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ORIGINAL ARTICLE

Effects of smoking on trace metal levels in saliva

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OBJECTIVES: To compare the salivary levels of trace metals between non-smokers and smokers using inductively coupled plasma mass spectroscopy (ICP-MS). The effect of pretreatment methods on the accuracy of ICP-MS analysis and daily variations in trace metal levels in saliva were also investigated.

SUBJECTS AND METHODS: The participants were 10 male non-smokers (mean age: 27.4 ± 3.4 years) and 30 male smokers (mean age: 26.5 ± 4.1 years). Unstimulated whole saliva was collected. Salivary flow rate, the number of metal restorations in the oral cavity, the level of blood contamination in the saliva and the levels of cotinine and trace metals in the saliva of each participant were determined.

RESULTS: Direct dilution of saliva samples with nitric acid showed the most accurate ICP-MS results. Trace metal levels in saliva showed wide daily variations. They were not affected by the number of metal restorations. Trace metal concentrations of saliva samples without blood contamination were much lower than the previously reported values. Salivary levels of cotinine and aluminum were significantly increased in smokers.

CONCLUSIONS: Saliva can be a medium for trace metal analysis. Salivary levels of cotinine and aluminum can be useful markers to evaluate smoking status.

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Keywords: smoking; trace metal; saliva; ICP-MS

Introduction

Saliva is a non-invasive, readily collectible and low-cost material (Nriagu *et al*, 2006; Esteban and Castaño, 2009). Recent technological advances in immunology, molecular biology, and analytical chemistry have facilitated the clinical use of saliva for drug monitoring and diagnosis of hereditary disorders, autoimmune diseases, infectious diseases, endocrine disorders, and cancers (Kaufman and Lamster, 2002; Streckfus and Bigler, 2002; Wong, 2006).

Saliva can also be used for biomonitoring to estimate environmental and occupational exposure to toxic trace metals (Barbosa *et al*, 2006; Nriagu *et al*, 2006; Wang *et al*, 2008; Costa de Almeida *et al*, 2009). Although the composition of saliva is originated from typical extracellular fluids such as plasma, the active transport and secretion mechanisms in the salivary glands can change the ionic composition of saliva. Therefore, saliva is not a simple surrogate of blood or other body fluids, but rather it has its own distribution of trace metals.

Analysis of trace metals in saliva still has some problems such as widely varying salivary compositions, frequent blood contamination of samples, very low concentrations of analytes, the lack of a standardized analytical method, and the absence of reliable reference values (Barbosa *et al*, 2005; Koh and Koh, 2007; Esteban and Castaño, 2009). To minimize the variability of salivary composition and the influence of blood contamination, it has been recommended to determine salivary flow rates (or salivary secretion rates) of participants and the levels of blood contamination in saliva samples (Koh and Koh, 2007). For the accurate measurement of trace metal concentration, inductively coupled plasma mass spectroscopy (ICP-MS) has been regarded as the optimum technique, as it is extremely sensitive and can be used for simultaneous quantification of multi-elements.

Smoking is one of the major environmental risk factors related to many serious systemic diseases, including respiratory diseases, heart diseases, and cancers (Fowles and Dybing, 2003; Pappas *et al*, 2006). Smoking is also associated with the prevalence of periodontal diseases and oral cancers (Tomar and Asma, 2000; Varela-Lema *et al*, 2009). Many toxic trace metals are found in tobacco, cigarette paper, filters, and cigarette smoke. It has been reported that toxic trace metals in cigarette smoke can accelerate damages and inflammations in the human body (Chiba and Masironi, 1992). During the act of smoking, toxic trace metals present in tobacco and cigarette smoke are transferred to the oral cavity, the lungs, the peripheral circulation,

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and other organs including salivary glands (Pappas *et al*, 2006). They are incorporated into the saliva and excreted in the oral cavity. Saliva has previously been used as a material for biomonitoring to estimate the amount of trace metals in smokers' bodies (Catalanatto and Sunderman, 1977; Zuabi *et al*, 1999; Monaci *et al*, 2002; Erdemir and Erdemir, 2006; Nriagu *et al*, 2006). However, salivary flow rates (or salivary secretion rates) and the levels of blood contamination in the saliva samples were not measured in these previous studies.

