Journal of Interdisciplinary Histopathology
www.scopmed.org
DOI: 10.5455/jihp.20150717124034

Original Research

A retrospective study on qualitative assessment of copper content in oral leukoplakia, submucous fibrosis, and squamous cell carcinoma with rhodamine stain

Priya Shirish Joshi, Bhagyalaxmi Praveen Hongal, Neha Shashank Agnihotri, Rutuja Shridhar Kempwade

Department of Oral Pathology and Microbiology, Vasantdada Patil Dental College and Hospital, Kavalapur, Sangli, Maharashtra, India

Address for correspondence:
Priya Shirish Joshi, Department of Oral Pathology and Microbiology, Vasantdada Patil Dental College and Hospital, Kavalapur, Sangli - 416 306, Maharashtra, India.
Phone: +91-9890712016,
E-mail: sangeetakov@yahoo.co.in

Received: May 24, 2015
Accepted: July 17, 2015
Published: July 22, 2015

ABSTRACT

Background: Trace elements are required in small concentrations as essential active components of biological enzyme systems. They are in the form of metalloenzymes and copper is one such component. Copper metabolism is profoundly altered in neoplastic disease. Copper plays an important role in tumor angiogenesis especially in early stages and is also necessary for endothelial cell activation. Copper content in tissues of oral leukoplakia (OL), oral submucous fibrosis (OSMF), and oral squamous cell carcinoma (OSCC) has not been studied extensively. Hence, we attempted this retrospective study to analyze copper content in the tissues by using rhodamine stain for better understanding of pathogenesis. **Aim and Objectives:** To evaluate and compare the copper content and its depth of penetration in tissue sections of diagnosed cases of oral precancer and cancer by using rhodamine staining method. **Materials and Methods:** This retrospective analytical study was conducted on histologically diagnosed 10 cases each of OL, OSMF, and OSCC. Ten cases of normal mucosa served as control. The selected cases were subjected to staining with rhodamine for qualitative analysis of copper content. **Results:** Paired *t*-test showed a significant increase in copper content in the study group ($P = 0.516$) as compared to control group. Krushall–Wallis non-parametric test showed higher mean value of copper content in cases of OSMF (2.00) than the cases of OL (1.20) and OSCC (1.70). **Conclusion:** Copper content by rhodamine staining technique was found to be higher in OSMF than OL and OSCC this staining technique can be used as a prognostic indicator for assessment of disease progression.

KEY WORDS: Areca nut, copper, leukoplakia, rhodamine, squamous cell carcinoma, submucous fibrosis

INTRODUCTION

Copper is one of the important trace elements existing in the form of metalloenzymes and is required in small concentration as an essential component of biological enzyme systems. Copper plays a vital role in several physiological functions such as erythropoiesis, leucopoiesis, skeletal mineralization, connective tissue synthesis, myelin formation, melanin pigment synthesis, and may also influence cell growth. Among the various cupro-enzymes, tyrosinase, ceruloplasmin, amine oxidase, and cytochrome oxidase are said to be involved in oxidation reaction. The impairment of cellular and physiological functions is caused by the altered activities of these metalloenzymes.

Several studies in recent years have linked association between micronutrient levels and various forms of cancer. India has one of the highest incidences of oral cancer in the world. The development of cancer is multistep process arising from pre-existing potentially malignant lesions [1]. Copper metabolism is profoundly altered in neoplastic disease and level of ceruloplasmin, the principal copper transporting protein, increases four to eight folds during malignant progression.

There is strong epidemiological evidence associating the neoplastic disease with the habit of chewing areca nut. Areca nut has shown to have a high copper content (302 nmol/g) compared to other commonly eaten nuts (22-173 nmol/g) [1]. The formulation in which areca is consumed varies according to geographical location and is often associated with cultural and religious practices. The basic constituent areca nut is either raw, dried, boiled or in baked form. Other diverse agents including lime, tobacco, catechu, cloves, saffron, and leaf of piper betel leaves may form a part of the formulation. Chewing areca nut for 5-30 min significantly increases soluble copper in whole mouth saliva and will further lead to increased tumor angiogenesis, endothelial cell activation, and proliferation, activation

of several angiogenic factors (vascular endothelial growth factor, tumor necrosis factor-α, interleukin 1) [1]. Copper demonstration, so far has been carried out either by cytology of exfoliated squamous cells [1], biochemical analysis of saliva [2], serum [3], aliquot preparation of biopsy tissue [4] or by mass absorption spectrometry (MAS) on buccal mucosal biopsies [5]. We are attempting to demonstrate copper in paraffin embedded tissue sections of oral leukoplakia (OL), oral submucous fibrosis (OSMF), and squamous cell carcinoma (OSCC). Rhodamine staining on tissue section demonstrates the protein to which the copper binds rather than the copper itself. So, it is considered to be a sensitive method for copper demonstration [6]. As the tissue copper analysis in oral precancer and cancer has not been performed extensively; we have attempted this retrospective analysis to evaluate the copper content in tissue sections by using rhodamine staining.

