

Evaluation of Serum Copper Level in Naswar (Smokeless Tobacco) Addicts Using Flame Atomic Absorption Spectroscopy

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Abstract

Background: Substance abuse and its consequences are a matter of great concern in South-East Asian countries, especially Pakistan and Afghanistan. Due to contamination of Naswar with copper, or by any other physiological mechanism, there is a great chance of copper poisoning in addicts. Keeping in view the literature, there is no comparative study on serum copper levels (SCuL) in Naswar addicts. Therefore, it is very important to evaluate SCuL in this population.

Methods: The current study was performed in September 2016 where a total of 75 volunteers (selected from villages near the University of Peshawar, Pakistan) were grouped as Naswar addicts (n = 45) and the control group (n = 30). The addicts were compared to controls for age, weight, BMI, FBS and creatinine clearance. For serum analysis, about 3mL of blood was collected from which the serum was separated, digested, and then evaluated for SCuL using flame atomic absorption spectroscopy at the Department of Pharmacy and Centralized Resource Laboratory of University of Peshawar.

Results: The SCuL in addicts had a range of 2.6 to 11.1 µg/dL with a mean of 5.1 ± 2.4 µg/dL. In the healthy control group, SCuL was between 1.7 to 3.9 µg/dL with a mean of 2.6 ± 0.1 µg/dL. The mean difference between the two study groups was statistically significant (P = 0.005); the duration of addiction and quantity of drug consumptions per day correlated positively with SCuL.

Conclusion: Serum level of copper in Naswar addicts is elevated compared to the Control group. The exact cause of SCuL elevations and the mechanisms involved must be studied in large group samples.

Keywords: Addiction, Naswar, oxidative stress, Pakistan, poisoning, smokeless tobacco

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Introduction

Copper is an important heavy metal mostly found in a variety of rocks and minerals. It is one of the essential micronutrients present inside the human body with an oxidation state of Cu(I) and Cu(II).¹ Normally, copper is bound to proteins, but it may be released resulting in the catalysis of reactive hydroxyl radicals.² As a cofactor of many enzymes involved in redox reactions, it can induce oxidative stress by either catalyzing the ROS formation directly via Fenton like reaction or by decreasing the glutathione concentration in the body. Glutathione is a powerful cellular antioxidant that functions in intracellular copper metabolism and detoxification.³⁻⁶

Manifestations of copper poisoning (copperiedus) may be acute or chronic. Acute symptoms include hematemesis, melena, jaundice and anemia while chronic manifestations are renal and hepatic damage. Penicillamine and dimercaprol are the antidotes of choice for treating copperiedus.⁷

Drug addiction is a relapsing and chronic disorder characterized by persistence in compulsive drug taking and seeking behavior regardless of significant negative outcomes.⁸ It has afflicted mankind for centuries; however, the exact mechanism by which

certain substances cause addiction is still elusive.⁹ Advancements in neuroscience research is unscrambling, in general, the process complexity underlying drug addiction and, in particular, drug abuse.¹⁰ Use of addictive substances has led to an increase in local and systemic complications in drug abusers.¹¹⁻¹³ One of the abused drugs is Naswar, which is smokeless tobacco snuffed in the floor of mouth under the lips; it is mostly consumed in South-East Asian countries such as Pakistan, Afghanistan, Iran, Turkmenistan, India, etc. It has been estimated that more than 200 million people in South Asian countries use smokeless tobacco products with a prevalence of about 13% of population in Pakistan using Naswar, Gutkha, Paan, etc. which are a major cause of upper gastrointestinal tract malignancies and cardiovascular disorders.¹⁴⁻¹⁶

The basic aim of the present study was to analyze the serum levels of copper in Naswar addicts, and to compare their values with those of a healthy control group.

Materials and Methods

This cross-sectional study was performed in September 2016 at the Department of Pharmacy, University of Peshawar. Subjects for this study were selected from different villages surrounding the University of Peshawar, Pakistan. A total of 75 subjects were enrolled in the study using non-probability convenience sampling technique. The subjects were enrolled according to these criteria; male, age >20 years, no history of medicine use in past two weeks, no major clinical ailment, and willingness to give written informed consent after discussion of the study protocol.

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Table 1. Spectrometric parameters for SCuL analysis.

Parameter	Value
Air (L/min)	17
Acetylene (L/min)	2.3
Lamp current (A)	15
Slit width (H)	0.7
Wavelength (nm)	283.3

Table 2. Characteristics of the study subjects.

Parameters	Naswar addicts (n = 45)	Control group (n = 30)	P-Value
	Mean ± SD		
Age (Years)	33.53 ± 5.75	36.83 ± 1.39	0.127
Body mass index (kg/m ²)	24.83 ± 5.09	22.32 ± 3.73	0.023*
Fasting blood sugar (mg/dL)	95.5 ± 27.0	91.83 ± 8.8	0.454
Creatinine clearance (mL/min)	119.1 ± 27.35	105.4 ± 19.2	0.063
Systolic blood pressure (mm of Hg)	119 ± 10.13	126.9 ± 9.19	0.031*
Diastolic blood pressure (mm of Hg)	79.67 ± 9.93	82.26 ± 5.49	0.042*
Duration of addiction (years)	14.37 ± 7.23	—	—
Quantity of Naswar per day (grams)	60.5 ± 16.10	—	—

*Statistically significant

Table 3. SCuL analyses in Naswar addicts and control group.

Study Groups	N	Serum Cu levels (µg/dL)				P-Value
		Min	Max	Mean	SD	
Naswar addicts	45	2.6	11.1	5.1	2.4	0.005*
Control group	30	1.7	3.9	2.6	0.1	

* Statistically significant

Exclusion criteria included: age <20 years, multiple addictions, malignancies, hematological disorders, and diabetes. Duration of Naswar consumption among the subjects ranged 4–30 years. Controls included age and area-matched non-addicts with neither current nor past history of regular addiction.

