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Salivary Copper Element Activity in Oral Submucous Fibrosis: A Biochemical and Clinicopathological Study

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Abstract

Introduction: There has been a subtle yet steady augmentation in oral submucous fibrosis, a potentially malignant disorder, both in India and across the world. Various efforts are being made to develop dependable and non-invasive biochemical tests for the early detection and diagnosis of this disorder. Of late, the role of specific salivary biomarkers has been studied comprehensively.

Methods: In this study, the level of copper element, a specific salivary biomarker, was assessed among 160 subjects (i.e. 80 oral submucous fibrosis patients and 80 healthy controls). Their levels were compared with different grades of oral submucous fibrosis and the duration of habit.

Result: The results revealed a positive correlation between the parameters in saliva samples of the two studied groups. There was significant increase in salivary copper levels in oral submucous fibrosis patients when compared to normal healthy individuals ($P < 0.001$). Positive relation was observed between those who had submucous fibrosis and with the duration of areca nut chewing.

Keywords: *Saliva; Salivary Biomarkers; Oral Submucous Fibrosis; Areca nut; Copper element*

Abbreviations

SCC: Squamous Cell Carcinoma; HNCA: Head and Neck Cancers; OSMF: Oral Submucous Fibrosis; Cu: Copper element

Introduction

In the present scenario of urbanization and technical revolution, mankind appears to have been placed on a roller coaster of up- to- the minute technology and development. In order to keep pace with the express escalation, he/she is subject to anxiety and pressure. Most of them, unfortunately, react to this stress and become enslaved to regretful habits like paan, betel chewing with or without tobacco, smoking and alcoholism. These habits, though believed to alleviate tension to some extent, inevitably produce detrimental and addictive effects on the body- especially the oral cavity [1,2].

Squamous cell carcinoma (SCC) of the head and neck region is the sixth most common cause of cancer-related deaths in the world [3]. In India, head and neck cancers (HNCA) account for 30-40% cancers at all sites with about 700,000 - 900,000 new cases diagnosed every year with SCC of the head and neck region is the third most common cancer, which affects the oral cavity, oropharynx, hypopharynx, and larynx [4,5].

In Madhya Pradesh alone, almost 15% cancer cases had been reported in just 9.8% of the total population [6]. The risk of an individual with pre-cancer for developing oral cancer is 69 times higher compared to those who do not have precancer [7]. Clinically visible lesions

are non- cancerous to begin and with a varying length of time may precede oral cancer. Thus, "Potentially Malignant Disorders"/ "Epithelial Precursor Lesions" are described as lesions which may an increased potential for malignant transformations [8].

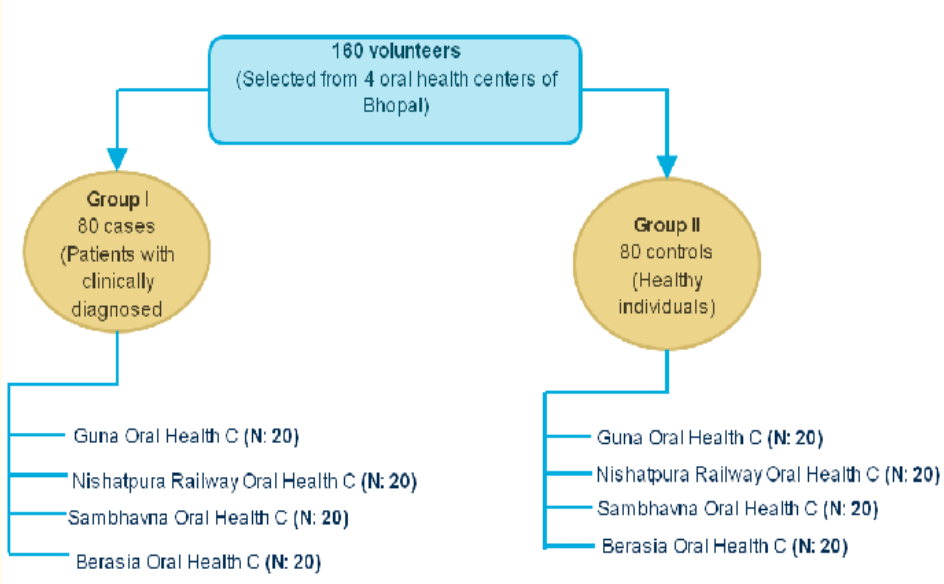
Oral Submucous Fibrosis is a potentially malignant disorder prevalent among Indian population and has a higher risk in developing carcinomas than those without this disease [8]. There is therefore, an escalating concern to recognize this disorder in its early stages for effectual management, better prognosis and helps to reduce mortality and morbidity of such cases.

