Brief Report

Determinants of Salivary Cotinine Concentrations Among Smokeless Tobacco Users

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Received May 25, 2011; accepted October 27, 2011

Abstract

Introduction: Factors associated with cotinine concentrations have been studied in smokers. Based on these studies, cotinine is recommended as a biomarker to assess severity of tobacco dependence. Evidence of association between cotinine concentrations and various factors among smokeless tobacco (ST) users is limited and mostly comes from tobacco cessation studies. The present study describes the relationship of salivary cotinine concentrations to sociodemographic factors, tobacco use characteristics, and ST products among ST users.

Methods: Data are from a community-based sample of 95 current adult ST users. Study participants provided a saliva sample for cotinine analysis and completed a mail survey that included questionnaires regarding sociodemographic information, tobacco use characteristics, and tobacco dependence measures. Crude and adjusted associations between cotinine and other variables were calculated.

Results: Age, years of ST use, cans per week, and swallowing of tobacco juices were signifcantly associated with salivary coti - nine concentrations in the multiple regression model. Fine-cut ST products resulted in higher cotinine concentrations as compared with long-cut ST products when adjusted for age and tobacco use characteristics ($p = .029$). The Fagerström Test for Nicotine Dependence for ST users ($r = .58$, $p < .0001$) and the modifed Tobacco Dependence Screener ($r = .24$, $p < .0001$) were both correlated with cotinine concentration.

Conclusion: The fndings suggest some similarities in the deter - minants of cotinine concentrations in ST users and smokers. Swallowing of tobacco juices and type of ST product are unique to ST users and are associated with higher cotinine concentrations.

Introduction

Nicotine, a major alkaloid in tobacco, has a stimulant effect at low doses and is responsible for dependence among tobacco users. Unlike smoking, where nicotine is frst inhaled and then enters the blood through alveoli, smokeless tobacco (ST) products directly deliver nicotine into the venous blood through sublingual absorption, and also the ingested tobacco juices result in additional nicotine entering the bloodstream through intestinal absorption. Nicotine undergoes frst-pass elimination through the liver and is metabolized by cytochrome P450 enzymes. C-oxidation of nicotine by CYP2A6 yields cotinine as a major metabolite (Messina, Tyndale, & Sellers, 1997; Nakajima et al., 1996). Other primary metabolites of nicotine due to oxidative metabolism and glucuronide conjugation include nicotine N′-oxide, nicotine glucuronide, nornicotine, and 2-hydroxynicotine (Benowitz et al., 1999; Nakajima et al., 1996). Nicotine has a shorter half-life of 2 hr; therefore, cotinine, with a longer half-life of 16–20 hr, is a comparatively stable indicator of nicotine intake (Jarvis, Russell, Benowitz, & Feyerabend, 1988).

Cotinine concentrations have widely been studied among smokers. Consequently, it has been recommended as a biomarker to assess severity of dependence in tobacco control studies (Society for Research on Nicotine and Tobacco, 2002). Previously conducted research focused on ST users reported association of cotinine concentration with tobacco use characteristics, such as years of ST use, quantity used per day, total dipping time, swallowing of tobacco juices, quit attempts, and demographic factors, including age, marital status, and occupation (Ebbert et al., 2004; Ferketich, Wee, Shultz, & Wewers, 2007b; Hatsukami, Keenan, & Anton, 1988). The ST dependence measure based on the Fagerström Tolerance Questionnaire was inconsistently associated with cotinine concentrations (Boyle, Jensen, Hatsukami, & Severson, 1995; Ebbert, Patten, & Schroeder, 2006; Ferketich, Wee, Shultz, & Wewers, 2007a). However, the Fagerström Test for Nicotine Dependence for ST (FTND-ST) users was signifcantly associated with serum cotinine concentrations (Ebbert et al., 2006). Studies of cigarette smokers investigating the cigarette favor and cotinine concentrations found that menthol cigarette smokers had higher cotinine concentration (Williams et al., 2007). Menthol infuences the rate of nicotine metabolism, thus affecting the cotinine concentrations. Such association among ST users has not been explored. (Hersey, Nonnemaker, Ghada Homsi, & Jane Allen, 2010; Jain & Bernert, 2010; Wackowski & Delnevo, 2007).

