Original Article / *Klinik Çalışma - Araştırma*

Evaluation of Cytological Alterations in Normal-Appearing Oral Mucosal Epithelia of Smokers and Non-Smokers Via AgNOR Counts and Nuclear Morphometry[*]

Sigara İçen ve İçmeyenlerin Normal Görünümlü Oral Mukozasındaki Sitolojik Değişikliklerin AgNOR Sayımı ve Nükleer Morfometri ile Değerlendirilmesi

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Objectives: We planned this study to evaluate the proliferative activity of the oral mucosal epithelial cells of smokers and non-smokers via nuclear morphometry and AgNOR counts.

Patients and Methods: Smears were collected from normal-appearing mouth floor mucosa of 40 non-smokers and 40 smokers between ages of 50 and 70. AgNORs were counted in the first 50 well-fixed, nucleated squamous cells and nuclear areas were calculated via computerized image analyzing system.

Results: Statistically mean AgNOR numbers per nucleus in the nonsmoking group (3.47±0.30) was lower than the smoking group (4.22±0.39, p<0.001), and mean nuclear areas of squamous cells of smokers (94.32±10.08) was also significantly higher than non-smokers (87±9.4, p<0.05). The mean number of nuclei having more than 5 AgNORs was 14.6% and 36.8% in non-smokers and smokers, respectively.

Conclusion: Our results support that smoking is a severe risk factor for oral mucosal proliferative lesions and exfoliative cytology can be the preferred method for screening of oral mucosal lesions.

Key Words: Cytology; AgNOR; morphometry; smoking; oral mucosa.

Amaç: Sigara içen ve içmeyen kişilerde normal oral mukozaya ait epitel hücrelerindeki proliferatif aktivite AgNOR boyama tekniği ve nükleer morfometri ile değerlendirildi.

Hastalar ve Yöntemler: Yaymalar 50-70 yaş arasında sigara içen ve içmeyen, 40'ar hastanın normal görünümlü ağız taban mukozasından elde edildi. İyi tespit edilmiş nükleuslu ilk 50 skuamöz epitel hücresinde AgNOR'lar sayıldı ve bilgisayarlı görüntü analizi ile nükleer alanlar hesaplandı.

Bulgular: İstatistiksel olarak sigara içmeyen grupta nukleus başına düşen ortalama AgNOR sayısı (3.47± 0.30) sigara içenlerden daha azdı (4.22±0.39, p<0.001). Ayrıca sigara içenlere ait hücre çekirdeklerinin alan ortalamaları (94.32±10.08) içmeyenlerden daha yüksek bulundu (87±9.4, p<0.05). Beş taneden fazla AgNOR'a sahip olan nukleusların ortalama sayısı sigara içmeyen ve içenlerde sırasıyla %14.6 ve %36.8 olarak bulundu.

Sonuç: Bulgularımız sigara içiminin oral proliferatif lezyonların oluşmasında önemli bir risk faktörü olduğunu ve bu lezyonların taranması için oral eksfolyatif sitolojinin tercih edilebilecek bir yöntem olduğunu ortaya koymaktadır.

Anahtar sözcükler: Sitoloji; AgNOR; morfometri; sigara içimi; oral mukoza.

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Squamous cell carcinoma (SCC), the most frequent malignancy of the mouth, is among the ten most common cancers in the world, accounting for more than 90% of all oral malignancies.[1,2] Being lifestyle-related, oral cancer is an avoidable disease by quitting smoking habits.[3] The contents of tobacco have been identified as mutagenic in vitro and in vivo.[4] Thus, smoking is known to be the most important risk factor for development of oral SCC.[5,6] Other most frequent agents associated with the development of oral cancer are exposure to the sun (lower lip) and chronic alcohol consumption.[7] Prognosis of oral SCC is poor with 56% and 41% of 5- and 10-year survival rates respectively.[8] Squamous cell carcinoma is more frequent in men older than 40 years and most frequent locations of the tumor are the lower lip, border of the tongue and floor of the mouth.[9]