Studies have reported the diagnostic significance of saliva in identifying certain systemic diseases including oral cancer and precancers. Copper is one such important trace element that may play a significant role as a salivary tumor marker. Saliva, unlike blood, is an easily obtainable, highly sensitive and non-invasive medium for investigation. It could further aid as a deterrent in continuing the habit [9]. There is, however, a dearth of adequate literature in the proposed field of investigation i.e. salivary copper activity in Oral Submucous Fibrosis patients of Bhopal.

The Oral Physician is usually consulted for a wide-range of dental and oral health problems and is therefore the best clinician to screen patient's oral mucosa for early signs of oral cancer or precancer. He or She has the sole responsibility to differentiate benign from precancerous and malignant lesions and also to detect a malignant neoplasm while they are still asymptomatic, innocuous and unsuspected.

Materials and Methods

Bhopal, the capital city of Madhya Pradesh, India, was selected as the area of research. The patient pool comprised of volunteers from the of oral health centres of Bhopal. The study was approved from the university's institutional review board and the participants had signed a consent form authorizing their voluntary involvement in the research. The survey period extended over a period of two years and six months, i.e. from June 2013 to December 2015. The total sample size of 160 patients was divided equally into two groups i.e. Group I and Group II. Group I/Case comprised of patients with clinically diagnosed oral submucous fibrosis while Group II/ Control comprised of 80 apparently healthy volunteers. Both, case and control were selected from 4 oral health centers where 40 individuals (20 case and 20 controls) were taken from each center.



Both Group I and Group II patients were in age group of 15 to 65 years. Group I/Study Group had a habit history of areca nut chewing and clinically confirmed oral submucous fibrosis. They were clinically categorized into three stages based on the classification given by Khanna JN and Andrade NN, Bailoor D, and Nagesh KS and More CB, Gupta S, Joshi J and Varma SN [10-12]. Patients with any other oral mucosal change other that those related with oral submucous fibrosis. Subjects suffering from systemic conditions and with a history of previous malignancy were excluded. Group I was further divided into 4 groups based on the duration of habit: < 6 months, 6-12 months, 12-24 months and > 24 months.

Sialo chemical Analysis for Copper Element

Unstimulated whole saliva was collected between 9 am to 12 pm from each volunteer by the spit method into sterile test tubes [13]. The samples were collected over a period of 10 minutes. These were stored in a polystyrene box containing dry ice and sent to the laboratory for analysis. Here they were centrifuged at 3,000 rpm at 4°C for 30 minutes and assayed for copper element using by colorimetric method with the help of copper kit manufactured by TULIP Diagnostics (P) ltd. Coral Clinical Systems. It was based on the principle of reduction of pyruvate to lactate in the presence of NADH by the action of LDH. The pyruvate that remains unchanged reacts with 2, 4-dinitro phenyl hydrazine to give the corresponding phenyl hydrazone, which is determined colorimetrically in an alkaline medium.

Results and Discussion

The study design was a *Prospective Case-Control* study where *Stratified Random Allocation Sampling Method* was used. The sample size was calculated using the following formula:

$$n = \frac{t^2 \times p(1-p)}{m^2}$$

where

n = required sample size.

m^2 t = confidence level at 95% (standard value of 1.96)

p = estimated prevalence of depressive illness in the project area

m = margin of error at 5% (standard value of 0.05)

The data was analysed by using SPSS statistical software version 20.0. One-way ANOVA and Unpaired T-test was used to compare the data between the 2 groups and the difference was considered to be statistically significant if 'p' values were 0.05 or less. Tukey's Post HOC tests were used to compare variations within the groups.

Age wise distribution of Salivary Copper

A positive correlation of salivary copper with age was observed in the study (r value = 0.068). However, correlation coefficient was not significant statistically (P = 0.391). (Table 1) In Group I, the levels of copper element in saliva was maximum in the age range of 15-40 years. Salivary copper showed a negative correlation with age in Grade I OSMF (r value = -0.38). However, the correlation of age with copper was not statistically significant among the 3 grades of OSMF. This showed that age had no relation with copper in the saliva.

