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Evaluation of Serum Copper (Cu), Serum Iron (Fe) and Serum Copper (Cu) / Iron (Fe) Ratio in Oral Submucous Fibrosis in Karachi

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Author's contribution

The sole author designed, analyzed and interpreted and prepared the manuscript.

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Original Research Article

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ABSTRACT

Aims: To evaluate serum copper, serum iron, and copper/iron ratio in oral submucous fibrosis disease in Karachi.
Study Design: A cross-sectional study.
Place and Duration of Study: Ziauddin Hospital (Dental OPD), Karachi, Pakistan. In between the period of January 2014 to August 2015.
Methodology: This cross-sectional study included 130 oral submucous fibrosis patients, which were divided into 50 stage 1 OSMF, 50 stage 2 OSMF and 30 healthy individuals between the age range of 6-60 years (91 males and 37 females). Detailed questionnaire followed by sample collection of serum copper and iron from each patient was administered. Colorimetric assay analysed the samples.
Results: Mean age of OSMF patients was 34 years (15- 44 years). Most of them were males (70%), and less were females (30%). The highly affected group was of Muhajirs (Urdu speaking people) with the frequency of 40.8% as compared to other ethnicities. In the present study, the mean serum levels for copper (Cu) and iron (Fe) in healthy individuals were found to be 87 ± 20.2927 µg/dL and 73.7 ± 29.594 µg/dL respectively. In stage 1 OSMF patients, mean serum

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copper and iron levels were 149.56 µg/dL ± 10.8009 and 28.32 µg/dL ± 3.04 respectively. In stage 2 OSMF patients, mean serum copper and iron levels were 178.58 µg/dL ± 6.60887 and 18.82 µg/dL±2.21. Copper/ Iron ratio was also compared among different stages OSMF patients and healthy individuals. Among healthy individuals, copper/iron ratio was 1.2 which rose to 5.3 in OSMF stage 1, and the highest ratio was found in OSMF stage 2 with 9.5. It showed that the copper/iron ratio significantly increased with the severity of the disease ($p < 0.0001$).

Conclusion: Copper (Cu) and iron (Fe) could be used as biological markers in oral submucous fibrosis. Serum Cu to Fe ratio could be a helpful tool and a reliable biomarker in the diagnosis and prognosis of OSMF disease.

Keywords: OSMF; serum; Cu; Fe; ratio.

1. INTRODUCTION

High incidence of oral pre-cancer and cancer cases have increased the need of in-depth probing of various contributory factors for its early and proper diagnosis [1]. After a follow-up period of 17 years, the incidence rate of patients with oral submucous fibrosis (OSMF) leading to oral squamous cell carcinoma (OSCC) has been found to be 7.6% [2]. Areca-associated oral squamous cell carcinoma is the third most common malignancy in the developing world [3]. Oral submucous fibrosis (OSMF) is a deliberating collagen disorder. This chronic disease initiates by burning sensation of oral mucosa followed by vesicles, ulcers, pigmentations, xerostomia, tongue depapillation and gradual stiffening of oral mucosa with fibrous bands of leathery consistency in the buccal mucosa and ultimately trismus. This is due to the chronic inflammation and irreversible fibrosis of lamina propria and deep connective tissues [4]. Pindborg JJ observed epithelial changes in the biopsy samples of 118 patients and concluded OSMF disease as a precancerous condition [5]. It commonly occurs in India, Bangladesh, Sri Lanka, Pakistan, Taiwan, Southern China, Polynesia and Micronesia [6]. The genotoxic effects of the major etiological factor betel nut cause chromosomal instability resulting in a cancerous process that can be investigated by easy, rapid and sensitive sister chromatid exchange (SCE) method [7]. The biomarker is a measurable characteristic which is used to indicate the severity of particular disease (pre-cancers or cancers) or some other physiological state [8]. Betel nut alkaloids and polyphenols are causative carcinogens, while tobacco and slaked lime assist as co-carcinogens [9]. The most highlighted mediator in OSMF is transforming growth factor-beta (TGF-β) which plays an important role in repair and fibrosis. The genes CoL1A2, COL3A1, CoL6A1, COL6A3 and COL7A1 have been confirmed as definite TGF-β targets in fibroblasts at initial stages of the disease [10]. Activation of collagen 1 and 6 genes expression by TGF-beta, results in excessive collagen synthesis in OSMF patients [11]. Arecoline, the major alkaloid present in areca nut is highly responsible for increased level of mRNA, protein expression of cystatin C (an MMP- non-glycosylated basic protein) [12], upregulated TIMP 1, reduction in MMP 2 [13], 13 and stimulation of heat shock protein (HSP47) mRNA expression in human BCL-2 modifying factor (BMF) in OSMF disease. All these factors perform a significant role in collagen accumulation in the extracellular matrix [14].

