Original Article

Estimation and Comparison of Serum Levels of Copper, Zinc and Cu/Zn Ratio as Markers of Disease Activity in Oral Submucous Fibrosis (OSMF) and Oral Squamous Cell Carcinoma (OSCC) Patients

Abstract

Context: Trace elements such as copper (Cu) and zinc (Zn) are involved in vital biochemical reactions, including different redox reactions and free radical formation and in maintaining cellular homeostasis. Several studies have been carried out on Cu, Zn, and iron level in the serum, plasma, and tissue of oral pre-malignant and malignant lesions. **Aim:** The aim of this study was the estimation and comparison of serum levels of Cu, Zn, and Cu/Zn ratio in oral submucous fibrosis (OSMF) and oral squamous cell carcinoma (OSCC) patients. **Subjects and Methods:** Sera of OSMF (n = 30) and OSCC (n = 30) patients as also of healthy controls was analyzed for the estimation of Cu and Zn using atomic absorption spectrophotometry. **Statistical Analysis:** Analysis of variance was used to compare the results of patients of OSMF, OSCC patients, and the controls. Independent sample's t-test was used to compare the mean values between the two groups. Comparison of mean values between different groups was carried out using Student's t-test using appropriate level of significance and degrees of freedom. **Results:** Sera levels of Cu were found to be increased while those of Zn were decreased in OSMF and OSSC patients as compared to the healthy controls. **Conclusion:** It could be concluded that altered sera levels of these trace elements can be helpful in early detection, management, and monitoring the efficacy of treatment in OSMF and OSCC patients.

Keywords: *Copper, oral squamous cell carcinoma, oral submucous fibrosis, Trace elements, Zinc*

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Introduction

Most of the oral cancer lesions are usually preceded by the occurrence of premalignant lesions and/or conditions. Oral submucous fibrosis (OSMF) is a precancerous condition and shows a significant tendency to develop frank cancer. OSMF is an chronic, insidious and debilitating, high-risk, precancerous condition affecting patients, especially, of South-East Asian origin. The exact etiology of OSMF is poorly understood but is believed to be closely related to the habit of chewing areca nut.[1] Furthermore, OSMF patients are strongly associated with a risk of developing frank oral squamous cell carcinomas (OSCCs) with a malignant transformation rate of 7.6% over a period of 17 years. The prevalence of oral precancerous lesions and/or conditions is much higher than that of oral cancer, and these lesions provide useful clinical markers for oral cancer.[1] The most common malignant lesion seen in the oral cavity is OSCC and it is a major problem in regions where tobacco habits, in the form of chewing and/or smoking, either with or without alcohol intake, are common. Its distribution and occurrence vary by age, ethnic groups, culture, and lifestyle-associated factors.[1] Timely recognition of oral cancers and precancers improves the quality of life of the affected patients. At present, the diagnosis of oral precancerous lesions and conditions and oral cancer is done by histopathology. Therefore, there is a need for the development of susceptible, precise, and quicker tests for the early diagnosis of the primary tumor and its recurrence or malignant conversion in premalignant states. Hence, detection of trace elements which can forecast disease succession becomes an absolute necessity for the better management of such patients. Serum levels of trace elements are significantly altered in the head and neck cancers, lung and breast carcinomas, and they also have an imperative role in carcinogenesis.[2] Cytological studies have confirmed the important role of copper (Cu) in the pathogenesis of OSMF showing

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intense staining in smears of OSMF patients as compared to smears from the nonchewers. Thus, it can prove an efficient marker of early diagnosis of malignant transformation.[3] Zinc (Zn) is an important part of biomembranes that manage membrane stability and lipid peroxidation-related injuries. It has an important role in modulating activities of various highly significant enzymes including ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) polymerase, inhibitory effect on phosphodiesterase, activation of membrane-bound adenyl cyclases, and thus has a possible role in carcinogenesis, too.[4] In the assessment for several trace elements, the concentrations in serum are often used as an index. The importance of these trace elements in cancer was reported to be of significance first by Schwartz M[5] which opened the door for almost a new diagnostic and therapeutic endeavor in many areas of medicine and particularly, in the areas of oncology. Immunological and biochemical alterations in the serum of such patients can help not only in the early diagnosis and treatment but also in assessing the prognosis, as well as the disease progression, and response to treatment.[6]

