Environ Sci Pollut Res (2014) 21:12017–12027
DOI 10.1007/s11356-014-3091-5

RESEARCH ARTICLE

Determination of nickel in blood and serum samples of oropharyngeal cancer patients consumed smokeless tobacco products by cloud point extraction coupled with flame atomic absorption spectrometry

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Received: 22 March 2014 / Accepted: 23 May 2014 / Published online: 13 June 2014

Abstract Oropharyngeal cancer is a significant public health issue in the world. The incidence of oropharyngeal cancer has been increased among people who have habit of chewing smokeless tobacco (SLT) in Pakistan. The aim of present study was to evaluate the concentration of nickel (Ni) in biological samples (whole blood, serum) of oral (n=95) and pharyngeal (n=84) male cancer patients. For comparison purposes, the biological samples of healthy age-matched referents (n=150), who consumed and did not consumed SLT products, were also analyzed for Ni levels. As the Ni level is very low in biological samples, a preconcentration procedure has been developed, prior to analysis of analyte by flame atomic absorption spectrometry (FAAS). The Ni in acid-digested biological samples was complexed with ammonium pyrrolidinedithio carbamate (APDC), and a resulted complex was extracted in a surfactant Triton X-114. Acidic ethanol was added to the surfactant-rich phase prior to its analysis by FAAS. The chemical variables, such as pH, amounts of reagents (APDC, Triton X-114), temperature, incubation time, and sample volume were optimized. The resulted data indicated that concentration of Ni was higher in blood and serum samples of cancer patients as compared to that of referents who have or have not consumed different SLT products (p= 0.012–0.001). It was also observed that healthy referents who consumed SLT products have two to threefold higher levels of Ni in both biological samples as compared to those who were not chewing SLT products (p<0.01).

Keywords Nickel · Oropharyngeal cancer · Smokeless tobacco products · Blood · Serum · Cloud point extraction

Responsible editor: Philippe Garrigues

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Introduction

Oral and pharyngeal cancers are the sixth most common cancers in the world (Khandekar et al. 2006). Oral cancer is common malignancies among people who have tobacco smoking and chewing habits (Oji and Chukwuneke 2007). The annual estimated incidence is around 275,000 for oral and 130,300 for pharyngeal cancers excluding nasopharynx; two thirds of these cases are occurring in developing countries (Ferlay et al. 2004).

Smoking tobacco in different forms is very common all over the world, while smokeless tobacco (SLT) products are

not very common in Europe. The SLT is indeed used by various cultures in many parts of the world, including Middle East and Asian subcontinent (Pakistan and India) (Trivedi et al. 1996; Kazi et al. 2013). In some cases, 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 (Thomas and MacLennan 1992; Kazi et al. 2010b).

Several studies have found that consumption of SLT can increase the risk of oral cancer and adjacent sites (Blot et al. 1988; Muscat et al. 1996; Schildt et al. 1998). Cancer caused by chewing habit of SLT is often beginning as leukoplakia or erythroplakia. Erythroplakia is generally more severe than leukoplakia and has a higher chance of becoming cancerous over time (Bouquot 1991). Several chemical classes, such as polyaromatic hydrocarbons, specifically nitosamines and heavy metals, are present in tobacco and have long been associated with risk of cancers (Pappas et al. 2006).

Tobacco carcinogenicity is more than evident, and about one fourth of oral cancer cases is attributable to cigarette smoking (Hashibe et al. 2007). Environmental factors may influence the uptake of heavy metal by tobacco plants including soil pH and heavy metal-containing sludge or fertilizers applied to crops (Adamu et al. 1989; Xiao et al. 2004). Tobacco plants easily take up heavy metals from soil and concentrate them in leaves. It was reported that tobacco leaves contain a large amount of Ni in the range of 0.64 to 1.15 μg/g per dry leaf (Bache et al. 1985; Lugon-Moulin et al. 2006).

Epidemiological, cell culture, and animal experimental studies have shown an association of cancer incidence with chronic exposure to Ni(II), Cr(VI), and As(III) (Domingo et al. 1996; Brahman et al. 2014), although a direct correlation between the ability of Ni to produce oxidative stress and carcinogenicity is not yet fully understood. There is evidence that the genotoxic effects of Ni compounds may indirectly inhibit the DNA repair systems (Rothenberg et al. 1994; ATSDR 2005). Carcinogenic actions of Ni compounds are thought to be mediated by oxidative stress, DNA damage, epigenetic effects, and the regulation of gene expression by activation of certain transcription factors (Leonard et al. 2004).