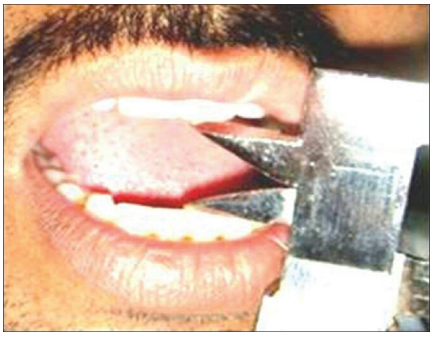
Trace elements are being used as biomarkers presently in cancer and pre-cancer detection as they are found highly altered in head and neck cancers [1]. Khana et al. [15] in 2005 observed that immune complexes, serum copper, iron and selenium act as a predictive marker in the causation of pre-cancer and cancer lesions [15].

Areca nut is high in copper and activates the enzyme lysyl oxidase which leads to excessive collagen cross-linking and organization of extracellular matrix [16]. Iron deficiency affects the development, maintenance and defence abilities of oral mucosa [17]. Iron plays an important role in the maturation of epithelium with the help of cytochrome oxidase (iron-dependent enzyme) in the oral mucosa [18]. OSMF is also termed as an “Asian version of sideropenic dysphagia” which results in mucosal susceptibility to many irritants [19]. Biochemical investigations of blood, serum, and tissues which are considered as “earliest form of interventions” have highly assisted in localizing parameters that are responsible for the malignant transformation of pre-cancer conditions [20]. OSMF is clinically divided into three stages. Stage 1 is known as stomatitis, and it includes erythematous mucosa, mucosal ulcers, vesicles, mucosal petechiae, and melanotic mucosal pigmentation. Stage 2 is termed as fibrosis, and it occurs in ulcers and healing vesicles. Stage 3 occurs as the sequela

of Stage 1 and 2 [21]. The researcher only focused on stage 1 and 2 because stage 3 would significantly extend the scope of the study and require analyzing the issues such as leukoplakia, speech deficits, and hearing deficits.

2. MATERIALS AND METHODS

This cross-sectional study was carried out in Ziauddin University located in Karachi city of Pakistan from January 2014 to April 2015, after approval by the Ethical Review Committee (ERC) of Ziauddin University, Karachi. The study was based on total 130 subjects including 50 OSMF stage 1, 50 OSMF stage 2 and healthy individuals with age range between six to sixty-five years. OSMF patients were staged from Pindborg [21] and Khanna [22]. Exclusion criteria included patients with any other oral precancerous lesion or condition other than oral submucous fibrosis and individuals taking anti-oxidants / multivitamin preparations. A detailed questionnaire was filled up regarding medical history along with the frequency of habits of causative agents like ghutka, pan, betel nut, tobacco and alcohol that has been briefly discussed in the article of "Pakistan Journal of Medicine and Dentistry" in 2016. Sample collection procedure was explained to the subjects, and written consent was signed by them. With due consideration, a 5ml venous blood sample was collected from subjects through a 5cc syringe, was transferred to a yellow topped (gel separating) serum tube and transferred to Ziauddin Hospital laboratory for the estimation of serum iron and copper levels. The blood sample was centrifuged at 3000 rpm for 15 minutes to get serum for further testing. Serum for iron estimation was stored at room temperature and processed immediately, while serum for copper estimation was stored at – 20° C to analyse later. Figures below show the pictures of materials and methods:



Materials Used

OSMF with Ulcer

2.1 Serum Copper Estimation

Copper present in serum sample was reacted with the Randox reagent at acid pH (4.7) to form stable coloured material on Hitachi 902 automated chemistry analyser. Colour intensity was directly proportional to the concentration of copper concentration in the sample that was measured photometrically at 570 nm wavelength.

