

# Salivary estimation of copper, iron, zinc and manganese in oral submucous fibrosis patients: A case-control study

Akshata Raghavendra Okade, Kaveri Satish Hallikeri, Dhiraj Jayantilal Trivedi<sup>1</sup>

Department of Maxillofacial Pathology and Microbiology, SDM College of Dental Sciences and Hospital, <sup>1</sup>Department of Biochemistry, SDM College of Medical Sciences and Hospital, Dharwad, Karnataka, India

## ABSTRACT

**Background:** Trace elements (TEs) are required for physiological functioning and alterations are noted in potentially malignant disorders and oral cancer. These TEs are used in early diagnosis, treatment and also as an indicator of disease progress and prognosis. **Aims:** To estimate the TEs such as copper (Cu), zinc (Zn), iron (Fe), manganese (Mn) and Cu/Zn ratio in the saliva of oral submucous fibrosis (OSF) patients and controls. **Settings and Design:** The hospital-based study was conducted to estimate salivary TEs using atomic absorption spectrometry (AAS) in 60 individuals. **Methods and Material:** 5 ml saliva was collected from OSF cases (n=30) and controls (n=30) and was centrifuged and prepared by using the Wet Ashing method. The TEs were estimated in parts per million (ppm) by using AAS. **Statistical Analysis Used:** The data obtained was statistically analyzed using non parametric tests such as Mann Whitney U and Kruskal Wallis tests. **Results:** Significant difference in the mean salivary Zn, Mn and Fe levels in OSF when compared to that of controls. Mean salivary Cu levels were increased and Cu/ Zn ratio was decreased in OSF when compared to the controls. **Conclusions:** To conclude TEs play a role in the pathogenesis and progression of OSF. Betel quid and areca nut chewing habits are frequently associated with OSF and alters the salivary TE levels. Concerted efforts would, therefore, help in early detection, management and monitoring the efficacy of treatment.

**Key words:** Trace elements, Oral submucous fibrosis, Saliva, Copper, Zinc, Manganese, Iron

## INTRODUCTION

Trace element (TE) refers to the chemical elements, which are present or required in minute quantities.<sup>[1]</sup> These TEs play an imperative role in numerous physiological and metabolic processes in humans. Metal ions are necessary for humans as >25% of the enzymes need to be activated by them.<sup>[2]</sup>

Schwartz reported the importance of TEs as a resourceful anti-cancer agents, which thereby led to the discovery of new diagnostic and therapeutic events in the fields of medicine and specifically in oncology.<sup>[3,4]</sup> Shockingly, the

oral potentially malignant disorder (PMDs) and cancer are spreading like an epidemic in India. Oral PMDs occurs much higher than oral cancer and these lesions have been predicted to be a useful clinical marker for the detection of oral cancer.<sup>[4]</sup>

Research workers have studied the role of TEs in serum or blood, tissues, and saliva. Olmez *et al.* and Borella *et al.* stated that collection of saliva is the most convenient, easy, and noninvasive procedure, which serves the purpose of being a useful tool for diagnosing any alterations in trace metal metabolism in the human body.<sup>[5-7]</sup> Studies done on the estimation of TEs in saliva is sparse and the results obtained by few studies were also not conclusive. Hence, the present study was undertaken to estimate the role of copper (Cu), zinc (Zn), manganese (Mn), and iron (Fe) in the unstimulated whole saliva of control and oral submucous fibrosis (OSF) patients.

## SUBJECTS AND METHODS

The present hospital-based study comprised of 60 patients visiting Department of Oral Medicine and Radiology at our

### Access this article online

#### Quick Response Code:



#### Website:

www.cci-journal.org

#### DOI:

10.4103/2278-0513.156075

**Address for correspondence:** Dr. Kaveri Satish Hallikeri, Department of Maxillofacial Pathology and Microbiology, SDM College of Dental Sciences and Hospital, Dhavalanagar, Sattur, Dharwad, Karnataka, India. E-mail: drcauveri2005@gmail.com

college. The study groups comprised of Group 1–30 cases of age and sex-matched healthy individuals (control) and Group 2–30 cases of OSF (study group). Clinically proven OSF patients were included. The individuals selected for the control group were free from the habits and oral lesions.