doi: 10.1093/ntr/ntr279
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To date, the evidence of an association between cotinine concentrations and various factors among ST users is limited and mostly comes from tobacco cessation studies (Ebbert et al., 2006; Ferketich et al., 2007b). Due to the specifc characteristics of the subjects enrolled in cessation programs, such as higher levels of motivation to quit and the fact that the users are already in a process of behavioral change, a study of a community-based sample of ST users will contribute to a better understanding of the factors associated with cotinine concentrations and ST use. In the present study, we examined the relation of salivary cotinine concentrations with tobacco use characteristics and demographic factors. In addition to investigating the previously studied factors associated with cotinine concentrations, other covariates, such as characteristics of ST products, which may have direct or indirect effects, were assessed.

Methods

Recruitment for this study was conducted from May 2010 to December 2010. Individuals eligible for participation were adult current ST users aged 18–64 years, who were currently nonsmokers (never-smokers or former smokers who refrained from smoking for a year or more), had used ST products for at least one year, consumed a minimum of one can or pouch of ST per week, and did not have a history of psychiatric illness or other substance abuse. ST users were recruited from Oklahoma through various techniques, including emails, fyers, and web - site postings. The initial screening was conducted through phone calls, and a self-administered questionnaire along with saliva collecting tubes was mailed to the eligible ST users. The consent form and information sheet describing the study protocol and confdentiality information were also included with the mail questionnaire. Participants were required to sign the consent form and mail it back to the study center together with the completed questionnaire and the saliva sample. The study was approved by the Institutional Review Board of the University of Oklahoma Health Sciences Center.

Measures

Participants completed a structured questionnaire that obtained information on the following items: (a) sociodemographic characteristics, including age, race, ethnicity, occupation, education, income, height, and weight; (b) past cigarette smoking information, such as age of smoking onset, duration (years), quantity (average number of cigarettes smoked per day), age at quit, and quit method (ST, nicotine replacement therapy, smoking cessation medication, other, or none); and (c) ST use characteristics, including age of regular ST use, everyday or someday ST use, number of dips/chews per day, number of cans/pouches consumed per week, number of lifetime quit attempts, number of quit attempts during the last 12 months, type of ST product used, and brand of ST product.

ST dependence was measured with the help of self-administered dependence measures, Fagerström Test for Nicotine Dependence (FTND-ST) and modifed Tobacco Dependence Screener (TDS). The FTND-ST, six-item scale has previously demonstrated a signifcant association with cotinine concentrations (Ebbert et al., 2006). Apart from providing a continuous score for ST dependence, two of its questions in FTND, time to frst chew (tobacco use in the morning) and number of chews/dips per day, have been used as independent indicators of dependence in cigarette-smoking studies. However, its question about swallowing the tobacco juices is of importance for exploring the determinants of cotinine concentrations. The TDS is based on *DSM-IV* and ICD-10 criteria of tobacco dependence (Kawakami, Takatsuka, Inaba, & Shimizu, 1999). The original scale was modifed for use in this study by replacing smoking-related terms with ST use.

Saliva Specimen

For the saliva sample, an oral swab and swab storage tube were sent to the ST users. Participants were instructed to rinse their mouths before providing the saliva sample through passive drool method. Specimens were sent back to the study center by the U.S. Postal Service. Cotinine remains stable in unfrozen saliva, and saliva samples sent through U.S. mail have comparable cotinine concentrations to the samples that are immediately frozen (Foulds, Bryant, Stapleton, Jarvis, & Russell, 1994; Greeley, Valois, & Bernstein, 1992) Specimens were frozen and stored at −20 °C and then shipped to Salimetrics laboratories in Pennsylvania for cotinine analysis. Cotinine concentration was determined using a highly sensitive enzyme immunoassay. This technique can detect cotinine as low as 1 ng/ml (the test used 20 µl of saliva sample per determination, has a lower limit of sensitivity of 0.15 ng/ml, a range of standard curve from 0.8 to 200 ng/ml, an average intra-assay coeffcient of variation of 6.4%, and an average interassay co - effcient of variation of 6.6%. Method accuracy determined by spike recovery averaged 99.6%, and linearity determined by serial dilution averaged 98%). Cross-reactivity was detected with nicotine (0.0293%) and 3-OH-cotinine (24.82%); however, other compounds such as nicotinic acid and nicotinamide were not found to cross-react (Salimetrics, 2010). Salivary cotinine assays were run in duplicate, and the mean of the two values were used for the analysis.

