

Assessment of cytological atypia, AgNOR and nuclear area in epithelial cells of normal oral mucosa exposed to toombak and smoking

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Abstract

The purpose of this study was to assess cellular proliferative activity of clinically healthy oral mucosal epithelial cells of toombak dippers and smokers by means of AgNOR counts and nuclear areas via nuclear morphometry. Smears were collected from normal-appearing mouth floor mucosa and tongue of 75 toombak dippers, 75 smokers and 50 non-tobacco users between the ages of 20 and 70 with a mean age of 36 years. AgNORs were counted in the first 50 well-fixed, nucleated squamous cells and nuclear areas were calculated via microscopic stage micrometer. Cytological atypia was ascertained in 6 tobacco users and could not be ascertained in non-tobacco users. Statistically mean AgNOR numbers per nucleus in the non-tobacco users (2.45 ± 0.30) was lower than the toombak dippers (3.081 ± 0.39 , $p < 0.004$), and the smokers (2.715 ± 0.39 , $p < 0.02$), and mean nuclear areas of epithelial cells of toombak dippers (6.081 ± 0.39 , $p < 0.009$) and smokers (5.68 ± 10.08 , $p < 0.01$) was also significantly higher than non-smokers (5.39 ± 9.4). The mean number of nuclei having more than 3 AgNORs was 28%, 19% and 7% in toombak dippers, smokers and non-tobacco users, respectively. These findings support the view that toombak dipping and smoking are severe risk factors for oral mucosal proliferative lesions and exfoliative cytology is valid for screening of oral mucosal lesions.

Introduction

The prevalence of potentially malignant oral mucosal lesions and conditions shows wide variations between developed and developing countries.¹ The primary risk factors for devel-

opment of both OSCCs or potentially malignant lesions are considered to be similar.^{1,2} However, social habits of tobacco use and alcohol consumption have been strongly attributed to development of these lesions.^{1,2} In the Sudan, the high incidence of OSCCs and an equally high prevalence of potentially malignant oral mucosal lesions have been strongly attributed to the habit of snuff use, locally known as toombak.³ Toombak has been in use in the Sudan for centuries and its use is widespread.^{3,4} A close relationship between use of toombak and development of OSCCs has been reported.³ In addition, use of toombak has been shown to produce a variety of oral mucosal changes such as dysplasia and hyperkeratosis.^{3,5} An unusually high level of the carcinogenic tobacco-specific nitrosamines (TSNAs) was found in toombak.⁶ In the Sudanese population, however, and due to several reasons, both cigarette smoking and alcohol consumption are uncommon.^{3,5} Early diagnosis is of great importance for oral SCC, oral exfoliative cytology, a simple, painless and inexpensive method has become a preferred method for both early diagnosis of the lesion and for establishing quantitative techniques.^{7,8} The presence of two or more of the following features were consistent with atypia: nuclear enlargement, associated with the increased nuclear/cytoplasmic ratio, nuclear hyperchromatism, chromatin clumping with prominent nucleation, irregularity of nuclear membranes, bi- or multinucleation, increased keratinization.⁹ Nucleolar organizer regions (NORs) are located in the cell nucleoli during interphase. They are loops of DNA in which ribosomal RNA is encoded.¹⁰ They are located in the acrocentric chromosomes 13, 14, 15, 21 and 22.⁷ Their number per nucleus has been shown to be correlated with the rate of ribosomal RNA transcription, cell proliferation and DNA ploidy.¹⁰ Nucleolar organizer regions can be demonstrated with the use of AgNOR technique which is the silver staining technique for showing NORs as black dots inside the nucleus when examined under a light microscope.⁷

Materials and Methods

A total of 200 clinically healthy volunteers seen in our Dental Clinic, aged between 20 and 70, with a mean age of 36 years, of which 75 were toombak dippers, 75 were cigarette smokers and 50 were non-tobacco users, were initially selected for this study. All toombak dippers and cigarette smokers were chosen among persons who have dipped toombak or smoked cigaretteS at least for the last five years and at least 5 cigarettes per day. Patients with clinically apparent oral mucosal lesions, and previous benign or malignant lesions were

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excluded from this study. Also individuals who had drunk any alcoholic beverage for the last ten years were not included in the study.