The purpose of this study was to compare the salivary levels of trace metals between non-smokers and smokers. We used salivary secretion rate as a measurement unit, estimated the levels of blood contamination in saliva samples, and accepted ICP-MS as a multi-element quantification method. In addition, recovery tests for the various pretreatment methods were performed to determine the most accurate pretreatment for ICP-MS analysis. Daily variations in trace metal levels in saliva were also investigated. Our hypothesis was that smokers have elevated levels of trace metals in their saliva.

Materials and methods

Participants

The study participants were 10 male non-smokers (mean age: 27.4 ± 3.4 years) and 30 male smokers (mean age: 26.5 ± 4.1 years), who were recruited from among office workers in a dental hospital and students in a college of dentistry.

First, five non-smoking participants (mean age: 28.0 ± 3.7 years) were selected to compare the effects of the sample pretreatments on the accuracy of ICP-MS analysis. Second, seven (mean age: 26.7 ± 3.4 years) of the non-smoking participants were chosen to estimate daily variations in trace metal levels in saliva. Finally, salivary trace metal levels were investigated in all the non-smoking and smoking participants. Participants were divided into four groups according to the number of cigarettes smoked per day. Group 1 comprised non-smokers, who had never smoked a cigarette in their life-time. Group 2 included occasional smokers who smoked ≤10 cigarettes per day. Group 3 comprised regular smokers, who smoked 11–19 cigarettes daily. Group 4 included heavy smokers who smoked ≥20 cigarettes per day. To take the smoker's life-time smoking into account, the pack-year (PY) was calculated as the 'number of cigarettes smoked per day' multiplied by the 'years of smoking' divided by 20 (Bernaards *et al*, 2001). We also classified all the participants into three pack-year groups according to the PY values: PY 0 (non-smokers; $n = 10$, PY = 0), PY 1 ($n = 14$, $0 <$ PY ≤ 5), and PY 2 ($n = 16$, $5 <$ PY ≤ 11).

Our research proposal was approved by the Institutional Review Board of Seoul National University Dental Hospital (CRI#08012).

Questionnaire

A trained interviewer (YJK) administered several face-to-face questionnaires to all the participants. The questions were designed to explore the participants' smoking status, environmental situations, and levels of environmental exposure to trace metals (Nriagu *et al*, 2006). None of the participants showed evidence of environmental exposure to trace metals.

Collection of saliva

For accurate sample collection and analysis, cleansed low-density polyethylene (LDPE) bottles were used. Unstimulated whole saliva was collected between 9:00 AM and 12:00 noon to minimize variability in salivary composition. All subjects were requested to refrain from oral activities, such as chewing, drinking, tooth brushing, and cigarette smoking for at least 1 h before sample collection. After two oral rinses with 20 ml of distilled water for 1 min, the subject carefully spat his saliva into a 15 ml LDPE bottle once or twice per min. This procedure was performed for approximately 10–20 min to obtain 5–10 ml of saliva per participant. To estimate daily variations in salivary levels of trace metals, sample collection was performed twice at one-day intervals.

After collection, salivary flow rate (ml min^{-1}) and the number of metal restorations in the oral cavity were recorded. To determine the level of blood contamination and salivary cotinine concentration, saliva samples were centrifuged immediately at 4000 $\times g$ for 15 min. Supernatants of each sample was sub-sampled into two aliquots of 100 μl, and these aliquots were stored at −70°C until use. Seventy percentage of supra-puric nitric acid was added to the rest of the sample (0.05 ml of nitric acid per 1 ml of saliva sample). These samples were then stored at −20°C until ICP-MS analysis.

Samples used to evaluate the effect of pretreatment method on the accuracy of analytical results and those used to evaluate daily variations in salivary levels had nitric acid added to them directly without centrifugation.

Pretreatments for ICP-MS analysis

To determine the pretreatment method that yielded the most accurate ICP-MS results, we compared three pretreatment methods: microwave-assisted acid digestion, direct dilution with nitric acid, and direct dilution with distilled water. A total number of 11 elements (Mg, Al, Mn, Cu, Zn, Rb, Sr, Mo, Cd, Tl, and Pb) in the saliva sample were monitored simultaneously for the comparison.