Aim and Objectives

To evaluate and compare the copper content and its depth of penetration in paraffin embedded tissue sections of diagnosed cases of OL, OSMF, and OSCC by using rhodamine staining method.

MATERIALS AND METHODS

This retrospective observational study was conducted on two groups. Group I consisted of ten cases of normal mucosa (control). Gingival tissue obtained during third molar surgical removal procedure from healthy patient's without a history of chewing areca nut or any other habits acted as a control. Group II consisted of histologically diagnosed ten cases each of OL (Group II a), OSMF (Group II b), and OSCC (Group II c). The selected cases were subjected to staining with rhodamine for qualitative analysis of copper content.

Review of clinical history revealed that in all the selected cases patients had a history of areca nut chewing (areca nut only or in combination with pan masala, tobacco, gutkha), for minimum period of 4-5 years, 5-6 times daily and placed it in the mouth for more than 15-20 min. Cases having a habit of areca nut chewing without having lesions were excluded from the study, as well as a control group.

All the paraffin embedded tissue sections were deparaffinized and dehydrated through 95% alcohol. Positive controls were dipped into copper sulfate for 15 min and then placed in working rhodamine solution (0.2 g) for 2 h at 70°C. The sections were then washed in distilled water, blot dried, placed in hematoxylin stain for 2 min and then placed in Borax for 10 s to remove excess of rhodamine stain. Lastly, the slides were cleaned and mounted with DPX [6].

Rhodamine stained sections were observed under oil emersion and categorized by using two qualitative parameters and cases were divided into three categories as mild, moderate, severe for each parameter [Table 1].

RESULTS

Tissue sections from negative control group showed no evidence of copper granules [Figure 1a] whereas sections from positive control group, which were dipped into the saturated copper sulfate solution showed intense reddish brown colored copper granules [Figure 1b].

Detailed qualitative analysis of study group according to two parameters was carried out and findings were correlated with the clinical type and presence or absence of dysplasia in OL and OSMF and histologically graded cases of OSCC [Table 2].

In Group II a (OL), pale brownish colored copper granules were observed in stratum corneum layer of the epithelium [Figure 2].

In Group II b (OSMF), yellowish brown colored copper granules were observed in stratum corneum and granulosum layers of epithelium, as well as in the deeper portions of the connective tissue and surrounding the lumen of the blood vessels in the advanced cases [Figure 3a-c].

In Group II c (OSCC) pale brownish colored copper granules were observed with relation to stratum corneum and granulosum layer of the surface epithelium in well differentiated squamous cell carcinoma cases [Figure 4], whereas in moderately differentiated cases intense reddish brown copper granules were observed in deeper portions of the connective tissue [Figure 5].

Analysis of color intensity in cases of OL, OSMF, and OSCC is shown in [Table 3].

Table 1: Criteria for qualitative analysis and scoring of copper content

Qualitative parameters → scores ↓ Criteria	Mild	Moderate	Severe
Color intensity of copper granules Depth of penetration	Pale brownish Stratum corneum	Yellowish brown Stratum corneum, granulosum and juxta-epithelial	Intense reddish brown Epithelial and deeper portions of the connective tissue

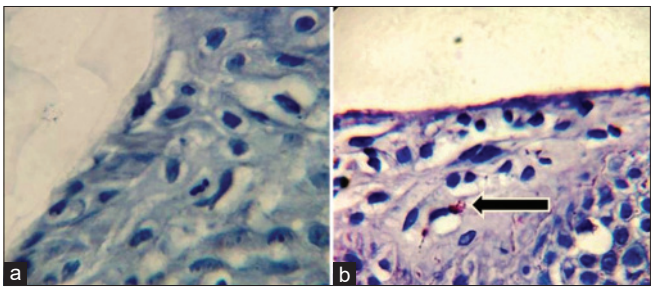

Figure 1: (a) Negative control, (b) Positive control - Intense reddish colored copper granules. Rhodamine stain (×100)