All cytological smears were collected by the same examiner from the mucosa of the mouth floor and tongue (in smokers) or from the dip site (in toombak dippers). Participants were asked to rinse their mouth with saline solution for a minute before collection of the samples. The specimen collection site was dried by a smooth wipe so as to avoid silver staining of the mucoid material of saliva during application of AgNOR method to the slides. The material was collected by a smooth brush after brushing the floor of the mouth and tongue or dip-site two times, and rinsing and cleaning the brush each time in a saline solution. This was done so as to collect cells from the inner layers of the oral mucosa. The material collected was smeared on two slides and immediately fixed in 95% ethyl alcohol for 15 minutes. One slide was stained according to the Papanicolaou staining method⁷ and the other was stained according to AgNOR staining method described by Ploton *et al.*¹¹

Papanicolaou staining method

Ethyl alcohol fixed smears were hydrated in descending concentrations of 95% alcohol through 70% alcohol to distilled water, for two minutes in each stage. Then the smears were treated with Harris' hematoxylin for five min-

utes to stain the nuclei, rinsed in distilled water and differentiated in 0.5% aqueous hydrochloric acid for a few seconds, to remove the excess stain. They were then immediately rinsed in distilled water, to stop the action of discoloration. Then the smears were blued in alkaline water for a few seconds and dehydrated in ascending alcoholic concentrations from 70%, through two changes of 95% alcohol for two minutes for each change. The smears were next treated with Eosin Azure 50 for four minutes. For cytoplasmic staining, they were treated with Papanicolaou Orange G6 for two minutes, rinsed in 95% alcohol and then dehydrated in absolute alcohol. The smears were then cleared in Xylene and mounted in DPX (Distrene Polystyrene Xylene) mount. All the reagents used were from Thermo Electron Corporation, UK.

Atypia was assessed cytologically by using the criteria described elsewhere.¹² The presence of two or more of the following features were consistent with atypia: nuclear enlargement associated with increased nuclear cytoplasmic ratio, hyperchromatism, chromatin clumping with moderately prominent nucleoli, irregular nuclear membranes and bi- or multi-nucleation, scant cytoplasm, and variation in size and/or shape of the cells and nuclei.

AgNOR staining method

The smears were stained according to the AgNOR staining method. Working solution was freshly prepared by mixing one volume of 2% gelatin in 1% formic acid solution and two volumes of 50% aqueous silver nitrate solution. All smears were incubated with this silver solution for 30 minutes at room temperature in a dark medium and they were protected in the dark until each slide was analyzed.

Two investigators, blind to the study groups, analyzed the silver-stained cells under light microscope (Olympus BX-51, Japan) at 1000x magnification. All smears were screened horizontally from left to right and AgNORs were counted in the nuclei of the first 50 non-overlapping, inner layers, nucleated epithelial cells. Superficial cells with pyknotic nuclei were not counted. The AgNOR count was made adopting the method described by Crocker *et al.*¹³

AgNORs, which were visible as black-dark brown dots located within the nuclei of the cells, were counted; overlapped black dots were counted as one structure. To calibrate the examiners, ten smears from each group (toombak dippers, smokers and non-tobacco users) were counted three times in a non-consecutive way. Mean number of AgNORs and mean percentage of nuclei with more than three AgNORs was also calculated.

For calculating the nuclear areas of the epithelial cells, the smears were further assessed. Fifty cells, having the same proper-

ties as the cells whose AgNORs were counted, were selected and the nuclear area of each cell was calculated via the microscopic stage micrometer.

Statistical analysis

SPSS version 12 statistical software was used for statistical analysis. The numeric results (AgNOR counts and nuclear areas) were expressed as mean \pm SD, and the 95% confidence intervals (CIs) of the means were calculated. The χ^2 test was used to compare the differences in categorical variables between the groups. Relationships between variables were analyzed using Pearson's correlation analysis. A $p < 0.05$ was considered statistically significant.

Ethical consent

Each participant was asked to sign a written ethical consent form during the interview, before the specimen was taken. The informed ethical consent form was designed and approved by the ethical committee of the Faculty of Medical Laboratory Research Board, Sudan University for Science and Technology.

Results

Mean age was 36.2 \pm 10.7 in the study population; 34.9 \pm 11.6 in non-tobacco users, 34.7 \pm 8.5 in smokers and 38.5 \pm 11.8 in toombak dippers. All the population were males and there were no statistically significant differences between non-smokers and toombak dippers ($p=0.09$) or smokers ($p=0.94$) in age. Cytological atypia was ascertained in 6 tobacco users and could not be ascertained in non-tobacco users. Among the 6 subjects with cytological atypia, there were 4 (67%) toombak dippers and 2 (33%) cigarette smokers.

