ORIGINAL ARTICLE

SALIVARY BIOMARKER INTERLEUKIN-8 LEVELS CORRELATED WITH DIFFERENT PARAMETERS OF NASWAR (SMOKE LESS TOBACCO) USAGE

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ABSTRACT

Use of smokeless tobacco is more dangerous than smoked tobacco. Objective of current cross sectional study was to find out the levels of interleukin-8 cytokine in naswar users and its stratification according to age of user, type, frequency, duration and site of placement of naswar. A total of 30 naswar users were recruited. Un-stimulated saliva samples were collected and analyzed by using an enzyme-linked immunosorbent assay (ELISA) technique. Data was entered in SPSS version 22. Age, duration of use of naswar, frequency of use, type of naswar used and site of placement of naswar in oral cavity were analyzed and one way ANOVA test followed by post hoc tukey analysis and Pearson correlation was also applied.

Levels of salivary IL-8 were found to be 173.48±46.52pg / ml. The mean duration of naswar usage was 7.17±6.33 years. No statistical significant difference was found in any parameter other than frequency of naswar usage in which p value (0.037) is less than 0.05. Pearson correlation analysis revealed that the age and duration of naswar usage, were not correlated to the levels of salivary biomarker IL-8. A higher level of IL-8 was found in naswar users. The statistically significant association was found between the levels of salivary biomarker IL-8 and frequency of naswar usage. On the other hand no association or correlation were found between IL-8 and age, duration, type and site of naswar placement

Key Words: *Interleukin 8, dipping tobacco, association, biomarker, oral cancer, ELISA*

INTRODUCTION

Oral cancer, being a major concern for the dentists for the past many decades, is a public health issue in both developed and under developed countries. World Health Organization has declared that oral carcinoma holds the place of being sixth and tenth most prevalent cancer in men and women respectively in the underdeveloped nations.[1] It is evident from the literature that 5% of all the human malignancies is constituted by oral cancers.[2] Among all the cancers 40% of oral cancer occur in South Asia but particularly in India and Pakistan it is up to 8-10%.[3] The prevalence of oral malignancy in Karachi, Pakistan is ranked at a highest level around the globe.[4] According to a study an increasing trend of oral cancer has been observed in Pakistan, Taiwan and Thailand.[5] The World Health Organization (WHO) predicts that the prevalence of oral cancer will increase worldwide in the next coming few decades.[6] Moreover, erythroplakia and leukoplakia are the commonly occurring potentially malignant epithelial lesions of the oral cavity with increased malignant transformation rate as 15–20% progress to oral cancer.[7]

The causative agents of this silent predator includes pan, chalia, naswar, smoking, alcohol and various viral agents, nutritional deficiencies and genetic predisposition.[8] Tobacco is one of the known carcinogenic agents for oral cancer. The risk of development of oral cancer in the people who use tobacco is 11 times more than the non-users. Moreover, smokeless tobacco (SLT) users are at a higher risk as compared to those who use smoking

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tobacco.[5] One third of the world's population consume tobacco products which are divided into smoking (bidis, cigarette, shisha, hookah) and smokeless tobacco. In Pakistan, 40% of the population of Karachi has shown to use at least any one form of tobacco products on a daily basis.[4] Naswar or oral snuff, a type of smokeless tobacco is described as an important risk factor. The dry snuff is in a powdered form used by inhaling while the moist form of tobacco called as dip or quid placed in the mouth. In Pakistan, the Pushtoon ethnic group use it widely.[9] About 28 known carcinogens are shown to be the constituents of smokeless tobacco. These carcinogens are the nonvolatile and volatile tobacco related nitrosamines, aldehydes and poly nuclear substances.[10]

Despite advances in the diagnostic and prognostic aids, the five year survival rate of the patients suffering from squamous cell carcinoma has not been increased above 60%.[7] More than two third of the oral and pharyngeal cancers are diagnosed at the advanced stage which then require a very aggressive treatment leading to reduced quality of life.[11] The detection of oral cancer in initial stages is very necessary to enhance the survival rate of the patient. Thus, the absence of definitive early cautionary signs and symptoms for cancer recommends the need to discover sensitive and specific biomarkers for screening high risk population.