Subjects and Methods

The study sample comprised ninety participants that was divided into four groups as:

1. Group A: Patients with histopathologically diagnosed OSMF, $n = 30$
2. Group B: Patients with histopathologically diagnosed OSCC, $n = 30$
3. Group C: Healthy controls without any habit and without lesion (tobacco, betel nut, and alcohol consumption), $n = 15$
4. Group D: Healthy controls with habit but without any lesion (tobacco and/or betel nut and/or alcohol consumption), $n = 15$.

The selection of the patients for the study was done after obtaining their written consent. Ethical clearance was obtained from the Institutional Ethics Committee.

Inclusion criteria

- Group A: Patients suffering from OSMF diagnosed clinically and histopathologically
- Group B: Patients suffering from OSCC diagnosed clinically and histopathologically
- Group C: Healthy controls without any habit (tobacco and/or betel nut and/or alcohol consumption) and without any clinically observable potentially malignant epithelial lesion (PMEL)
- Group D: Healthy controls with habit (tobacco, betel nut, and alcohol consumption) but without any clinically observable PMEL.

Methodology

A detailed oral examination of the patients was carried out using diagnostic instruments (mouth mirror and probe), and findings were recorded. A clinical diagnosis of OSMF or the malignant ulcer was made based on the clinical appearance of the lesion, and a detailed case history was taken. Biopsy was taken from the site of the lesion. Biopsy tissue was processed and stained, and the diagnosis was histopathologically confirmed. Then, sera samples of patients afflicted with OSMF and OSCC as also of the healthy controls were collected, processed, and analyzed for the estimation of Cu and Zn using atomic absorption spectrophotometry (AAS).

Procedure

After the patients were clinically and histopathologically diagnosed to have OSMF or OSCC, 2 ml of intravenous blood was drawn under aseptic precautions. The blood was allowed to clot and centrifuged at 1000 rpm for 15 min to separate serum. Serum was subjected to digestion using concentrated nitric acid and perchloric acid. For the determination of serum Cu, the sample was diluted with an equal volume of deionized water. For the determination of serum Zn, the sample was diluted in a ratio of 1:5 with deionized water. Estimation of serum Cu and Zn levels was done using AAS (Perkin Elmer, Shelton, CT 06484, USA Analyst 200/4000 spectrometer).

Statistical analysis

Data were entered into Microsoft Excel sheet and analyzed using Statistical Package for Social Sciences (Graph pad prism, Version 6.07) (SPSS Inc., Chicago, USA). Analysis of variance (ANOVA) was used to compare the results of patients of OSMF, OSCC patients, and the controls. Independent sample's *t*-test was used to compare the mean values between the two groups. Comparison of mean values between different groups was done using the Student's *t*-test using appropriate level of significance and degree of freedom.

Results

The results of the patients with OSMF, OSCC, and individuals the control groups (both with habit and without lesion and without habit and without lesion) were analyzed using descriptive statistical analysis. Data were entered into Microsoft Excel sheet and analyzed using Statistical Package for Social Sciences (Graph Pad Prism, version 6.07). ANOVA was used to compare the results of patients of OSMF, OSCC patients and the control groups. Independent sample's *t*-test was used to compare the mean values between the two groups. Comparison of mean values between different groups was done using Student's *t*-test using the appropriate level of significance and degree of freedom. Serum samples were analyzed for estimation and comparison of Cu, Zn, and Cu/Zn ratio. As per Table 1, in Group A, the mean serum levels in OSMF and OSCC was significantly increased as compared to healthy control with habit or without habit. In Table 2, statistical analysis by ANOVA test showed a mean serum Zn level in OSMF patients of 80.40 ± 6.610 μg/dl and in OSCC patients of 59.63 ± 6.846 μg/dl which were

