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Evaluation of copper salivary level in oral squamous cell carcinoma, occupationally copper exposed, and its normal population and its association with cytomorphologic changes of oral mucosa

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ABSTRACT

Background: Oral squamous cell carcinoma (SCC) is one of the most common malignancies in oral cavity. Hence, presenting methods for early diagnosis and find the etiologic factors of oral SCC are important. Saliva analysis can be used to discover various conditions because of its noninvasive methods. Copper as a useful metal has been used by men since ancient times. The level of copper increases when the cancerous changes occur in addition to biopsy, an alternative method for examining oral lesions is exfoliative cytology. The primary objective of this study was to determine the salivary copper level and cytomorphologic changes of oral mucosa among three study groups.

Materials and Methods: This cross-sectional study included 15 individuals with oral SCC, 15 workers exposed to copper, and 15 healthy individuals. Saliva samples were collected and analyzed by atomic absorption spectrophotometer. The exfoliative smears were prepared by brush biopsy and stained by Papanicolaou and argyrophilic nucleolar organizer region (AgNOR) staining methods. Data analysis using one-way ANOVA and Kruskal–Wallis test. $P < 0.05$ was considered significant.

Results: There was a significant difference in mean salivary copper ($P = 0.008$), cytomorphology of oral mucosa, and AgNOR among the three groups ($P < 0.001$).

Conclusion: The results suggested that occupational exposure to copper increases the salivary levels of this element and causes changes in mucosal cells. Since this increase was very high and evidence of nuclear activity was seen in this group and in oral SCC patients, exposure to copper should be considered an important risk factor for oral mucosal changes.

Key Words: Copper, cytology, saliva, silver nitrate staining, squamous cell carcinoma

INTRODUCTION

Head-and-neck squamous cell carcinoma (SCC), which involves oral cavity, oropharynx, and hypopharynx, is the fifth most commonly occurring cancer in the world and is one of the most frequent causes of death in humans.[1] SCC usually arises

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from a dysplastic mucosa, but dysplastic criteria and early invasive SCC grading are discussable.[2] Several etiologic factors, such as the use of tobacco (smoking and nonsmoking), occupational exposure, environmental pollutants, dust and particles of metals, sunlight, vitamin deficiencies, hot foods, and bacterial and viral infections, have been identified for SCC.[3,4] Several studies have investigated the role of occupational exposures in the occurrence of head-and-neck cancer.[5]

Copper is one of the most useful metals, mined and worked by men since ancient times, because of its essential use in various spheres of life.[6] The level of copper increases significantly when cancerous changes occur in the body. Ceruloplasmin of serum, which is the main protein of copper, rises four to eight times during malignancy.[7] Workers exposed to melting, welding, cutting, or casting copper are also involved with this element.[8]

Recently, saliva analysis had been adverted due to its simple, noninvasive collection, and low-cost storage methods. Oral fluid sampling is safe for the operator and patient.[9]

These days, exfoliative cytology as an alternative method for oral lesions examination is simple, noninvasive, and inexpensive and can be used to conveniently identify biomarkers in laboratories. It is routinely done in medical and dental offices, which may increase the early diagnosis of oral mucosal malignancies in clinical trials.[10] Changes in surface epithelium cells can be reserved in nuclei and it would be a real marker for dysplastic or neoplastic changes.[11]

Papanicolaou (PAP) staining is a common method used for cytology analysis and allows it to identify primary inflammation, dysplastic, or malignant changes.[12]

Over time, the ability of other staining methods and markers to enhance the reliability of exfoliative cytology has been investigated, one of which is silver nitrate staining (argyrophilic nucleolar organizer region [AgNOR]).[13] This technique uses nuclear organizer regions (NORs) as an indicator for the diagnosis of various cancers.[14] These particular portions of DNA are associated with nonhistone, argyrophilic, and acidic proteins.[15] NORs are seen by staining under the optical microscope as the black spots on the nucleus.[16] Given the close relationship between NORs and cellular activity, the size and number of NORs can reflect or predict cell proliferation, transformation, or malignancy.[17] Malignant tumor cells show large numbers per nucleus along with small size, scattered distribution, and an irregular shape of AgNOR. However, a small number of AgNORs per nucleus as well as large size, clustered distribution, and roundness are observed in benign tumor cells.[18]

The aim of this study was to determine the level of salivary copper and cytomorphologic changes of oral mucosa in patients with oral SCC, healthy people, and people with occupational exposure to copper.