Nanobiomaterials in clinical dentistry have made saliva a new emerging bio fluid for clinical diagnosis making this an era of salivary diagnostic assay. Within the last 15 years genomics, transcriptomics, proteomics, metabolomics and metagenomics have assisted in figuring out different veiled configurations, components and mechanisms of dental hard and soft tissues.[12] Various studies have undergone the process of investigating the circulatory tumor biomarkers for OSCC and have shown that a moderately acceptable sensitivity and specificity exists for the role of biomarkers in diagnosis, prognosis prediction and management protocols.[13, 14] Although, in comparison to the soft and hard tissue biopsy the body fluids have gained more importance to identify reliable and suitable biomarkers but histopathology remains the gold standard in the diagnostic realm. However it is imperative to examine a molecular biomarker profile of an individual in combination with other clinic-pathological information which can assist to better characterize the oral cavity tumors and then predicting the individual's treatment progress.[15]

Cytokines are cell to cell signaling proteins that demonstrate a significant part in normal cell growth and multiplication, angiogenesis and repairing of tissue. Interleukin 6 and interleukin 8 are the much studied cytokines as they are essential mediators of cancer development and powerful activators of apoptotic and anti-apoptotic signaling cascade.[16] IL-8 is found to stimulate angiogenesis, influence tissue remodeling, leukocyte chemotaxis, dysregulated cell cycle, suppressed immune system and regulating of cell proliferation and differentiation.[17] Various stimuli for example chemical and environmental stresses including chemotherapy and hypoxia, proinflammatory cytokines like TNF-α and IL-1, hormones and bacterial and viral products are known to regulate the manifestations of IL-8.[18]

In light of the previous study done comparing the levels of interleukin-8 in naswar users and non-users to propose an early screening test for oral squamous cell carcinoma in high risk groups, the present study was planned to correlate any change in the levels of interleukin-8 cytokine with different parameters like age of user, type, frequency, duration and site of placement of naswar in our population.

MATERIALS AND METHODS

A cross sectional study approved by the Institutional Review Board was conducted from August 2016 to July 2017 in a multicenter setting. The total sample size of the naswar users was calculated using WHO calculator at confidence level of 95%, anticipated population proportion of 40% with absolute precision of 0.20 and found to be 24 which was rounded to 30. Non-probability, convenience sampling technique was used. The study included males between the ages of 20 to 60 years using naswar for at least past one year or more. The study excluded the individuals having previous history of dermal, rheumatic, cancerous, diabetes, cardiovascular, dysplastic or any acute viral condition, immunodeficiency, periodontal disease (Basic Periodontal Examination score 3 and 4), insufficient salivary flow rate, history of consumption of alcohol or tobacco (any form: dry snuff, paan, smoking) and any previous or ongoing radiotherapy and chemotherapy.

After taking the consent from the participants, history and clinical examination was carried out to fulfill the inclusion criteria. Age, duration of use of naswar, frequency of use, type of naswar used and site of placement of naswar in oral cavity were noted. A morning sample of unstimulated whole saliva was collected by saliva collecting device according to manufacturer's instructions (Pure•SAL™, Oasis Diagnostics® Corporation, Vancouver, USA). Collected samples were tested for levels of interleukin-8 cytokine by enzyme-linked immunosorbent assay (ELISA) procedure according to the manufacturer's instructions (PicoKine™, Boster Biological Technology, Pleasanton, USA).

Age of the participants were stratified into the brackets of 10 years. Duration of naswar usage were further divided into participants using naswar for <5 years, 6 to 10 years and >10 years. Similarly, type of naswar used were further categorized into black type,

green type and a new filtered type of naswar. Frequency of naswar use were noted as 10 times or less per day, more than 10 times per day, and whole day. Site of naswar placement in oral cavity was also noted.

The gathered data was entered and analyzed in SPSS version 22.0. Descriptive analysis was done for the age, IL-8 levels and duration of use of naswar. Frequencies were also calculated regarding the type, frequency of naswar used, and site of placement. One way analysis of variance (ANOVA) followed by post hoc analysis was used to find any association between the IL-8 levels and the under study naswar usage parameters. Pearson correlation coefficient was also calculated to evaluate the correlation between the levels of IL-8 and duration of naswar usage and age of the participants. A p-value equal or less than 0.05 was considered significant at 95% confidence interval.

RESULTS

Thirty male participants between the aged from 22 to 49 were recruited in this study (n=30). The mean age and standard deviation was 27.67±5.69 years. Levels of salivary IL-8 were found from 100.73pg/ml to 263.93pg/ml with mean and standard deviation of 173.48±46.52pg/ml. The mean duration of naswar usage was 7.17±6.33 years as shown in table 1. The black type of naswar was used by 83.3% as compared to green type (13.3%) and new filtered type (3.3%). Frequency

TABLE 1: DESCRIPTIVE ANALYSIS OF AGE AND INTERLEUKIN 8 (IL-8) LEVELS AND DURATION OF NASWAR USAGE

	Age of Participant (Years)	IL-8 levels (pg/ml)	Duration of Naswar Usage (Years)
n	30	30	30
Mean	27.67	173.48	7.17
Std. Deviation	5.63	46.52	6.33
Range	27	163.20	29
Minimum	22	100.73	1
Maximum	49	263.93	30