2.2 Serum Iron Estimation

Iron present in the sample was reacted with Roche reagent to form a coloured iron complex on Hitachi Modular P800, automated chemistry analyser. Ferric form was converted into ferrous, which when combined with ferrozine present in the reagent, forms a red coloured complex. The colour intensity was directly proportional to the iron concentration in serum that was measured photometrically at 570 nm wavelength.

The collected data was sorted, tabulated and statistically analysed.

2.3 Statistical Analysis

Data was entered on "SPSS version 20". Quantitative data was presented as the mean and standard deviation. In qualitative data, frequencies and percentages were calculated. The study has been performed to determine the association between the quantitative variables of three groups, i.e. healthy individuals, OSMF stage 1 and OSMF stage 2 patients. The level of significance was less than 0.05. One-way Analysis of Variance (ANOVA) was used to compare the means between healthy, OSMF stage 1 and OSMF stage 2 group and to compare serum copper/ iron ratio of different stages of OSMF. Chi-square test was applied to see the association of categorical variables with each other.

3. RESULTS

The present study confirmed that the highest age frequency percentage of oral submucous fibrosis (114; 87.5%) was found among individuals in the age group of 15-44 years. Out of 130 subjects, 91 (70%) were males and 39 (30%) were females. The study population in the present study included various ethnic groups namely Muhajirs (Urdu speaking people), Punjabis, Balochis and Pathans. A small number of Siraikis, Bengalis and Gujaratis were grouped as "Others". The highly affected group was of Muhajirs with the frequency of 40.8% followed by Balochis (36.2%), Pathans (11.5%), Others (6.2%) and Punjabis (2.3%) ($p < 0.05$) (Table 1). In the present study, the mean serum levels for copper (Cu) and iron (Fe) in healthy individuals were found to be 87 ± 20.2927 µg/dL (range = 38 µg/dL– 150 µg/dL) and 73.7 ± 29.594 µg/dL (range = 56 µg/dL-124 µg/dL) respectively. In stage 1 OSMF patients, mean serum copper and iron levels were 149.56 µg/dL± 10.8009 (range = 91µg/dL-169 µg/dL) and 28.32 µg/Dl ± 3.04 (range = 22 µg/dL-33 µg/dL) respectively. In stage 2 OSMF patients mean serum copper and iron levels were 178.58µg/dL ± 6.60887 (range = 165 µg/dL-195 µg/dL) and 18.82 µg/dL ± 2.21 (range= 14 µg/dL-24µg/dL) (Table 2) (Fig. 1 & Fig. 2). Copper/ Iron ratio was also compared among different stages OSMF patients and normal healthy individual. Among healthy individuals, copper/iron ratio was 1.2 which rose to 5.3 in OSMF stage 1 and the highest ratio was found in OSMF stage 2 as 9.5. It showed that the copper/iron ratio significantly increased with the severity of disease ($p < 0.0001$) (Table 3).

Table 1. Age ranges and risk distribution of oral sub mucous fibrosis by gender

Factor	Characteristics of OSMF patients		
	Age Range (years)	**n (%)**	**P-value**
	1-14	10 (7.70)	≤ 0.05
	15-44	84 (87.50)	
	45-65	6 (4.60)	
	Mean age (30 years)		
	Gender		
	Male	70 (70)	≤ 0.05
	Female	30 (30)	
	Ethnicity		
	Urdu speaking	53 (40.80)	
	Sindhi	4 (3.10)	≤ 0.05
	Balochi	47(36.20)	
	Pathan	15 (11.50)	
	Punjabi	3 (2.30)	
	Others	8 (6)	