Table 2: Clinical and histological analysis of OL, OSMF and OSCC

N=10	OL		OSMF			OSCC		
Clinical grading	Homogenous	Non homogenous	Early	Moderately advanced	Advanced	-	-	-
Number of cases	9	1	5	4	1	-	-	-
Histopathological grading	With dysplasia	Without dysplasia	-	-	-	Well differentiated	Moderately differentiated	Poorly differentiated
Number of cases	7	3	-	-	-	5	5	0

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

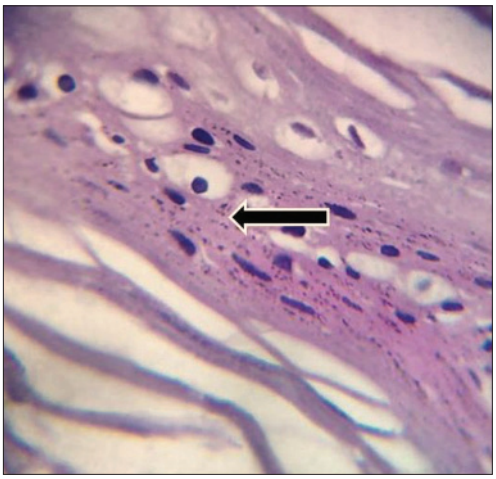

Figure 2: Oral leukoplakia showing brownish colored copper granules in stratum corneum and granulosum layers of surface epithelium. Rhodamine stain (×100)

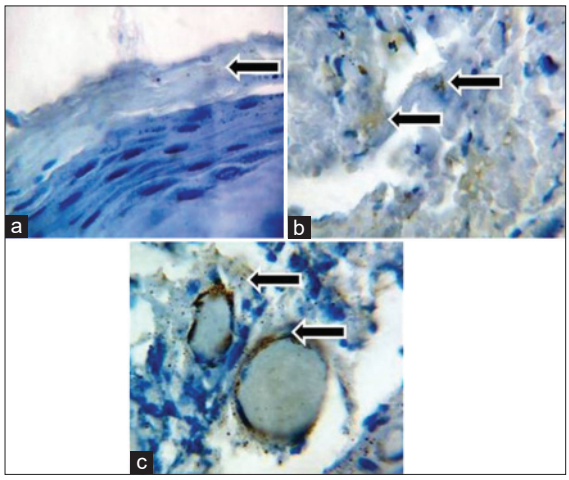

Figure 3: (a) Early oral submucous fibrosis (OSMF) showing pale brownish colored copper granules in stratum corneum and granulosum layers of surface epithelium, (b) Moderate OSMF showing yellowish brown colored copper granules in deeper portions of connective tissue, (c) Advanced OSMF showing intense yellowish brown colored copper granules surrounding the blood vessels. Rhodamine stain (×100)

Statistical Analysis

Paired *t*-test showed significant increase in copper content in study group ($P = 0.516$) as compared to control group. Krushall–Wallis non-parametric test showed significantly higher mean value of copper content in OSMF cases (2.00) than the cases of OL (1.20) and OSCC (1.70).

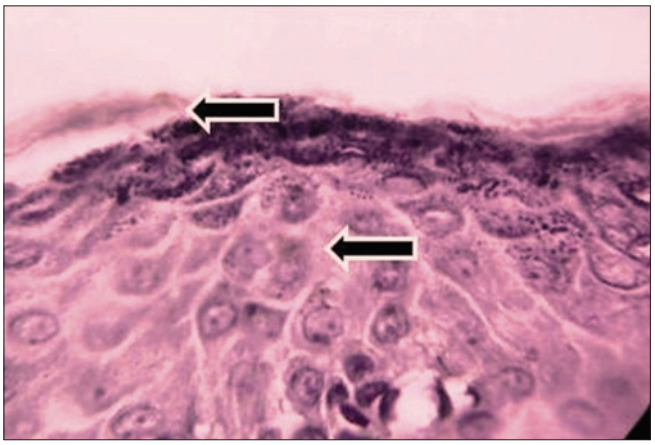

Figure 4: Well-differentiated oral squamous cell carcinoma showing pale brownish colored copper granules in stratum corneum and granulosum layers of surface epithelium. Rhodamine stain (×100)

