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Chromium Exposure in the Adult Population, Consuming Different Types of Smokeless Tobacco Products in Pakistan

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Abstract The pervasive smokeless tobacco (SLT) consumption and diseases related to its use is a hot topic for the public discussion. In this study, concentrations of chromium (Cr) were measured in different SLT products [snuff (dry and moist), mainpuri, and *gutkha*] offered and used in Pakistan. The current study was also designed to assess the Cr levels in the biological (scalp hair and blood) samples of male and female subjects, age ranged from 25 to 60 years, chewing different SLT products. For comparative purpose, the healthy persons of the same age group, who did not consume any SLT products, were selected as referents. The concentrations of Cr in SLT products and biological samples were measured by electrothermal atomic absorption spectrophotometer after microwave-assisted acid digestion. The validity and accuracy of the methodology were checked by certified reference materials (CRMs). The resulted data indicated that the adult persons, who consumed different SLT products, have 2–3 fold higher levels of Cr in biological samples as compared to referent subjects ($p < 0.01$). The persons, who chew/sniff different SLT products, have 50–80 and 42–82 % higher levels of Cr in their scalp hair and blood samples as related to referents. The daily intake of Cr is lower as compared to the recommended value of 50–200 μg/day. It was expected that 10 g consumption of various kinds of SLT products (snuff, mainpuri, and *gutkha*) may subsidize 21.2–220, 17.7–122, and 18.4–273 % of the recommended daily intake of Cr, respectively.

Keywords Chromium · Smokeless tobacco products · Electrothermal atomic absorption spectrometry · Daily intake

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Abbreviations

BM	Brown moist
Cr	Chromium.
Cr(III)	Trivalent chromium.
Cr(VI)	Hexavalent chromium.
CRM	Certified reference material.
DB	Dry brown.
DBk	Dry black.
GM	Green moist.
GU	*Gutkha* users.
MU	Mainpuri users.
SLT	Smokeless tobacco.
SU	Snuff users.

Introduction

Currently, numerous forms of smokeless tobacco (SLT) products are accessible that can be ingested orally or nasally. Twist, plug, and scrap leaf are the common forms of chewing tobacco [1]. The practice of SLT is restricted to some areas in all continents. At present, the forms, circulation, ingestion, and formulations of SLT products appeal the consumers [2]. The practice of SLT is common in Asia and its consumers have been assessed to be more than 100 million [3].

The SLT is not homogeneous, since the tobacco is often combined with betel leaf (*Piper betle*), sliced areca nut (*Areca catechu*), powdered slaked lime, and additives that enhance the toxicity as well as the psychotropic effect of tobacco [2, 4–6]. Among different SLT products, mainpuri contains pieces of tobacco leaves, finely cut betel nut and other ingredients, which are thoroughly mixed with lime [7]. Its name emerges from a district in the Indian state of Uttar Pradesh named Mainpuri [3]. Mainpuri is chewed gently and its aqueous extract in saliva is absorbed locally and also circulates in other parts of the body [3, 8, 9]. Another SLT product *gutkha* is a dry preparation of tobacco, areca nut, catechu, and slaked lime with different spices, which have stimulating and relaxing effects [10–12].

A very popular SLT product snuff is frequently used in Asian and European countries. The different snuff products contain commonly moist/dry particles of tobacco leaves treated with diverse flavors, such as mint or menthol, and placed between the lip or cheek and the gum. The brown moist (BM) snuff is prepared by adding different constituents such as processed tobacco leaves, quicklime, gum, and water. Whereas in green moist (GM) snuff, fresh tobacco leaves, menthol, oil, and water are used [13]. The dry brown (DB) snuff is made from different ingredients such as tobacco, cotton or sesame seed oil and ash, tobacco leaves, and $Ca(OH)_2$ [14]. However, indigo is also added in dry black (DBk) snuff. Different types of SLT products such as loose or plug chewing tobacco leaves, dry and moist snuff are available in developed and developing countries [13].

The usage of snuff is more common among all societies from Europe to Asia without any restriction of class. Snuff is locally named as *naswar* in Pakistan; however, we follow the term snuff as its international name. Generally, small pouches which contain approximately 5–10 g of snuff are available. Interest of women and teenagers has been developed towards this habit due to overall belief against tobacco smoking [13].

The SLT products have been recognized to enhance the risk of cancer including oral cancer [3, 4, 8, 9]. It has been observed by surveying the consumption of SLT products in Pakistan and India that consumers taking different chewing products are mostly affected by oral cancer [2, 15]. The addicted people have developed oral submucous fibrosis, which become more dangerous in youngsters [16]. In Pakistan, oral cancer is the second most common reason of death among both genders, with the maximum informed incidences all over the world. The habit of chewing SLT products persist main etiological threat issues even without use of alcohol [15, 16].

The tobacco plant is accumulated by different metals frequently based on soil characteristics and plant diversity [17, 18]. Tobacco-specific nitrosamines are one of the several carcinogenic ingredients in tobacco which is related to the carcinogenesis due to consumption of SLT products. The mutual experience of nitrosamines and toxicants including metalloids, increase the severity of damage. The biological half-lives of these elements vary and amounts excreted can reflect a combination of recent and past exposures [19, 20].

Chromium exist mostly in two oxidation states (III) and (VI) in the environmental and biological samples. Cr (III) is crucial for health at trace levels, whereas its high concentration might causes allergic sensitization. However, Cr (VI) is a more poisonous form because it possesses high oxidizing potential [21]. The tobacco absorbs Cr from soil where Cr (III) oxidizes to Cr (VI) by manganese oxides present in growing media [22]. The deposition of Cr in lung tissue has been linked with smoking habit, which confirms that Cr, in some chemical forms, reaches the lung. It was reported in literature that five lobes of a smokers' lungs contain higher concentration of Cr than a nonsmokers' lungs. It is still not clear that what proportions of both species, accumulate in the lungs tissues [23, 24]. However, no information about absorption of Cr via chewing different SLT products was reported.