Statistical Analysis

Exploratory analysis of all the variables in the study was performed, and descriptive statistics were calculated. Simple linear regression analysis was conducted to test associations between cotinine concentrations and individual variables. Residuals from the regression analysis were analyzed to verify the model assumptions of normality, linearity, and homoscadasticity. Variables found to be associated at a signifcance level of .05 with cotinine concentrations from simple linear regression were used in multivariate regression analysis. A stepwise selection procedure was applied to fnd a parsimonious solution to this association. Confounding and effect modifcation of the covari - ates were also checked.

We examined the relationship of salivary cotinine with ST type, brand of ST product, and its favor. Analysis of variance was conducted to evaluate differences in mean salivary cotinine concentrations across ST types (chewing tobacco, snuff, and snus). Similar analyses were performed for the brand and favor of the ST products.

Assumption of normality was not met for the bivariate analysis; therefore, a square root transformation of the cotinine concentration was performed. This transformation met the assumptions of the regression models. All analyses were conducted using SAS v. 9.2, and a level of .05 was used for statistical signifcance.

Results

Hundred male ST users participated in the study, and data related to cotinine concentration were obtained from 95 participants. The mean age of study subjects was 31.9 (*SD* = 12.2) years, and the majority was White (93%) and non-Hispanic (95%). This group had high levels of education with 82% having some college education or college degree and the remaining 18% having a high school education. The median salivary cotinine concentration was 350.5 (min = 15.5, max = 1,772.1) ng/ml.

In order to explore the association of cotinine with sociodemographic factors and tobacco use characteristics, initially, simple linear regression analysis was performed. Among the tobacco use characteristics, years of ST use, everyday use, number of chews/dips per day, swallowing of tobacco juices, age at regular ST use, and number of cans used per week were signifcantly related to the transformed cotinine concentration in the univariate analysis. ST quit variables such as history of lifetime quit, ST quit during the past 12 months, and number of quit attempts during the past 12 months were not associated with cotinine concentration (Table 1).

Multivariate analysis was performed by including the abovementioned signifcantly related tobacco use characteristics and age as independent variables in regression models. We noted positive correlations between predictor variables. Most notably, ST use characteristics were correlated with each other and with age. Multiple regression analysis with highly correlated predictors would affect the regression coeffcients and infate *SE*s as a result of multicollinearity. Collinearity diagnostics, tolerance, and variance infation factor (VIF) for years of ST use, age of regular ST use, and age were beyond the recommended limits. It indicated that these variables provided the same information and were redundant. We omitted age of regular ST use from the model, and the assumption of collinearity was met. The stepwise selection procedure retained age, cans per week, swallowing of tobacco juices, and years of ST use in the overall fnal model that had an adjusted R^2 of .466. Transformed salivary cotinine concentration was positively associated with increasing age, consuming more cans or pouches of ST, and swallowing of tobacco juices, when adjusted for each other and number of years of ST use (Table 2). These results suggested that the salivary cotinine concentration increased by 20.88 ng/dl among those who swallowed tobacco juices as compared with those who did not swallow when adjusted for other covariates.

Results regarding the tobacco dependence measures indicated that ST dependence measures were signifcantly associated with transformed cotinine concentrations. FTND-ST had the highest correlation with salivary cotinine concentration ($r = .58$, $p < .0001$). Similarly, TDS was also signifcantly associated with the transformed cotinine concentrations ($r = .237$, $p < .0001$).