Oral carcinogenicity studies with water-soluble Ni compounds in animals have examined the potential of Ni compounds to induce systemic tumors. The water-soluble Ni compounds have the highest bioaccessibility in gastric fluids and systemic absorption as compared to water-insoluble Ni compounds (sulfidic, oxidic, and metallic nickel). This fact represents the worst case scenario for systemic carcinogenicity associated with Ni exposures (Haber et al. 2000; Seilkop and Oller 2003). A number of comprehensive reviews discussed the mechanisms of Ni carcinogenesis (Haber et al. 2000; Denkhaus and Salnikow 2002; Seilkop and Oller 2003). The Ni compounds were shown to act synergistically with many mutagenic carcinogens in enhancing cell transformation both in vitro and in vivo (Schwerdtle et al. 2002). Recent studies have shown that carcinogenicity due to metals is, in general, the result of the production of reactive oxygen species (Lee et al. 2012; Khlifi et al. 2013a).

The SLT products are mostly produced in Asian countries, especially in India and Pakistan. Mainpuri product contains pieces of tobacco leaves, finely cut betel nut, and other ingredients, which are mixed thoroughly with lime (Gupta and Ray 2004). Gutkha, a dry preparation commercialized since 1975, is basically a flavored and sweetened dry mixture of areca nut, catechu, and slaked lime with tobacco. Spices such as saffron, cardamom, cloves, mustard, turmeric, paraffin, lime, anise seeds, or sweeteners are also added for flavor. For the last couple of decades, gutkha has been available in several brands (Nair et al. 2004).

The analysis of trace element concentrations in biological media, especially fluids, might be considered a difficult analytical task, due to complexity of the matrix and low concentration of analytes, which requires sensitive instrumental techniques (Manzoori and Nezhad 2004; Naeemullah et al. 2014). Preconcentration can solve these problems and allows easy determination of the trace elements by less sensitive but more accessible instrumentation such as flame atomic absorption spectrometry (FAAS) (Soylak et al. 2002). Various preconcentration methods including liquid–liquid extraction, ion exchange, cloud point extraction, and solid phase extraction have been proposed for preconcentration of trace elements (Soylak and Tuzen 2006; Ghaedi et al. 2009; Wadhwa et al. 2014). The traditional liquid–liquid extraction and separation methods are usually time-consuming and require quite large volumes of high-purity solvents. Of additional concern is the disposal of the solvents used, which creates a severe environmental crisis. In this sense, cloud point extraction (CPE) is an interesting and efficient option, as it reduces the use and exposure to solvents, the disposal costs, and extraction time (Citak and Tuzen 2012; Soylak et al. 2012; Arain et al. 2013).

The rate of oropharyngeal cancer is increased in Pakistan; although an extensive list of risk factors has been well characterized in its pathogenesis, the very common habit of chewing SLT products is also one of the main reasons. This hospital-based study is aimed to evaluate the concentration of Ni in whole blood and serum samples of male oropharyngeal cancer patients. For comparison purposes, referents of same age group (range 30–60 years), socioeconomic status, localities, and dietary habits were selected. The multiple logistic regression analysis was applied to evaluate the correlation of Ni level in biological samples related to consumption of SLT products and mortality rate of both types of cancer patients.

Materials and methods

Reagents and glassware

Ultrapure water was obtained from ELGA LabWater system (Bucks, UK). Concentrated nitric acid (65 %) and hydrogen peroxide (30 %) were obtained from Merck (Darmstadt, Germany). The nonionic surfactant (Triton X-114) was obtained from Sigma (St. Louis, MO, USA) and used without further purification. The 0.1 mol/L of acetate buffer was used to control the pH of the solutions. The pH of the samples was adjusted to the desired pH (3–8) by the addition of 0.1 mol/L HNO_3/NaOH solution in acetate buffer. Working standard solutions of Ni was prepared prior to their use by stepwise dilution of certified standard solutions (1,000 ppm), Fluka Kamica (Buchs, Switzerland), with 0.2 mol/L of HNO_3. For the accuracy of methodology, certified reference materials (CRMs) of Clincheck control-lyophilized human whole blood (Recipe, Munich, Germany) and Clincheck control-lyophilized human serum (Recipe, Munich, Germany) were used. Glasswares and polyethylene containers were soaked in 10 % (*v*/*v*) HNO_3 for 24 h, washed with distilled water, finally with de-ionized water, and dried in such a manner to ensure that no any contamination from glasswares occurs.

Instrumentation

The determination of Ni was carried out by means of a PerkinElmer model AAnalyst 700 (Norwalk, CT) flame atomic absorption spectrophotometer. The hollow cathode lamp of Ni was run under the conditions suggested by the manufacturer. A single-element hollow-cathode lamp was operated at 30 mA and spectral bandwidth of 0.7 nm. The analytical wavelength was set at 232.0 nm. The acetylene flow rate and the burner height were adjusted in order to obtain the maximum absorbance signal. A pH meter (Ecoscan Ion 6, Malaysia) was employed for pH adjustments. A PEL domestic microwave oven (Osaka, Japan), programmable for time and microwave power from 100 to 900 W, was used for digestion of whole blood and serum samples. The phase separation was assisted with a centrifuge ROWKA Laboratoryjna type WE-1, nr-6933 (Mechanika Phecyzyjna, Poland). A programmable ultrasonic water bath, model no. SC-121TH (Sonicor, Deep Park, NY, USA), was used for incubation with temperature ranging from 0 to 80 °C at intensification frequency of 35 kHz.