MATERIALS AND METHODS

In this cross-sectional study, 15 individuals with oral cancer, 15 workers exposed to copper (workers of the copper melting factory who had at least 5 years of work experience), and 15 healthy volunteers in dental school (control group) were included in the study. The participants were asked to wash their mouths with a physiological serum before starting saliva collection. Then, each individual sat in a comfortable position starting the saliva collection with open eyes, bending slightly forward, and evacuating 5 ml of saliva in the test tube. The specimens were then stored at −20°C and sent to the laboratory for evaluation of salivary copper. After the oral examination, using a disposable cytobrush on the mucosa (in the healthy group and workers, from the buccal mucosa and oral SCC from the lesion area), the cell samples were collected and transferred to a clean and dry slide. Before drying, the specimens were fixed immediately by Pathofix spray (Iran's antibacterial medicine company), which contains 95% ethanol. Then, the slides were stained separately by PAP and AgNOR.

Saliva samples were analyzed by atomic absorption spectrophotometer, and the results were expressed as micrograms per liter (μg/L). The PAP-stained slides were analyzed under a microscope (Olympus BX41TF, Tokyo, Japan) at (×100) magnification, and the parameters mentioned in the smears were analyzed and grouped. The parameters analyzed in the smears by PAP stain included large nuclei, nuclear changing in size and shape, pleomorphism, nuclear borders, nucleus-to-cytoplasm ratio, number of nuclei, and hyperchromatism. Dysplastic changes were registered and grouped in sequence[19]:

- Class I: Normal
- Class II: Atypical
- Class III: Indeterminate
- Class IV: Suggestive of cancer
- Class V: Positive for cancer.
- Then, the AgNOR-stained slides were evaluated for NOR count. In each slide, several cells were explored at (×100) magnification, and one cell was randomly selected. The brown–black spots in the nucleus were counted. The interconnected and nucleolus points were considered one point. The areas of necrosis, severe inflammation, and artifacts were not calculated.[20] Data were analyzed by one-way ANOVA, Tukey's test, Kruskal–Wallis test, and Mann–Whitney test, and $P < 0.05$ was considered significant.

Ethics

This study has been approved by the Research and Ethics Committee of Isfahan University of Medical Sciences by No: 396089.

RESULTS

One-way ANOVA showed a significant difference in mean salivary copper ($P = 0.008$) and AgNOR ($P < 0.001$) among the three groups [Table 1 and Figure 1]. Tukey's test showed that the mean amount of copper was significantly higher in the workers' saliva workers than in those with oral cancer ($P = 0.001$), and it was significantly higher in oral SCC patients than in the control group [$P = 0.04$, Diagram 1]. The average number of AgNOR was significantly higher in oral SCC patients than in workers ($P < 0.001$) and was significantly higher in workers than in the control group [$P < 0.001$, Diagram 2].

Kruskal–Wallis test showed that the cytomorphology of oral mucosa was significantly different between the three groups ($P < 0.001$). To compare the cytomorphology of oral mucosa between two groups, Mann–Whitney test was used. This test showed that oral mucosal cytomorphology classes were significantly higher in people with oral SCC than in workers ($P < 0.001$) and in workers was significantly higher than in the control group [$P < 0.001$, Table 2 and Figure 2].

Table 1: Average salivary copper with µg/L and Ag nucleolar organizer region count in three groups

Class of cytomorphology of oral mucosa	Control group, *n* (%)	Worker, *n* (%)	Oral SCC patient	*P*
I	15 (100)	5 (33.3)	0	<0.001
II	0	4 (26.6)	0	
III	0	6 (40)	0	
IV	0	0	0	
V	0	0	15 (100)	

SCC: Squamous cell carcinoma

DISCUSSION

Oral SCC involves half of the oral cavity malignancies and is one of the major health problems in developing countries that leads to death.[21]

Several factors are involved in the development of oral SCCs, including lifestyle and environmental factors. However, efforts have been made by clinical epidemiologists to illustrate the link between

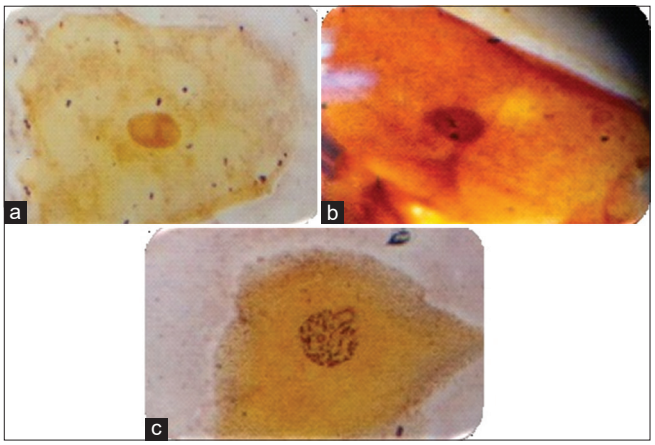

Figure 1: Argyrophilic nucleolar organiser region stain (×100) (a) Control group, (b) Worker, (c) Oral squamous cell carcinoma patient.