The practice of chewing SLT products is common in the people of Pakistan, especially in laborers of different fields and teenage boys. The chewing and snuffing of SLT products by these people is usually due to interplay of several social factors including domestic stress, peer pressure, media advertisements, low price, and convenient accessibility. Chewing/sniffing SLT product affects oral health in several ways; it can result in bad breath, yellowish stains on the teeth, and mouth sores. The consequences are bleeding and receding gums and lips. Chewing tobacco has also been proposed as a risk factor for periodontitis [25]. The other major consequences of frequently consumed SLT products, imposes an enormous economic burden on society.

The analysis of toxic elements at trace level is considered as a difficult analytical job because it requires sensitive instrumental techniques and a preconcentration step [26–29]. A number of analytical techniques are used to determine trace elements in tobacco products with appropriate sensitivity including graphite furnace atomic absorption spectrometry (GFAAS) [30], inductively coupled plasma-atomic emission spectroscopy [31], and inductively coupled plasma mass spectrometry [32]. To avoid effects of complex matrices in SLT products on metal analysis, wet acid digestion methods using either open systems or microwave ovens have been employed

to achieve the task of analysis [33, 34]. In microwave-assisted acid digestion, less volume of acid reduces the formation of nitrous vapors, which is one of the main advantages [35, 36].

The purpose of this study was to explore the practices of various types of SLT products among the people in the south eastern province of Pakistan. For this purpose, chromium concentration was examined in various types of SLT products, snuff (dry and moist), mainpuri, and *gutkha*, prepared and consumed frequently in India and Pakistan. The impact of Cr concentrations was evaluated by analysis of blood and scalp hair samples of both gender age ranged 15–60 years, consuming different types of SLT products. For comparison purposes, population who does not consume any SLT products was selected as referent subjects.

Materials and Methods

Reagents and Glassware

Ultra-pure water has been taken from ELGA labwater system (Bucks, UK) for the experimental work. Concentrated HNO_3 (65 %) and H_2O_2 (30 %) have been acquired from Merck (Darmstadt, Germany). Series of Cr standard solutions were prepared by successive dilution of certified standard solutions (1000 ppm), Fluka Kamica (Buchs, Switzerland), with HNO_3 (0.2 mol L^{-1}). The matrix modifier [0.0015 mg Mg $(NO_3)_2$] was employed for the analysis of Cr, prepared from Mg $(NO_3)_2$ (Sigma, St. Louis, MO). Polyethylene bottles were used to store the solutions at 4 °C. Virginia tobacco leaves (ICHTJ-cta-VTL-2) was used as certified reference material (CRM), to assure the methodology. Glassware and polyethylene containers were washed with distilled water after soaking them in 10 % (*v*/*v*) HNO_3 for 24 h and dried to remove the remaining contaminants.

Instrumentation

The analysis of Cr was conducted by using a double-beam Perkins-Elmer Atomic Absorption Spectrometer Model 700 (Norwalk, CT) equipped with the graphite furnace HGA-400, pyrocoated graphite tubes with integrated platform, an autosampler AS-800, and deuterium lamp as background correction system. Hollow cathode lamp (Perkin Elmer) was used as radiation source, operated at suggested current. All instrumental conditions were followed as recommended by the manufacturer. Portions of each standard/sample and modifier had been transferred into autosampler cups, and 20 μL (equal volumes of standard/sample and modifier) was delivered to the electrothermal atomizer. Integrated absorbance signals computed by the AA spectrometer were employed throughout the analysis. In order to shake the samples, a horizontal flask electrical shaker (220/60 Hz, Gallenkamp, England) was used. A pH meter (781-pH meter, Metrohm) was used for pH measurement. The analytical wavelength was set at 357.9 nm. Other parameters were as follows: drying temperature (°C)/ramp/hold (s) (140/15/15), ashing temperature (°C)/ramp/hold (s) (1400/10/20), atomization temperature (°C)/ramp/hold (2500/0/5), and cleaning temperature (°C)/ ramp/hold (s) (2600/1/3). For acid digestion of samples, a PEL domestic microwave oven (Osaka, Japan) was used that was programmable for time and microwave power from 100 to 900 W.

Smokeless Tobacco Users and Methods

Before starting this study, approval was obtained from ethical review committee of the University of Sindh, Jamshoro, Pakistan.

Study Population

A questionnaire was designed for research purpose to conduct a survey about the consumption habits of studied smokeless tobacco (SLT) products by both male and female, age ranged from 15 up to 60 years, belonging to different cities of Pakistan. The number of participants was given in Table 1. All participants (SLT users) were informed about the purpose of the study, most of them agreed to participate voluntary and signed the form. The questionnaire provided the complete information of consumer, his/her trends in frequency, and duration of consuming SLT products. Ethics committee of NCEAC, University of Sindh Jamshoro, has approved this study. By analyzing 468 questionnaires, it was observed that about 40 % of people are snuff users, 38 % consumed mainpuri (mostly laborers and drivers), while 22 % use *gutkha*. For all smokeless tobacco users, serum ferritin, hemoglobin, hematocrit, and red blood cell count were measured using standard methods (Table 2). It was noted that the levels of serum ferritin, hemoglobin, hematocrit, and red blood cell count were found to be lower in the blood samples of all smokeless tobacco product users than referent subjects (Table 2).