TABLE 2: ASSOCIATION BETWEEN THE IL-8 LEVELS AND DIFFERENT PARAMETERS OF NASWAR USAGE

		n	IL-8 (pg/ml)	P-value*
Duration of Naswar usage	<5 years	15	163.97	0.547
	6-10 years	10	181.97	
	>10 years	5	185.15	
Frequency of use of Naswar	10 times or less/day	6	165.79	0.037
	More than 10 times/day	11	200.82	
	Whole Day	13	153.90	
Type of naswar	Black	25	171.12	0.200
	Green	4	202.93	
	New (filtered)	1	114.84	
Age of Naswar users	20-30 years	24	171.64	0.154
	31-40 years	5	196.89	
	41-50years	1	100.73	
Site of Placement of Naswar	Lower left buccal mucosa	5	158.77	0.341
	Lower right buccal mucosa	3	175.14	
	Upper left buccal mucosa	13	171.36	
	Upper right buccal mucosa	8	194.60	
	Lower labial mucosa	1	100.73	

* One way ANOVA test applied

TABLE 3: PEARSON CORRELATION BETWEEN IL-8 LEVELS AND AGE, DURATION OF NASWAR USAGE

	r*	P-value
Age	-0.099	0.604
Duration of use of naswar	0.007	0.970

*r= Pearson correlation coefficient

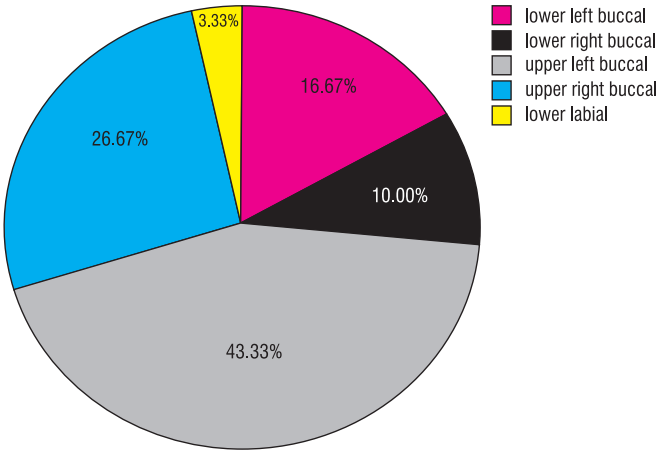

Fig 1: Frequency of site of naswar placement

analysis revealed that 43.3% kept throughout the day while 36.7% more than 10 times a day and 20 % kept it 10 times or less per day. Site of naswar placement is shown in figure 1.

When one way analysis of variance (ANOVA) followed by post hoc tukey analysis was used to find any association between the IL-8 levels and the understudy naswar usage parameters, no statistical significant difference was found in any parameter other than frequency of naswar usage in which p value (0.037) is less than 0.05 as shown in table 2. Post hoc analysis showed difference in more than 10 times and whole day.

Correlation (Pearson correlation) analysis revealed that the age and duration of naswar usage, were not correlated to the levels of salivary biomarker IL-8 as shown in table 3.

DISCUSSION

The predictive indicators of oral cancer can be divided into morphological and molecular level. These biomarkers can be considered in proteomics or genomics targets. Different proteomic tools have been designed to analyze the body fluids like blood, saliva, serum, gingival crevicular fluids etc. Interleukins stimulate cancer cell growth. IL-8 functions as an important regulator of the tumor microenvironment because its expression is enhanced via cancerous cells. IL-8 is also associated with other local and systemic illnesses as well for example periodontitis, diabetes and heart disease.[19] Most studies have been conducted regarding the assessment of the levels of interleukins in oral precancerous and cancerous lesions but less is known about the association of the risk factors of the oral cancer like naswar with the interleukins particularly IL-8. In literature very few clinical studies are present which involve the smokeless tobacco products in relation with IL-8. Other studies trying to correlate smokeless tobacco use with the pro inflammatory cytokines showed mixed results depending on the increased or decreased levels of cytokines.

The habit of using snuff is a part of the culture and inculcated in the lifestyle of the pushtun ethnic group in Pakistan. A pinch of naswar is about 1g and it is placed several times during the day. It is made from grounded or pulverized sun dried tobacco leaves which can either be used in dry or moist form and these are mixed with calcium hydroxide. By adding water this substance is given a form of balls and packed into a tea bag sized polythene bags. It is placed between the labial or buccal mucosa and gingiva, allowing absorption of nicotine through the oral tissues. This is available in tin cans or pouches.[9] The present study focused on finding any association between the levels of IL-8 with different parameters regarding the naswar use in our society. Levels of salivary IL-8 in naswar users were found to be 173.48±46.52 pg/ml.