As early diagnosis is of extreme 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.[9] Most challenging lesions of the oral cavity are the dysplastic lesions which are clinically often diagnosed as leukoplakias.[10] Leukoplakia, as a clinical entity, may have varied histological presentations, ranging from mildly hyperkeratotic lesions to the lesions that exhibit severe dysplastic features. Tobacco smoking and tobacco chewing are the known risk factors for oral leukoplakia.[11] However, dysplasia is not necessarily limited to leukoplakia.[12] It was found that, 4.5% of clinically benign-appearing lesions have dysplastic or carcinomatous features.[7] Thus, cytological screening of the patients, with high risk of oral neoplastic lesions and without any macroscopically apparent oral lesion, can be of extreme importance.

Nucleolar organizer regions (NORs) are located in the cell nucleoli during interphase. They are loops of DNA in which ribosomal RNA is encoded.[1] They are located in the acrocentric chromosomes 13, 14, 15, 21 and 22.[9] Their number per nucleus has been shown to be correlated with the rate of ribosomal RNA transcription, cell proliferation and DNA ploidy.[1] Nucleolar organizer regions can be visualized 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.[5,9] This technique has been used to identify various benign and malignant lesions, to establish prognoses and to determine the proliferative activity of the cells.[9]

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 are known to be the most important signs of cellular atypia in the squamous epithelium.[13] Proliferative activity is also prominent in the atypical or dysplastic cells of the epithelium.[1] Thus, nuclear areas of the proliferating squamous epithelial cells can be expected to widen.

The purpose of this study was to evaluate cellular proliferative activity by means of AgNORs per nucleus quantification and to assess the correlation of nuclear areas with mean AgNOR counts of the nuclei in clinically normal oral mucosal epithelium.

PATIENTS AND METHODS

A total of 116 patients seen in the Sevgi Dental Clinic, aged between 50 and 70, of which 55 were smokers and 61 were non-smokers, were initially selected for this study. All smokers were chosen among patients who have smoked at least for the last 20 years and at least 20 filtered cigarettes per day. Patients with systemic disease, clinically apparent oral mucosal lesions, and previous benign or malignant lesions were excluded from this study. Also patients who had drunk any alcoholic beverage more than 1 glass a week for the last 20 years were not included in the study. After the initial examination of 116 slides, 28 patients with hypocellular slides or with slides having severe fixation or overlapping or crashing artifacts were also excluded. Aiming to obtain better statistical results, eight non-smoker patients were excluded randomly to make the smoking and non-smoking groups

Table 1. There was no statistical significance between smokers and non-smokers in age and sex. Difference between mean±SD of AgNOR counts and nuclear areas were statistically significant

	Non-smokers (n=40)	Smokers (n=40)	*p*
Age	60.2±6.1	57.6±7.3	0.091
Sex, male	18 (45.0)	22 (55.0)	0.502
AgNORs	3.5±0.3	4.2±0.4	<0.001
Nuclear areas	87.6±9.4	94.3±10.1	0.003

equal to each other. Thus, of the 116 patients initially examined, 80 (18 smoker women, 22 non-smoker women, 22 smoker men and 18 non-smoker men) were included in the study (Table 1). Brushing habits were also asked to the patients, and patients were also grouped according to their brushing habits; 29 patients were brushing their teeth once a day or less (non-brushers), whereas 51 patients were brushing their teeth twice a day or more (brushers). 18 of non-brushers were also smokers (45%) and of non-smokers only 11 (27.5%) were non-brushers.

All cytologic smears were collected by the same examiner from the mucosa of the mouth floor. Partial or complete removable dentures were removed before the collection of the smears. Patients were asked to rinse their mouth with saline solution for a minute before collection of the samples. The area which will be subject to the specimen collection 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 3 times, and rinsing and cleaning the brush each time in a saline solution. This was done so as to collect cells from the basal and parabasal layers of the oral mucosa. The material collected was smeared on a slide and immediately fixed in absolute alcohol for 30 minutes.

The slides were stained according to the AgNOR staining method described by Ploton et al.[14] 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 slides were incubated with this silver solution for 30 minutes at room temperature in a dark medium and they were protected in dark until analyzing each slide (Fig. 1a, b).

