ORIGINAL ARTICLE

Salivary Cotinine Levels as a Biomarker of Tobacco Use - A Biochemical Study

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Abstract:

Background: Routine exposure to cigarette smoke has conventionally been assessed by questionnaire. The correctness of this method has been limited by incorrect reporting. Rejection and underrating the extent of smoking are common practices especially among youth and proclaimed quitters. Biochemical validation is the recommended choice in interventional studies where cessation results have to be evaluated. Cotinine measurement is the most common used method in population studies. It validates the use of tobacco consumption when compared to other markers available. *Aim and Objectives*: This study was designed to estimate the levels of salivary cotinine in tobacco smokers and chewers and compare them with the levels in subjects who do not report of any tobacco related habits. The study was also conducted to validate the self-report of tobacco use with a biological marker for tobacco exposure. *Material and Methods:* The study was performed in 200 study subjects divided into 4 groups (C, G1, G2 and G3) of 50 each. The saliva samples were collected from subjects who had no previous history of tobacco consumption, subjects with smoking, pan chewing habit, subjects who had both smoking and pan chewing habits were included in the study. The results were then compared and co-related between the groups. *Results:* The mean salivary cotinine levels in groups C, G1, G2 and G3 was found to be 10.74 ng/ml, 92.29 ng/ml, 108.80 ng/ml and 117.01 ng/ml respectively. When the mean values were compared between the groups the values were found to be statistically highly significant. *Conclusion:* The results of this study recommend that biochemical authentication of self-reported tobacco use must be done in any prevention and tobacco cessation programs. It is thus, highly recommended to use biological markers such as salivary cotinine to approve the information provided by the patients in terms of tobacco use.

Keywords: Smoker, Pan Chewer, Cotinine, Self-Report

Introduction:

Tobacco consumption is the chief cause of avoidable deaths in many developing countries [1]. Cigarette smoke is found to be the main risk factor for various ailments [1, 2]. Intake of tobacco subdues the response of the immune system. The individual is then prone to infection which in turn hampers wound healing [3]. The smokers have various oral lesions like smoker's melanosis, erythroplakia, leukoplakia, stomatitis nicotina, impaired gingival bleeding, periodontal diseases, halitosis, failure of the implant, excessive stains and calculus [4]. Tobacco intake also is a risk factor for cancer of the oral and congenital defects in offspring whose mothers consumed tobacco during gestation [3].

Habitual exposure to tobacco smoke has conventionally been evaluated by questionnaire.

The correctness of this method has been restricted by false reporting [5]. Rejection and under-estimating the extent of tobacco use are common practices especially among young population [6]. Most of the estimation of tobacco use in youth are based on self-reports [7]. In developed countries like the United States, several studies have confirmed self-reported tobacco use using actual biochemical validation [8]. Biochemical validation is very important where cessation outcomes have to be measured [9]. Cotinine measurement is most commonly used in population studies to validate the use of tobacco use.

Cotinine is a metabolite of nicotine with a long half-life of 15–19 hours and can be assessed in body fluids such as plasma, urine and saliva. The biochemically estimated cotinine levels is found to be an indicator of active smoking, use of smokeless tobacco, second hand smoke exposure or use of therapeutic nicotine [10].

In the developing countries like India, youth are mainly susceptible to initiation and dependence on tobacco products [7]. In India, studies have revealed that among 4 to 75 % of 13 to 15year old individuals report use of some form of tobacco or other in their lifetime [11]. It is also been found that individuals from lower socio economic sections are more likely to use tobacco products [12].

Therefore, this study was designed to estimate the levels of salivary cotinine in tobacco smokers and chewers and compare them with the levels in subjects who do not report of any tobacco related habits. The study was also conducted to validate the self-report of tobacco use with a biological marker for tobacco exposure.

Material and Methods:

A case control study was conducted on subjects reporting to the Department of Oral Medicine and Radiology. After obtaining the institutional ethical clearance, the nature and purpose of the study was explained and informed written consent was acquired from the subjects who were to be included in the study. A detailed case history was recorded and a thorough oral examination was performed for all the subjects included in the study. Sample size computation was done based on a pilot study conducted on 10 patients.

$\alpha = 5\%$, z value = 1.96

$\beta = 10\%$, $1 - ß = 90\%$, z value = 1.28

$n = 2*3.24*3.24*16*16/(11*11)$

= 44.42 rounded off to 50 per group

[Where, α = significance level, $1 - ß$ = power]

The study consisted of four groups with 50 samples each between the age group of 15-70 years.