The studies suggested that the frequency and duration of use of SLT has strong association with development of oral cancer.[10,20] In present study frequency of naswar usage also showed statistically significant association with the levels of IL-8. Post hoc analysis revealed that difference is in more than 10 times and whole day users.

The levels of IL-8 increased with the increased duration of use of naswar. The black and green types of naswar are more commonly used with different brand names, with black one being more strong and injurious to health as it contains more amount of nicotine and high pH value. A new form of naswar by the brand name of Tara is now circulating in the market which is even more filtered but serves the purpose. As from the literature when tobacco is placed adjacent to the gingiva, gingival recession occurs frequently resulting in epithelial attachment loss. Smokers experience widespread periodontal destruction but in case of SLT use adverse effects are seen at the site of placement resulting in gingival recession and appearance of white lesions.[21] Although the site of placement is different among SLT users but this epithelial attachment loss occurs adjacent to mandibular buccal vestibule in most of the SLT users because smokeless tobacco products are most commonly being placed in this location unlike the result of our study in which upper left buccal

vestibule is the most common site.[22] Keeping in mind that IL-8 levels are increased in periodontal disease, good periodontal status was given importance in our study to remove any alteration in the levels of salivary IL-8 due to periodontal diseases.

Jacob et al. examined the effect of another smokeless tobacco product named gutka on certain cytokines including IL-1β and IL-8. The IL-1β and IL-8 levels among the gutka users with periodontitis subjects were 369.01±273.44 pg/μL and 205.97±196.78 pg/μL respectively. The study by evaluating the level of interleukin-1β and 8 in the rural Indian population documented that there is no effect on the proinflammatory cytokines. The study also documented that the average duration of gutka chewing was 11.3±9.6 years and the duration of gutka placement in the mouth was 32.6±7.9 minutes. A positive correlation was found between the IL-8 levels and the duration of gutka chewing ($r=-0.64, P<0.01$), unlike the present study.[23]

Johnson et al reported that the presence of elevated Interleukin-1 levels at the habitual site of smokeless tobacco placement suggests that tobacco components exert a localized effect on IL-1 production. SLT contains numerous irritants including nicotine and known carcinogens. The mechanical and chemical injury caused by these irritants can induce cytokine synthesis. The raised levels of IL-1 at the habitual placement sites of ST may signify its possible role in ST-induced oral mucosal lesions. The smokeless tobacco users in present study had used snuff for an average of 14.11±1.5 years and retained tobacco orally for 10.42±1.21 hours per day.[24]

Another study by Pandey et al evaluated the use of snuff on the levels of IL-1 □ and IL-8 in the gingival crevicular fluid of periodontal patients. In this study the average consumption of snuff by snuff users group was 17.1±8.4 years and average duration of snuff placement was 90.5±8.6 minutes per day.[25] Similarly a local study assessed the proinflammatory interleukins IL-1 □ and IL-6 and thyroid function in naswar users. The study also assessed the impact of duration of naswar usage on the levels of the interleukins. There was not any association found.[9]

Some in vitro studies showed results in contrast to the findings of our research, stating that nicotine didn't affect IL-8 levels.[26] The effect of nicotine on reconstituted oral mucosa model was demonstrated by an in vitro study in which application of different doses of nicotine after 5 min and 24 hours showed that there was no effect of different concentrations of nicotine on IL-1□, IL-6, IL-8 release but GM-CSF showed some peculiar changes at different levels of high dosage nicotine exposure.[27] In this study the effect of nicotine on self-made human epithelium model was negligible because of the fact that in human oral cavity nicotine is absorbed in the deeper epithelial layers displaying many adverse effects. As in the case of our study, the subjects were significantly exposed to high nicotine levels in naswar for prolonged duration of several years thus resulting in high levels of salivary IL-8 demonstrating a vast difference between the human oral mucosa and experimental epithelial model.

In present study sample size was less but increased level of IL-8 were found. It is recommended that study with larger size and wide spread sample with controls will be conducted to evaluate the accurate association and correlation of IL-8 and naswar specially frequency of naswar placement daily.

CONCLUSION

A higher level of IL-8 was found in naswar users. The statistically significant association was found between the levels of salivary biomarker IL-8 and frequency of naswar usage. On the other hand no association or correlation were found between IL-8 and age, duration, type and site of naswar placement.

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