Saliva samples of five subjects (10 ml per person) were pooled to obtain a 50 ml sample. After centrifugation at 4000 $\times g$ for 15 min, the pooled saliva was sub-sampled. For the microwave-assisted acid digestion, the 3 ml aliquots were diluted with 4 ml of 70% supra-puric nitric acid and 4 ml of tertiary distilled water. Then, they were acid-digested in a microwave for 20 min (180°C, 200 psi). After this, the aliquots were diluted 16.7-fold with tertiary distilled water. To test the accuracy of this method, two more aliquots of 3 ml were spiked with known metal quantities (6.7 and 13.8 $\mu g\ l^{-1}$). For samples that were diluted directly with nitric acid, 1 ml aliquots were diluted 10-fold with 1% supra-puric nitric acid. Two more aliquots of 1 ml were spiked with known metal quantities (5 and 10 $\mu g\ l^{-1}$). In

the case of direct dilution with distilled water, the same procedures used for direct dilution with nitric acid were performed with tertiary distilled water.

To evaluate the daily variation in trace metal levels in saliva or to compare the levels of trace metals in saliva between non-smokers and smokers, samples were diluted directly with nitric acid, as this was determined to be the pretreatment method that yielded the most accurate ICP-MS results. All experiments were performed in duplicate.

Determination of trace metal concentrations in saliva samples

The concentrations of trace metals ($\mu g\ l^{-1}$) in saliva samples were measured by ICP-MS (Thermo X-series II; Thermo Fisher Scientific Inc., Waltham, MA, USA). The instrumental operating conditions recommended by the manufacturer were used. Each test sample was analyzed three times in the same manner. The limit of detection (LOD) for each element was defined as three times the standard deviation of the blank samples (Menegário *et al*, 2001; Nriagu *et al*, 2006).

Determination of blood contamination in saliva samples

To determine the blood contamination of saliva samples, the transferrin concentration in the saliva samples was measured using a salivary blood contamination enzyme immunoassay kit (Salimetrics, State College, PA, USA).

Determination of cotinine concentration in saliva samples

To determine the cotinine concentration in saliva samples, a high sensitivity salivary continue quantitative enzyme immunoassay kit (Salimetrics) was used.

Data analysis

Before statistical analysis, concentrations below the LOD were assigned the value of half of the LOD (Nriagu *et al*, 2006).

Differences between groups were analyzed using the Mann–Whitney *U*-test or Kruskal–Wallis test. *Post hoc* test procedures were performed using the Mann–Whitney *U*-test with Bonferroni correction. Spearman's rho was used to analyze the relationship between various parameters. In the Mann–Whitney *U*-test, Kruskal–Wallis test, and Spearman's rho, $P < 0.05$ was considered significant. In the Mann–Whitney *U*-test with Bonferroni correction, $P < 0.013$ for four groups or $P < 0.017$ for three groups was accepted as significant.

Results

The recovery tests for the three pretreatments for ICP-MS analysis are shown in Table 1. Among the pretreatments tested, recoveries after direct dilution with nitric acid ranged from 86.1% to 117.7%. Therefore, this method was considered to be the most accurate and was used for all further analyses.

Daily variations in trace metal levels in saliva are presented in Table 2. Salivary secretion rate was calculated by multiplying the mean salivary concentration by the salivary flow rate (Nagler and Hershkovich, 2005; Koh and Koh, 2007). To estimate the daily variation in the levels of trace metals in saliva, the change in concentration or secretion rate was taken as a percent ratio (%) of the mean salivary concentration or the salivary secretion rate of the next day sample *vs* the first day sample. Concentrations of each element varied widely across days, as did the salivary secretion rate.

Table 3 shows age, salivary flow rate, the level of blood contamination, salivary cotinine concentration, and salivary trace metal concentrations in Groups 1–4. There were significant differences in salivary flow rate and cotinine concentrations between groups. The transferrin values of all participants were below 1.0 mg dl^{-1}. With regard to trace metal concentrations, only Al showed a significant difference between Group 1 and Group 2, whereas there were significant differences in Mg and Al concentrations between Group 1 and Group 3.

Only the salivary secretion rate of Al was significantly different between non-smokers and smokers (Table 4).