The groups comprised of:

Group C: 50 subjects who did not report of any tobacco related habits.

Group G1: 50 subjects who were smokers of tobacco products.

Group G2: 50 subjects who were pan chewers with tobacco

Group G3: 50 subjects who were smokers and pan chewers of tobacco products

Inclusion and Exclusion Criteria:

Strict inclusion and exclusion criteria were followed. All the four groups in the study included subjects between the ages of 15 to 70 years. The control Group C comprised of subjects who do not have a positive history of smoking of hand rolled

or manufactured tobacco products or pan chewing habit. The study group G1 comprised of subjects who used to smoke one or more than one pack of hand rolled or manufactured tobacco products per week since the last 1 year or more or at least from the last 30 days. The study group G2 included subjects who regularly chewed pan since the last 1 year or more or at least from the last 30 days. The study group G3 comprised subjects who regularly smoke one or more than one pack of hand rolled or manufactured tobacco product per week and chew pan with tobacco since the last 1 year or at least from the last 30 days.

Individuals with history of any other substance abuse other than smoking and pan chewing with tobacco products, recent infection, subjects with systemic illness and subjects on any medication were omitted from the study.

Method of Data Collection:

The study was conducted in the time period of 2014 to 2016. Informed consent was obtained from all the individuals included in the study.The demographic parameters were recorded using a detailed case history emphasizing the presence of any deleterious habits such as smoking and or tobacco chewing. Convenience sampling technique was used for selection of the participants.

Saliva Collection:

Saliva sample was collected from the subjects through 'Spit Technique'. The subjects were instructed to sit on the dental chair with the head tilted forward and asked not to speak or swallow any saliva. The subject was then instructed to spit into a sterile graduated container every minute for 8-10 minutes. The salivary sample represented whole mouth fluid (saliva from major and minor salivary glands and gingival crevicular fluid). The collected sample was centrifuged at 3000 rpm for 10 minutes and the supernatant collected was stored at -20^{o}C. Salivary cotinine analysis was done using ELISA method (Calbiotech Laboratories).

Measurement of Cotinine:

Tobacco usage in any form can be detected by measuring nicotine and its metabolites. Cotinine due to its longer half- life has been used in research as a reliable marker for smoking status. The measurement of cotinine was done by competitive ELISA technique [13]. The cotinine levels were measured and were given in ng/ml.

The data collected was entered into Microsoft excel spreadsheet and analysed using IBM SPSS Statistics, Version 22(Armonk, NY: IBM Corp). Descriptive data were presented in the form of mean and standard deviation. The cotinine levels were compared between the study groups using One Way ANOVA followed by Post hoc tukey test. Pearson's correlation test was used to test the correlation between the cotinine levels and tobacco habits. P value < 0.05 was considered as statistically significant.

Results:

The demographic data analysis of control group (C), study group (G1) and study group (G2) and (G3) (Table 1). In the control group 49 were males and 1 was a female subject. In the study group (G1) all the subjects were males. In the study group (G2) 38 were males and 12 were females. In the study group (G3) all the 50 subjects were males. The mean salivary cotinine levels in groups C, G1, G2 and G3 was found to be 10.74 ng/ml, 92.29 ng/ml, 108.80 ng/ml and 117.01 ng/ml respectively. When the mean values were

compared between the groups the values were statistically highly significant (Table 2). Pairwise comparison of cotinine levels between the study groups showed statistically highly significant results (Table 3). In the study group 1 there was excellent positive correlation between salivary cotinine levels and number and duration of habits (r=0.72). In the study group 2 there was moderate positive correlation between salivary cotinine levels and number (r= 0.53) and duration of habits (r=0.51). In the study group 3 there was weak positive correlation between salivary cotinine levels and number of beedi/cigarettes smoked. (r=0.30) There was no significant correlation between salivary cotinine levels and duration of habits and number of pan chewed (Table 4). The duration of the habit and the number of cigarettes / pan chewed were not found to be significant predictors of cotinine levels in smokers and smoker and pan chewer group (Table 5 and 7). The number of pan chewed per day and duration of habit were significant predictors of salivary cotinine levels in pan chewers (Table 6).