Study group

This hospital-based case–control study consisted of 179 oropharyngeal cancer patients, age range 30–60 years, which were consecutively recruited between 2011 and 2012, admitted in Nuclear Institute of Medicine and Radiotherapy Jamshoro (NIMRA), Larkana Institute of Nuclear Medicine and Radiotherapy (LINAR), and Muhammad Medical Hospital Mirpurkhas (MMH), and situated in different cities of Sindh, Pakistan. All subjects were male and chewing different SLT products. Both types of cancer patients were grouped according to their habits of chewing different SLT products. The oral cancer patients consumed gutkha (OPG), mainpuri (OPM), and both mainpuri and gutkha (OPB), whereas the pharyngeal cancer patients were termed as PPG, PPM, and PPB who consumed gutkha, mainpuri, and both SLT products, respectively. Among male referent subjects, 89 consumed SLT, while 61 had none of this chewing habit. Referents were divided into four groups, gutkha-consuming referents (RG), mainpuri-user referents (RM), consumers of both SLT products (RB), and referents that have none of any SLT chewing habit (RN).

The all patients have histologically confirmed oropharyngeal cancers (oral 95 and pharyngeal 84). Clinical characteristics including basic medical data were obtained from medical records with the help of paramedical staff. The referents were recruited simultaneously (mostly the relatives of patients); among them, 60 % consumed different SLT products and 40 % none of these products. Before the start of this study, all referents and oropharyngeal cancer patients were informed through a consent form and explained about the aim of study, and all agreed to participate and signed the form. A questionnaire was also administered to them in order to collect details concerning physical data, consumption of different tobacco products, ethnic origin, health, dietary habit, age, and consent. All participants (referents and patients) were interviewed personally by the authors and coauthors. Participant's history of cigarette smoking, SLT chewing habit, including initiation age, quantities, and years consumed was also collected.

The occupational history included jobs held for more than 1 year over the lifetime; the study subjects were mostly drivers, working in workshops (automobile, battery recycling), and labors in construction buildings. The 34 % of patients consumed gutkha, 26 % mainpuri while 40 % consumed both mainpuri and gutkha products. The criteria for the collection of biological samples (whole blood, serum) of biopsy proved cases of oral and pharyngeal cancer patients, prior to any treatment, i.e., surgery, chemotherapy, or radiotherapy, and were not taking any mineral supplement during last 3 months. The exclusion criteria for patients and referents were smoking or drinking alcohol habit.

Among study groups, more than 70 % of patient's condition was apparently worse in terms of chronic illnesses, malnutrition, poverty, and ignorance of disease for long time. Physical examinations were performed in the cancer hospitals to measure participant's weight, height, blood pressure, and biochemical data. The biochemical tests of patients and referents (hemoglobin, red blood cells, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, mean

corpuscular volume, and transferrin iron-binding capacity in the blood) are shown in Table 1. The histological distribution is not shown in the present study. Lifetime consumption of SLT products was also calculated. The average number and weight of SLT products (~2 g) consumed per day and the total number of years of chewing were used to calculate cumulative chewing dose as pack years.

$$\text{Pack years} = (\text{number of SLT sachet consumed per day}) \times (\text{years consumed})$$

Sampling

Blood sampling was carried out at NIMRA and LINAR hospitals and MMH. Venous blood samples (5 mL) were collected by using metal-free vacutainer EDTA tubes (Becton Dickinson, Rutherford, NJ, USA). Then, 2 mL of venous blood sample of each study subjects was stored at −4 °C until required for analysis, while the remaining (3 mL) was used for separating the sera.

Sample preparation

Duplicate samples of whole blood and serum (0.5 mL) were directly taken into polytetrafluoroethylene (PTFE) flasks. Then, we added 3 mL of a freshly prepared mixture of concentrated HNO_3–H_2O_2 (2:1, *v*/*v*) and kept at room temperature for 10 min. Then, we placed the flasks in 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 0.1 mol/L concentrated HNO_3. A blank extraction (without sample) was carried out through the complete procedure.