Table 1 Recovery tests for the pretreatment methods for ICP-MS analysis in saliva

	Microwave-assisted acid digestion		*Direct dilution with nitric acid*		*Direct dilution with distilled water*	
Spike level ($\mu g\ l^{-1}$)	*6.7*	*13.8*	*5*	*10*	*5*	*10*
Recovery (%)						
Mg	257.7	135.6	114.6	114.0	90.0	445.0
Al	381.1	132.1	116.9	115.6	143.0	57.0
Mn	300.1	246.9	109.4	99.4	90.4	110.3
Cu	302.9	195.4	96.2	99.4	115.7	94.0
Zn	145.8	188.1	86.1	91.9	419.9	43.5
Rb	3373.1	1907.2	117.0	117.7	N.D.	N.D.
Sr	220.7	189.2	102.6	95.8	105.1	95.7
Mo	N.D.	N.D.	90.6	90.4	N.D.	N.D.
Cd	114.6	98.9	89.3	86.8	89.1	88.0
Tl	95.0	85.4	87.9	95.1	63.4	62.1
Pb	89.3	81.0	86.2	94.8	60.5	59.5

$n = 5$; for the recovery tests, saliva samples of five subjects were pooled and sub-sampled.
N.D., not detected; ICP-MS, inductively coupled plasma mass spectroscopy.

Table 2 Daily variations in salivary concentrations and salivary secretion rates of trace metals

	Change in concentration (%)		*Change in secretion rate (%)*	
Trace metal	*Minimum*	*Maximum*	*Minimum*	*Maximum*
Mg	73.8	166.9	58.5	178.9
Al	18.5	213.5	22.2	207.1
Mn	29.0	215.2	22.0	215.2
Cu	38.6	166.0	29.3	166.1
Zn	28.1	343.7	21.4	343.7
Rb	83.6	109.0	81.1	135.7
Sr	48.7	225.1	37.0	218.4
Mo	42.6	327.0	42.6	392.4
Cd	33.3	340.9	25.3	330.7
Tl	50.0	175.0	48.5	210.0
Pb	85.8	420.3	75.9	407.7

$n = 7$
'Change in concentration or change in secretion rate' was defined as the percent ratio of the mean concentration or the secretion rate of the next day sample *vs* the first day sample.

The salivary secretion rates of trace metals and cotinine in Groups 1–4 are shown in Table 5. All trace metals, except Tl, showed significant differences between groups. There were significant differences in the salivary secretion rates of Al, Rb, Sr, Mo, and Cd between Group 1 and Group 2. The salivary secretion rate of Al varied significantly between Group 1 and Group 4. The salivary secretion rates of Mg, Mn, Cu, Zn, Rb, Sr, Mo, Cd, and Pb were significantly different between Group 2 and Group 4. Cu was the only trace element that had significantly different salivary secretion rates between Group 3 and Group 4. Salivary secretion rates of cotinine showed statistically significant differences between groups.

When the participants were divided into three pack-year groups (Table 6), significant differences in salivary secretion rates of Al, Mo, and Cd were found between PY 0 and PY 1, and there were significant differences in the salivary secretion rates of Mg, Mn, Zn, Sr, Cd, and Pb between PY 1 and PY 2.

Regarding the relationships between the concentrations of trace metals and salivary flow rate in non-smokers, the salivary concentration of Cu displayed a significant negative correlation ($r = -0.632$, $P = 0.050$) with salivary flow rate, whereas Sr had a significant positive correlation ($r = 0.705$, $P = 0.023$) with it. The concentrations of all elements in non-smokers' saliva were not significantly correlated with the number of metal restorations (data not shown). When the correlations were analyzed using the salivary secretion rates of trace metals (Table 7), the secretion rate of cotinine in smokers' saliva was significantly correlated with all elements except Al. All elements had no significant correlations with the number of metal restorations in both non-smokers and smokers.

Discussion

Rapid progress in the development of analytical techniques has allowed more accurate determination of the salivary concentrations of trace metals. ICP-MS is accepted as one of the best biomonitoring tools

Table 3 Age, salivary flow rate, the level of blood contamination, salivary cotinine concentration, and salivary concentrations of trace metals