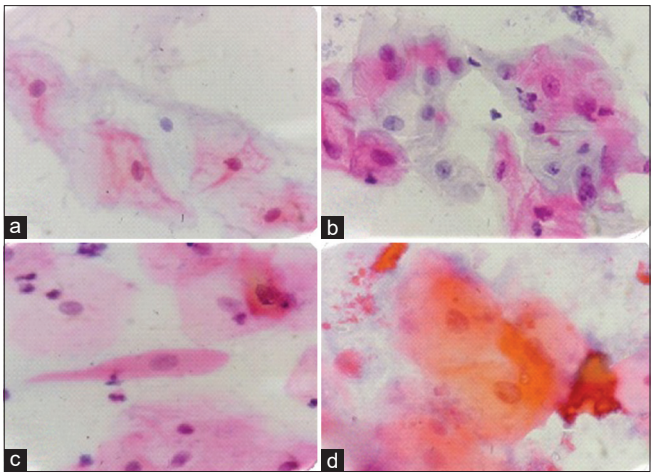

Figure 2: Papanicolaou stain (×100) (a) Normal cells (Class I), (b) Atypical cells (Class II), (c) Indeterminate cells (Class III), (d) Positive for cancer (Class V).

Table 2: Frequency distribution of oral mucosa cytomorphology classes with Papanicolaou staining in three groups

Variable	Control group		Worker		Oral SCC patient		*P*
	Average	SD	Average	SD	Average	SD	
Average salivary copper	31.5	8.7	1215.5	483.4	143.3	32.7	0.008
AgNOR count	1.9	0.8	4	1.8	8.8	1.4	<0.001

AgNOR: Argyrophilic nucleolar organizer region; SD: Standard deviation; SCC: Squamous cell carcinoma

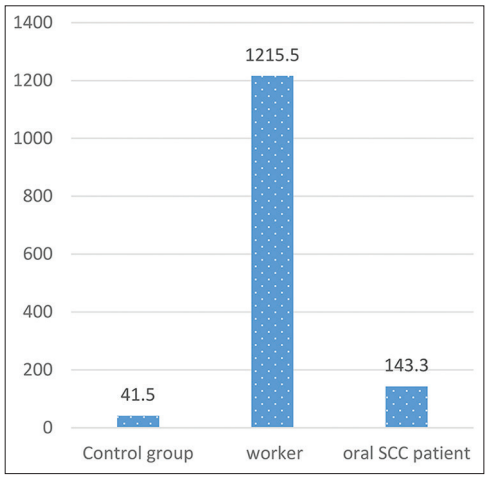

Diagram 1: Average salivary copper in three groups.

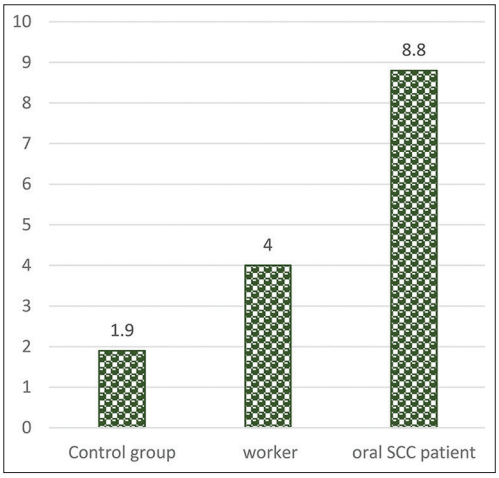

Diagram 2: Average AgNOR count in three groups.

malignancies and scarcity or excessive presence of rare elements.[22]

Some evidence and studies have shown that long-term exposure to an average amount of copper, as in various occupational environments, affects people's health. Moreover, some studies have reported a positive relationship between copper levels in the serum, saliva, and malignancy outbreak.[23,24]

In this study, the copper level in saliva and cytomorphological changes in the oral mucosa of subjects with occupational exposure to copper in comparison with healthy people and those with oral cancer were evaluated using exfoliative cytology and AgNOR staining.

The salivary copper level in this study showed a significant difference between the three groups. It was 1215.5 in the workers, which was higher than normal and oral SCC patients. However, it was higher in people with SCC 143.3 than in healthy individuals 31.5.

Several studies have investigated the serum copper level in various oral, head, and neck lesions, but few studies have examined the salivary copper level in patients.