CPE procedure

Aliquots of 10 mL of standard solutions containing Ni in the range of 4.0–20.0 μg/L and duplicate samples of each digested real samples of whole blood and serum (10 mL) were transferred into graduated centrifuge tubes with glass stopper. We added 0.2–1.0 mL of APDC (0.1–0.5 %), 2 mL of different buffers to adjust a pH range of 3–8 with 0.1 mol/L HNO_3/NaOH, and 2 mL of Triton X-114 (0.1–1 %, *v*/*v*). The tubes were placed in an ultrasonic bath between 40 and 60 °C for 10–30 min. Thus, after different time intervals, the phase separation was accelerated by centrifuging for 5 min at 3,500 rpm. After cooling in an ice bath, the surfactant-rich phase became viscous and the upper aqueous phase was decanted. To decrease the viscosity of surfactant-rich phase, 0.5 mL of acidic ethyl alcohol (0.1 mol/L HNO_3) was added, and the contents of Ni were then measured by FAAS. Blank determination was simultaneously carried out on matrix-matched solution of standards (0.2 mol/L HNO_3).

Statistical analysis

All statistical analyses were performed using computer program Excel X State (Microsoft Corp., Redmond, WA, USA) and Minitab 13.2 (Minitab Inc., State College, PA, USA). ANOVA was used to assess the significance of differences between the concentrations of Ni observed in the biological samples of patients and the referent subjects, calculated by the unpaired two-sample *t* test. A $p<0.05$ was considered as significant difference. A chi-square test was conducted for discrete variables (biochemical parameters of referents and patients). Association between Ni level and habits of chewing SLT products on the development of oropharyngeal cancer was assessed using odds ratio (OR) and 95 % confidence interval (CI) by multiple logistic regression analysis.

Analytical figures of merit

The calibration graph for preconcentration of Ni with APDC was linear with correlation coefficients 0.992–0.998, at the range of 4.0–20 μg/L. The equation for the linear range of Ni calibration curve after preconcentration was found to be [y=(17.821) (Ni)−(0.0905)], where y is integrated absorbance and the concentration is expressed as microgram per liter. The limit of detection (LOD) and limit of quantification (LOQ) were calculated as below 3 and 10s/m, respectively, where s is the standard deviation of ten measurements of the blank and m is slope of the calibration graph. The LOD and LOQ were calculated for Ni as 0.52 and 1.58 μg/L, respectively. The accuracy of proposed analytical method (CPE) was validated with CRM, Clincheck control-lyophilized human whole blood (Recipe, Munich, Germany) and Clincheck control-lyophilized human serum (Recipe, Munich, Germany). The % recoveries of certified and observed values in CRM samples (blood and serum) were greater than 98 % (Table 2), which indicated that the proposed method was reliable and efficient. The experimental enhancement factor (EF) calculated as the ratio of slopes of calibration graphs with and without preconcentration of Ni was 46, which may increase with high initial volume of sample solution. The high sensitivity and low detection limits of present CPE method are efficiently applied for the determination of very low concentrations of Ni in whole blood and serum samples by FAAS.

Results

Nickel in biological samples of cancer patients

The demographic distributions of oropharyngeal cancer patients and referents are shown in Table 3. The mean concentrations with standard deviations of Ni in

Table 1 Biochemical tests of referents and SLT-chewing male referents and oropharyngeal cancer patients

Parameters	Normal range	Referents				Oral cancer patients			Pharyngeal cancer patients		
		RN	RG	RM	RB	OPG	OPM	OPB	PPG	PPM	PPB
Hb (g/dL)	11–14.5	13.1±1.95	12.3±1.23	12.1±1.21	11.9±1.32	9.89±1.21*	9.82±1.44*	9.75±1.57*	9.34±0.96**	9.56±0.79**	9.21±0.61**
RBC ($\times 10^{12}$/L)	3.5–4.5	4.11±0.43	3.98±0.23	3.69±0.34	3.53±0.47	3.48±0.35*	3.41±0.41*	3.39±0.28*	3.27±0.21*	3.23±0.39*	3.11±0.46**
MCH (pg)	27–32	30.8±1.1	31.4±1.85	31.2±1.45	32.1±1.21	34.3±1.72**	35.6±1.42**	37.8±1.32**	37.3±1.54**	37.9±1.96**	38.3±1.41**
MCHC (g/dL)	32–36	35.3±1.2	32.1±0.97	31.1±0.67	30.1±1.23	27.5±1.12**	26.8±1.31**	24.4±1.11**	24.7±0.91**	25.8±0.49**	24.8±0.53**
MCV (fL)	92.6±2.6	93.9±2.8	94.2±3.11	95.7±2.97	97.8±3.54	123±3.43**	129±4.21**	139±5.65**	134±4.87**	138±6.61**	141±5.12**
Serum Fe (μg 100/mL)	60–160	137±14.1	118±7.23	119±5.89	115±7.65	101±6.99	99.8±7.32	97.8±4.53	98.9±6.53	92.9±6.53	97.1±5.76
TIBC (μg 100/mL)	280–400	369±36.1	351±25.1	342±23.7	327±29.7	245±19.8**	252±18.6**	224±27.9**	234±25.9**	231±19.8**	245±24.7**