In this study, fasting blood samples were collected from the study subjects, i.e. Naswar addicts (n = 45) and the controls (n = 30) in the ethylene diaminetetraacetic acid (EDTA) tube (≈3 mL) by venous puncture. Verbal and written consents on informed consent form were taken and the protocols complied with the Helsinki Declaration 1964¹⁷ and guidelines for good clinical practice E6 (R1)¹⁸ that were approved by the Ethical Committee of Department of Pharmacy, University of Peshawar, Pakistan.

The collected blood samples were centrifuged for serum separation using Centurion Centrifuge machine (Centurion Scientific Ltd), UK. The serum was then digested by adding 5 mL of HNO₃ to 1 mL of serum and heated at 60°C in water bath for 2 hours; then, 5 mL of Perchloric acid was added to the same sample and heated again at the same temperature for 30 minutes. Final volume of the samples (=10 mL) was made with de-ionized water before analyses. Keeping in view the sensitivity and simplicity, an atomic absorption spectrophotometer (Model Analyst 700 Perkin Elmer, Inc, U.S.A.) with automatic background correction and hollow cathode lamp (C-HCL lamp) was used for measurement of serum copper level (SCuL). Other specifications of the spectrometer are given in Table 1.

Statistical analyses were carried out using the GraphPad Prism biostatistical software package (version 5.01, GraphPad Software, Inc., CA, USA). All results are expressed as mean and standard deviation (mean ± SD) and were compared between the two

groups using unpaired two-tailed student *t*-test. P value < 0.05 was considered as statistically significant.

Results

In the current study, a total of 75 volunteers were enrolled that were grouped in two categories; the Naswar Addicts (n = 45) and the control group (n = 30). Significant differences were observed in height, weight, BMI, systolic blood pressure (SBP), and diastolic blood pressure (DBP) while no significant difference was observed in age, fasting blood sugar and creatinine clearance. Minimum duration of addiction was five years with the lower limit of quantity of *Naswar* consumption being 40g/day (Table 2). *Naswar* from different brands was used by the addicts and the mostly used *Naswar* was “Bannusi *Naswar*”.

Spectrophotometric analysis via flame atomic absorption spectrophotometry of the serum sample revealed that a significant increase ($P = 0.005$) in SCuL in Naswar addicts compared to the healthy volunteers with serum levels of 5.1 ± 2.4 µg/dL and 2.6 ± 0.1 µg/dL, respectively (Table 3). A significant correlation was observed between the duration of addiction and quantity of *Naswar* consumption per day to SCuL with R-squared linear values of 0.2465 ($P = 0.027$) and 0.08 ($P = 0.044$), respectively.

Discussion

Naswar is actually smokeless tobacco that consists of damped tobacco leaves, calcium oxide, cardamom and slaked lime. It is prepared by pressing and rolling the constituents repeatedly until a soft mass is obtained. Sometimes, flavors and coloring agents

are also added to increase its palatability. The use of Naswar relies on its venous absorption from the buccal mucosa and thus has slow action systemically. However, the nicotine effect can occur within 5–10 minutes.¹⁹ Due to its affordability and availability, Naswar is commonly used by almost every socioeconomic group of people. Other factor that encourage the people to use smokeless tobacco is some misconceptions that it is safer than cigarette smoking with some medicinal effects such as for treatment of headache and toothache.¹⁹

Various studies have been published worldwide to measure SCuL for copper toxicity in different populations such as patients with Rheumatoid arthritis,²⁰ Alzheimer's disease,²¹ Tobacco smokers,²² etc. This study focuses on measuring SCuL in Naswar addicts and correlating its toxic effects in the living system. Though pure Naswar does not contain any major ingredients with copper, its contamination is possible due to many reasons. Either it is contaminated by the smugglers or drug dealers with lead or copper to increase its weight or during its production, certain other unintentional factors are involved due to which Naswar is contaminated.²³ Furthermore, nicotine or other constituents of smokeless tobacco or their metabolites may alter the SCuL by altering the metabolic pathways of copper or its binding to the respective sites such as proteins and enzymes. Excess copper concentrations in body may results in aging, liver cirrhosis, Alzheimer's disease, and Wilson's disease.²⁴

Excess copper can induce oxidative stress by many mechanisms but two major mechanisms have been reported extensively. First, it can catalyze the formation of reactive species through Fenton like reaction that causes peroxidation of lipids resulting in increased levels of Hydroxynonal (HNE) that acts as a second messenger in oxidative stress damage^{5,25} and secondly, its increased levels may decrease the levels of glutathione, homocysteine and other thiol oxidants which results in an imbalance between the oxidants and antioxidants.^{26–28} Such mechanisms result in different disease conditions such as cancer, Parkinson's disease, diabetes, and atherosclerosis.¹

The major limitation of this study was the identification of confounders of copper intake by the addicts. Keeping in view the strict matching of the two study groups, the effect of confounders shall be neglected as both groups belonged to same area using the same resources with almost matching lifestyle. We were also unable to access the female population due to cultural limitations. The findings of the current study should be generalized carefully because increased SCuL in Naswar addicts depends on the contamination of Naswar by copper or there should be a further study conducted for the possible molecular mechanism involved in increasing the levels of copper either by decreasing its metabolism and excretion or by removing the bounded copper from the binding sites.

In conclusion, the findings of this study reveal an elevation in serum levels of copper in Naswar addicts compared to healthy controls. Therefore, the risk of copper toxicity is higher in Naswar addicts and copper screening may be helpful in any type of diseased status of the subjects. Further investigations are required in large population samples to confirm the significance of the study.

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