Al-Rawi and Talabani reported that the salivary levels of copper and iron were higher in malignant individuals than in healthy people.[25] Ayinampudi and Narsimhan also showed a significant difference in the salivary copper level in malignant and premalignant oral lesions.[7]

The results of this study were along with those of the above studies, indicating that the amount of salivary copper increased when malignancy occurred. Although it cannot be argued with certainty that this increase is a secondary response to malignant changes or the patients; for some other reasons, had increased levels of salivary copper before malignancy occurrence. This increase stimulates the proliferation of endothelial cells and plays an important role in angiogenesis, which can be effective in the process of malignancy. Hence, increasing salivary copper content in the workers exposed to this element needs special consideration. Although saliva was easily accessible and could reflect the presence of rare elements in the body, in this study, salivary levels of copper were investigated using a precise method, atomic absorption.

The results of the exfoliative cytology evaluation showed a significant difference among the three groups. The exfoliated cells in patients with SCC were classified as Class V, which were positive for malignancy and showed malignant diagnostic criteria. Cellular changes in the oral mucosa of workers were significantly different from those in normal people. In this group, the cells showed atypical changes (Class II) and intermediate changes (Class III), while in the healthy people group, all cells were normal (Class I) without any changes.

In 2014, Gonzalez Segura *et al.* examined the relationship between PAP staining and

cytomorphometric analysis in exfoliative cytology of oral mucosa and observed that these methods had high accuracy in the diagnosis of malignancy.[26]

Verma *et al*. (2015) conducted a study to evaluate the usefulness of this method in the diagnosis of oral malignant lesions and observed that assessment of changes in the nucleus and cytoplasm by cytomorphometry could be utilized as a very useful complement in the diagnosis and prognosis of dysplastic lesions which could be transformed to malignancy.[27]

The results of this study by this method were in agreement with those of previous studies. Cellular changes that have been made and their transformation from normal to atypical and intermediate cells can indicate the effects of copper on these cells, which requires a special follow-up despite the absence of clear tissue changes.

The mean number of AgNOR showed a significant difference between the three groups. The mean number of AgNORs in oral malignant lesions was 8.8, which was significantly higher than that of the workers with a mean of 4. In the normal group, the mean number of AgNOR was 1.9, which was significantly lower than those of the other two groups.

In a similar study in 2014, Mansoor Samadi *et al*. showed the mean staining dots by AgNOR in specimens of normal mucosa, leukoplakia, and SCC increased, respectively.[28] In 2017, Jajodia *et al*. evaluate the role of brush cytology in the screening of oral malignant suspicion lesions and showed that the sensitivity of oral SCC diagnosis by conventional cytology and liquid-based cytology was 75% and 85%, respectively, which improved on combining with the AgNOR count, with a cutoff of 6.5.[29] The results of these studies were in line with those of our study.

Since the diagnosis of dysplastic changes and suspected malignant lesions is difficult, and sometimes, there is no strong evidence to proof malignancy, evaluation of cytomorphological changes, especially the use of AgNOR staining can be helpful.[30]

The results of this study and other similar studies showed that salivary levels of copper increased in the people exposed to various levels of copper due to the occupational environment, and despite the absence of any apparent changes in the tissue, the mucosal cells exhibited cellular changes. These changes consisted of Class II and Class III of cytomorphology classes, which were confirmed by counting AgNOR-positive points and were higher than the control group. An interesting point in this study was that having longer working experience and more exposure period to copper, increased the amount of salivary copper, and also the changes in epithelial cells. The cytology changes and the number of colored points by AgNOR were higher in these workers than in the others with less work experience. This could indicate that the harmful effects of copper would increase due to the increasing duration and amount. Therefore, populations that are exposed to the particles and dust of this element require special attention.

CONCLUSION

According to the results of the study, it can be concluded that occupational exposure to copper increases the salivary levels of this element and causes some changes in mucosal cells. These changes consist of nuclear activity enhancement and an increase in the number of NOR in the nucleus. Since this increase is very high in salivary levels of copper and nuclear activity in oral SCC patients, exposure to copper should be considered an important risk factor for oral mucosal changes.

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Author's contribution

NT contributed in the conception of the work, conducting the study, definition of intellectual content, interpretation of data for the work, manuscript preparation, editing and review, approval of the final version of the manuscript, and agreed for all aspects of the work. AA contributed in the design of the study, definition of intellectual content, literature search, clinical and experimental, data acquisition, data analysis, statistical analysis, manuscript preparation, editing and review, approval of the final version of the manuscript, and agreed for all aspects of the work. NK contributed in the conducting the study, clinical and experimental and data acquisition, manuscript preparation, editing and review, approval of the final version of the manuscript, and agreed for all aspects of the work. AM contributed in the in the conducting the study, clinical, experimental and data acquisition, revising the draft, approval of the final version of the manuscript, and agreed for all aspects of the work.

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Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or non-financial in this article.

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