For the effect of ST product characteristics on cotinine, analysis of variance was used to explore if the cotinine concentrations differed across the brand, type, and favor of the smokeless tobacco products. Two popular brands used by the participants were Copenhagen (41%) and Grizzly (23%). Fifty-seven percent used long-cut products; whereas, Wintergreen was the most commonly (55%) used favor. Salivary cotinine was not associated with the brand or the favor of the ST product. However, ST type was signifcantly related to the cotinine concentrations ($p = .0459$). In order to adjust the association of cotinine concentration with ST type for the determinants of salivary cotinine concentrations, analysis of covariance was performed. These results indicated that use of fne-cut ST products led to higher salivary cotinine concentrations as compared with long cut ($p = .0286$) when adjusted for age, years of ST use, number of cans used per week, and swallowing of tobacco juices.

Discussion

Cotinine is a major metabolite of nicotine, and its concentration in body fuids is determined by the rate of nicotine metabolism and cotinine clearance. Although individual variability might exist in salivary cotinine concentrations because of these parameters, it is still an important indicator of nicotine dependence. This study examined a number of factors that could determine cotinine concentrations among ST users. Unlike past ST studies, which were based on participants enrolled in tobacco cessation programs, this study used a community-based sample of ST users.

For the sociodemographic factors, age was identifed as the most important factor related to cotinine. Age accounted for 34% of variation in salivary cotinine concentrations of ST users. Although income and employment were also signifcant factors in the univariate analysis, only age was retained based on the multivariate analysis. This positive association between age and cotinine is well-established in past ST and smoking studies (Ferketich et al., 2007b; Figueiredo et al., 2007; Swan, Habina, Means, Jobe, & Esposito, 1993). While employment status and income were not retained in the fnal model, those who were employed full-time for wages had signifcantly higher cotinine concentrations as compared with students and to part-time workers or unemployed in the univariate analysis. Similar to these fndings, a study of rural ST users reported higher cotinine concentrations among full-time workers as compared with part-time workers or unemployed in the univariate model, which was not signifcant when adjusted for other covariates (Ferketich et al., 2007b).

Previously conducted research studies have reported distinct tobacco use characteristics associated with cotinine among ST users. These variations in fndings might be due to the discrep - ancies in the tobacco use characteristics studied in these studies. Despite the inconsistencies in the variables used in these studies, some of the tobacco use characteristics, such as cans per week, inter dip interval, dip duration, and years of ST use, were measured in at least two studies. Ebbert et al. (2004) documented that swallowing tobacco juices was related to higher cotinine concentration. A study of rural ST users identifed number of quit attempts in the past year to be associated with cotinine (Ferketich et al., 2007b). Hatsukami et al. (1988) reported number of dips per day, duration of dip, total duration of ST use in a day, and interdip interval as signifcant predictors. The Hatsukami study of 56 ST users employed only univariate analysis, and most of these factors point toward redundancy, as these are apparently highly correlated indicators. In the current study, several tobacco use characteristics were signifcantly associated with cotinine in the univariate analysis. These included years of ST use, dips per day, cans per week, age of onset of regular ST use, swallowing of tobacco juices, and daily or occasional ST

Table 1. Univariate Association of Salivary Cotinine[a] With Sociodemographic Factors and Tobacco Use Characteristics