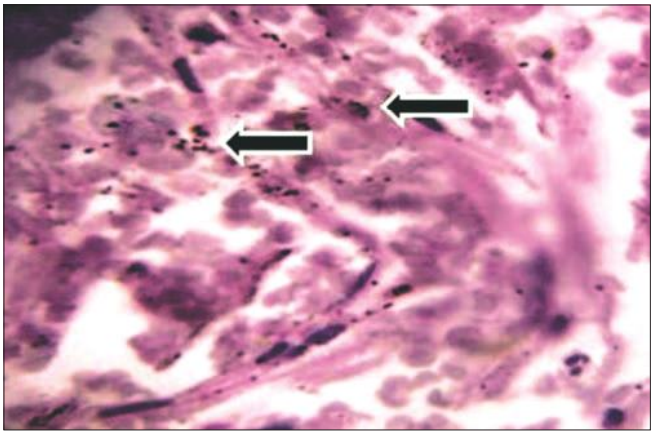

Figure 5: Moderately differentiated oral squamous cell carcinoma showing intense reddish brown colored copper granules in deeper portions of the connective tissue. Rhodamine stain (×100)

DISCUSSION

Recent rise in cases of OSMF has been attributed to consumption of commercially available areca nut/gutkha with very high copper content. Copper levels have been analyzed by using various techniques, so far such as exfoliative cytology [1], biochemical analysis of saliva [2], serum [3], and tissue through aliquot preparation [4], or even by MAS of biopsy tissues [5]. Jain *et al.* [7] have compared the results of liver copper measurement by neutron activation analysis with copper staining by rubeanic acid and rhodanine, and with staining of copper-associated protein by the orcein method, in

Table 3: Analysis of copper content in OL, OSMF, OSCC cases

Study cases (n=10)	Color intensity of copper granules			Depth of penetration of copper granules		
	Mild	Moderate	Severe	Mild	Moderate	Severe
Oral leukoplakia	9	0	1	9	1	0
OSMF	1	8	1	0	6	4
OSCC	1	4	5	1	4	4

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

order to evaluate different methods of demonstrating excess copper in patients with chronic liver disease. But, copper content in the paraffin embedded tissue sections of oral precancer and cancer tissues have not been studied extensively till now. Our study is the first of its kind to demonstrate copper content in paraffin embedded tissue sections of OL, OSMF, and OSCC cases.

Copper released during areca nut chewing is brought in direct contact with the oral mucosa. Rhodamine method is applicable for the evaluation of tissue copper and provides a satisfactory screening method for the identification of the abnormal tissue copper levels. Frequent chewing of areca nut or areca nut products for a longer duration will raise the salivary copper allowing greater absorption through the mucosa. Penetration of the copper granules form the various layers of epithelium to the connective tissue supports the hypothesis of transmucosal transport of the copper to reach the connective tissue which contributes to the further disease progression [1].

Trivedy *et al*. have reported significantly raised copper levels in OSMF tissues by using MAS. They are of the opinion that this fact has important implications in the aetiopathogenesis of OSMF and its progression to malignancy [5]. First, copper has been implicated in tissue fibrogenesis via the copper-dependent enzyme lysyl oxidase, which has a crucial role in the cross-linking of collagen and elastin. Lysyl oxidase has been implicated in other fibrotic disorders such as hepatic and pulmonary fibrosis and scleroderma. Copper is an essential co-factor required for the expression of lysyl oxidase. This enzyme appears to be an intrinsic protein of connective tissue that is induced at detectable levels during fibrogenesis and fibro proliferative processes. The mechanism of this upregulation by fibroblasts in OSMF is not fully understood, but upregulation of this enzyme in OSMF tissues has been demonstrated by immunocytochemical methods [8]. Second, there is evidence to suggest that trace metals such as copper may play an important role in the development and progression of neoplasia [9]. The mutagenicity of trace metals such as copper has been well documented in head and neck cancer as well in cancers of the gastrointestinal tract, pancreas, and cervix [10]. The exact mechanism of copper-induced mutagenesis is not fully understood. Copper-induced DNA damage has been reported [11] and there is evidence to suggest that copper may bind to the protein product of p53, resulting in alteration of its conformation. Aberrations of p53 have also reported in OMSF tissues [12] and P53 stabilization may be critical in the progression of potentially malignant lesions to squamous cell carcinoma which may thus arise from the DNA damage inflicted by chewing copper-containing areca nut [13].

Our study proves the significance of rhodamine stain in copper demonstration which correlates with the study done by Irons *et al*. [14], who concluded that the rhodamine method was found to be effective method for the demonstration of abnormal tissue copper levels in the paraffin embedded tissue sections of liver. In our study, copper content was appreciated as yellowish to reddish brown colored granules. Similar copper demonstration was noted in the study done by Jain *et al*. [7] on histological demonstration of copper in chronic liver diseases by rhodamine staining method.