		Non-smokers	*Smokers*			
		Group 1	*Group 2*	*Group 3*	*Group 4*	P-*value*[†]
Age (years)		27.4 ± 3.4	25.4 ± 4.7	26.6 ± 4.6	27.4 ± 3.1	0.634
Flow rate (ml min^{-1})		0.68 ± 0.20[a]	0.36 ± 0.25[a,b]	0.44 ± 0.24	0.94 ± 0.52[b]	0.002**
Blood contamination[‡] (mg dl^{-1})		0.12 ± 0.04	0.13 ± 0.10	0.10 ± 0.13	0.15 ± 0.15	0.353
Cotinine concentration (ng ml^{-1})		1.5 ± 0.2[a,b,c]	15.0 ± 18.0[a,d,e]	162.5 ± 107.3[b,d]	300.4 ± 155.3[c,e]	< 0.001**
Salivary concentration ($\mu g\ l^{-1}$)	LOD ($\mu g\ l^{-1}$)					
Mg	0.9	171.2 ± 141.5[a]	195.5 ± 111.9	252.8 ± 67.2[a]	173.3 ± 81.7	0.044*
Al	1.45	1.39 ± 0.85[a,b]	12.8 ± 6.8[a]	6.36 ± 7.15[b]	6.23 ± 8.40	0.001**
Mn	0.34	2.94 ± 2.82	2.26 ± 1.90	3.18 ± 2.02	3.40 ± 2.37	0.410
Cu	0.13	1.53 ± 1.33	2.05 ± 1.29	1.39 ± 0.45	1.72 ± 1.16	0.405
Zn	3.0	13.5 ± 12.2	10.6 ± 14.7	11.5 ± 4.60	15.2 ± 16.5	0.136
Rb	0.03	64.2 ± 12.2	69.1 ± 19.9	69.1 ± 19.9	62.9 ± 13.4	0.872
Sr	0.05	2.16 ± 0.96	1.85 ± 1.15	3.46 ± 3.48	2.04 ± 1.53	0.423
Mo	0.13	0.29 ± 0.24	0.14 ± 0.08	0.21 ± 0.12	0.35 ± 0.28	0.309
Cd	0.002	0.023 ± 0.015	0.016 ± 0.016	0.021 ± 0.012	0.021 ± 0.015	0.239
Tl	0.007	0.012 ± 0.009	0.020 ± 0.013	0.015 ± 0.011	0.012 ± 0.009	0.191
Pb	0.064	0.080 ± 0.037	0.088 ± 0.113	0.121 ± 0.083	0.078 ± 0.033	0.403

Data represent mean ± s.d.; $n = 10$ in each group.
Group 1, non-smokers; Group 2, smoke ≤10 cigarettes on a daily basis; Group 3, smoke 11–19 cigarettes on a daily basis; Group 4, smoke ≥20 cigarettes on a daily basis.
The limit of detection (LOD) for each element was calculated as three times the standard deviation of the blank samples.
[‡]The level of blood contamination was determined by measuring the transferrin level in the saliva sample.
[†]Kruskal–Wallis test statistics for comparison, $*P < 0.05$ and $**P < 0.01$.
[a,b,c,d,e]Significantly different according to the Mann–Whitney *U*-test with Bonferroni correction ($P < 0.013$).

Table 4 Salivary secretion rates (ng min^{-1}) of trace metals in non-smokers and smokers

Trace metal	*Non-smokers (n = 10)*	*Smokers (n = 30)*	P-*value*[†]
Mg	111.2 ± 86.6	106.1 ± 72.1	0.914
Al	0.99 ± 0.69	3.76 ± 4.18	0.003**
Mn	1.86 ± 1.70	1.87 ± 2.85	0.508
Cu	0.95 ± 0.77	0.88 ± 0.69	0.548
Zn	8.19 ± 7.11	7.36 ± 8.89	0.396
Rb	43.4 ± 15.6	37.3 ± 26.4	0.209
Sr	1.56 ± 1.08	1.14 ± 0.87	0.233
Mo	0.18 ± 0.15	0.16 ± 0.21	0.089
Cd	0.016 ± 0.012	0.011 ± 0.012	0.072
Tl	0.008 ± 0.007	0.009 ± 0.010	0.590
Pb	0.056 ± 0.035	0.049 ± 0.040	0.508

Data represent mean ± s.d.
The 'Salivary secretion rate' was calculated by multiplying the 'mean salivary concentration' by the 'salivary flow rate'.
[†]Mann–Whitney *U*-test statistics for comparison, $^{**}P < 0.01$.

currently in use. In this study, the LOD of each element ranged from 0.002 to 3.0 $\mu g\ l^{-1}$, and almost no interference in the multi-element analysis was observed, highlighting the sensitivity and the utility of ICP-MS. The direct dilution with nitric acid showed the most stable recovery range among the methods tested. Microwave-assisted acid digestion can result in sample contamination during handling and direct dilution with distilled water may not completely remove organic components in saliva sample. Therefore, a simple pretreatment, such as direct dilution with nitric acid, is preferred to control sample contamination and to remove impurities in saliva effectively.