Table 1 The number of subjects as referent and SLT product users, age group (25–60 years)

	NU		SU		MPU		GU	
	Male	Female	Male	Female	Male	Female	Male	Female
	142	95	115	72	109	70	63	40
Total	237		187		179		103	

NU not use smokeless tobacco, *SU* snuff users, *MPU* mainpuri users, *GU* Gutkha users

Table 2 Clinical and biochemical characteristics of referents, different SLT product users

	Serum ferritin (μg/L)	Hemoglobin (g/dL)	Hematocrit	Red blood cell count
Male				
Normal range	30	11.5–16.5	35–55	3.5–5.5
Referent/NU	33.5 ± 4.06	13.8 ± 0.82	46.5 ± 2.98	4.45 ± 0.35
GU	28.5 ± 2.24	11.5 ± 1.32	36.2 ± 1.95	3.12 ± 0.49
MPU	27.3 ± 1.82	10.9 ± 0.46	32.7 ± 2.05	2.95 ± 0.37
SU	25.9 ± 1.42	10.7 ± 0.50	33.6 ± 2.14	2.75 ± 0.65
Female				
Referent/NU	32.8 ± 2.50	12.5 ± 0.54	44.8 ± 1.52	4.34 ± 0.28
GU	27.3 ± 1.96	11.0 ± 1.51	33.9 ± 1.09	3.08 ± 0.55
MPU	25.9 ± 2.09	10.4 ± 0.39	31.9 ± 1.72	2.73 ± 0.48
SU	24.3 ± 1.07	10.2 ± 0.41	30.5 ± 1.85	2.52 ± 0.40

NU not use smokeless tobacco, *SU* snuff users, *MPU* mainpuri users, *GU* Gutkha users

Sampling of SLT Products

A total of 46 samples/brands of different SLT products including 23 of different moist and dry snuff, 12 brands of mainpuri, and 11 products of *gutkha* (from different cities of Pakistan) were purchased from local markets during January 2014–December 2015. The packed samples were placed in washed dried plastic bags separately and kept at 4 °C until tested. Five composite samples were prepared from each brand of SLT products (*gutkha*, mainpuri, and snuff) after making uniform mixture. The sample preparation step was performed carefully in a clean environment to avoid any source of impurity. The samples were dried at 80 °C. After drying, the samples were pulverized using a mortar and pestle. Then, nylon sieves, with mesh sizes of 125 μm, was used to sieve the samples. Finally, the samples were stored in the prewashed and labeled sample bottles.

pH Determination of SLT Products

pH of the samples was determined by taking the weighed amount (1 g) of each brand of SLT products, *gutkha*, mainpuri, and snuff ($n = 3$ of each), in flask (100 mL capacity). Then, 10 mL of ultra-pure water was added in each flask and placed in an electrical shaker at 30 rpm for 30 min; the resulted solution was then filtered through Whatman No. 42 filter paper then determined the pH of each extract.

Biological Samples

Venous blood samples (5 mL) were collected by 7-mm heparinized lithium Vacutainer® tubes (Becton Dickinson). About 2 mL of venous blood samples were stored at −20 °C until elemental analysis. The scalp hair samples were taken from the nape of the neck. The first 5 cm of hair from the root was used for analysis. Hair samples were put into separate plastic envelopes for each participant, tightly sealed, and attached with identification number of the participant and questionnaire. In the laboratory, hair specimens were further cut into pieces, approximately 0.2 to 0.3 cm, and washed four times with a 1:200 *v*/*v* dilution of Triton X-100, then rinsed with ultra-pure water and acetone. Then, samples were dried in an oven at 80–85 °C.

Microwave-Assisted Acid Digestion

Six replicate samples of each CRM (0.5 mL Clincheck® control-lyophilized human whole blood, 0.2 g of Virginia tobacco leaves, and BCR 397 human hair) and duplicate samples of different types of SLT products (0.2 g), whole blood (0.5 mL), and scalp hair (0.2 g) were taken separately in polytetrafluoroethylene (PTFE) flasks (25 mL in capacity). Then, 3 mL of a freshly prepared mixture of concentrated HNO_3–H_2O_2 (2:1, *v*/*v*) was added and kept the flask at room temperature for 10 min. Then, the flasks were placed in a covered PTFE container and heated at 80 % of total power (900 W) for 3–4 min. The digested samples were diluted up to 10 mL with HNO_3 (0.1 mol L^{-1}). A blank extraction (without sample) was carried out through the complete procedure.

Statistical Analysis

Data processing and statistical analysis were conducted by using computer program Excel 2003 (Microsoft Office ®), XLState (Addinsoft, NY, USA), and Minitab 13.2 (Minitab Inc., State College, PA, USA) software packages. The ANOVA was used to assess the significance of differences between the concentrations of Cr in biological samples of study subjects chewing different SLT products and referents, calculated by the unpaired two-sample *t* test. Significant difference of $p < 0.05$ was considered at 95 % confidence interval. Student's *t* test was used to assess the significant difference of Cr concentration in certified and experimental values.

Analytical Figures of Merit

The concentration range of Cr for calibration curve reached from the detection limit up to 10 μg L^{-1}. LOD = 3 × *s/m* and LOQ = 10 × *s/m* represent the detection and quantification limits respectively, where the standard deviation *s* of ten measurements of a reagent blank and the slope *m* of the calibration graph were calculated for graphite furnace atomic absorption spectrometer determination. The LOD and LOQ, calculated for Cr were 30.0 and 92.0 μg L^{-1}, respectively. Certified values of biological and Virginia tobacco leaves (ICHTJ-cta-VTL-2) were used to check the validity and efficiency of the MAD method (Table 3). Microwave-assisted digestion method required less time (<10 min) for complete digestion of samples. Less than 1–2 % difference for the mean values of Cr from the certified values was observed. The coefficient of variation differed <2 %. When obtained values were compared with both methods, nonsignificant differences ($p > 0.05$) were perceived.