Patients with systemic disease like myocardial infarction, hepatitis, cirrhosis or with a history of consuming drugs containing Cu, Zn, Fe, and Mn were excluded. OSF patients undergoing treatment and OSF patients with malignant transformation were excluded.

All the patients were explained about the study, and informed consent was acquired. An approval was obtained by the Ethical Committee of the Institutional Review Board to proceed with the research. A detailed case history including demographic information, general history, details of diet, habits, and socioeconomic status of the patients were recorded. The clinical examination of the lesion which included various parameters pertaining to the symptoms of the patient such as mouth opening, palpable bands, tongue protrusion, and deviation of the uvula were noted. The interincisal distance was measured according to Ranganathan *et al.*,<sup>[8]</sup> using a divider and scale to stage the disease. 5 ml of unstimulated whole saliva specimen from the selected patients was collected by draining method between 9 a.m. and 12 p.m. without mouth rinse to avoid any washing out of TEs and diurnal variation. Subjects were instructed to bend forward their head so that saliva will move toward the anterior region of the mouth. The pooled saliva was allowed to drool into the wide bore sterile container.

Saliva sample was centrifuged at 1200 g for 5 min at the cold centrifugation. This process provides a saliva sample free of large debris and of reduced viscosity, allowing more accurate and reproducible analysis.

An acid digestion (nitric acid + perchloric acid + hydrochloric acid) procedure was used for sample preparation in the determination of TEs. After the digestion, the samples are diluted with deionized water with a specific volume. The main advantage of this method is that it eliminates elemental loss by volatilization because the digestion takes place at a low temperature. Later, oxidation of the samples was carried out by Wet Ashing method. Samples were boiled with a mixture of nitric acid and perchloric acid to achieve oxidation. The proteins and other organic substances were eliminated by placing the samples in a furnace at a temperature of 350°C. After the solutions were prepared, the TEs were estimated using GBC flame atomic absorption spectrometry. The values were obtained in parts per million (ppm). Statistical analysis was done using a nonparametric test such as Mann–Whitney U-test.

## RESULTS

The age ranged from 16–60 yrs for both Groups I and II with a mean age of  $24.3 \pm 2.6$  and  $29.2 \pm 8.5$  respectively. A male proclivity is observed in Group I i.e. OSF group consisting of 96.66%, who had arecanut, tobacco, betel quid chewing and other habits. The mean salivary TE of OSF group (Cu  $0.08 \pm 0.16$ , Zn  $0.1 \pm 0.21$ , Mn  $0.06 \pm 0.07$ , and Fe  $0.14 \pm 0.24$ ) were compared with control group (Cu  $0.05 \pm 0.06$ , Zn  $0.1 \pm 0.29$ , Mn  $0.03 \pm 0.028$ , and Fe  $0.52 \pm 0.14$ ) [Table 1]. Statistical significance was observed between OSF and control group with respect to Zn, Mn and Fe as  $p < 0.05$ . Although salivary Cu levels were increased in OSF compared to the control group, no statistical significance was observed. ( $p > 0.05$ ) [Graph 1].

Non parametric test such as Mann Whitney U Test was done to qualitatively analyze the ratio of Cu/Zn in OSF and control groups. (Mean values –Cu/Zn in OSF  $-3.78 \pm 4.13$ , Cu/Zn in control  $-10.9 \pm 15.8$ ) [Table 2]. Statistically significant results were observed between the two groups at  $p < 0.05$  [Graph 2].