In toombak dippers, AgNOR numbers per nucleus varied from 1 to 9, in smokers from 1 to 7, while the range was between 1 and 4 in the non-tobacco users. The mean \pm SD of AgNOR numbers per nucleus in the non-tobacco users (2.45 \pm 0.30) was lower than the toombak dippers (3.081 \pm 0.39, $p < 0.004$), and the smokers (2.715 \pm 0.39, $p < 0.02$), and mean \pm SD nuclear areas of the oral epithelial cells of toombak dippers (6.081 \pm 0.39, $p < 0.009$) and smokers (5.68 \pm 10.08, $p < 0.01$), were also significantly higher than non-tobacco users (5.39 \pm 9.4). The mean number of nuclei having more than 3 AgNORs was 28%, 19% and 7% in toombak dippers, smokers and non-tobacco users, respectively.

Discussion

In this study we have taken the smears from the mouth floors and tongue of the study subjects. It has been found that the mean AgNOR counts in smokers are the highest in epithelial cells of floor of the mouth when compared with the edge of the tongue and lower lip,⁷ although some studies have reported strong association between smoking and carcinoma of the tongue. Consequently, our cytological materials were collected from the mouth floors and tongues of the smokers. Concerning toombak dippers we planned to take the cytological materials from the application site (dip site). Generally toombak is dipped and retained between gum and lip or cheeks or floor of the mouth, and sucked slowly for about 10 \pm 5 minutes.⁴ Toombak dippers develop a clinically and histologically characteristic lesion at the site of dipping.³

In the present study it can also be seen that cytological atypia among the tobacco users is significantly higher than the non-tobacco users, which is a similar finding with the study of Ahmed *et al.*¹² Their findings suggested that toombak dipping and cigarette smoking are associated with a risk for occurrence of oral epithelial atypia, which can be detected by use of simple cytological methods. The findings further suggested that tobacco components, specially the TSNAs, may stimulate the epithelial cells to undergo squamous differentiation/or cellular morphological changes that might lead to malignancy as suggested by others.^{6,14}

AgNOR counts have been of great value for the assessment of cellular proliferative activity that is frequently encountered in pre-malignant and malignant changes.¹⁵ A number of studies have pointed out that the AgNOR count is a rapid and an easily reproducible method which permits a clear distinction between malignant and benign cells.^{15,16}

There are many previous AgNOR studies of oral mucosa with benign, pre-malignant and malignant lesions, and only a few studies have been conducted on exfoliative cells obtained from oral mucosa exposed to cigarette smoking or alcohol.^{7,17-19} It is noteworthy that to the best of our knowledge there has been no AgNOR study of normal oral mucosa exposed to toombak dipping. Mean AgNOR counts and keratinization are the only parameters that are sought in most of these studies. Though some cytological studies have assessed the oral epithelial atypia of toombak dippers,^{8,12} to the best of our knowledge, this is the first study including cytological atypia, mean AgNOR counts and nuclear areas of oral mucosal cells exposed to toombak dipping and cigarette smoking, and a comparison of all these parameters.

However, there is no study from the Sudan

measuring AgNOR or NA. Similar outcomes in the effect of toombak in previous studies reported that the use of toombak plays a major role in the etiology of oral cancer in the Sudan.^{3,8}

Significant elevation in the mean AgNOR number of the smoker population was previously documented by some similar studies.^{7,19,20} In the present study it can also be seen that mean nuclear area of the smoker group is significantly higher than the non-tobacco users, which is a similar finding to another study reported elsewhere.²¹

Results of both mean AgNOR counts and mean nuclear area show that cellular proliferation is significantly higher in tobacco users and this causes an increase in the nuclear atypical changes of oral epithelial cells, which can be accepted as a progression towards features of dysplastic cellular changes. It is well established that, changing of a normal cell to a malignant cell requires the occurrence of a precursor non-malignant cell, which exhibits increased DNA changes, cell proliferation and apoptosis.²²

Another remarkable outcome of our study was the percentage of cells with AgNOR counts of more than three. It was greater in tobacco users (toombak (28%) and smokers (19%) than in non-tobacco users (7%), which is a consistent finding with the study that compared the mean AgNOR count between smokers and non-smokers.¹⁷ Nevertheless, mean AgNOR counts of both tobacco users and non-tobacco users are relatively lower than that of some previous studies.^{7,10,20} Mean nuclear areas of both groups are also smaller than the mean nuclear areas calculated in the previous studies.^{10,23} Although there might be negligible differences in counting and calculating techniques, we believe that the variation in age of the study population may be an explanation for the differences in our results from previous studies. It is well-known that the risk of cytological atypia increases with increasing age.¹² In conclusion, our results support the view that tobacco use is a risk factor for the development of oral epithelial proliferative changes, and exfoliative cytology can be the preferred method for screening for oral mucosal lesions. As oral cancer is a major health problem in the Sudan, strategy for prevention comprising comprehensive public educational program is highly recommended. All those involved in the habit of toombak use should undergo a continuous screening program.

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