Parameter	Mean	Std. Deviation	r Value	P value
Age	30.3938	11.34374	0.068	0.391
S. Cu µg/L	54.9410	24.35342		

Table 1: *Overall correlation of age with salivary copper µg/L.*

Group I	Age		Copper µg/L		r value	P value
	Mean	Std. Deviation	Mean	Std. Deviation		
Grade I	29.0476	9.50514	21.0698	1.99265	-0.380	0.089
Grade II	29.6364	10.17294	27.3012	6.66938	-0.053	0.770
Grade III	31.9615	12.68221	51.7881	7.31197	-0.085	0.679

Table 2: *Correlation of age with Salivary Copper µg/L in group I.*

Gender wise distribution of Copper in Group I: (Table 3)

OSMF	Gender	N	Salivary Copper µg/L		T Value	P Value
			Mean	Std. Deviation		
Grade I	Male	19	21.1147	2.09510	0.312	0.759
	Female	2	20.6425	0.00354		
Grade II	Male	30	27.8517	6.74157	1.530	0.136
	Female	3	21.7967	1.63967		
Grade III	Male	19	52.2453	7.58755	0.518	0.610
	Female	7	50.5471	6.89925		

Table 3: *Distribution of Gender with Salivary Copper (µg/L) in Group I.*

In males' patients suffering from Grades I, II and III OSMF the mean salivary Copper level was 21.1147 ± 2.0951, 27.8517 ± 6.74157, 52.2453 ± 7.58755 µg/L respectively. The mean salivary LDH levels of female subjects in the corresponding grades were 20.6425 ± 0.00354, 21.7967 ± 1.63967 and 50.5471 ± 6.89925 µg/L respectively. Salivary copper was statistically higher in male participants when compared to the females in all the 3 grades of OSMF. However, the values were not statistically significant.

Distribution of Copper levels according to Groups: (Table 4)

Group		N	Mean	Std. Deviation	F value	P value
Group I OSMF	Grade I	21	21.0698	1.99265	6.3277	< 0.0001*
	Grade II	33	27.3012	6.66938		
	Grade III	26	51.7881	7.31197		
Group II	Healthy Control	80	8.9727	2.37885		

Table 4: Distribution of Salivary Copper (µg/dl) according to groups.

Estimation of unstimulated salivary copper level in OSMF patients showed high copper level with mean salivary copper level of 21.0698 µg/dl in Grade I, 27.3012 µg/dl in Grade II and 51.7881 µg/dl in Grade III as compared to healthy controls which was 8.9727 µg/dl. ANOVA test results showed significant difference ($p < 0.0001$) between the patients and the control group.

Pair wise comparison of salivary copper levels between Healthy control and the 3 Grades of OSMF: (Table 5)

Group (I)	Group (J)	Mean Difference (I-J)	Std. Error	Significance
Healthy Control	OSMF Grade I	12.0971*	1.79768	< 0.0001*
	OSMF Grade II	16.4285*	1.51685	< 0.0001*
	OSMF Grade III	42.8154*	1.65511	< 0.0001*
OSMF Grade I	OSMF Grade II	6.23145*	2.04661	0.014*
	OSMF Grade III	30.71832*	2.15109	< 0.0001*
OSMF Grade II	OSMF Grade III	24.48686*	1.9226	< 0.0001*

Table 5: *Pair wise comparison of salivary copper levels µg/L between Groups (post HOC tests).*

The pair wise comparison of the mean copper level between healthy controls and OSMF (grade I, II and III) was 12.0971 µg/dl, 16.4285 µg/dl, and 42.8154 µg/dl respectively. Post Hoc tests comparison between the 2 groups were highly statistically significant ($p < 0.0001$). Intra group variations between all the 3 grades of OSMF were also highly significant ($p < 0.0001$).