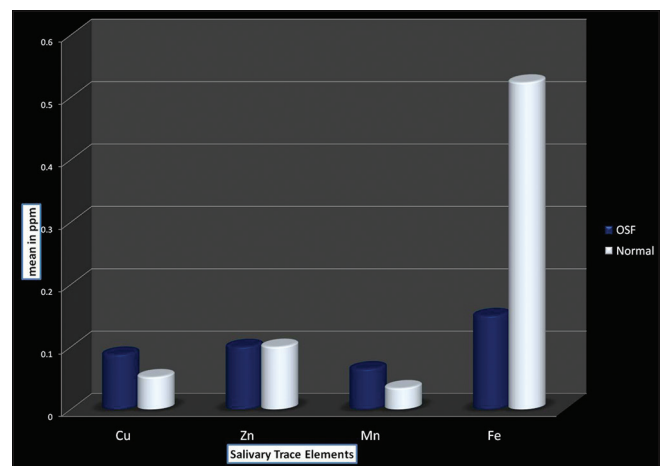
## DISCUSSION

Amongst the various PMDs known, OSF is gaining importance because of the numerous cases reported in the

**Table 1: Mean and SD of OSF and control group**

Group	Copper	Zinc	Manganese	Iron
OSF				
Mean	0.087	0.100	0.064	0.149
SD	0.162	0.214	0.077	0.242
Control				
Mean	0.051	0.100	0.034	0.524
SD	0.060	0.295	0.028	0.143

SD: Standard deviation, OSF: Oral submucous fibrosis

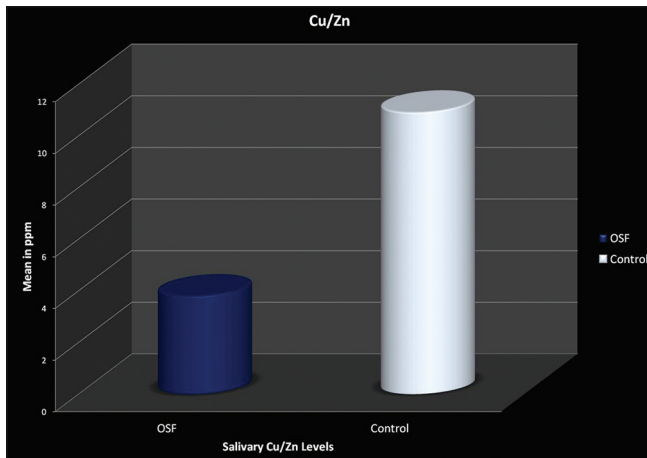


**Graph 1:** Comparison between oral submucous fibrosis group and control group by means of salivary trace elements. OSF: Oral submucous fibrosis, Cu: Copper, Zn: Zinc, Mn: Manganese, Fe: Iron

**Table 2: Mean and SD of Cu/Zn in OSF and control groups**

Group	Cu/Zn
OSF	
Mean	3.78
SD	4.13
Control	
Mean	10.91
SD	15.89

SD: Standard deviation, OSF: Oral submucous fibrosis, Cu: Copper, Zn: Zinc



**Graph 2:** Comparison between oral submucous fibrosis group and control group by means of salivary copper/zinc ratio

recent years in the younger age group and also because of its multifactorial origin. Several factors such as areca nut chewing, chilies consumption, nutritional deficiency, genetic susceptibility, autoimmunity, and collagen disorders have proposed to be implicated in the pathogenesis of OSF. At present, chewing of areca nut is considered to be the most important etiologic factor of OSF.<sup>[4]</sup>

A male proclivity is observed in the present study group, i.e. OSF group consisting of 96.66%, who had arecanut, tobacco, betel quid chewing and other habits.