Results

Determination of Cr in Different Smokeless Tobacco Products

The pH of all SLT products was highly basic, found in the range of 8.6–8.9. Toxicity of these products may link with the formation of tobacco-specific amines. As we examined various samples for each type of SLT (snuff, *gutkha*, mainpuri) products, only the mean concentration with the standard deviation is provided for each brand (Table 4). The range of Cr levels in moist snuff (brown and green) was found to be 4.23–10.64 and 6.5–11.0 μg g^{-1}, respectively. Whereas, the ranges of 4.96–7.05 and 5.17–7.85 μg g^{-1} were observed for Cr in dry snuff (brown and black) products, respectively. An increasing order as DB > DBk > BM > GM has been observed for chromium level in various types of snuff. The contents of Cr in all samples of mainpuri ($n = 12$) and *gutkha* ($n = 11$) were found in the range of 3.53–6.05 and 3.68–13.6 μg g^{-1}, respectively.

The variation in Cr concentration was determined to be higher within all SLT products. The obtained Cr levels in various SLT products (snuff, mainpuri, and *gutkha*) are compared with the present data from previous studies. Clinical and biochemical tests of participants are given in Table 2. For all snuff products users, male to female ratio is found as 5:1. Nowadays, consumption of these SLT products became usual among adolescents.

Table 3 Determination of chromium in certified sample of human hair, blood, and Virginia tobacco leaf by conventional (CDM) and microwave-assisted digestion method (MAD) ($n = 6$)

Certified values	MWD Mean ± SD	Recovery (%)	Paired *t* test[a] $t_{Experimental}$
Virginia tobacco leaves (μg g^{-1})			
1.87 ± 0.16	1.859 ± 0.132 (7.10)[b]	99.4	0.875314
Certified sample of whole blood (μg L^{-1})			
2.0 ± 0.5	1.94 ± 0.09 (4.64)	97.0	0.422
Certified sample of human hair (μg g^{-1})			
91.0 ± 10	90.94 ± 5.99 (6.59)	99.9	0.528

[a] Paired *t* test between certified values versus found values, degree of freedom (n − 1) = 5. $t_{Critical}$ at 95 % confidence limit = 2.57

[b] Values in parenthesis RSD

Chromium Concentration in Scalp Hair and Blood Samples

This study shows a clear relationship between chewing habit of SLT products with Cr contents in scalp hair and blood samples of adult population, who chew/sniff SLT products. The mean values of Cr along with standard deviations in biological samples are shown in Table 5. The concentration of Cr in scalp hair and blood samples of male GU, MPU, and SU was higher at 95 % confidence interval [(CI 5.02–5.29 μg/g, CI 85.7–95.3 μg/l) , (CI 5.71–6.05 μg/g, CI 94.6–102 μg/l), and (CI 5.98–6.43 μg/g, 110–122 μg/l), respectively] than referents (CI: 3.28–3.63 μg/g, CI: 58.2–67.6 μg/l). The resulted data indicated that the values of Cr in both biological samples GU and MPU were insignificant ($p = 0.253$–0.342).

The significantly higher level of Cr was observed in scalp hair and blood samples of female GU, MPU, and SU [(CI 4.81–5.08 μg/g; 78.6–85.6 μg/l), (CI 5.57–5.85 μg/g; 94.7–101 μg/l), (CI 5.96–6.25 μg/g; 104–116 μg/l), respectively] as compared to female referents [CI 3.02–3.35 μg/g, 54.7–61.0 μg/l] (p 0.001).

The distribution of the Cr resulted data of referents and adults chewing different types of SLT products was checked by the Shapiro–Wilk test for normality. There is no significant difference observed between normal and log normal distribution. So for comparative purpose, we use data of Cr in referents and adult populations, consuming different types of SLT at normal distributions. The unpaired student *t* test at different degrees of freedom between study subjects chewing different types of SLT products and referents was calculated at different probabilities. Our calculated $t_{Experimental}$ exceeds that of $t_{critical}$ value at 95 % confidence intervals, which indicated the significant differences between the mean values of Cr in biological samples of study subjects who have consumed different SLT than referents ($p < 0.01$).

Regression analyses have been carried out between the Cr concentrations in different types of SLT (snuff, *gutkha*, and mainpuri) products and biological (scalp hair, blood) samples

Table 4 Chromium concentrations in snuff, *gutkha*, and mainpuri samples, intake of Cr by consuming 10 g of each smokeless products (μg/g)

Moist snuff			Mainpuri			Gutkha		
No.	$x \pm ts/\sqrt{n}$ [a]	[b]μg/10 g	No.	$x \pm ts/\sqrt{n}$ [a]	[b]μg/10 g	No.	$x \pm ts/\sqrt{n}$ [a]	[b]μg/10 g
[d]BM1	4.23 ± 0.22	39.8–45.2[c]	MP1	4.31 ± 0.60	36.9–49.0	G1	5.25 ± 0.30	48.9–55.9
BM2	10.64 ± 0.22	104–109	MP2	4.39 ± 0.47	39.2–48.7	G2	12.82 ± 1.28	115–141
BM3	7.29 ± 0.33	69.9–76.8	MP3	4.86 ± 0.31	45.3–51.9	G3	6.42 ± 1.40	50.1–78.4
BM4	9.05 ± 0.51	89.9–91.1	MP4	4.70 ± 0.39	42.9–51.1	G4	5.01 ± 1.83	31.6–68.6
BM5	10.5 ± 0.82	96.6–114	MP5	4.31 ± 0.68	36.1–50.2	G5	5.09 ± 1.65	34.2–67.7
BM6	8.07 ± 0.60	80.1–81.3	MP6	4.78 ± 0.37	43.9–51.8	G6	3.68 ± 0.89	27.3–45.8
BM7	9.82 ± 1.07	87.2–109	MP7	4.85 ± 0.57	42.5–54.9	G7	4.39 ± 1.06	33.2–54.4
[e]GM8	6.5 ± 0.71	56.7–72.9	MP8	3.53 ± 0.63	28.9–41.8	G8	4.46 ± 1.02	34.1–54.9
GM9	7.04 ± 0.81	62.2–78.8	MP9	4.62 ± 0.70	39.1–53.3	G9	4.85 ± 1.65	31.9–65.5
GM10	6.75 ± 1.02	57.0–78.0	MP10	4.93 ± 0.36	45.3–53.4	G10	13.60 ± 0.84	127–145
GM11	9.34 ± 1.07	82.2–104	MP11	5.28 ± 0.64	46.3–59.7	G11	8.29 ± 1.65	66.3–99.8
GM12	11.0 ± 1.15	98.2–121	MP12	6.05 ± 0.58	54.3–66.6			
GM13	9.85 ± 0.70	91.1–106						
GM14	8.65 ± 0.51	81.2–91.8						
Dry snuff								
No.				No.				
[f]DB1	4.46 ± 0.68	37.5–51.7	[g]DBK6	5.17 ± 0.45	47.2–56.7			
DB2	4.95 ± 1.03	39.0–59.8	DBK7	5.96 ± 0.72	52.3–66.8			
DB3	6.83 ± 1.25	55.4–80.6	DBK8	6.35 ± 0.98	53.4–73.7			
DB4	7.05 ± 0.98	60.2–80.5	DBK9	7.85 ± 1.07	67.2–89.7			
DB5	5.72 ± 0.81	48.9–65.8						