Two-sided chi-square test indicates that statistical difference between biochemical parameters of referents and oropharyngeal cancer patients was *$p<0.01$ and **$p<0.001$

RBC red blood cells, *Hb* hemoglobin, *MCH* mean corpuscular hemoglobin, *MCHC* mean corpuscular hemoglobin concentration, *MCV* mean corpuscular volume, *TIBC* transferrin iron-binding capacity, *RN* referents not chewing smokeless tobacco products, *RG* referents chewing gutkha, *RM* referents chewing mainpuri, *RB* referents chewing both gutkha and mainpuri, *OPG* oral cancer patients chewing gutkha, *OPM* oral cancer patients chewing mainpuri, *OPB* oral cancer patients chewing both gutkha and mainpuri, *PPG* pharyngeal cancer patients chewing gutkha, *PPM* pharyngeal cancer patients chewing mainpuri, *PPB* pharyngeal cancer patients chewing both gutkha and mainpuri

Table 2 Comparison of the certified and experimental values of Ni in certified sample of human whole blood and serum (n=6) (μg/L)

Ni	Certified value $\bar{x} \pm s$ [a]	Experimental value $\bar{x} \pm s$ [a]	Paired t test[b] $t_{experimental}$	% Recovery[c]
Whole blood	7.5±1.8	7.41±0.78	0.396	98.8
Serum	8.2±1.9	8.14±1.2	0.487	99.2

[a] Average value with standard deviation

[b] Paired t test between certified values versus found values, degree of freedom ($n-1$)=5, $t_{critical}$ at 95 % confidence limit=2.56

[c] % Recovery = [Experimental values] / [Certified value] × 100

biological samples of referents and patients are shown in Table 4. The resulted data indicated that the concentration of Ni was higher in both biological samples (whole blood and serum) of oropharyngeal cancer patients as compared to that of referents who consumed different SLT products ($p<0.01$) and who have none of these habits ($p<0.001$), whereas the biological samples of referents who have not consumed any SLTs have 2–3 times lower levels of Ni than those of referents who consumed different SLT (gutkha and mainpuri) products ($p<0.01$).

The concentrations of Ni in the whole blood samples of oral cancer patients who consumed SLT products (OPG, OPM, and OPB) were found at 95 % CI (7.97, 8.47), (8.01, 8.46), and (9.21, 10.1), respectively, while pharyngeal cancer patients, PPG, PPM and PPB, have Ni levels (CI 7.91–8.61), (CI 7.80–8.56), and (CI 9.49–10.5 μg/L), respectively, as compared with those values obtained for referents who consumed SLT products; RG, RM, and RB correspond to (CI 3.67, 3.88), (CI 3.31, 3.47), and (CI 3.90, 4.39 μg/L), respectively, ($p\leq0.01$).

An elevated level of Ni content was observed in the serum samples of oropharyngeal cancer patients. The ranges of Ni in the serum samples of oral cancer patients (OPG, OPM, and OPB) and pharyngeal cancer patients (PPG, PPM, and PPB) were found at 95 % confidence limit (CI 6.75–7.15), (CI 6.09, 6.98), and (CI 7.68, 8.45) and (CI 6.75–7.15), (CI 7.48–7.92), and (CI 6.92, 7.42 μg/L), respectively. The levels of Ni in referent group consumed SLT products, RG, RM, and RB, were found to be 2.5–3 times lower as compared to those values obtained for patients ($p<0.01$). The studied biological samples (whole blood and serum) can be used alone or in conjunction with each other, while they both still can be used as a surrogate measure of metal exposure (Barany et al. 2002).

The high levels of Ni was observed in biological samples of oropharyngeal cancer patients consuming both types of SLT

Table 3 Comparison of demographic and lifestyle characteristics between referents and oropharyngeal cancer patients

Characteristics	Total number (%)	Referents	Oral cancer	Pharyngeal cancer
Gender				
Male	32 (100)	150	95	84
Occupation				
Driver	89 (27.1)	33	32	24
Workshop workers	118 (35.8)	51	34	33
Labor	122 (37.1)	66	29	27
Habits[a]				
Chewing gutkha (G)	86 (26.1)[b]	30	29	27
Chewing mainpuri (MP)	69 (20.9)	24	26	19
Chewing both G and MP	114 (34.6)	36	40	38
Chewing none of them	60 (18.2)	60	–	–
Pack year consumption[c]		<10	>25	>25
Gutkha consumer	101 (30.7)	40	31	30
Mainpuri consumer	89 (27.4)	38	30	21
Both GT and MP consumers	139 (42.2)	72	34	33