When we compared salivary levels of trace metals from saliva samples collected on two different days, we found a wide range of variation in all subjects evaluated. These results imply that salivary concentrations and salivary secretion rates of trace metals may be as variable as the nutritional and hormonal status of an individual (Barbosa *et al*, 2005).

With regard to the analysis of salivary biomarkers, several methodological issues need to be addressed (Barbosa *et al*, 2005; Koh and Koh, 2007). To control these limitations, we recorded the followings, which have not been considered in previous studies: the salivary flow rate, the salivary secretion rate, and the amount of blood contamination in the saliva samples. The salivary secretion rate is defined as the amount of salivary trace metals secreted into the oral cavity per min (Nagler and Hershkovich, 2005; Koh and Koh, 2007). In non-smokers, there were significant correlations between salivary flow rate and salivary concentrations of Cu and Sr, which may support that salivary flow rates can change the salivary concentrations of these metals, and that salivary secretion rate is an important measurement unit to estimate the actual salivary levels of trace metals.

Blood contamination of saliva samples can artificially elevate the concentrations of trace metals (Koh and Koh, 2007). The amount of blood contamination was determined by measuring the concentration of transferrin in the saliva samples. Generally, values greater than 1 mg dl^{-1} indicate blood contamination of the saliva sample. In this study, the salivary transferrin concentrations of all participants were below 1 mg dl^{-1}. These results indicate that blood contamination of the saliva samples in this study was minimal. In addition, we found no significant relationship between the number of metal restorations and salivary levels of all the elements in non-smokers. This is consistent with the previous studies that have reported that the amount of trace metals released from metal restorations is very small and far below the amount acquired from food and drink (Brune, 1986; López-Alías *et al*, 2006; Melchart *et al*, 2008).

Table 5 Salivary secretion rates (ng min^{-1}) of trace metals and cotinine

	Non-smokers	*Smokers*			
	Group 1	*Group 2*	*Group 3*	*Group 4*	P-*value*[†]
Trace metal					
Mg	111.2 ± 86.6	65.5 ± 57.1[a]	102.6 ± 44.2	150.2 ± 86.7[a]	0.014*
Al	0.99 ± 0.69[a,b]	4.35 ± 3.73[a]	2.20 ± 2.74	4.73 ± 5.54[b]	0.006**
Mn	1.86 ± 1.70	0.75 ± 0.89[a]	1.32 ± 0.98	3.54 ± 4.42[a]	0.009**
Cu	0.95 ± 0.77	0.67 ± 0.44[a]	0.54 ± 0.21[b]	1.43 ± 0.88[a,b]	0.015*
Zn	8.2 ± 7.1	4.3 ± 7.7[a]	4.9 ± 3.0	12.9 ± 11.7[a]	0.005**
Rb	43.4 ± 15.6[a]	24.0 ± 13.6[a,b]	29.5 ± 13.9	58.4 ± 33.2[b]	0.003**
Sr	1.56 ± 1.08[a]	0.55 ± 0.31[a,b]	1.17 ± 0.70	1.71 ± 1.06[b]	0.013*
Mo	0.18 ± 0.15[a]	0.05 ± 0.03[a,b]	0.10 ± 0.10	0.32 ± 0.30[b]	0.002**
Cd	0.016 ± 0.012[a]	0.005 ± 0.007[a,b]	0.009 ± 0.007	0.018 ± 0.016[b]	0.002**
Tl	0.008 ± 0.007	0.008 ± 0.010	0.006 ± 0.006	0.012 ± 0.013	0.391
Pb	0.056 ± 0.035	0.028 ± 0.033[a]	0.051 ± 0.039	0.069 ± 0.039[a]	0.029*
Cotinine	1.0 ± 0.3[a,b,c]	5.8 ± 8.1[a,d,e]	79.1 ± 72.2[b,d]	261.3 ± 171.3[c,e]	< 0.001**

Data represent mean ± s.d.; $n = 10$ in each group.
Group 1, non-smokers; Group 2, smoke ≤10 cigarettes on a daily basis; Group 3, smoke 11–19 cigarettes on a daily basis; Group 4, smoke ≥20 cigarettes on a daily basis.
The 'salivary secretion rate' was calculated by multiplying the 'mean salivary concentration' by the 'salivary flow rate'.
[†]Kruskal–Wallis test statistics for comparison, $^{*}P < 0.05$ and $^{**}P < 0.01$.
[a,b,c,d,e]Significantly different according to the Mann–Whitney *U*-test with Bonferroni correction ($P < 0.013$).