The mean age of the OSF group is 29.2yrs  $\pm$  8. 51 and youngest patient recorded was 16 yrs and older was 60yrs. Similar findings reported by Sinor *et al.*, in their study, that 79 per cent of the OSF cases were less than 35 years of age group and by the Shah and Sharma showed a preponderance of cases from 21-40 years of age group.<sup>[9,10]</sup>

The duration and frequency of habits and its association with the development of OSF have been well documented. The Eipe study reported that the habitual use of betel quid for 5 years predisposes the oral mucosa to oral potentially malignant disorders including OSF.<sup>[11]</sup> In the present study, the mean duration of habits is 5.1  $\pm$  3yrs. The frequency of Gutkha consumption in our study, among individuals with OSF ranged from twice a day to 25 times daily (average frequency 8 times daily). Sinor *et al.*, 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.<sup>[9-11]</sup>

Cu is a TE which is essential for the function of several basic enzymes, which are involved in maintaining the physiological homeostasis as well as metabolism in humans. They comprise diverse enzymes such as cytochrome-c oxidase, superoxide dismutase (SOD), metallothionein, and lysyl oxidase (LO).<sup>[12]</sup> The uniqueness in the structure of Cu permit it to serve as a cofactor in redox reactions of enzymes. For example: SOD involved in detoxification of reactive oxygen species (ROS).<sup>[13]</sup>

Trivedy *et al.*, demonstrated that arecanut products contain a high level of Cu (mean 302 nmol/g) when compared to other commonly eaten nuts (22  $\pm$  173 nmol/g). The Cu which is released from these nuts while chewing is brought in direct contact with the oral epithelium or keratinocytes and is also dissolved in the saliva. In saliva, Cu remains for a prolonged period of time (up to 30 min) following chewing. As, Cu is present for a longer period of time, it is taken up by the epithelial cells, either by non-energy dependent diffusion or may be transferred across by the basolateral membrane.<sup>[14]</sup> 15 min after the ingestion of these arecanut and its products, absorbed Cu appears in the blood streams.<sup>[13]</sup>

In the present study, the mean salivary Cu levels in OSF (0.087ppm  $\pm$  0.162) is increased as compared to that of the control mean salivary Cu (0.051ppm  $\pm$  0.06). But this increase in the concentration was not statistically significant ( $p > 0.05$ ). The results were similar to the study done by Kode *et al.*<sup>[15]</sup> Ayinampudi *et al.*, observed that salivary Cu levels were significantly increased in OSF and malignant lesions compared to the control groups. These variations could be due to small sample size included in their group and also due to the use of inductively coupled plasma mass spectrometry (ICP-MS) for the estimation of Cu.<sup>[2]</sup>

Lately, the major role of Cu in the pathogenesis of OSF has been well recognized. Various studies done by Ma *et al.*, and Trivedi *et al.*, have indicated that Cu dependent extracellular enzyme LO is upregulated in OSF. This upregulation in turn results in the excessive cross linkage of collagen, which is resistant to the degradative action of collagenase enzyme.<sup>[14]</sup>

Zn is physiologically and biologically essential for the normal development, growth and function in mammals.<sup>[16]</sup> Zn is basically involved as a cofactor in carbonic anhydrase, carboxy peptidase, leucine peptidase and SOD. Zn is also an essential component for regulating cell cycle and cell division and is also an essential ion needed for the activation of DNA polymerase enzyme.<sup>[2]</sup>

In our study, a significant difference is observed in the mean salivary Zn levels between OSF and controls ( $p < 0.05$ ). The mean salivary Zn levels in OSF ( $0.10 \pm 0.21$ ) were decreased compared to that of the control group ( $0.10 \pm 0.29$ ). Zn is a cofactor for Cu – Zn SOD enzyme which forms an integral part of the primary antioxidant system. Inflammatory process has a significant role in the disease progression of OSF. Prolonged inflammation in OSF induces stress known as oxidative stress. This stress can also be induced by the products of gutkha packets. Due to the unnecessary build up of oxidative stress, excessive consumption of Zn is required to counter react oxidants. Thus, significant depletion in Zn as observed in the present investigation might be an important signal to reflect the interplay between the underlying oxidant – antioxidant status during progression of OSF.<sup>[17,18]</sup> Varghese *et al.* and Toke *et al.*, had similar observations where the serum Zn levels were decreased in OSF and oral cancer.<sup>[16,19]</sup>