[a] Average value ± confidence interval ($P = 0.05$)

[b] Intake of toxic metals per 10 g of each snuff product

[c] Range

[d] Brown Moist

[e] Green Moist

[f] Dry Brown

[g] Dry Black

of adults, who consumed these SLT Products. The linear regression showed correlation (r) between the Cr concentrations in SLT versus G was higher (r = 0. 84–0.94) than Cr concentrations in SLT versus MPU and GU (r = 0.70–0.73 and 0.52–0.67, respectively) (Table 6).

Table 5 Chromium concentration in scalp hair and blood samples of referent and study subjects, consuming different types of SLT products

Elements	Referents	GU	MPU	SU
Scalp hair (μg/g)				
Male	3.44 ± 0.86	5.16 ± 0.76	5.87 ± 0.71	6.21 ± 0.91
Female	3.19 ± 0.78	4.94 ± 0.64	5.70 ± 0.68	6.09 ± 0.87
Blood (μg/l)				
Male	63.0 ± 8.44	90.0 ± 8.29	98.4 ± 7.49	116 ± 9.63
Female	57.9 ± 7.29	82.5 ± 7.29	97.9 ± 6.63	110 ± 10.9

Discussion

The resulted data of Table 3 indicated that Cr level in different brands of *gutkha* and mainpuri did not possess any uniformity. These inconsistencies rely on the quality of different constituents/ingredients and the accessibility of tobacco foliage cultivated in different regions, as well as the course of processing and wrapping [13]. Several additives such as the areca nut, a known carcinogen, are commonly used in these products in Southeast Asian countries including India and Pakistan [10, 37, 38].

In different types of snuff, varied range of Cr concentration was observed. It is of prime importance to note that the processed raw tobacco leaves and other spices are mixed in different ratio to produce various forms of snuff. Moreover, different companies have their own formulations to produce a variety of snuff products. In literature, very few information are

Table 6 Linear regression and Pearson coefficient (*r*) between chromium concentrations in biological samples of SLT users versus their contents in each SLT product

GU vs. G	MPU vs. MP	SU vs. S	GU vs. G	MPU vs. MP	SU vs. S
Male			Female		
Scalp hair					
0.717× + 0.887	0.900× + 0.857	1.692× + 1.052	2.353× + 1.53	0.11× + 1.35	0.272×- 2.71
$r = 0.57$	$r = 0.73$	$r = 0.87$	$r = 0.56$	$r = 0.73$	$r = 0.84$
Blood					
1.257× + 0.986	0.651× + 1.476	1.609× + 1.476	2.085× + 1.119	0.115× + 0.983	0.272×- 2.71
$r = 0.52$	$r = 0.70$	$r = 0.94$	$r = 0.62$	$r = 0.73$	$r = 0.84$

GU Gutkha user, *G gutkha*, *MPU* Mainpuri user, *MP* Mainpuri, *SU* Snuff user, *S* Snuff

present about intake of metals from different types of SLT products by the population of all age groups in Asian countries including Pakistan. It was noticed that addicted consumer used up different kinds of snuff, as 1–2 g of dry snuff may be sniffed quickly into the nostrils or 2–3 g of snuff is placed or dipped in the middle of the lower lip and gum or cheek and gum. Both dipping and sniffing habits are repeated several times a day (mostly 2–15), conditional to the severity of the addiction [13].

The resulted values for both dry and moist snuff were found to be lower than the reported values [39]. It is found that Cr concentration in understudy moist snuff (green and brown) was found to be higher than the former studies [40–42], while Cr content in GM and BM snuff product was lower than the previously reported values [32], as shown in Table 7. Chromium content in dry snuff products has been found to be higher and comparable with other studies [32, 42–44]. Whereas Cr content in different brands of *gutkha* products was higher or comparable with the reported values [32, 40, 44–47]. The values of Cr in mainpuri products have been higher than those data reported in previous studies [46].

It is documented in the literature that tobacco plants have a capability to absorb certain heavy metals/toxic elements from soil [36]. The levels of the heavy metals in tobacco plant may vary due to its cultivation in various geographical regions [48]. It was reported that Cr in tobacco found in the range of 0.8–2.4 mg/g which is higher than those values (0.01–0.1 mg/g) reported for other plants [49].