Table 2. Serum iron and copper estimation in different stages of OSMF

Groups		95% CI					
		N	**[a]Mean (±SD) (µg/dL)**	**LB**	**UB**	**Min**	**Max**
Serum Iron	Healthy Individuals	30	73.7± 29.594	62.65	84.75	38	175
Serum Copper	Stage 1 OSMF	50	28.32± 3.04	27.46	29.18	22	34
	Stage 2 OSMF	50	18.82± 2.219	18.19	19.45	14	22
	Normal	30	87± 20.2927	79.4226	94.5774	56	124
	Stage 1 OSMF	50	149.5± 10.801	146.49	152.63	135	169
	Stage 2 OSMF	50	178.58±6.608	176.702	180.458	169	195

[a]*Unit used for serum copper and iron was microgram per deciliter (µg/ dL)*

Table 3. Comparison of serum copper and iron ratio

Groups	Serum copper and iron ratio	
	n	**(Cu/ Fe ratio)**
Healthy Individuals	30	1.2
Stage 1 OSMF	50	5.3
Stage 2 OSMF	50	9.5

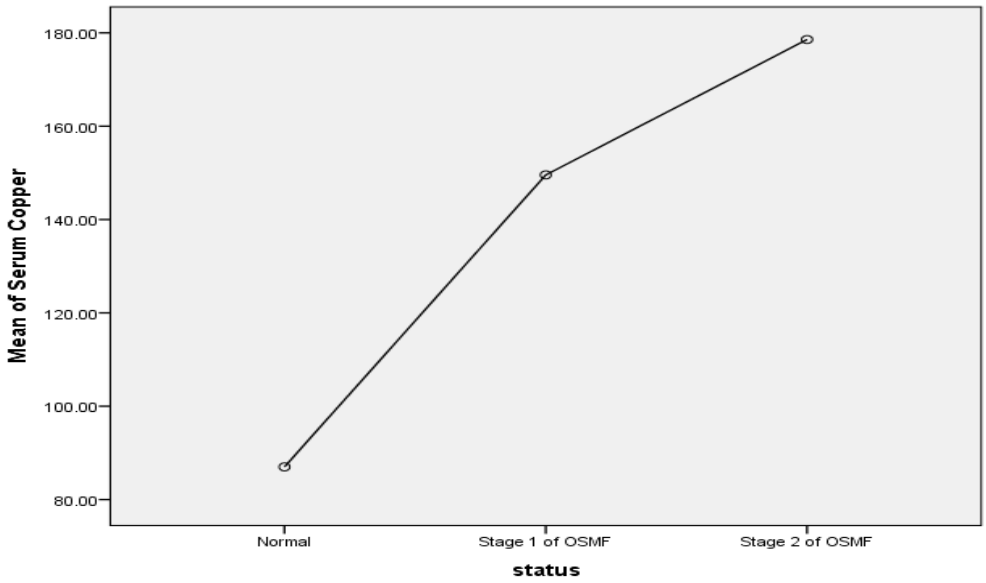


Fig. 1. Comparison of serum copper levels with stages of oral sub mucous fibrosis