Our results correlate with the cytological studies done by Rooban *et al*. [1] and Gupta *et al*. [15], who showed significant increase in the copper content in OSMF cases as compared to normal healthy individuals. Nayak *et al*. [4] have stated that in OSMF patients, oral mucosal tissue serves as a better medium for the evaluation of the trace elements as compared to serum and even the three clinical stages of OSMF can be correlated to the progressive increase in tissue copper levels. They have commented favorably on the efficacy of the biochemical analysis as a prognostic indicator for OSMF.

In the biochemical study done by Shetty *et al*. [3] on role of trace elements in serum of patients with oral precancer and cancer, higher serum copper levels in OSMF patients were found than in the cases of OL and OSCC. Similar results were observed in our study. However, our results are in contrast to the findings of Varghese *et al*. [16] who have noted a significant reduction in the serum copper in OSMF patients.

Since, ours is a pioneer study, we propose future studies in this direction with larger sample size in patients with OL, OSMF, and OSCC especially in those having a habit of areca nut chewing.

CONCLUSION

Copper granules in tissue sections of OL, OSMF, and OSCC can be stained excellently by rhodamine staining method. Copper content was higher in OSMF than OL and OSCC. This staining technique can be used as prognostic indicator for assessment of disease progression.

REFERENCES

1. Rooban T, Saraswathi TR, George A, Joshua E, Ranganathan K. Cytological study of copper in oral submucous fibrosis. Indian J Dent Res 2004;15:129-32.
2. Raja KB, Hazarey VK, Peters TJ, Warnakulasuriya S. Effect of areca nut on salivary copper concentration in chronic chewers. Biometals 2007;20:43-7.
3. 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.
4. Nayak AG, Chatra LK, Prashanth S. Analysis of copper and zinc levels in mucosal tissue and serum of oral submucous fibrosis patients. World J Dent 2010;1:75-8.
5. 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.
6. Copper – Rhodamine Method. Staining Manual – Mineral and

Pigments. Surgical Pathology – Histology.1-2, downloaded [Last accessed on 27 October 2014]. Available from: http://library.med.utah.edu/WebPath/HISTHTML/MANUALS/COPPER.PDF

7. Jain S, Scheuer PJ, Archer B, Newman SP, Sherlock S. Histological demonstration of copper and copper-associated protein in chronic liver diseases. J Clin Pathol 1978;31:784-90.
8. Trivedy C, Warnakulasuriya KA, Tavassoli M, Steingrimsdottir H, Penhallow J, Maher R, *et al.* p53 aberrations in oral submucous fibrosis and oral squamous cell carcinoma detected by immunocytochemistry and PCR-SSCP. J Oral Pathol Med 1998;27:72-7.
9. Margalioth EJ, Schenker JG, Chevion M. Copper and zinc levels in normal and malignant tissues. Cancer 1983;52:868-72.
10. Gupta SK, Shukla VK, Vaidya MP, Roy SK, Gupta S. Serum and tissue trace elements in colorectal cancer. J Surg Oncol 1993;52:172-5.
11. Tkeshelashvili LK, McBride T, Spence K, Loeb LA. Mutation spectrum of copper-induced DNA damage. J Biol Chem 1991;266:6401-6.
12. Murti PR, Warnakulasuriya KA, Johnson NW, Bhonsle RB, Gupta PC, Daftary DK, *et al.* p53 expression in oral precancer as a marker for malignant potential. J Oral Pathol Med 1998;27:191-6.
13. Trivedy C, Warnakulasuriya KA, Hazarey VK, Tavassoli M, Sommer P, Johnson NW. The upregulation of lysyl oxidase in oral submucous fibrosis and squamous cell carcinoma. J Oral Pathol Med 1999;28:246-51.
14. Irons RD, Schenk EA, Lee JC. Cytochemical methods for copper. Semiquantitative screening procedure for identification of abnormal copper levels in liver. Arch Pathol Lab Med 1977;101:298-301.
15. Gupta N, Saxena S, Gupta S, Gupta S, Singh V, Yadav J. Role of copper in oral submucous fibrosis: A cytological correlation. Indian J Dent Sci 2010;5:29-32.
16. Varghese I, Sugathan CK, Balasubramoniyan G, Vijayakumar T. Serum copper and zinc levels in premalignant and malignant lesions of the oral cavity. Oncology 1987;44:224-7.

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