Variable		Parameter estimate	*SE*	Test stat	*p* Value
Age *M* (*SD*)	31.9 (12.2)	0.475	0.061	7.73	<.0001
Race *n* (%)					
White	88 (92.6)	Referent			
Other	7 (7.4)	−0.390	3.62	0.01	.914
Ethnicity *n* (%)					
Hispanic	5 (5.3)	−4.861	4.206	1.34	.248
Non-Hispanic	90 (94.7)	Referent			
Education *n* (%)					
High school	18 (18.9)	Referent			
Some college	46 (48.4)	−0.251	2.56	0.01	.922
College graduate	18 (18.9)	0.665	2.72	0.06	.807
Occupation *n* (%)					
Student	34 (35.8)	Referent			
Employed	53 (55.8)	8.103	1.804	20.17	<.0001
Other	8 (8.4)	11.414	3.227	12.51	.0004
Income *n* (%)					
Less than $25,000	19 (22.9)	Referent			
$25,000–$35,000	13 (15.7)	8.101	3.124	6.72	.0095
$35,000–$50,000	12 (14.5)	3.287	3.201	1.05	.3044
$50,000–$75,000	21 (25.3)	6.788	2.748	6.10	.0135
More than $75,000	18 (21.7)	4.045	2.855	2.01	.1566
Past smoking history, *n* (%)					
Yes	42 (44.2)	3.30	1.89	1.74	.0847
No	53 (55.8)	Referent			
Quit method, *n* (%)					
ST	30 (71.4)	5.266	3.32	2.51	.1130
NRT	2 (4.76)	−5.72	7.05	0.66	.4174
None	10 (23.8)	Referent			
ST use, *n* (%)					
Everyday	87 (91.6)	9.31	3.30	2.82	.0059
Someday	8 (8.4)	Referent			
Ever quit, *n* (%)					
Yes	68 (71.6)	2.495	2.081	1.44	.2305
No	27 (28.4)	Referent			
Quit during past 12 months, *n* (%)					
Yes	30 (68.4)	−1.603	2.028	−0.78	.4294
No	65 (31.6)	Referent			
Swallowing of tobacco juices, *n* (%)					
Yes	44 (46.3)	7.496	1.752	4.28	<.0001
No	51 (53.7)	Referent			
Age at regular ST, *M* (*SD*)	18.8 (7.8)	0.403	0.116	3.47	.0008
Cans/pouches per week, *M* (*SD*)	3.6 (2.2)	1.223	0.413	2.96	.0039
No. of dips/chews per day, *M* (*SD*)	6.6 (4.5)	0.896	0.191	4.68	<.0001
Years of ST use, *M* (*SD*)	13.1 (11.1)	0.378	0.077	4.88	<.0001

Note. NRT = nicotine replacement therapy; ST = smokeless tobacco.
[a]Square root transformation of cotinine.

use. However, the multivariate analysis specifed only years of ST use, cans per week, and swallowing of tobacco juices as signifcant determinants of salivary cotinine. These tobacco use characteristics along with age jointly explained 47% of the variation in cotinine concentrations in ST users. Due to our sample size of 95 and possibly other unique population characteristics, we had a smaller proportion of occasional ST users; therefore, it was not included in the multivariate model. Similarly age of regular ST use was excluded from the multivariate analysis because of its multicollinearity with age and years of ST use. It is important to note that quantity of ST use and swallowing of tobacco juices were positively associated with cotinine when adjusted for other covariates, but years of ST use had a negative association, indicating that increased years of ST use resulted in lower cotinine concentrations. This change in association of years of ST use with cotinine concentrations indicates the confounding effect of age. This effect is possibly due to the increased metabolism and elimination of cotinine from the body with increased

Table 2. Results of Multiple Regression Model for Salivary Cotinine[a] Among 95 ST Users

Variable	Parameter estimate	*SE*	Test stat	*p* Value
Age	0.555	0.090	6.12	<.0001
Cans/pouches per week	1.210	0.316	3.82	.0001
Swallowing of tobacco juices	4.57	1.424	3.21	.0019
Number of years of ST use	−0.220	0.102	−2.15	.0340

Note. ST = smokeless tobacco.
[a]Square root transformation of cotinine.

use of tobacco, as reported in the studies of cigarette smoking (Istvan, Nides, Buist, Greene, & Voelker, 1994). It also explains development of tolerance with increased number of years of tobacco use. These tobacco use characteristics associated with cotinine concentration are more biologically plausible as compared with the previously identifed cotinine predictors.