Salivary copper levels in OSMF in relation to duration of habit: (Table 6)

Duration (Months)	Grade I	Grade II	Grade III	T Value	P value
<6	20.17 ± 1.37	24.9 ± 4.20	No cases recorded	2.6233	0.0305*
6-12	21.64 ± 2.60	25.32 ± 2.83	No cases recorded	4.2202	0.0003*
12-24	No cases recorded	25.39 ± 3.53	51.53±6.83	10.9744	<0.0001*
>24	No cases recorded	26.96 ± 3.11	52.72±7.75	9.4593	<0.0001*

Table 6: *Salivary copper (µg/dl) in relation to duration of OSMF.*

It was observed that the salivary copper level in those with a habit history of less than 6 months was 20.17 µg/dl in Grade I and 24.9 µg/dl in Grade II OSMF. In those with habit duration of 6-12 months, it was 21.64 µg/dl in grade I and 25.32 µg/dl in grade II OSMF. Those with duration of 12-24 months, lactate dehydrogenase levels were increased to 25.39 µg/dl in grade II and 51.53 µg/dl in grade III OSMF and in those with a habit duration of 24 months or more it was 26.96 µg/dl and 52.72 µg/dl in grade II and grade III OSMF respectively. There were no cases of grade III OSMF up to 1 year. It was observed that there was an increase in copper levels in saliva with increase in duration of the disease. Student T test showed that the relation was statistically significant.

Discussion

Oral submucous fibrosis is defined as a chronic disease of oral cavity which is characterized by sub epithelial inflammatory reaction followed by fibroelastic changes in the submucosa [14]. There has been a slow yet steady rise in oral submucous fibrosis both in India and across the world; the reason being attributed to the consumption of areca nut or gutkha [15,16]. Salivary analysis is a non-invasive and

reliable biochemical test for the early detection and diagnosis of a variety of diseases. In this study, a specific salivary biomarker, i.e. copper, was assessed among 160 subjects. Concentrations of this biomarker were compared in different grades of oral submucous fibrosis.

The mean age in the current study was approximately 29.0 years in Grade I and II OSMF and about 32 years in grade III OSMF. Although a correlation of salivary copper with age and gender was observed in the study, the results were not statistically significant i.e. age and gender were not associated with changes in salivary copper. This could be compared to related studies done by Sangeeth S., *et al*. [17] and Sinor PN., *et al*. (1990) where the mean ages in the study group were 25.0 ± 2.7 years and 29.1 years respectively [18].

Among the 80 Oral Submucous Fibrosis patients, 68 were males (i.e. 85%). Okade AR., *et al*. reported a male proclivity of 96.66% in their study done on the salivary copper and OSMF patients [19]. A male predominance was also reported in similar studies by Sangeeth S., *et al*. [17] and R Rajendran., *et al*. [20] where the male to female ratio was 1:0 and 9:5 respectively.

The increasing occurrence of oral submucous fibrosis in our youth can be blamed on economical convenience for procuring the product, the negligible cost, advertisements by celebrities, peer pressure and to the ready habituation to areca chewing habit which in turn which makes them susceptible for this dreaded disease.

Significant associations were observed between Group I (OSMF patients, n = 80) and Group II (healthy control, n = 80). Severity of changes in the qualitative analysis of copper among Group I patients was observed. Akshata RO., *et al.* (2015) in their study found that the mean salivary copper trace element in the OSMF group was 0.08 ± 0.16 ppm. Although salivary Cu levels were increased in OSF compared to the control group, no statistical significance was observed [19]. In a study done by Sangeeth S., *et al.* (2014) it was observed that salivary copper could not be detected in the control when compared to study group. The reason suggested was the presence of copper was in very minute quantities (i.e. parts per billion) and the sensitivity of atomic absorption spectrophotometer was to detect parts per million [17]. Reddy MS., *et al.* (1980) found significantly higher concentrations of soluble copper in saliva ($p < 0{\cdot}001$) than stimulated salivary samples collected without areca-nut chewing. They suggested that the amount of copper released from areca products induces lysyl oxidase activity upregulating collagen synthesis by fibroblasts, facilitating its cross linking and thereby inhibiting its degradation [21]. Rajalalitha P., *et al.* (2005) stated that copper is the main constituent of areca nut, which in turn is one of the main constituent of gutkha. This copper is incorporated into lysyl oxidase during its biosynthesis. [22] Trivedy CR., *et al.* (2000) stated that areca nuts have a high copper content which in turn significantly increases soluble copper levels in oral fluids. This increased soluble copper could act as an important factor in oral submucous fibrosis by stimulating fibrogenesis through up-regulation of Lysyl oxidase activity [23]. In another study by Kishor B., *et al*. (2007) the mean salivary copper levels were higher than the control group [24]. However, the level of copper in these studies was found to be a little lesser than the present study where a comparatively larger sample size was taken. Also, the differences may be attributed to other factors like geographical variation, dietary habits and the usage of the various areca nut products.