significantly lower ($P < 0.0001$) as compared to the healthy controls in Group C, wherein a mean serum Zn level of 97.67 ± 4.865 μg/dl and Group D wherein a mean of 100.1 ± 4.559 μg/dl were found. As per Table 3, statistical analysis using ANOVA test showed mean serum Cu/Zn ratio in OSMF patients of 1.682 ± 0.1433 μg/dl and in OSCC patients of 2.323 ± 0.3225 μg/dl which were found to be significantly greater with a $P < 0.05$ as compared to the healthy controls in Group C, wherein a mean 1.106 ± 0.0678 μg/dl and group D, wherein a mean of 1.057 ± 0.0677 μg/dl were found. Statistical analysis by ANOVA test showed Mean serum Zn levels between OSMF and OSCC to significantly decrease as compared to the control groups. Mean serum Cu/Zn levels in OSMF and OSCC were significantly increased as compared to the control groups As per Table 4, mean serum Cu levels in OSMF and OSCC were significantly increased as compared to the control groups.

Discussion

Oral cancer is one of the leading causes of death in the Indian subcontinent. Similarly, the existence of precancerous lesions and conditions such as OSMF and leukoplakia is also very high due to various adverse oral habits.[1] Recently, much attention is being provided toward the detection of trace elements in oral cancer and the various precancerous lesions and conditions due to the encouraging results of the studies on head and neck carcinoma, lymphomas, lung and breast carcinomas.[7] Many studies have reported that these trace elements play an inhibitory role in cancers. Trace elements play, directly or indirectly, an important role in various physiological metabolic processes in the humans. Cu and Zn are involved in vital biochemical activities, including the different redox reactions and free radical formation and in maintaining cellular proton homeostasis. Cu is present in many enzymes involved in oxidation (tyrosinase, ceruloplasmin, amine, and cytochrome oxidases).[8] In this study, AAS was used for the analysis of serum levels of Cu and Zn which is a highly specific and sensitive analytical method currently available allowing measurement of micro-concentrations of trace elements in the serum. In the present study, serum Cu levels were analyzed among OSMF, OSCC, and the select control groups. Study results revealed that

Table 1: Comparison of mean serum copper levels between groups

Groups	*n*	Minimum value (μg/dl)	Maximum value (μg/dl)	Mean±SD (μg/dl)
Group A (OSMF)	30	127	140	134.8±4.197
Group B (OSCC)	30	126	144	136.8±5.499
Group C (control with habit or without lesion)	15	98	115	108.3±5.080
Group D (control without habit and without lesion)	15	96	112	106.1±5.257

$P \leq 0.0001$. OSMF: Oral submucous fibrosis, OSCC: Oral squamous cell carcinoma, SD: Standard deviation

Table 2: Comparison of mean serum zinc levels between groups

Groups	*n*	Minimum value (μg/dl)	Maximum value (μg/dl)	Mean±SD (μg/dl)
Group A (OSMF)	30	72	90	80.40±6.610
Group B (OSCC)	30	49	73	59.63±6.846
Group C (control with habit or without lesion)	15	90	104	97.67±4.865
Group D (control without habit or without lesion)	15	92	107	100.1±4.559

$P \leq 0.0001$. OSMF: Oral submucous fibrosis, OSCC: Oral squamous cell carcinoma, SD: Standard deviation

Table 3: Comparison of mean copper/zinc ratio between groups

Groups	*n*	Minimum value (μg/dl)	Maximum value (μg/dl)	Mean±SD (μg/dl)
Group A (OSMF)	30	1.47	2.05	1.682±0.1433
Group B (OSCC)	30	1.78	2.93	2.323±0.3225
Group C (control with habit or without lesion)	15	1.02	1.24	1.106±0.0678
Group D (control without habit or without lesion)	15	0.93	1.17	1.057±0.0677

