EVALUATION OF AgNOR STAINING IN EXFOLIATIVE CYTOLOGY OF NORMAL ORAL (BUCCAL) MUCOSA: EFFECT OF SMOKING

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Introduction: There are many studies regarding the ability of AgNOR staining to show cellular proliferative activity; including its ability to distinguish the thyroid follicular adenoma and follicular carcinoma; comparison of dysplastic, and non-dysplastic mucosa; and the SCC grade I, II, III study of curettage normal endometer, hyperplastic and malignant comparison of different types of amelobelastoma. But regarding the comparison of normal buccal mucosa in cigarette smokers and nonsmokers with this staining and in this cytological study, there is only one experiment done by Sampo in Brazilia, and the samples in their study are less than ours.

Materials & Methods: In this basilar-applied study, 60 patients were chosen from patients referred to the Surgical Department of the Mashhad Dental School. Thirty of them were cigarette smokers (smoking at least 20 cigarettes per day for 10 years) and the other 30 were nonsmokers. None of the patients in both groups had any oral lesions, systemic diseases, or stimuli in their mouth. After rinsing with 0.9% sodium chloride for 15 minutes, cytologic samples were prepared, dried, and fixed. The samples were stained using the AgNOR method and observed in immersion oil at 1000 x magnification. Finally, 100 cells were randomly selected; the AgNOR dots counted and their means recorded. The student t-test was used for data analysis.

Results: In smokers, the mean AgNOR count was 3.6 and in nonsmokers it was 1.96 (P<0.0001). The percentages of cells, which showed at least 3 AgNOR dots in their nucleuses, were 83.6% in smokers and 21.46% in nonsmokers (P<0.0001).

Conclusion: 1-Cigarette smoking increases cellular proliferation significantly. 2-This proliferation is observable with AgNOR staining before any clinical symptom has appeared.

Key words: Cytology, AgNOR, smoking, oral mucosa.

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A strong causal relationship exists between cigarette smoking and the development of oral squamous cell carcinoma.1-4 Nevertheless, there is little reference in the literature to a cytologic assessment of the effects of smoking upon normal oral mucosa.

The nuclear organizer regions (NORs) represent loops of DNA actively transcribing to ribosomal RNA and then to ribosome and finally to protein.5,6 NORs are associated with acidic, argyrophilic, nonhistonic proteins that are visualized with the use of a silver-staining AgNOR technique.7,8 Studies using this technique have suggested that quantitative analysis of AgNORs can be useful in estimating proliferative activity of neoplasms in surgical pathology.9 In cytologic preparations, the AgNOR technique has not been frequently used. However, some studies have demonstrated good results with this technique using ethanol-fixed cytologic smears.10-12

The purpose of this study was to compare the AgNOR count of cells collected from normal buccal mucosa by exfoliative cytology in smokers and nonsmokers.

Materials & Methods

A total of 60 smokers and nonsmokers with an age range of 42-71, were selected for the study. These were patients of the Surgical Department of the School of the Mashhad University of Medical Sciences for routine dental treatment. The smokers had been using a minimum of 20 cigarettes a day for at least 10 years. None of the patients in both groups had any oral lesions, systemic diseases, or stimuli in their mouth. The smears were taken from clinically normal buccal mucosa.

First, the patients used a mouthwash with 0.9% sodium chloride solution for 15 minutes, and the area to be smeared was wiped of excessive saliva and surface debris. The smears were obtained with a wooden tongue spatula scraped firmly across the mucosa and the cells were scattered on a dry glass slide, and then fixed in 95% ethanol for 12 hours.
Fixed smears were subjected to the silver-staining method for AgNOR proteins according to Ploton's method. The final 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. Slides were incubated in darkness with this silver solution for 30 minutes at 45°C. The AgNOR count was established in 100 cells for each cytologic smear. The cells were examined at 1,000 x magnification under immersion oil. The difference between the AgNOR count of smokers and nonsmokers was assessed using the student t-test. The mean percentage of cells with three or more AgNOR-stained dots per nucleus in each group was also determined.

Results
AgNOR dots were located strictly within the nuclei and were clearly visible as black dots. The mean and range of AgNOR dots and also the mean percentage of cells with three or more AgNOR dots per nucleus in both groups is shown in Table 1. The mean±SD of AgNOR dots per nucleus in the smoking group was statistically higher than the nonsmoking group (p<0.001). The mean percentage of cells with three or more AgNOR dots per nucleus in the smoking group was statistically higher than the nonsmoking group (p<0.001).