Since abnormal levels of Cu and Zn have been encountered in various malignancies, Cu/Zn ratio has been suggested as a better prognostic marker than the individual levels of either of the two TEs.<sup>[16,19]</sup> Homeostasis of Zn is not as well regulated as that of Cu therefore the Cu / Zn ratio can be considered as a more consistent key for determining the status of these elements.<sup>[16]</sup> In various studies done by Varghese *et al.*, Toke *et al.*, and Ray *et al.*, they observed that serum Cu/Zn ratio was elevated in oral cancer and OSF. The suggestion given for this increment in the ratio was because of the Cu which is discharged from the commercially available gutkha; the Cu levels are generally increased and as Zn is consumed in counteracting the oxidant levels, the levels are usually decreased.<sup>[16,17,19]</sup>

In our study, we observed that mean salivary Cu/Zn ratio was decreased in OSF ( $3.74 \pm 4.13$ ) when compared to that of control group ( $10.9 \pm 15.8$ ) and the difference was statistically significant ( $p < 0.05$ ). This result was similar to the studies conducted by Ayinampudi *et al.* and Desai *et al.*<sup>[2,13]</sup> An important facet of our research indicated that mean salivary Zn levels in OSF group ( $0.10 \pm 0.21$ ) were more than the mean salivary Cu levels ( $0.08 \pm 0.16$ ). Zn bears an inverse relationship with Cu and has been involved in the modulation of mucosal metallothionein, hence it interferes with the level of Cu. Excess Zn particularly impairs the absorption of Cu because metallothioneins absorb both metals.<sup>[2,13]</sup>

The function of Fe in the growth, maintenance, and defense abilities of the oral mucous membrane has been cited. Fe is a necessary TE found in virtually all living organisms. Fe-containing proteins and enzymes ex: heme prosthetic groups, take part in many biological oxidations and transportation.<sup>[20]</sup>

Arecanut products release various harmful substances which when comes in direct contact with the oral tissues produces

a hypersensitivity reaction. Thus, the disease process is initiated. Alkaloids, which are soluble in nature, are the major irritant in arecanut. This irritant initiates the formation of juxtaepithelial inflammatory reaction which later leads to clinical symptoms and signs such as burning sensation and ulceration in the oral mucous membrane resulting in impaired food consumption and poor nutrition. The lack of nutrition leads to the paucity of important vitamins and TEs such as Fe in the body which could initiate anemia in the patients. Anemia acts as a promoting agent for the advancement of the disease. Later on, after the establishment of the lesion, anemia may further be responsible for inadequate intake of food due to the formation of fibrous bands and trismus, thus becoming a never ending loop.<sup>[4,21]</sup> Salivary mean Fe levels in the present study were significantly decreased in OSF patients when compared to that of control groups ( $p < 0.05$ ). The mean salivary Fe level in OSF is  $0.14 \pm 0.24$  and the mean salivary Fe level in controls was  $0.52 \pm 0.14$ . Reduced Fe levels in OSF patients might be due to utilization of Fe in collagen synthesis. Hydroxyproline, an amino acid, is one of the ingredients which are necessary for collagen synthesis. The incorporation of this amino acid occurs by a process of hydroxylation which requires cofactors such as ferrous and ascorbic acid. Hence, increased formation of collagen leads in the increased consumption of Fe leading to a reduction in Fe levels. There is increased production of insoluble crosslinked Type I collagen in OSF patients and there is also loss of soluble procollagen Type III and Type IV in this lesion.<sup>[4,18]</sup>

Reduction in the levels of Fe in OSF leads to a noticeable variation in epithelium and connective tissue of the oral cavity. Cytochrome oxidase, an Fe dependant enzyme, is essential for the normal maturation of the keratinocytes. Scarcity of Fe in the body leads to a reduced activity of cytochrome oxidase, which thus hampers the maturation of the oral keratinocytes leading to the formation of an atrophic epithelium. As the thickness of the epithelium is reduced, the epithelium becomes more susceptible to the chemical irritants of Areca nut and its products.<sup>[4,18]</sup>