An observer, blind to the study groups, analyzed the silver-stained cells under light microscope (Olympus BX-51, Japan) at 1000x magnification. All slides were analyzed horizontally from left to right and AgNORs were counted in the nuclei of the first 50 non-overlapping, well-fixed, nucleated squamous cells. Superficial cells with picnotic nuclei, though very scarce, were not examined. The AgNOR count was performed according to the technique described by Crocker et al.[15] The black dots inside the well-defined

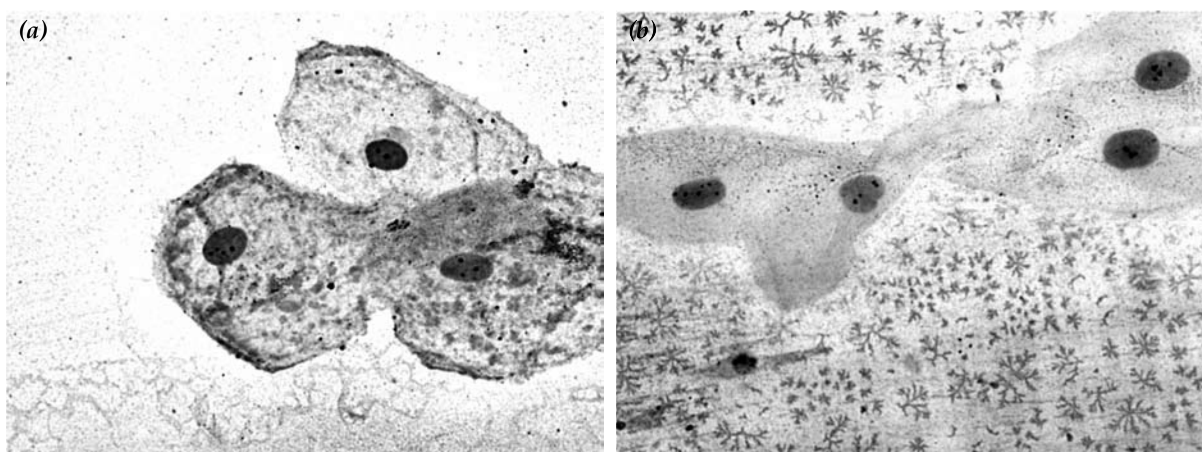

Fig. 1- ***(a)*** *Squamous epithelial cells of a non-smoker (AgNOR x 400).* ***(b)*** *Squamous epithelial cells of a smoker (AgNOR x 400).*

nuclei were counted; overlapped black dots were counted as one structure. To calibrate the examiner, the first ten slides were counted three times in a non-consecutive way.[16] Mean number of AgNORs and mean percentage of nuclei with more than five AgNORs were also calculated.

Images were captured by a Sony D-73431 Aalen 3CCD color video camera which was attached to Zeiss MC 80 DX Axioplan 2 imaging microscope and for calculating the nuclear areas of the squamous cells, the images were transferred to the connected computer using KS300 environment with software of Autocyte Image Management System. Fifty cells, having the same properties with the cells which AgNORs were counted, were chosen and nuclear area of each cell was calculated via the image analyzing system.

The numeric results (AgNOR counts and nuclear areas) were expressed as mean±SD, and the 95% confidence intervals (CIs) of the means were calculated. Categorical results were expressed as number (percentage). Normality distribution of the variables was tested using one sample Kolmogorov-Smirnov test. Differences between groups were assessed using Student's t test due to the normal distribution. The chi-square test was used to compare the differences of categorical variables between the groups. Relationships between variables were analyzed using Pearson correlation analysis. A p value <0.05 was considered statistically significant. Statistica 7.0 statistical software was used for statistical analyses.

RESULTS

Mean age was 60.2±6.1 in non-smokers and 57.6±7.3 in smokers. Male gender was 45% in non-smokers and 55% in smokers (Table 1). However, there were no statistical significance between smokers and non-smokers in age and sex (p=0.091 and p=0.502, respectively).