$P \leq 0.0001$. OSMF: Oral submucous fibrosis, OSCC: Oral squamous cell carcinoma, SD: Standard deviation

Table 4: Comparison of mean serum copper, zinc and copper/zinc ratio between groups

Elements	Group A (OSMF)	Group B (OSCC)	Group C (control with habit or without lesion)	Group D (control without habit or without lesion)
Copper (μg/dl)	134.8±4.197	136.8±5.499	108.3±5.080	106.1±5.257
Zinc (μg/dl)	80.40±6.610	59.63±6.846	97.67±4.865	100.1±4.559
Copper/zinc ratio	1.682±0.1433	2.323±0.3225	1.106±0.0678	1.057±0.0677

OSMF: Oral submucous fibrosis, OSCC: Oral squamous cell carcinoma

increase in mean serum Cu levels were observed in OSCC and OSMF groups when compared to the control groups. Similarly, Haines *et al*.[9] presented data from a study of 28 cases in the Northwick Park Heart Study and 84 controls to assess the relationship between prediagnostic serum Cu levels and subsequent cancer risk assessment where serum Cu levels among the 28 cases was higher than the control group. Khanna S[6], Ayinampudi BK and Narsimhan M[8], Jayadeep A *et al*.[10], Haider SM *et al*.[11] and Shetty SR *et al*.[12], also reported increased Cu levels in the sera of patients with oral premalignant and malignant lesions. Margalith *et al*.[13] suggested that role of Cu ions in biological damage is due to superoxide radicals or other reducing agents such as ascorbate, which eventually leads to damage to proteins, ribonucleic acid (RNA), and deoxyribonucleic acid (DNA), that are not repairable by cellular mechanisms, thereby initiating the malignant process. Ma RH[14] suggested that the initiating role of Cu in OSMF is stimulation of fibrogenesis by upregulation of lysyl oxidase, thereby causing an increased synthesis and correspondingly, decreased degradation of the mature collagen. Jayadeep A *et al*[10], in their study, reported that the rise in serum Cu levels might be due to increased turnover of ceruloplasmin (a Cu carrying globulin with essential oxidase activity) in carcinoma patients.[1,5] Following chewing, the uptake of Cu into the epithelial cells occurs probably by a nonenergy dependent diffusion where it is either bound to the proteins (mainly metallothioneins) or transferred across the basement membrane. The absorbed Cu appears in the blood stream in as little as 15 min after its ingestion.[15] The mutagenicity of trace elements such as Cu has been well documented in head and neck cancers as well in cancers of the gastrointestinal tract, pancreas, and cervix. The exact mechanism of Cu-induced mutagenesis is not fully understood. Cu-induced DNA damage has been reported, and there is evidence to suggest that Cu may bind to the protein product of p53, the major tumor suppressor gene, resulting in alteration of its conformation.[9] Trivedy CR *et al*.[16] have also reported Cu-induced mutagenesis through the p53 aberrations in OSMF which might be critical in the progression of the potentially malignant lesion to OSCC. Copper activates several angiogenic factors including vascular endothelial growth factor, tumor necrosis factor alpha, interleukin-1, and basic fibroblast growth factor which bind to endothelial cells switch from G0 into G1 phase and force cellular proliferation. The level of ceruloplasmin, the principal Cu transporting protein, increases four to eight folds during malignant progression as reported by Nasulewicz A *et al*.[17] However, the findings of the present study contradict the findings of the study conducted by Varghese *et al*.[18] who found a significant reduction in serum Cu levels in OSMF patients. In the present study, the mean serum Zn levels were analyzed among three groups. The study results revealed that there was decrease in mean serum Zn levels among group A and group B compared to the control groups (C, D). The results were in accordance with the various studies conducted by Varghese I *et al*[18] in OL, OSF, OSCC, Abdulla M *et al*[19] in head and neck cancers, Jha IN *et al*[20] in oral cancers and Toke GB and Dhamne BK[21] in head and neck cancers, in the past. Decreased sera levels of zinc have, also, been reported in patients suffering from gastrointestinal cancers, gynecological tumors, lymphomas, and breast and lung carcinomas. Altered sera zinc levels have, also, been correlated with decreased appetite in patients with advanced malignancies, especially, oral cancers. Similar findings were found in the study conducted by Vashistha A *et al*[2] on patients with oral cancer (oral squamous cell carcinoma) and oral submucous fibrosis (OSMF) associated with habit of tobacco consumption in any form as compared to the healthy controls. Shetty SR *et al*[12], also, found decrease in sera levels of zinc in patients with oral pre-cancers and cancers as compared to the controls.