In Figures 1 and 2, one sample of nucleus with AgNOR dots in smokers and nonsmokers is clearly visible as black dots.

Discussion
Exfoliative cytology has not been frequently used to evaluate the effects of cigarette smoking on the normal oral mucosa. The karyopyknotic index,13,14 and nuclear and cytoplasmic area15 of oral mucosa have so far been investigated, and alterations were observed in normal oral mucosa of smokers. It has been shown smokers have an increased number of leukoplasic lesions.16 In addition, the proportion of smokers among patients with oral squamous cell carcinoma is two to three times higher than that of the general population.17

The silver-staining method for identification of NORs has been recently introduced into pathology18 and has been frequently utilized in formalin-fixed, paraffin-embedded specimens. Work using the AgNOR technique has demonstrated a correlation between the number of AgNORs per nucleus and proliferative activity on a variety of pathologic entities.19-23 Frequently, malignant tumor cells show large numbers per nucleus along with small size, scattered distribution, and an irregular shape of AgNOR. While small numbers of AgNORs per nucleus as well as large size, clustered distribution, and roundness are observed in benign tumor cells.

The AgNOR technique in cytologic smears has not been used frequently. Sujathan et al11 used this technique to distinguish reactive mesothelial cells from malignant cells in serous effusions applied to cytologic preparations. The author demonstrated that malignant cells presented large numbers per nucleus and irregular-shaped AgNORs, while in benign cells the AgNOR number per nucleus was comparatively fewer, and the AgNORs had a regular shape. Cardilho10 also demonstrated a correlation between AgNORs in benign and malignant cells in fine needle aspiration cytology of salivary gland masses. In cytologic smears, the analysis of AgNORs is more accurate because the whole nucleus can be assessed as it occurs in tissue sections. This present study successfully used the AgNOR technique in cytologic smears of oral buccal mucosa.

Table 1. Statistical analysis of AgNOR dots in normal buccal mucosa cytology of smokers and nonsmokers

<table>
<thead>
<tr>
<th>Study group</th>
<th>Number of cases</th>
<th>Mean of AgNOR</th>
<th>Range of AgNOR dots</th>
<th>SD</th>
<th>Mean percentage of cells with*</th>
<th>Range of cells with*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonsmokers</td>
<td>30</td>
<td>1.96±0.14</td>
<td>1.72-2.18</td>
<td>0.14</td>
<td>22.13±3.82</td>
<td>16%-30%</td>
</tr>
<tr>
<td>smokers</td>
<td>30</td>
<td>3.6±0.43</td>
<td>2.92-4.80</td>
<td>0.43</td>
<td>83.6±8.72</td>
<td>64%-98%</td>
</tr>
</tbody>
</table>

*Three or more AgNOR dots per nucleus

Fig 1. Six AgNOR dots in each of the squamous cells of normal buccal mucosa in smokers. (AgNOR staining, 1,000x).

Fig 2. Two AgNOR dots in the squamous cell of normal buccal mucosa in nonsmokers. (AgNOR staining, 1,000x).
In this study, low AgNOR numbers per nucleus, clustered distribution, and a round shape in both groups were characteristic of benign cells. This is compatible with clinical aspects of the normal buccal mucosa of smokers and nonsmokers. However, the AgNOR number in smears of smokers was higher than those of nonsmokers indicating increased proliferative activity in cells of normal buccal mucosa in smokers. The higher frequency of cells with three or more AgNORs per nucleus in smokers was indicative of higher proliferative activity.

Zimmermann and Zimmermann\textsuperscript{14} observed that age, smoking habit, and oral and systemic disease modified the degree of keratinization of oral mucosa at certain sites. The patients in our study did not show any systemic or oral diseases.

**Conclusion**

1. Cigarette smoking increases cellular proliferation significantly.

2. This proliferation is observable with AgNOR staining before any clinical symptom has appeared.

3. Conducting more studies regarding the effect of age, sex, the number of cigarettes per day, and on other areas of the mouth is necessary. It is recommended that other proliferative indices, as ki-67, be compared with AgNOR staining as well.

**References**