Table 1: Comparison of Age between the Study Groups

Groups	N	Mean ± SD	Min	Max	ANOVA	
					F	p-value
C	50	36.740 ± 13.37	18.0	62.0	29.256	<0.001*
G1	50	43.160 ±11.91	22.0	70.0		
G2	50	52.760 ±9.91	21.0	70.0		
G3	50	54.500 ±7.61	41.0	69.0		

Table 2: Comparison of Cotinine Levels between the Study Groups

Groups	N	Mean ± SD	Min	Max	ANOVA	
					F	p-value
C	50	10.74 ± 2.59	5.24	15.39	1311.172	<0.001*
G1	50	92.29 ± 14.42	40.43	113.75		
G2	50	108.80 ± 9.30	73.50	119.38		
G3	50	117.01 ± 7.83	99.88	129.76		

Table 3: Pair wise Comparison of Cotinine Levels between the Study Groups

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	p-value	95% CI	
					Lower Bound	Upper Bound
C	G1	-81.55	1.90	<0.001*	-86.49	-76.62
	G2	-98.06	1.90	<0.001*	-102.99	-93.13
	G3	-106.27	1.90	<0.001*	-111.20	-101.34
G1	G2	-16.51	1.90	<0.001*	-21.44	-11.57
	G3	-24.72	1.90	<0.001*	-29.65	-19.79
G2	G3	-8.21	1.90	<0.001*	-13.15	-3.28

(I) and (J): represents the pair wise comparison between groups that has been symbolically represented in the first 2 columns

Table 4: Correlation of Coefficient and p Value between the Salivary Cotinine Levels and Habits in Each Study Group

Groups	Habits	Salivary Cotinine Value	
G1	Number of beedi/cigarette per day	r	0.72
		p-value	<0.001*
	Duration in years	r	0.72
		p-value	<0.001*
G2	Number of pan per day	r	0.53
		p-value	<0.001*
	Duration in years	r	0.51
		p-value	<0.001*
G3	Number of beedi/ cigarette per day	r	0.30
		p-value	0.03*
	Duration in years	r	0.04
		p-value	0.79(NS)
	Number of pan per day	r	0.17
		p-value	0.23(NS)
	Duration in years	r	-0.12
		p-value	0.42(NS)

Table 5: Multiple Linear Regressions to Predict Cotinine Levels in Smokers Groups

	Unstandardized Coefficients		Standardized Coefficients	t	p-value
	B	Std. Error	Beta		
Constant	74.96	5.67	-	13.22	<0.001*
Age	0.03	0.12	0.02	0.21	0.84^{NS}
Number of Beedi/ Cigarette per day	1.09	1.10	0.31	0.99	0.33^{NS}
Duration in years	1.26	0.94	0.42	1.34	0.19^{NS}

F (3, 46) = 17.58, P<0.001, R^2 = 0.53, p>0.05, NS-non-significant

Table 6: Multiple Linear Regressions to Predict Cotinine Levels in Pan Chewers Groups

	Unstandardized Coefficients		Standardized Coefficients	t	p-value
	B	Std. Error	Beta		
Constant	86.43	6.40	-	13.51	<0.001*
Age	-0.11	0.11	-0.12	-1.03	0.31^{NS}
Number of pan per day	1.31	0.34	0.43	3.83	<0.001*
Duration in years	1.15	0.30	0.46	3.85	<0.001*

F (3, 46) = 12.71, P<0.001, R^2 = 0.45, p>0.05, NS-non-significant

Table 7: Multiple Linear Regressions to Predict Cotinine Levels in Smokers+Pan Chewers Groups

	Unstandardized Coefficients		Standardized Coefficients	t	p-value
	B	Std. Error	Beta		
Constant	120.14	13.66	-	8.79	<0.001*
Age	-0.14	0.15	-0.14	-0.92	0.36^{NS}
Number of Beedi/ Cigarette per day	0.70	0.35	0.29	2.00	0.052^{NS}
Duration in years	-0.08	0.25	-0.05	-0.30	0.76^{NS}
Number of pan per day	0.48	0.56	0.12	0.85	0.40^{NS}
Duration in years	-0.86	0.64	-0.20	-1.36	0.18^{NS}

*F (5, 44) = 1.51, p = 0.21(NS), R^2 = 0.15 *p<0.05 statistically significant p>0.05, NS-non-significant*

Discussion:

It is of great significance to precisely assess the amount of consumption of tobacco products in monitoring programmes. It is extremely beneficial especially in the youth population [14]. Cotinine is the major metabolite resulting from nicotine which is a by product of tobacco [15]. Cotinine and its metabolites represent about 80% of the metabolic products resulting from the nicotine absorbed by a smoker. It is excreted mainly by the kidneys, maternal milk, sweat and oral fluids [16].