To manufacture the SLT products, dried tobacco leaves are used as main raw material. So, variation of elemental level would be predictable in any form of SLT products because of their concentration in tobacco plants (leaves) [50]. International Agency for Research on Cancer (IARC) has declared SLT as a group 1

Table 7 Chromium concentrations in *gutkha*, mainpuri, and snuff products as reported in literature (in micrograms per gram)

Author, year	Products	Range	Country
Musharraf et al., 2012 [32]	*Gutkha*	0.69–10.44 μg/g	Pakistan
	Dry snuff	6.33–7.14 μg/g	Pakistan
	Moist snuff	2.12–78.8 μg/g	Pakistan
Nawal Al-Mukhaini, 2014 [39]	Snuff	15.02–16.38 μg/g	Oman
Rickert, 2009 [40]	Moist snuff	0.837–1.144 μg/g	Canada
	Gutkha	0.818 μg/g	India
Pappas, 2008 [41]	Moist snuff	0.86–3.20 μg/g	USA
Mishra and Shaikh, 1986 [43]	Dry snuff	3 μg/g	India
	Gutkha	6.3 μg/g	India
Mishra and Shaikh, 1983 [45]	*Gutkha*	6.6 μg/g	India
Shaikh et al., 2002 [44]	Dry snuff	3.8 μg/g	India
Verma, 2010 [47]	*Gutkha*	6.97 μg/g	India
Garg et.al., 2012 [46]	Mainpuri	0.99–2.64 μg/g	India
	Gutkha	1.75–3.44 μg/g	India
Borgerding, 2012 [42]	Moist snuff	0.877–2.285 μg/g	Products from US market
	Dry snuff	1.184–5.740 μg/g	Products from US market
	Moist snuff	0.870–1.822 μg/g	Products from Swedish market
	Dry snuff	0.955–4.452 μg/g	Products from Swedish market

carcinogen. The injurious constituents of *gutkha* and snuff can originate cancer and other problems in the mouth because these products remain in contact with the inner lining of the oral cavity for several hours [51].

The absorption and biological fate of metals such as Cd and Cr after entering into the body, governs much of the disorders and serious health effects. These elements could accumulate in the lung tissues and persist for a lifetime [23]. The reported guidance level tolerable daily intake for Cr is 150 μg/kg/day [52]. The recommended daily intake of Cr is 50–200 μg [53]. The US Food and Drug Administration have selected a 120 μg/day as reference daily intake for chromium [54]. The resulted data on the intake of Cr via chewing/snuffing different SLT products are at the lower end of the estimated safe and adequate dietary daily intake or chromium of 50–200 μg/day identified by the US National Research Council, which corresponds to 0.83–3.33 μg/kg/day for a 60-kg adult. The US Food and Drug Administration have selected a reference daily intake of 120 μg/day for Cr [54].

The intake of Cr by consumption of different types of SLT products is shown in Table 4. Cr intake via chewing/sniffing 10 g of studied snuff products was found in the range of 39.8–114, 56.7–121, 37.5–80.6, and 47.2–89.7 μg $day^{-1}person^{-1}$ for BM, GM, DB, and DBK respectively, which contribute 21.2–220 % of reference daily intake of Cr for adults (60 kg) as recommended range by the US National Research Council, 50–200 μg/day [54]. The intake of Cr via consuming 10 g of different types of *gutkha* and mainpuri products found in the range 27.3–145 and 28.9–66.6 μg $day^{-1}person^{-1}$ respectively, contributing 18.4–273 and 17.7–122 % of the daily intake values of Cr in adults (60 kg). The intake of Cr via consuming different SLT products is far below than reported tolerable daily intake value for Cr, 150 μg/kg/day [52].

The frequent tobacco users are mostly affected by periodontal disease. The most important periodontal alteration includes local gingival recession. In general, 25–30 % of these consumers are effected by gingival recession while 50–60 % from white mucosal lesions [55]. For this reason, the health risks associated with SLT products arises not only due to tobacco leaves but also the more complex mixture of ingredients that may further increase the risk. Several salts (i.e. sodium chloride) are added to SLT as a flavor enhancer and antimicrobial agent, which may harm the gastric epithelium and also intensify the absorption of carcinogens and contribute to chronic irritation and tumor promotion [56]. Severe oral mucosal lesions have been faced by the consumers who begin to use these SLT products at an earlier age, for more hours per day, greater dosages, or more days per month. The lesions usually resolve when people quit the habit of smokeless tobacco [57].

Conclusion

The Cr exposure through consumption of different types of SLT products was confirmed by the analysis of biological (scalp hair and blood) samples of human subjects. The obtained levels of Cr in different SLT products are nearly comparable to the existing data with few exceptions. It was observed that higher level of Cr was obtained in biological samples of study subjects consuming different SLT products as compared to referents. Whereas significantly higher value of Cr was observed in both biological samples of snuff users as compared to those consuming other SLT products. During survey, it was observed that the consumers may not be aware that they are affected by the hazardous constituents of SLT products, which affect their general and oral health. As the consumption rate of SLT product increases in addition to tobacco smoking, this may adversely affect human health. There is need for more detailed studies on other metals and their species. We consider that people consuming any type of SLT product, especially those consuming more than 10 g/day, are at risk of developing adverse health effects. Public health policy makers should seriously consider implementing policies to reduce or eliminate the consumption of snuff in populations where this practice is prevalent. A strategy involving parents, teachers, and local communities could be initiated to discourage the use of SLT products.

Compliance with Ethical Standards

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Conflict of Interest The authors declare that they have no conflict of interest.