[a] Consumed smokeless tobacco products

[b] Total referent, oral, and pharyngeal cancer patients

[c] Pack years = (number of SLT sachet consumed per day) × (years consumed). Pack year consumption index expressed as the number of sachet of SLT (~2 g) chewing per day for the number of years/person

Table 4 Adjusted odds ratios for potential risk factors for oral and pharyngeal cancers

Cancer types	Characteristics (whole blood Ni level)	Single-product consumer/both SLT products consumers	OR (95 %)	*p* value
Oral cancer	Low (≤8 μg/L)	15/7	1.00	
	High (≥8 μg/L)	14/33	5.05 (1.69–15.1)	<0.01
Pharyngeal cancer	Low (≤8 μg/L)	18/7	1.00	
	High (≥8 μg/L)	09/31	8.85 (2.81–27.8)	<0.001

(gutkha and mainpuri) products as compared to those patients who were chewing single product. We classified the cancer patients who consumed gutkha, mainpuri, and both SLT products based on the level of Ni in blood samples having value lower or higher than 8-ppb level as shown in Table 4. With multiple logistic regression model, the habits of chewing different SLT products, mainpuri, gutkha, and both, were characterized. Among these three factors, consuming both mainpuri and gutkha presented significant association with oral cancer (OR=5.05, 95 % CI 1.69–15.1), $p<0.01$, and pharyngeal cancer (OR=8.85, 95 % CI 2.81–27.8), $p<0.001$, as compared to consuming single SLT product.

The risk estimates for the consumption of SLT products were also evaluated with respect to intake of Ni via consumption of different SLT products. Due to the consumption of both SLT products (mainpuri and gutkha), the relative risk for oral cancer was OR= 2.28, 95 % CI 1.35–3.86, $p=0.019$, and pharyngeal cancer (OR=3.20, 95 % CI 1.71–5.93), $p=0.03$, as compared to that for single SLT product consumers. During study period, 25 oral cancer patients and 19 pharyngeal cancer patients died. The OR of mortality rate in oral cancer patients was 1.16 (95 % CI 0.598–2.26) as compared to that in pharyngeal cancer ($p=$ 0.65), indicated that there is no significant difference in death rate of both types of cancer patients.

The studied patients mostly belong to low socioeconomic status coupled with lack of health consciousness. This may be due to less social activities, poor quality of food, irregular screening, late diagnosis, and unequal access to health care, deficiency of vitamins, and essential elements especially iron and zinc (Spitz et al. 2004; Kolachi et al. 2012).

The level of Ni in blood and serum samples of pharyngeal cancer patients was slightly higher as compared to that of oral cancer patients, but the difference was not significant ($p>0.05$). The unpaired Student's t test at different degrees of freedom between male oropharyngeal cancer patients and referents was calculated at different probabilities. Our calculated t value exceeds that of $t_{critical}$ value at 95 % CIs, which indicated the significant differences between Ni mean values in oropharyngeal cancer patients and referents who have or have not consumed SLT ($p<0.01$).

Discussion

Optimization of CPE procedure

For the optimization of CPE method, five factors were selected to be examined, volume of surfactant, mass of complexing reagent, pH, incubation time, and temperature. The effect of pH upon the extraction of Ni ions was studied by using six replicate standard solutions of Ni (20.0 μg/L) in the pH range of 3–8, while other parameters were at their optimum levels. The maximum extraction efficiency of Ni was obtained at pH ranges of 5.5–6.5. For subsequent work, pH 6.0 was selected. The APDC was used as the chelating agent due to the high hydrophobic nature of its metal/metalloid complexes. The extraction efficiency of Ni as a function of APDC concentrations of 0.1 to 0.5 % (*w*/*v*) was used. The optimum recovery of Ni was achieved at 0.35 % APDC. The optimum quantity of Ni complex was entrapped in 0.2 % (*v*/*v*) of Triton X114.

The influence of temperature and time for equilibration was investigated by varying the temperature from 40 to 60 °C and 10 to 30 min, respectively. It was observed that optimum recovery of Ni was obtained at an equilibrium temperature of 50 °C after 20 min. To evaluate the selectivity of proposed method for the determination of Ni at trace levels, effect of different interfering ions was investigated. To perform this study, 10 mL of solution containing 20 μg/L of Ni at different interference to analyte ratios was subjected to the developed procedure. The tolerance limits of the interferent ions, the obtained errors, were <5 %. Therefore, the proposed method has good selectivity for studied element.