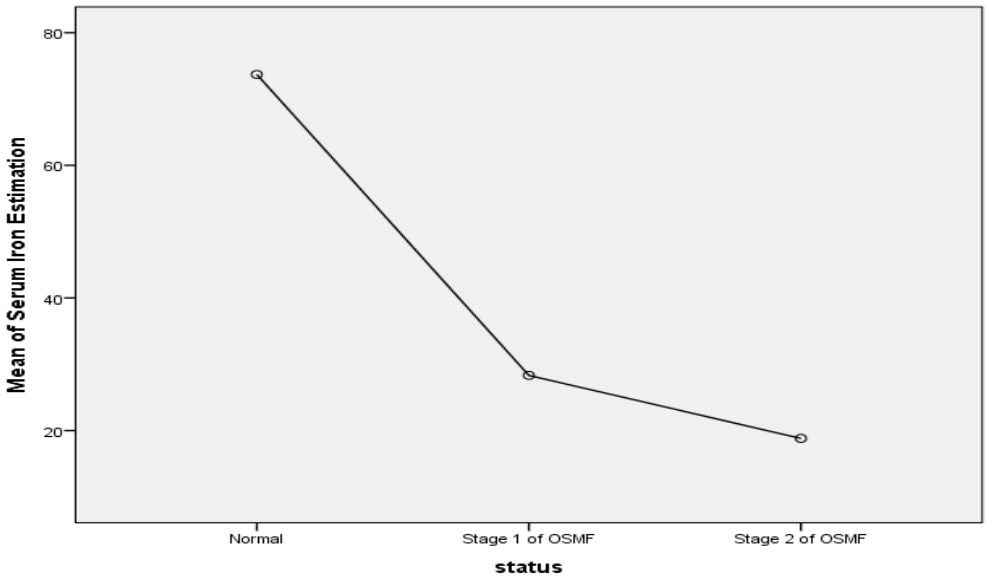


Fig. 2. Comparison of serum iron levels with stages of oral sub mucous fibrosis

4. DISCUSSION

Trace elements play a vital role in the management of physiological, metabolic processes in human body. More than 25% of body enzymes in the body are activated by metal ions to carry out their metabolic functions. The metal ions (trace elements) may function as inhibitory or causative agents of pre-cancer or cancer [23]. In the present study, OSMF, a precancerous disease, has been observed with the highest frequency percentage of 87.5% (n=114) among the young population between the age range of 15-44 years (mean age = 34 years). The present study was found in consonance with Ahmed et al. [24] in 2006, reported most of the OSMF patients with age range between 21-40 years while Hazarey et al. [25] in 2014, observed age range of 30-39 years among 1000 OSMF patients of Central India [25]. Young people were found to be more attracted towards the pan, ghutka, betel nut and tobacco

due to aggressive marketing and advertisements by actors and famous athletes that often claim these products safe and due to their easy availability in different colourful packing and low prices.

OSMF has been observed as a male predominant disease in our study with 70% males (n=91) and 30% females (n=39), with male/female ratio of 2.3:1. It has been found consistent with the study conducted in Karachi and concludes increased consumption of betel quid among young males as compared to females (Tanweer et al. (2011)). Other studies were also found consistent with the present study, such as those conducted by Ragnathan et al. [26], Rupak et al. [27] and Kapoor et al. [28]. Males were more addicted as compared to females due to less social restrictions and easy availability. They are less aesthetic conscious than females. Most of the OSMF patients were Muhajirs (Urdu speaking) (40.8%) as compared to other ethnicities. Similar was the finding by Akhlaq et al. [29] in showing 55% Muhajirs suffering from OSMF disease. Muhajirs are Indian immigrant and has adopted the habit of pan chewing from their ancestors, or it may be due to genetic disposition of this disease.

In this study, serum copper and iron has been estimated among different stages of OSMF disease and compared with healthy individuals. Healthy individuals were observed with the mean serum levels of 87 ±20.29 µg/dL. Mean serum copper levels in stage 2 OSMF (178.5± 6.6 µg/dL) was significantly higher than stage 1 OSMF patients (149.5 ± 10.8 µg/dL) ($p < 0.05$). The present study was found in conformity with the study of Tadakamadla et al. [30] with mean copper levels of 114 µg/Dl in OSMF stage 1 patients and 134 µg/Dl in OSMF stage 2 patients and confirmed OSMF stage 2 to be high in serum copper levels as compared to OSMF stage1. Shakya et al. [31] concluded that copper content had been significantly added in commercial areca nut as compared to the raw nut. The serum copper levels are directly proportional to the increase in the severity of OSMF [30]. Serum copper in OSMF patients vary in different regions. It may be due to the difference in the copper content present in betel nuts and its products and due to different concentration of copper in drinking water and natural environment. Copper ions produce free radicals that destroy RNA and DNA of the cell [31] and up-regulate lysyl oxidase that is responsible for intense cross-linkages of collagen and decreased collagen degradation leading to fibrosis. A study also confirmed that there is a link between copper and fibrosis in OSMF disease [32].