References

1. Melikian AA, Hoffmann D (2009) Smokeless tobacco: a gateway to smoking or a way away from smoking. Biomarkers 14:85–89
2. Subramanian SV, Nandy S, Kelly M, Gordon D, Smith GD (2004) Patterns and distribution of tobacco consumption in India: cross sectional multilevel evidence from the 1998–1999 national family health survey. Br Med J 328:801–806
3. Gupta PC, Ray CS (2003) Smokeless tobacco and health in India and South Asia. Respirology 8:419–431
4. Farrand P, Rowe RM, Johnston A, Murdoch H (2001) Prevalence, age of onset and demographic relationships of different areca nut habits amongst children in Tower Hamlets, London. Br Dent J 190: 150–154
5. Richter P, Spierto FW (2003) Surveillance of smokeless tobacco nicotine, pH, moisture, and unprotonated nicotine content. Nicotine Tob Res 5:885–889
6. Kazi TG, Wadhwa SK, Afridi HI, Kazi N, Kandhro GA, Baig JA, Shah AQ, Kolachi NF, Khan S (2010b) Evaluation of cadmium and zinc in biological samples of tobacco and alcohol user male mouth cancer, patients. Hum Exp Toxicol 29:221–230

7. Gupta PC, Ray CS (2004) Epidemiology of betel quid usage. Ann Acad Med Singap 33:31–36
8. Boucher BJ, Mannan N (2002) Metabolic effects of the consumption of *Areca catechu*. Addict Biol 7:103–110
9. Shah SM, Merchant AT, Luby SP, Chotani RA (2002) Addicted school children, prevalence and characteristics of areca nut chewers among primary school children in Karachi, Pakistan. J Pediatr Child Health 38:507–510
10. Nair U, Bartsch H, Nair J (2004) Alert for an epidemic of oral cancer due to use of the betel quid substitutes gutkha and pan masala: a review of agents and causative mechanisms. Mutagenesis 19:251–262
11. Arain SS, Kazi TG, Arain JB, Afridi HI, Brahman KD (2014) Preconcentration of toxic elements in artificial saliva extract of different smokeless tobacco products by dual-cloud point extraction. Microchem J 112:42–49
12. WHO (2004) Betel-quid and areca-nut chewing and some areca-nut-derived nitrosamines. World Health Organization, International Agency for Research on Cancer, Lyon, p. 85
13. Kazi TG, Arain SS, Afridi HI, Brahman KD, Kolachi NF, Mughal MA (2013) Analysis of cadmium, nickel, and lead in commercial moist and dry snuff used in Pakistan. Environ Monit Assess 185: 5199–5208
14. US Department of Health and Human Services (1986) Health consequences of using smokeless tobacco: a report of the Advisory Committee to the Surgeon General. Bethesda, Maryland
15. Mazahir S, Malik R, Maqsood M, Kanwal ARM, Malik F, Majeed A, Fatmi Z, Khawaja MR, Ghaffar S (2006) Socio-demographic correlates of betel, areca and smokeless tobacco use as a high risk behavior for head and neck cancers in a squatter settlement of Karachi, Pakistan. Substit Abuse Treat Prev 1:10–16Policy
16. Khawaja MI, Shafiq M, Nusrat R, Khawaja MR (2005) Preventing the oral cavity cancer epidemic. Asian Pac J Cancer Prev 6:420
17. Angelova V, Ivanov K, Ivanova R (2003) Effect of chemical forms of lead, cadmium and zinc in polluted soils on their uptake by tobacco. J Environ Prot Ecol 4:316–327
18. Lugon-Moulin N, Martin F, Krauss MR, Ramey PB, Rossi L (2006) Cadmium concentration in tobacco (*Nicotiana tabacum* L.) from different countries and its relationship with other elements. Chemosphere 63:1074–1086
19. Elinder CG (1991) Cadmium as an environmental hazard. IARC Sci Publ 118; 123–132.
20. Stepanov I, Hecht SS (2005) Tobacco-specific nitrosamines and their pyridine-N-glucuronides in the urine of smokers and smokeless tobacco users. Cancer Epidemiol Biomark Prev 14:885–891
21. Zhu X, Hu B, Jiang Z, Li M (2005) Cloud point extraction for speciation of chromium in water samples by electrothermal atomic absorption spectrometry. Water Res 39(4):589–595
22. Kim JG, Dixon JB, Chusuei CC, Deng Y (2002) Oxidation of chromium(III) to (VI) by manganese oxides. Soil Sci Soc Am J 66:306–315
23. Pääkkö P, Kokkonen P, Anttila S, Kalliomäki PL (1989) Cadmium and chromium as markers of smoking in human lung tissue. Environ Res 49:197–207
24. Tsuchiyama F, Hisanaga N, Shibata E, Aoki T, Takagi H, Ando T, Takeuchi Y (1997) Pulmonary metal distribution in urban dwellers. Int Arch Occup Environ Health 70(2):77–84
25. Robertson PB, Walsh M, Greene J, Ernster V, Grady D, Hauck W (1990) Periodontal effects associated with the use of smokeless tobacco. J Periodontol 61:438–443
26. Soylak M, Yilmaz E, Ghaedi M, Montazerozohori M (2011) Solid phase extraction on multiwalled carbon nanotubes and flame atomic absorption spectrometry combination for determination of some metal ions in environmental and food samples. Toxicol Environ Chem 93:873–885
27. Gopikrishna P, Rao KS, Rao TP, Naidu GR (2004) Solid phase extractive preconcentration of cobalt and nickel in hair samples using ethyl xanthate modified benzophenone. Microchim Acta 144:285–289
28. Yunes N, Moyano S, Cerutti S, Gasquez JA, Martinez LD (2003) On-line preconcentration and determination of nickel in natural water samples by flow injection-inductively coupled plasma optical emission spectrometry FI-ICP-OES. Talanta 59:943–949
29. Suvardhan K, Kumar KS, Krishnaiah L, Rao SP, Chiranjeevi P (2004) The determination of nickel II after on-line sorbent preconcentration by inductively coupled plasma atomic emission spectrometry using *Borassus flabellifer* inflorescence loaded with coniine dithiocarbamate. J Harzard Mater 112:233–238
30. Arain SS, Kazi TG, Arain JB, Afridi HI, Brahman KD, Shah F, Naeemullah, Arain S, Panhwar AH (2013) Simultaneous preconcentration of toxic elements in artificial saliva extract of smokeless tobacco product, mainpuri by cloud point extraction method. Ecotoxicol Environ Saf 92:289–296
31. Ryan J, Clark M (2010) Trace metal determination in tobacco and cigarette ash by inductively coupled plasma-atomic emission spectroscopy. J Anal Chem 1:34–41
32. Musharraf SG, Shoaib M, Siddiqui AJ, Najam-ul- Haq M, Ahmed A (2012) Quantitative analysis of some important metals and metalloids in tobacco products by inductively coupled plasma–mass spectrometry (ICP–MS). Chem Cent J 56:1–12
33. Ghaedi M, Shokrollahi A, Ahmadi F, Rajabi HR, Soylak M (2008) Cloud point extraction for the determination of copper, nickel and cobalt ions in environmental samples by flame atomic absorption spectrometry. J Hazard Mater 150:533–540
34. Demir TA, Akar T, Akyuz F, et al. (2005) Nickel and cadmium concentrations in plasma and Na^+/K^+ ATPase activities in erythrocyte membranes of the people exposed to cement dust emissions. Environ Monit Assess 104:437–444
35. Afridi HI, Kazi TG, Kazi GH, et al. (2005) Essential trace and toxic element distribution in the scalp hair of Pakistani myocardial infarction patients and controls. Biol Trace Elem Res 113:19–34
36. Arain MB, Kazi TG, Jamali MK, Jalbani N, Afridi HI, Kandhro GA, et al. (2008) Hazardous impact of toxic metals on tobacco leaves grown in contaminated soil by ultrasonic assisted pseudo-digestion: multivariate study. J Hazard Mater 155:216–224
37. Patidar KA, Parwani R, Wanjari SP, Patidar AP (2015) Various terminologies associated with areca nut and tobacco chewing: a review. J Oral Maxillofac Pathol 19:69
38. IARC (2004) Betel-quid and areca-nut chewing and some areca-nut-derived nitrosamines. IARC monographs on the evaluation of carcinogenic risks to humans, 85 edn. World Health Organization, International Agency for Research on Cancer, Lyon
39. Al-Mukhaini N, Ba-Omar T, Eltayeb E, Al-Shehi A (2014) Determination of heavy metals in the common smokeless tobacco Afzal in Oman. Sultan Qaboos Univ Med J 14:349
40. Rickert WS, Joza PJ, Trivedi AH, Momin RA, Wagstaff WG, Lauterbach JH (2009) Chemical and toxicological characterization of commercial smokeless tobacco products available on the Canadian market. Regul Toxicol Pharmacol 53:121–133
41. Pappas RS, Stanfill SB, Watson CH, Ashley DL (2008) Analysis of toxic metals in commercial moist snuff and Alaskan iqmik. J Anal Toxicol 32:281–291
42. Borgerding MF, Bodnar JA, Curtin GM, Swauger JE (2012) The chemical composition of smokeless tobacco: a survey of products sold in the United States in 2006 and 2007. Regul Toxicol Pharmacol 64:367–387
43. Mishra UC, Shaikh GN, Sadasivan S (1986) Trace elements in tobacco and tobacco smoke by X-ray fluorescence technique. J Radioanal Nucl Chem 102:27–35