AgNORs which were visible as black-dark brown dots were located within the nuclei of the cells (Fig. 1a, b). They were round and most were clustered in distribution. In smoking group AgNOR numbers per nucleus varied from 1 to 9 while the range was between 1 and 8 in the nonsmoking group.

The mean±SD of AgNOR numbers per nucleus in the nonsmoking group (3.47±0.30) was lower than the smoking group (4.22±0.39; p<0.001) (Fig. 2a), and mean±SD nuclear areas of the squamous cells of smokers (94.32±10.08) were also significantly higher than non-smokers (87±9.4; p=0.003) (Fig. 2b). AgNOR counts and nuclear areas showed a positive relationship among whole patients (n=80; smokers + non-smokers) (r=0.338; p=002). Likewise there was a positive relationship between AgNOR counts and nuclear areas among non-smokers (r=0.422; p=0.007). However, among smokers there was no significant relationship (r=–0.049; p=0.762).

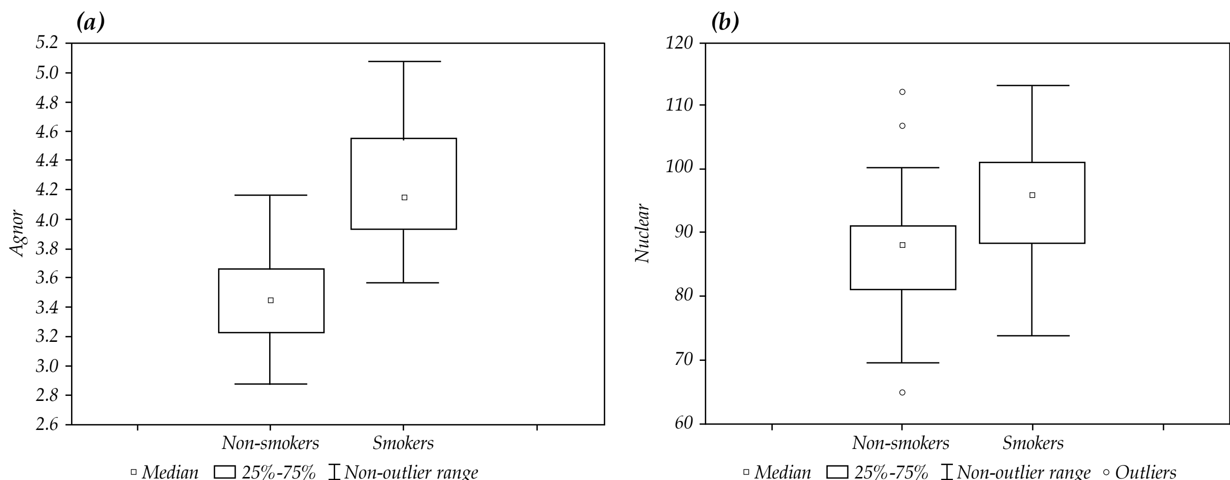

Fig. 2 ***(a)*** *Graph showing differences of AgNOR counts between non-smokers and smokers.* ***(b)*** *Graph showing differences of nuclear areas between non-smokers and smokers.*

Mean percentage of nuclei with more than five AgNORs were 14.6% and 36.8% in non-smokers and smokers respectively.

Among the brushers AgNOR counts were lower whilst nuclear areas were higher than non-brushers, however, there was no statistically significant difference (p=0.269 and p=0.682, respectively).

DISCUSSION

It has been documented that, the anatomic site that is particularly affected from smoking is the floor of the mouth. Paiva et al.[9] has found out that, mean areas of AgNORs per nucleus in smokers is the highest in mucosal cells of floor of the mouth when compared with border of the tongue and lower lip. It is also well established that, most frequent site of oral squamous cell carcinoma is the floor of the mouth.[9] Thus, we planned to take our smears from the mouth floors of the patients. We tried to keep the study groups big enough, and we counted AgNORs of a total of 2000 cells for each group and we calculated nuclear areas of the same total number. Counting AgNORs of each slide took approximately 20 minutes.