Table 6 Salivary secretion rates (ng min^{-1}) of trace metals in the three pack-year groups

	Non-smokers	*Smokers*		
Trace metal	*PY 0* (n = *10*)	*PY 1* (n = *14*)	*PY 2* (n = *16*)	P-*value*[†]
Mg	111.2 ± 86.6	74.7 ± 54.3[a]	133.5 ± 75.9[a]	0.017*
Al	0.99 ± 0.69[a]	4.36 ± 3.56[a]	3.24 ± 4.71	0.002**
Mn	1.86 ± 1.70	0.91 ± 0.91[a]	2.71 ± 3.65[a]	0.012*
Cu	0.95 ± 0.77	0.66 ± 0.42	1.07 ± 0.83	0.258
Zn	8.19 ± 7.11	4.44 ± 6.65[a]	9.91 ± 9.98[a]	0.012*
Rb	43.4 ± 15.6	27.7 ± 17.9	45.7 ± 30.1	0.033*
Sr	1.56 ± 1.08	0.74 ± 0.62[a]	1.49 ± 0.93[a]	0.013*
Mo	0.18 ± 0.15[a]	0.07 ± 0.08[a]	0.23 ± 0.27	0.011*
Cd	0.016 ± 0.012[a]	0.005 ± 0.006[a,b]	0.015 ± 0.014[b]	< 0.001**
Tl	0.008 ± 0.007	0.007 ± 0.009	0.010 ± 0.011	0.193
Pb	0.056 ± 0.035	0.028 ± 0.032[a]	0.067 ± 0.038[a]	0.003**

Data represent mean ± s.d.
The pack-year (PY) was calculated as the number of cigarettes smoked per day multiplied by the years of smoking divided by 20.
PY 1, $0 < PY \leq 5$; PY 2, $5 < PY \leq 11$.
The 'salivary secretion rate' was calculated by multiplying the 'mean salivary concentration' by the 'salivary flow rate'.
[†]Kruskal–Wallis test statistics for comparison, $*P < 0.05$ and $**P < 0.01$.
[a,b]Significantly different according to the Mann–Whitney *U*-test with Bonferroni correction ($P < 0.017$).

The concentrations of trace metals in saliva presented in this study were far lower than those reported previously (Vaughan *et al*, 1991; Menegário *et al*, 2001; Watanabe *et al*, 2005; Barbosa *et al*, 2006; Nriagu *et al*, 2006; Wang *et al*, 2008; Costa de Almeida *et al*, 2009). Methodological differences, different pretreatments, and blood and/or environmental contamination during sample collection and analysis in previous studies may all contribute to this discrepancy. It should also be noted that participants in our study had little risk of environmental exposure, as they lived in a region with low traffic density and very few factories. Based on our results, we suggest that the trace metal concentrations of saliva samples without blood contamination and environmental interference are much lower than the values previously reported.

Among the elements analyzed in this study, only the concentrations of Mg and Pb in smokers' saliva have been reported in previous studies (Zuabi *et al*, 1999; Monaci *et al*, 2002; Erdemir and Erdemir, 2006; Nriagu *et al*, 2006). We found a significant difference in the Mg concentration in the saliva between Group 1 and Group 3, which is not in accordance with the former studies (Zuabi *et al*, 1999; Monaci *et al*, 2002; Erdemir and Erdemir, 2006). This difference may be due to the fact that smoking is not a main contributor to the body burden of Mg, and salivary flow rate has some influence on the Mg concentration in saliva. Previous studies have shown that current smokers have significantly higher amounts of Pb in their saliva than non-smokers (Nriagu *et al*, 2006), which is not consistent with the results of this study. The reason for this discrepancy may be that other factors such as dietary intake and environmental pollution have a greater influence on the salivary concentration of Pb than smoking status.