Correlation of nickel in SLT products and cancer

The rising trend in oropharyngeal cancers incidence is a worldwide public health problem. Many epidemiological studies have reported that oral cancer is strongly associated with smoking and alcohol drinking (Kazi et al. 2010a; Duran et al. 2012). According to survey that carried out about the habits of chewing SLT tobacco, these products were analyzed for Ni contents as reported in our previous work (Arain et al. 2013, 2014). The mean concentrations of Ni in different brands of mainpuri and gutkha were found in the range of 10.1–17.3 and 1.01–2.69 μg/g, respectively. The cumulative chewing dose of Ni was calculated on the basis of these values (Arain et al. 2013). It was reported that the pH of studied SLT

Table 5 Trace Ni concentrations in biological samples (whole blood and serum) of referents and oropharyngeal cancer patients

Sample Ni (μg/L)	Referents				Oral cancer patients			Pharyngeal cancer patients		
Whole blood	RN	RG	RM	RB	OPG	OPM	OPB	PPG	PPM	PPB
	1.45±0.38	3.41±0.46	3.82±0.62	4.14±0.62	8.29±0.71	8.12±1.05	9.40±2.23	8.32±0.76	8.28±1.02	10.1±1.32
Serum	0.89±0.08	2.51±0.80	2.28±0.48	3.27±0.72	6.96±0.62	6.53±0.96	7.96±1.58	7.65±1.04	7.15±0.99	8.53±1.22

RN referents not chewing smokeless tobacco products, *RG* referents chewing gutkha, *RM* referents chewing mainpuri, *RB* referents chewing both gutkha and mainpuri, *OPG* oral cancer patients chewing gutkha, *OPM* oral cancer patients chewing mainpuri, *OPB* oral cancer patients chewing both gutkha and mainpuri, *PPG* pharyngeal cancer patients chewing gutkha, *PPM* pharyngeal cancer patients chewing mainpuri, *PPB* pharyngeal cancer patients chewing both gutkha and mainpuri

products were 8.1–8.7, and at this basic pH, the formation of tobacco specific amines occurs, which makes the SLT products potentially toxic to consumers (Nair et al. 1996). Chewing tobacco may cause local exposure of many organic and inorganic toxicants to oral mucosa (Nair et al. 1996; Petti 2009; Stepanov and Hecht 2005).

In the present study, 100 % of the reported cases (oropharyngeal cancer patients) have habit of chewing SLT products for many years. Results of logistic regression analyses indicated that Ni levels in both biological samples had stronger associations with the consumption of SLT in referents and patients. The Ni levels in blood and serum samples of referents who consumed SLT products were 1.5–2 times higher than those values obtained for nonconsumer referents (Table 5). The Ni levels of blood and serum samples of oropharyngeal cancer patients were significantly higher than those of referents (Table 5, Fig. 1a, b), suggesting a specific influence of carcinogenic effects of Ni on the development of both types of cancer in local population of Pakistan.

Cancer is the most dreaded disease, and it is the second leading cause of death in many developed and developing countries of the world (Asha et al. 2004). Worldwide, oral cancer is a serious cause of morbidity and mortality, and its incidence varies widely according to geographical location. In developing countries including Pakistan, early detection of oropharyngeal cancer, for example, as premalignant oral epithelial dysplasia or early oral squamous cell carcinoma, was not possible probably due to poverty and illiteracy and the

