users were found compared with tobacco non-users, also in tobacco chewers compared with tobacco smokers, as well as for dual tobacco smokers and smokeless tobacco users compared with smokers. These results are similar to study done by Rostron BL et al., which showed higher CTN concentration for tobacco users compared with non-tobacco users and for smokeless tobacco users compare with cigarette smokers [21]. Hecht SS et al., showed that CTN concentrations were same as among smokeless tobacco users as among cigarette smokers [22]. Hecht SS et al., analysed concentration of urinary CTN and found that smokeless tobacco users had significantly higher CTN compared with smokers [22]. On the other hand, Naufal ZS et al., concluded that biomarker (biomarkers of volatile organic compounds, halogenated aromatic hydrocarbons, polycyclic aromatic hydrocarbons etc.,) concentration were generally significantly lower among smokeless tobacco users compared with cigarette smoker [23].

The cause of this difference in serum CTN level is not completely known. Possible explanations include, differences in use of products with different nicotine content [24], diverse constituents [25], absorption of nicotine [22] etc. Smokeless tobacco remains in contact of buccal mucosa for a longer duration, and a large amount of nicotine is absorbed into the blood. Amount of nicotine absorption and speed of transfer across the oral mucosa is determined by a number of factors like pH value, nicotine concentration of the product, surface area exposed, time kept in the mouth etc. During smoking, nicotine absorption is primarily by pulmonary blood vessels, where as during chewing nicotine is absorbed both through buccal mucosa and gastrointestinal tract.

This study shows that serum CTN level in tobacco mishri users was lower than other tobacco users. So, it is not viewed as seriously as tobacco chewing and tobacco smoking. However, it can act as a silent killer as its use start at a very early age, mostly in childhood as toothpaste and hence needs evaluation.

This study showed that, in tobacco users serum CTN levels increased with increase in amount of tobacco use in grams per day. There was no significant correlation between serum CTN levels and duration of tobacco use in years. The study by Castelino RL et al., showed positive correlation between salivary CTN, number of cigarettes or bidis and duration of habit in smokers and moderate positive correlation between salivary CTN, number and duration of habits in pan chewers [26]. However, there was no significant correlation found between salivary CTN levels and duration of habit in subjects who had both smoking and pan chewing habit. The study by Figueiredo V et al., showed no significant association between CTN concentration and duration [27]. This study shows that in linear regression analysis adjusting demographic factors, in all tobacco users serum CTN levels have not been affected significantly at any duration of tobacco use. However, serum CTN levels have been affected significantly with change in amount of tobacco use.

LIMITATION

Limitation of this study is that we did not have exact information on amount of tobacco used per day, which is approximately calculated from number of packets used per day. In the present study the serum CTN levels were analysed as per the smokeless and smoking form of tobacco use and not as per the different brands of tobacco used by the subjects. Further research is recommended to include other smokeless products.

CONCLUSION

These results have shown that the mean serum CTN levels were significantly higher in tobacco users than tobacco non-users and in tobacco chewers than tobacco smokers. The mean serum CTN levels in tobacco mishri users were significantly lower than other tobacco user groups.

Concurrent use of chewing tobacco and tobacco mishri is more common in younger age group. Those who are dual tobacco smokers and smokeless tobacco users tend to have relatively high level of serum CTN, which suggests tobacco dependence.

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