Fe has an important role to play in the formation of vascular channel. Therefore, lack of Fe because of the disease process leads to the improper vascular channel formation leading to leaky channels and reduced vascularity. Due to this, the inflammatory response in the lamina propria is disturbed and hence results in a defective healing with scar formation. Overall, this entire feature together constitutes further fibrosis, which is a prominent feature in OSF.<sup>[4,18]</sup>

Mn acts as a main constituent as well as an activator of various enzymes and proteins in animals and humans. The three major primary Mn metalloenzymes are: Mn SOD, pyruvate carboxylase and arginase.<sup>[22]</sup> The enzyme

SOD is believed to be present in all oxygen-metabolizing cells because its physiological function is to provide a defense against the potentially damaging reactivity's of the superoxide radical (O<sub>2</sub><sup>-</sup>) and ROS generated by aerobic metabolic reactions.<sup>[22]</sup>

In the present study, we estimated the mean salivary Mn level because Mn is an essential TE required for the activation of SOD enzyme. In the literature, there are very few studies conducted on the estimation of Mn in OSF patients in serum, but a study in saliva has not been undertaken yet. We observed that the mean salivary Mn level in OSF (0.064 ± 0.24) was increased compared to that of the control groups (0.034 ± 0.028). Statistical evaluation showed the difference in levels of Mn between the OSF group and control group was statistically significant (*p* < 0.05). Similar results were observed by Ray *et al.*, were estimated of the serum Mn levels were conducted in leukoplakia and OSF patients. They proposed that significant Mn level observed in case of blood from OSF patients could be attributed to prolonged underlying inflammation in parallel to reports of Morton and Schwartz.<sup>[3,23]</sup> However, the precise role of Mn warrants further study.

## CONCLUSION

Results of our work suggest that the expression of TEs is altered in OSF when compared to that of the control group. Estimation of TE could be integrated in decision-making allowing it to individualize the treatment and to develop targeted therapies based on the TE expression. Our results also indicate that saliva may be used as a potential diagnostic tool which can be efficiently employed to evaluate the TEs in PMDs of the oral cavity. However, as there are controversial reports on the association of OSF and these TEs, future studies are anticipated on a larger heterogeneous population to confirm the hypothesis.

## ACKNOWLEDGMENTS

We wish to thank Dr. Srinath Thakur, Principal, SDM College of Dental Sciences and Hospital, Dharwad, Dr. J. V. Chowti, Principal, SDM College of Medical Sciences and Hospital, Dharwad, University of Agriculture Sciences, Dharwad and Dr. M V Muddapur, Statistician, SDM College of Dental Sciences and Hospital, Dharwad for their valuable support in the course of the study.