44. Shaikh AN, Negi BS, Sadasivan S (2002) Characterization of Indian cigarette tobacco and its smoke aerosol by nuclear and allied techniques. J Radioanal Nucl Chem 253:231–234
45. Mishra UC, Shaikh GN (1983) Determination of trace element concentrations of Indian cigarette tobacco by instrumental neutron activation analysis. J Radioanal Chem 78:385–390
46. Garg ANR, Choudhury RP, Acharya R, Reddy AVR (2012) Determination of minor, trace and toxic elements in chewing tobacco products by instrumental neutron activation analysis and identification of glutamic acid. J Radio Anal Nucl Chem 294:197–202
47. Verma S, Yadav S, Singh I (2010) Trace metal concentration in different Indian tobacco products and related health implications. Food Chem Toxicol 48:2291–2297
48. Regassa G, Chandravanshi BS (2016) Levels of heavy metals in the raw and processed Ethiopian tobacco leaves. Springer Plus 5:1
49. Pendias AK, Pendias H (1985) Trace elements in soils and plants, chap. 11. CRC Press, Boca, Raton, FL, p. 193
50. Asta J, Guillard E, Tissut M, Gaude T, Ravanel P (2003) Heavy metal transfer from atmosphere to plants. J Phys 107:65–67
51. Trivedi AH, Bakshi SR, Balart DB, Shah PM, Dinavahill VBR, Patel DD, Patel RK (1996) Carcinogenic and genotoxic effects of the tobacco substitute pan masala: present status and likely future impact on the Indian population. Cancer Treat Rev 22:345–354
52. EVM (2003) Safe upper levels for vitamins and mineral. Expert Group on Vitamins and Minerals, Report of the Expert Group on Vitamins and Minerals. Food Standards Agency. http://www.food.gov.uk/multimedia/pdfs/vitmin2003.pdf last accessed Aug., 2010)
53. RDA (1989) Recommended dietary allowance, 10th edn. National Academic Press, Washington, DC
54. US EPA (1998) Toxicological review of trivalent chromium. US EPA, Washington, DC
55. Johnson GK, Slach NA (2001) Impact of tobacco use on periodontal status. J Dent Educ 65:313–321
56. Stepanov I, Jensen J, Hatsukami D, Hecht SS (2008) New and traditional smokeless tobacco: comparison of toxicant and carcinogen levels. Nicotine Tob Res 10:1773–1782
57. IARC (2007) Smokeless tobacco and some tobacco-specific N-nitrosamines. IARC monographs on the evaluation of carcinogenic risks to humans, 89 edn. World Health Organization, International Agency for Research on Cancer, Lyon