AgNOR counts have been of great value for the last two decades and for counting purposes some criterion has been established by Crocker et al. in 1989.[15] Costa et al.[1] pointed out that, the number of NORs in oral squamous cell carcinoma is higher in cells immunolabelled by the antibodies against proliferative markers such as PCNA and Ki-67. Akhtar et al.[17] have applied AgNOR staining to malignant and benign pleural effusions and concluded that AgNOR counts, as a rapid and an easily reproducible method, permits a clear distinction between malignant and benign cells.

Though many histopathological studies showing the importance of AgNOR numbers in normal, reactive, dysplastic cells and in cells of various benign and malignant tumors have been held for years, there are fewer studies with cytological preparations.[1,2,5,9,10,12,16-21] If cut section of a paraffin block is accepted as 3μm, the microscopically analyzable volume of a nucleus with a diameter of 11 μm will be 1/2.4 of whole nuclear volume. Thus AgNOR counts from a tissue section of a paraffin block would be nearly two times lower than AgNOR counts of a cytological preparation. Thus, one can easily estimate that diagnostic criteria of AgNOR numbers per nucleus in histological sections should significantly differ from the criteria of cytological smears. We think that, previous studies from tissue sections of oral mucosal epithelia owe their lower counts of AgNORs to this fact.[1,10,12,18-20]

There are some previous AgNOR studies of oral mucosa with leukoplakia, dysplasia, benign and malignant tumors, but only a few studies have been held concerning normal oral mucosa exposed to smoking or some other carcinogenic effects, such as chronic alcohol consumption.[5,9,16,21-22] Numbers and mean areas of AgNORs are the only parameters that are sought in most of the studies. Orellana-Bustos et al[16] also sought keratinization of the oral mucosal cells besides AgNOR counts and found an increase in the number of enucleated or orthokeratinizated superficial cells in oral mucosa of smokers. To the best of our knowledge, this is the first study including both AgNOR counts and nuclear areas of oral mucosal cells exposed to smoking and comparing both parameters.

Significant elevation in the mean AgNOR number of the smoker population was previously documented by some similar studies.[5,9,16,21-22] In the present study it can also be seen that mean nuclear area of the smoker group is significantly higher than the non-smoker group, which is a similar finding with the study of Einstein et al.[23] Results of both AgNOR counts and nuclear morphometry show that cellular proliferation is significantly higher in smokers and this causes an increase in the nuclear dimensions of oral mucosal cells, which can be accepted as an ignition towards enlarged nuclei of dysplastic cells. It is well known that, to produce a malignant cell requires the presence of a precursor non-malignant cell, which exhibits increased DNA damage, content, cell proliferation and apoptosis.[6]

Another striking result of our study was the percentage of cells with AgNOR counts

more than five. It was greater in smoker group (36.8%) than in non-smoker group (14.6%), which is a consistent finding with results of Sampaio et al.[5]

Interestingly, mean AgNOR counts of both smoker and non-smoker group are somewhat higher than that of the previous cytological studies.[5,9,16,20-21] Mean nuclear areas of both groups are also greater than the mean nuclear areas calculated in the previous studies.[24,25] Though there can be minor differences in counting and calculating techniques, we do not believe that this is the only explanation for the differences of our results from previous studies. This can be a result of our technique of collecting cytological material as we applied brushing three times to the mouth floor, before smearing the fourth brushing on the slides. Thus, we think that we have reached the deepest cells of the stratum spinosum of the oral mucosa. An additional explanation may be the high tea consumption in Turkish population, as 86% of patients included in our study were heavy tea drinkers (more than 5 cups/day). We measured the temperature of tea three times just before drinking and found it between 65-75 °C. Thus, direct irritative effect of hot tea can be a reasonable cause, for higher proliferative abilities of mucosal cells and this will be a different interesting topic for us to study deeply and separately.

We also sought the brushing habits of the patients and tried to find some correlation between brushing habits and cellular proliferating features, but there was no statistically significant difference between AgNOR counts of brushers and non-brushers, though mean AgNOR count of brushers were lower than non-brushers.

As a conclusion, our results support that smoking is a severe risk factor for oral mucosal proliferative lesions, and exfoliative cytology can be the preferred method for screening of oral mucosal lesions.

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