To the best of our knowledge, this study is the first to report the salivary concentrations and salivary secretion rates of Al, Mn, Cu, Zn, Rb, Sr, Mo, Cd, and Tl in smokers. Only Al had a significantly elevated salivary concentration and salivary secretion rate in the smoker groups compared with the non-smoker group. Although it is not possible to make a straightforward comparison, no significant differences in Al levels between smokers and non-smokers have been found in urine, plasma, serum, or erythrocyte samples (Buratti *et al*, 1986; Bernhard *et al*, 2006). In contrast, the level of Al in the hair of smokers is generally much higher than that of non-smokers (Unkiewicz-Winiarczyk *et al*, 2009). These previous results support that most amount of Al transferred into the body is removed from the bloodstream by a first-pass clearance in liver, and the retained amount of Al is accumulated in hair or other tissues (DeVoto and Yokel, 1994). Based on these toxicokinetics, it is thought that increased levels of Al in smokers' saliva may not be the result of transferred Al into the body by smoking. Al is abundant in cigarettes, and a large proportion of

Table 7 Correlations between the salivary secretion rate of cotinine, the number of metal restorations, and the salivary secretion rates of trace metals[†]

	Non-smokers (n = *10*)		*Smokers* (n = *30*)	
Salivary secretion rate	*Secretion rate of cotinine*	*No. of restorations*	*Secretion rate of cotinine*	*No. of restorations*
Mg	0.042	−0.031	0.767**	0.177
Al	0.491	0.031	−0.080	0.052
Mn	−0.200	0.276	0.653**	0.212
Cu	0.042	0.178	0.504**	−0.019
Zn	−0.333	−0.141	0.706**	0.225
Rb	0.600	0.117	0.789**	−0.023
Sr	0.358	0.362	0.524**	−0.027
Mo	0.067	−0.018	0.621**	0.046
Cd	0.140	0.420	0.701**	0.141
Tl	0.360	−0.062	0.396*	−0.043
Pb	0.576	0.104	0.698**	−0.148

[†]Spearman's rho, $*P < 0.05$ and $**P < 0.01$.
The 'salivary secretion rate' was calculated by multiplying the 'mean salivary concentration' by the 'salivary flow rate'.

the Al remains behind in the cigarette ash after smoking (Kazi *et al*, 2009). Smokers are far more exposed to tobacco, filters, and cigarette ash than non-smokers. Thus, Al that remains on smokers' hands or lips may influence the level of Al in the saliva through hand-mouth or lip-mouth transfer. In this study, Group 2 had a much lesser mean value of salivary flow rate compared with Group 1 or Group 4. These differences of salivary flow rate can explain why Group 2 showed significant decreases in salivary secretion rates for the majority of elements analyzed. Therefore, as well as salivary concentrations, salivary secretion rates must be considered to evaluate actual levels of trace metals in saliva.

The daily number of smoked cigarettes has been shown to be an inaccurate estimate of the dose-related risk of smoking (Etter *et al*, 2000; Bernaards *et al*, 2001). Therefore, we included pack-year values and salivary cotinine levels in addition to the daily number of cigarettes smoked. Although most of the elements showed significant differences in salivary secretion rates between the pack-year groups, only Al showed a significantly elevated salivary secretion rate in the PY 1 smoker group compared with the non-smoker group. These results were comparable to the results obtained based on the daily number of smoked cigarettes. The concentration of cotinine in the saliva can be used as an indirect measure of recent exposure to cigarette smoke (Koh and Koh, 2007). We found significantly higher salivary concentrations of cotinine in smokers compared with non-smokers, consistent with previous reports (Etzel, 1990; Etter *et al*, 2000). Moreover, the cotinine concentration was not significantly correlated with the salivary flow rate in this study, which further supports that the cotinine concentration in saliva is independent of the salivary flow rate (Van Vunakis *et al*, 1989). Therefore, it is thought that the salivary concentration of cotinine may determine the salivary secretion rate of cotinine.

In conclusion, direct dilution of saliva samples with nitric acid was found to be the pretreatment method that yielded the most accurate ICP-MS results. Trace metal levels in saliva showed wide daily variations. They were not affected by the number of metal restorations. The trace metal concentrations of saliva samples without blood contamination and environmental interference were much lower than the values previously reported. We also found that the salivary levels of cotinine and aluminum were significantly elevated in smokers compared with non-smokers. In summary, we have demonstrated that saliva samples can be used to estimate the levels of trace metals in smokers' bodies. Smoking status has significant effects on cotinine and aluminum levels in human saliva.

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