Pair wise comparison of salivary copper levels between Healthy control and the 3 Grades of OSMF

The study showed a trend of progressive increase in the clinical grading of OSMF as salivary copper concentration as the salivary copper concentration increased. The mean salivary copper level in grade I oral submucous fibrosis was 21.0698 µg/dl, whereas it was relatively higher in grade II and III OSMF with mean value of 27.3012 µg/dl and 51.7881 µg/dl respectively.

There was significant difference between the clinical grades of OSMF (i.e. all 3 grades together) and salivary copper levels (i.e. P-value was less than 0.0001). Intra group variations between the individual grades of OSMF showed statistically significant difference between grade I and grade II (p value = 0.014), while the differences were significant between grade I and III and grade II and III with a p-value of less than 0.0001. From this observation the possible role of copper in different grades of OSMF can be inferred.

Similar studies were done by M Faraz., *et al*. (2015). The mean salivary copper level in grade I oral submucous fibrosis was 21.0698µg/dl, whereas it was relatively higher in grade II and III OSMF with mean value of 27.3012 µg/dl and 51.7881 µg/dl respectively. However, in that study the difference between grade I and grade II were not statistically significant [25].

Areca nuts have been shown to have a high copper content which in turn significantly increases soluble copper levels in oral fluids. This could act as an important factor in oral submucous fibrosis by stimulating fibrogenesis through up-regulation of Lysyl oxidase activity [26]. The role of copper in carcinogenesis is emphasized by its involvement in Fenton reaction i.e. H2O2 + Cu (I) →Cu (II) OH + OH−. Copper also activates angiogenic factors like vascular endothelial growth factor (VEGF), Tumour Necrosis Factor alpha (TNFα) and Interleukin-1 (IL-1) [29].

Salivary copper levels in OSMF in relation to duration of habit

In the present study patients with a habit history of less than 6 months to 1 year suffered from either grade I and grade II OSMF. The concentration of copper in saliva in those who chewed areca nut for more than 1-2 years was significantly increased. In Grade III the mean salivary copper levels were increased significantly. Sinor PN., *et al.* (1990) in his study found that the frequency of areca nut chewing habit among OSF patients was reported to be higher compared to the general population and in the controls [18]. Margalith EJ., *et al.* (1983) stated that copper is involved in the cell metabolism as a part of various enzymes tyrosinase, uricase and cytochrome oxidase, which are mainly concerned with oxidation reactions [28]. Okade AR, Hallikeri KS, Trivedi DJ (2015) in their study reported that the mean duration of habits is 5.1 ± 3yrs. The frequency of Gutkha consumption in our study, among individuals with OSF ranged from twice a day to 25 times daily [19].

Eipe N (2005) reported that the habitual use of betel quid for 5 years predisposes the oral mucosa to oral potentially malignant disorders including OSF [27].

Conclusion

Of late a number of studies involving serum levels of specific biomarkers in various malignancies have been done. The present study was a prospective case control study which was conducted in Madhya Pradesh. An attempt was done to assess and bring about a comparison of sialo chemical alterations in copper, a specific salivary biomarker, in oral submucous fibrosis patients (Group I) and in healthy controls (Group II). Oral submucous fibrosis has always been a challenging disease with high prevalence in India. The results of this study revealed that a majority cases with oral submucous fibrosis were in the third decade of life with a male predominance. A significant increase in the salivary copper levels was observed in Group I participants especially in Grade II and III cases. The present study showed statistically significant pair-wise comparison between healthy controls and OSMF. Post Hoc Intra group variations between 3 grades of OSMF were highly significant. The concentration of saliva copper element in those who chewed areca nut for more than 1-2 years was significantly increased and in Grade III the mean salivary copper levels were increased significantly.

Oral submucous fibrosis has always been a challenging disease with high prevalence in India. Copper is the transition metal ion that is involved in the catalytic process of reactive oxygen species generation. It can be concluded that salivary copper can be used as a potential diagnostic and prognostic marker in OSMF patients. It can also be used as a valuable aid in monitoring treatment outcomes in the OSMF patients. Also it would be highly desirable and beneficial if salivary tumor marker analysis could be performed on a routine basis. This is especially important for people who live far from treatment centers and for those at high risk for developing oral cancer.

Conflict of Interest

This was an unsponsored and self financed study. I declare that there exists no financial interest or any conflict of interest.

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