The present study also observed serum iron levels among OSMF stage 1 and 2 patients and healthy individuals. Mean serum iron level among healthy individuals was found to be 73.7 ± 29.594 µg/dL, in OSMF stage 1 was 28.32± 3.04 µg/dL and OSMF stage 2 was 18.82 ±2.22µg/dL respectively ($p < 0.0001$). A study conducted by Pathak et al. [33] concluded lowest levels of serum iron in OSMF stage 2 (12.23 µg/dL) as compared to OSMF stage 1 ((24.83 µg/dL) [33]. Other studies conducted by Saurabh et al. [34], Kode et al. [17] and Tadakamadla et al. [30] were also found consistent. The difference in serum iron levels in the present study and Indian studies is due to the difference in the type of diet intake as Indians preferred vegetarian diet as compared to the non-vegetarian diet which is common in the society. Iron deficiency may be due to its utilization by peptidyl lysine hydroxylase during the hydroxylation of proline and lysine in collagen synthesis that results in decreased serum iron [17]. Anemia will result in epithelial atrophy [35], oxidative stress [36] and decreased vascularity that will lead to increased destruction by arecoline on oral mucosa [30].

The ratio has a role in clear-cut clinical judgement [37]. Kapoor et al. concluded ratio to be a good indicator for oral cancer. The present study compared serum copper (Cu)/iron (Fe) ratio among healthy individuals, OSMF stage1 and OSMF stage 2. It was observed that OSMF stage 2 was found with the highest serum Cu/Fe ratio (9.5) as compared to the ratio in OSMF stage 1 (5.3) and healthy individuals (1.2). Oyama et al. [38] concluded in their study that the increase in the ratio of trace elements predicts tumour progression precisely as compared to the changes in the individual trace elements levels in the serum [38]. In this regard, some studies have observed Cu/Zn ratios in OSMF patients [1,17] but none has observed Cu/Fe ratio in such patients. Serum copper and serum iron level estimation have been done among oral submucous fibrosis patients by few researchers, with the conclusion of upregulated copper and downregulated iron levels in OSMF patients, highlighting them as factors responsible for causation and progression of disease [28,29, 39]. In this disease, the cellular and physiological functions have been lost due to loss of

equilibrium of metalloenzymes [28] and direct association with cancer mutations [40]. Angadi [41] presents contradictory evidence and highlights that there are significant limitations and ambiguities in the management of OSMF. The studies suffer from poor design, insufficient follow-up, lack of validated instruments, and inconsistent reporting. The author emphasizes on following strict guidelines for ensuring the validity of results. Some studies have presented other hypothesis and emphasized on the role of other biochemical parameters in the progression of OSMF. These parameters include genetic parameters and lipids glycoconjugates enzymes [42].

Iron has an inverse relationship with copper. A rise in copper levels in serum will result in low iron levels that showed iron levels are inversely proportional to copper levels in OSMF disease due to increased hydroxylation of proline and lysine during collagen formation. Estimation of serum copper and serum iron levels has been considered a helpful tool regarding the diagnosis of OSMF disease but copper/iron ratio can assist not only in the diagnosis and staging of oral submucous fibrosis but would also provide pertinent information regarding progression of the disease.

5. CONCLUSION

The present study concludes that imbalance in copper and iron levels may lead to the progression of OSMF disease. Although serum copper and iron levels are sensitive but not specific, Cu/ Fe ratio is a more reliable indicator in assessing the progression of malignancy and possesses the potential of the transformation of premalignant state to malignancy. Concerted efforts of determining serum levels of trace elements would, therefore, help in early detection, management and monitoring of the efficacy of treatment.

CONSENT

As per international standard or university standard, patient's written consent has been collected and preserved by the authors.

ETHICAL APPROVAL

As per international standard or university standard, written approval of Ethics committee has been collected and preserved by the authors.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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