Several studies have assessed the relationship between the type of cigarette smoked and cotinine. Flavored cigarettes, especially mentholated cigarettes have been documented to yield higher nicotine concentration (Hersey et al., 2010; Wackowski & Delnevo, 2007). Other studies of smokeless tobacco have reported an association between snuff brand (Red Seal) and cotinine concentration (Ferketich et al., 2007b). We did not fnd an association between cotinine and the brand of the ST products. These inconsistencies in fndings are most likely because of the differences in the ST products used by the study sample and methodological issues. Additionally, the current study had higher proportions of participants using Copenhagen and Grizzly brands, and we did not have a sufficient number of Red Seal users to perform a similar comparison. Therefore, we used Copenhagen as the comparison group. Unlike the association between mentholated cigarettes and cotinine concentration among smokers, there was no association between different fa - vors of ST products and cotinine concentrations in our study. Type of ST product was signifcantly associated with cotinine concentrations. Participants using fne-cut ST products were more likely to have higher cotinine concentration as compared with those who used long-cut ST products. This association remained signifcant when adjusted for other determinants of cotinine.

Cotinine is a biomarker that has been used to measure dependence among tobacco users (Society for Research on Nicotine and Tobacco, 2002). Consistent with the fndings of the past studies, dependence measures were associated with cotinine concentrations in our study (Boyle et al., 1995; Ferketich et al., 2007a; Kawakami et al., 1999; Smith et al., 2010). FTND-ST, which includes information about cans per week, time to frst dip, swallowing of tobacco juices, amount of tobacco used during the frst hours as compared with the rest of the day, and using tobacco despite being sick, had the highest correlation with cotinine. These results are in agreement with the previous studies that indicate an association between FTND-ST and cotinine. *DSM-IV*–based TDS was also associated with cotinine concentrations. Although its correlation was not as strong as observed for FTND-ST, the TDS items do not query about the frequency or duration of the ST use. These fndings support the use of cotinine as a criterion variable for the validation of tobacco dependence measures.

Although most of the fndings of this study are in agreement with past studies, there are a few discrepancies. These differences may be attributable to the variation in the populations across studies. This study had relatively younger participants with higher level of education and socioeconomic status. Variations in cotinine concentrations exist not only across but also within populations. Studies of cigarette smoking have shown that cotinine concentrations differ among people who smoked same number of cigarettes per day. We addressed a number of methodological issues in this study. A consistent approach was adapted for the statistical analysis, and continuous variables were not categorized only to fnd an association, which was not originally evident. Highly correlated predicators, which might jeopardize the validity of the estimates due to collinearity were handled accordingly. Assumptions of the statistical tests were checked, and as a result, the cotinine data were transformed.

The study has a few limitations. All the study participants were male, the majority was White and non-Hispanic, and had higher level of education, which limits the genralizability of the study. Although we had a comparable sample size to the previous ST studies, the power of the study for multiple regression analyses was compromised. Not all the respondents provided information about the type and favor of ST products. We did not obtain information about dip duration among our study subjects, which was identifed as a signifcant factor as - sociated to cotinine in other studies; however, Ferketich et al. did not fnd a signifcant association with this factor when controlling for other factors. The results for the association of cotinine with income were based on data from only 83 participants. These results should be interpreted with caution, as the smaller sample size might infuence some of the fndings. Our study was based on a self-administered questionnaire, which might lead to self-reporting bias, such as social desirability bias.

Conclusion

This study identifed the determinants of cotinine concentrations in ST users. Similar to the past ST studies and smoking studies, we found that sociodemographic factors and tobacco use characteristics infuence cotinine concentrations. Cotinine is not only correlated with FTND-ST scores but also with TDS that measures various aspects of nicotine dependence. These fnd - ings support its use as biomarker measure for nicotine dependence among ST users. Association of ST type with cotinine requires further investigation and potentially calls for policy development that regulates ST products. The fndings also suggested that some similarities in the determinants of cotinine concentrations exist between ST users and smokers. Swallowing of tobacco juices and type of ST products are unique to ST users and are associated with higher cotinine concentrations.

Funding

This study was funded by the Oklahoma Tobacco Research Center.

Declaration of Interests

The authors have no competing financial, professional, or personal interests that might have influenced the performance or presentation of the work described in this manuscript.

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