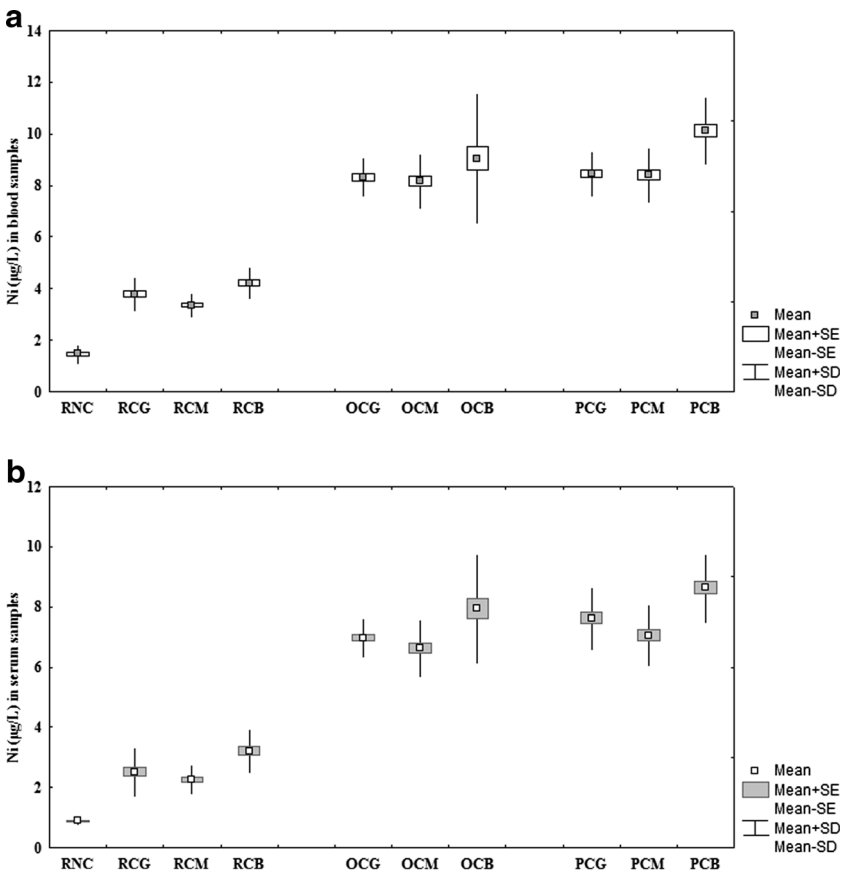

Fig. 1 **a** Concentration of Ni in blood samples of referents (RN, RG, RM, and RB), oral (OPG, OPM, and OPB), and pharyngeal (PPG, PPM, and PPB) cancer patients. **b** Ni levels in serum samples of referents (RN, RG, RM, and RB), oral (OPG, OPM, and OPB), and pharyngeal (PPG, PPM, and PPB) cancer patients. *RN* referents not chewing smokeless tobacco products, *RG* referents chewing gutkha, *RM* referents chewing mainpuri, *RB* referents chewing both gutkha and mainpuri, *OPG* oral cancer patients chewing gutkha, *OPM* oral cancer patients chewing mainpuri, *OPB* oral cancer patients chewing both gutkha and mainpuri, *PPG* pharyngeal cancer patients chewing gutkha, *PPM* pharyngeal cancer patients chewing mainpuri, *PPB* pharyngeal cancer patients chewing both gutkha and mainpuri

patients in initial stage not consulted medical quacks at the onset of the disease (Muir and Zaridze 1986; Trivedi et al. 1996). The International Agency for Research on Cancer recently concluded that there is "sufficient evidence" that the oral use of SLT is carcinogenic to humans (Cogliano et al. 2004). Chewing of SLT products is the most important risk factor for oropharyngeal cancer (Kazi et al. 2010b). From our study, we found that the increasing trend in consumption/prevalence of SLT products by every age group in the last many decades could be responsible for the substantial increase in oropharyngeal cancer incidence especially in Asian countries, including Pakistan and India. Poor oral cavity hygiene and ill-fitting denture and SLT consumption can increase the risk of developing oral cancer. People chewing SLT products are over five times more likely to be at risk of oral cancer (Savino et al. 2007).

The toxic elements (As, Cd, and Ni) are generally associated with increased risk of many cancers in human (Goyer 1996; Hu. 2002; Verougstraete et al. 2003), and each of them has been designated as a group 1 human carcinogen by the International Agency for Research on Cancer (Hayes 1997; IARC 1999). Chronic exposure to heavy metals has long been known to increase cancer incidence among affected individuals (Hu 2002). The mechanisms of heavy metal toxicity through electron transfer most often involve the cross-linking of sulfhydryl groups of proteins. Ni and other heavy metals can also generate free radicals directly from molecular oxygen in a two-step process to produce superoxide anion which combines with protons in the dismutation reaction to generate hydrogen peroxide in the process (Chen et al. 2002; Spitz et al. 2004). Certain metals which exist in SLT products (Al-Rmalli et al. 2011), ingested during chewing, can cause serious diseases, including head and neck cancer (Kazi et al. 2010b; Khlifi et al. 2013b).

With regard to the habit of chewing different SLT products (gutkha and mainpuri), the referents and patients gave information that they use 2–10 packets (2–5 g) per day, kept in the mouth for 30 min to 1 h, chewed, and mostly swallowed; only 5 to 10 % claimed that they spitted out while the patients suffering from oropharyngeal cancer informed that mostly at night, they kept SLT in mouth and sleep without using any mouthwash. Therefore, it appears that more amounts of SLT contents are absorbed by buccal mucosa or posterior region of the mouth. It was also observed that most of the oral cancer patients have fibrosis on one side of the buccal vestibule, where they kept SLT products for long time. It was observed that oropharyngeal cancer patients, who had consume gutkha and mainpuri products, were not aware of the symptoms till the severity got developed. Some of the patients left the habit after knowing about the harmful effects of gutkha and mainpuri.

Conclusion

This study provides some support for the hypothesis that intake of Ni through chewing different SLT products (gutkha and mainpuri), alone or in conjunction with each other for long time, may increase the risk of oropharyngeal cancer and related disorders. The levels of Ni in the blood and serum of oropharyngeal cancer patients were 2–3 and 4–5 times higher than those of referents who consumed different SLT products and who have none of this habit, respectively. However, the results of our study revealed the significant differences in the concentration of Ni in biological samples of oropharyngeal cancer patients between healthy referents who consumed or have not consumed any SLT products. Since the roles of Ni in the mechanism of oropharyngeal cancer development are still unclear, further detailed and comprehensive investigations are necessary.

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