[11] Kapil U *et al*[22] observed that 53% of oral cancer patients in Jharkand had serum zinc deficiency and the deficiency was higher in females as compared to the males. Barch DH and Iannaccone PM[23] reported that zinc deficiency causes upregulation of cyclooxygenase-2 (COX-2) and over expression of COX-2 enhances cell proliferation, inhibits apoptosis and modulates angiogenesis, thereby, contributing to carcinogenesis. The role of zinc in RNA and DNA polymerase functioning, its inhibitory effects on enzyme phosphodiesterase and its activating effect on membrane-bound adenyl cyclases, suggests a potential role of zinc in carcinogenesis. Zinc has, also, been shown to stabilize ribosomes and the DNA double helix. Louise Y Fong *et al*[24] concluded from their study that zinc deficiency leads to cancer initiation through activation of NF-κB and the consequent induction of tumorigenic signaling. In the present study, mean serum Cu/Zn ratio was analyzed amongst the three groups and the study results revealed that there was a significant ($p < 0.0001$) increase in mean serum Cu/Zn ratio in groups A and B when compared to the controls (Groups C, D). Similar findings were observed in earlier studies done by Jayadeep A *et al*10, Shetty SR *et al*[12], Varghese I *et al*[18], Abdulla M *et al*[19], Jha IN *et al*[20], Toke GB and Dhamne BK[21], and Altered copper zinc ratio has, also, been observed in patients suffering from pancreatic cancers, gastric cancers, lymphomas, malignant lung tumors and breast cancers. Thus, from the present study, it can be inferred that serum copper and Cu/Zn ratio was significantly higher in OSMF and OSCC patients while simultaneously, there was a significant reduction in mean serum zinc levels when compared with the controls. Thus, the alteration in serum copper and zinc and Cu/Zn ratio can be used as a potential biomarker in early detection of numerous oral pre-cancerous lesions and conditions and cancers as well as their malignant transformation and turning into frank cancers at an early enough stage.

Conclusion

Determination of sera levels of Cu and Zn is simple as well as an inexpensive procedure and can be used as an adjunct screening tool for determining risk in patients with potentially malignant OSMF and/or frank OSCC. The significant alteration in the levels of Cu and Zn in sera may be due to the cellular metabolic changes that occur during the pathogenesis of OSMF and OSCC. Thus, more studies need to be conducted on these trace elements. The potentially strong points of this study were that the controls with chewing habits without lesion as well as without habit and without lesion were included following stringent inclusion and exclusion criteria. Besides, AAS was used to analyze the elements which is more accurate in the elemental analysis than the traditional colorimetric methods. Altered serum trace element levels are documented in the malignant cases and they are considered good biomarkers of malignancies. The serum Cu and Zn levels and the Cu/Zn ratio in the OSMF patients can be considered the markers for susceptibility toward frank malignant transformation and the possibility of turning into OSCC. Concerted efforts of assessing serum levels of trace elements would, therefore, help in early detection, management, and monitoring the efficacy of treatment in these cases.

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Conflicts of interest

There are no conflicts of interest.

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