## REFERENCES

- Silva MP, Soave DF, Ribeiro-Silva A, Poletti ME. Trace elements as tumor biomarkers and prognostic factors in breast cancer: A study through energy dispersive x-ray fluorescence. *BMC Res Notes* 2012;5:194.
- Ayinampudi BK, Narsimhan M. Salivary copper and zinc levels in oral pre-malignant and malignant lesions. *J Oral Maxillofac Pathol* 2012;16:178-82.
- Schwartz MK. Role of trace elements in cancer. *Cancer Res* 1975;35:3481-7.
- Khanna SS, Karjodkar FR. Circulating immune complexes and trace elements (Copper, Iron and Selenium) as markers in oral precancer and cancer: A randomised, controlled clinical trial. *Head Face Med* 2006;2:33.
- Olmez I, Gulovali MC, Gordon GE, Henkin RI. Trace elements in human parotid saliva. *Biol Trace Elem Res* 1988;17:259-70.
- Borella P, Fantuzzi G, Aggazzotti G. Trace elements in saliva and dental caries in young adults. *Sci Total Environ* 1994;153:219-24.
- Schipper RG, Siletti E, Vingerhoeds MH. Saliva as research material: Biochemical, physicochemical and practical aspects. *Arch Oral Biol* 2007;52:1114-35.
- Ranganathan K, Mishra G. An overview of classification schemes for oral submucous fibrosis. *J Oral Maxillofac Pathol* 2006;10:55-8.
- Sinor PN, Gupta PC, Murti PR, Bhonsle RB, Daftary DK, Mehta FS, *et al.* A case-control study of oral submucous fibrosis with special reference to the etiologic role of areca nut. *J Oral Pathol Med* 1990;19:94-8.
- Shah N, Sharma PP. Role of chewing and smoking habits in the etiology of oral submucous fibrosis (OSF): A case-control study. *J Oral Pathol Med* 1998;27:475-9.
- Eipe N. The chewing of betel quid and oral submucous fibrosis and anesthesia. *Anesth Analg* 2005;100:1210-3.
- Arakeri G, Patil SG, Ramesh DN, Hunasgi S, Brennan PA. Evaluation of the possible role of copper ions in drinking water in the pathogenesis of oral submucous fibrosis: A pilot study. *Br J Oral Maxillofac Surg* 2014;52:24-8.
- Desai VD, Kumar MV, Mathi RJ, Gaurav I, Sharma R. Molecular analysis of trace elements in oral submucous fibrosis and future perspectives. *Univers Res J Dent* 2014;4:26-35.
- Trivedy CR, Warnakulasuriya KA, Peters TJ, Senkus R, Hazarey VK, Johnson NW. Raised tissue copper levels in oral submucous fibrosis. *J Oral Pathol Med* 2000;29:241-8.
- Kode MA, Karjodkar FR. Estimation of the serum and the salivary trace elements in OSMF patients. *J Clin Diagn Res* 2013;7:1215-8.
- Toke GB, Dhamne BK. A study of serum copper, serum zinc and Cu/Zn ratio as diagnostic and prognostic index in cases of head, neck and face tumors. *Indian J Pathol Microbiol* 1990;33:171-4.
- Ray JG, Ghosh R, Mallick D, Swain N, Gandhi P, Ram SS, *et al.* Correlation of trace elemental profiles in blood samples of Indian patients with leukoplakia and oral submucous fibrosis. *Biol Trace Elem Res* 2011;144:295-305.
- Shetty SR, Babu S, Kumari S, Shetty P, Hegde S, Karikal A. Role of serum trace elements in oral precancer and oral cancer – A biochemical study. *J Cancer Res Treat* 2013;1:1-3.
- Varghese I, Sugathan CK, Balasubramoniyam G, Vijayakumar T. Serum copper and zinc levels in premalignant and malignant lesions of the oral cavity. *Oncology* 1987;44:224-7.
- Kamath VV, Satelur K, Komali Y. Biochemical markers in oral submucous fibrosis: A review and update. *Dent Res J (Isfahan)* 2013;10:576-84.
- Karthik H, Nair P, Gharote HP, Agarwal K, Ramamurthy Bhat G, Kalyanpur Rajaram D. Role of hemoglobin and serum iron in oral submucous fibrosis: A clinical study. *ScientificWorldJournal* 2012;2012:254013.
- Oberley LW, Buettner GR. Role of superoxide dismutase in cancer: A review. *Cancer Res* 1979;39:1141-9.
- Kulkarni N, Kalele K, Kulkarni M, Kathariya R. Trace elements in oral health and disease: An updated review. *J Dent Res Rev* 2014;1:100-4.

**Cite this article as:** Okade AR, Hallikeri KS, Trivedi DJ. Salivary estimation of copper, iron, zinc and manganese in oral submucous fibrosis patients: A case-control study. *Clin Cancer Investig J* 2015;4:302-6.

**Source of Support:** Nil, **Conflict of Interest:** None declared.