Cotinine has long half-life when compared to nicotine and hence has been accepted as a short-term indicator of nicotine exposure. It is less prone to instabilities and can be easily measured in body fluids. Cotinine estimation from body fluids provide an estimation of recent exposure to tobacco products but not the duration of exposure [17, 18].

Cotinine from body fluids is the marker of choice for the assessment of absorption of tobacco related products. The method of collection and the type of specimen impacts the levels of cotinine during detection. The cotinine levels are found to be significantly higher in unstimulated than in stimulated saliva. Also, the quantitative and semi-quantitative evaluation methods have revealed that the cotinine levels from unstimulated saliva is the most specific and sensitive biomarker of tobacco exposure. The salivary cotinine can be evaluated in people with recent nicotine exposure, active/passive smokers and in people who are occupationally exposed to tobacco and its related products [19].

In the present study salivary cotinine levels were estimated in smokers, pan chewers and in subjects who had both smoking and pan chewing habit in order to assess the cotinine values in different groups with different tobacco related habits.

In the control group the subjects were between 18 to 62 years of age and the mean age was 36.740 years. In the study group GI the subjects were between 22 to 70 years of age and the mean age was 43.160 years. In the study group G2 the subjects were between 21 to 70 years of age and the mean age was 52.760 years. In the study group G3 the subjects were between 41 to 69 years of age and the mean age was 54.5 years. On comparison of the age between the study groups the difference between the groups were found to be statistically highly significant with $P < 0.001$ (Table 1).

The Society for Research on Nicotine and Tobacco Subcommittee (SRNT) on biochemical verification has defined non- smokers as people who have never smoked or people who are ex-smokers and who have a salivary cotinine concentration <15.0 nanogram per millilitre. They have further gone to define current smokers as people who have a self -reported habit or people with a salivary cotinine concentration of 15.0 nanogram per millilitre and above [20]. Based on these recommendations given by the SRNT subcommittee similar values were taken into consideration in the present study.

The mean salivary cotinine levels in control group, study group GI, GII and GIII was found to be 10.74 ng/ml, 92.29 ng/ml, 108.80 ng/ml and 117.01 ng/ml respectively. When the mean values were compared between the groups the values were found to be statistically highly significant. The pairwise comparison of cotinine levels between the study groups showed statistically

highly significant results which was in accordance with the study conducted by Dhavan *et al*. [21]. The present study also focussed on to validate self-reports of tobacco use with a biochemical marker to confirm the same which was in accordance with studies conducted by Dhavan *et al*. [21], Figueiredo *et al*. [17] , Nuca *et al*. [19]. In the present study we used a questionnaire to collect demographic, smoking behavioural data and then the collection of unstimulated saliva for the estimation of salivary cotinine.

Our study showed an excellent positive correlation between salivary cotinine levels and number of cigarettes or beedis and the duration of habits (r=0.72) in smokers which was consistent with the studies conducted by Binnie [22] and Etter *et al*. [23].

There was moderate positive correlation between salivary cotinine levels and number (r= 0.53) and duration of habits (r=0.51) in case of pan chewers. The number of pan chewed per day and the duration of their habit were significant predictors of salivary cotinine levels in pan chewers. The present study was also conducted on subjects who had both smoking and pan chewing habit. In these subjects there was weak positive correlation between salivary cotinine levels and number of beedi/cigarettes smoked per day. There was no significant correlation between salivary cotinine levels and number of pan chewed. The duration of the habit and the number of cigarettes or pan chewed were not found to be significant predictors of cotinine levels. The present study did not analyse the salivary cotinine levels in each of the different brands of tobacco used by the subjects. It also did not analyse salivary cotinine levels in patients exposed to second hand smoke or passive smokers or subjects whose close relatives are tobacco users which should be addressed in the future.

Conclusion:

This study was designed to estimate the levels of salivary cotinine in tobacco smokers and chewers and compare them with the levels in subjects who do not report of any tobacco related habits. The results of our study proved that cotinine from body fluids are the marker of choice for the assessment of absorption of tobacco related products. The results of this study strongly recommend the assessment of biochemical validation of self-reported tobacco usage to be done in any prevention and tobacco cessation programs among dental patients especially in developing countries like India. As the quitting process is of a long duration periodic assessment of salivary cotinine is highly recommended to track the progress of improvement for both the oral physician and the patient who is willing to quit the habit. It is therefore, highly suggested to use biological markers such as salivary cotinine which will confirm the information provided by the patients in terms of tobacco use.

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