View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Sunderland University Institutional Repository

Omar, Galal, Shamssain, Mohammad and McGarry, Kenneth (2015) Monitoring histological changes in oral mucosa using AgNORs as biomarkers for oxygenic stress in smokers and COPD patients. IOSR Journal Of Pharmacy, 5 (4). pp. 24-29. ISSN 2250-3013

Downloaded from: http://sure.sunderland.ac.uk/5375/

Usage guidelines

Please refer to the usage guidelines at http://sure.sunderland.ac.uk/policies.html or alternatively contact sure@sunderland.ac.uk.

IOSR Journal Of Pharmacy
(e)-ISSN: 2250-3013, (p)-ISSN: 2319-4219
www.iosrphr.org Volume 5, Issue 4 (April 2015), PP. 24-29

Monitoring histological changes in oral mucosa using AgNORs as biomarkers for oxygenic stress in smokers and COPD patients

[1]Galal A. S. Omar ,[2]Mohammed Shamssain , [3]Ken McGarry
[1]Lecturer, Department of Zoology, College of Sciences, University of Garyounis, Benghazi, Libya.
[2]Associate Professor, College of Pharmacy and Health Sciences, Ajman University of Science and Technology, Ajman, UAE.
[3]Senior Lecturer, Department of Pharmacy, Health and Wellbeing, Faculty of Applied Sciences, University of Sunderland, UK

Abstract
Background: It is well known that tobacco smoke causes cellular proliferation and chronic obstructive pulmonary disease (COPD), but changes in buccal mucosa cells in smokers and COPD patients remains unknown.
Aims: The aim of this study is twofold: (i) to assess the effect of smoking on cellular proliferative activity of buccal mucosa in healthy smokers and in smoking COPD patients (ii) to detect early cellular proliferative activity in buccal mucosa. Our hypothesis is that tobacco smoking induces changes in buccal mucosa cells that we can detect using AgNOR analysis.
Methods:879 male subjects; non-smokers (controls), smokers and COPD patients were studied. Cytogenic damage was defined using the argyrophilic nucleolar organiser regions (AgNOR). Buccal mucosa cells were obtained from swabs and brushing. The *respiratory symptoms were assessed by St. George's questionnaire* and lung function tests were measured by Vitalograph spirometer. Statistical analysis using ANOVA and discriminant analysis (classification) was used to determine differences between the three groups, regression models were built on the lung function data.
Results: The AgNOR count, nuclear area, nuclear volume, and percentage of cells with 5 or more AgNORs, were significantly higher in smokers than non–smokers (5.20 vs 3.27, 74.66 vs 55.65, 462.67 vs 304.01, 73.93% vs 14.75%, respectively). For COPD patients, the values were 6.77, 110.42, 813.26, 96.97%, respectively. Respiratory symptoms were higher in smokers and COPD patients than non-smokers. There was significant *relationship between respiratory symptoms and AgNORs count ($P \leq 0.001$). There was positive association* between cigarette smoking and enhanced cellular activity in oral mucosa. Pack years of smoking is significantly associated with cellular abnormality in smokers and COPD patients.
Conclusions: Strong correlations were found between AgNORs parameters and respiratory symptoms, pulmonary function tests and pack years.
Keywords: COPD, buccal mucosa, AgNOR staining, discriminant analysis.

I. INTRODUCTION

Smoking induces changes in the nucleoprotein of buccal mucosa cells which can be detected by a method of silver staining the argyrophilic nucleolar organiser regions (AgNOR)(Kadivar, and Attar, 2008). The NOR's are identified as black dots that appear throughout the nucleolar area and can be used as histological biomarker in smokers to predict who is at risk of developing chronic obstructive pulmonary disease (COPD). Our primary aim was to assess the relationship between smoking and cellular proliferative activity of buccal mucosa in smokers and heavy smokers who are at risk of developing COPD and in COPD patients using AgNORs as a biomarker (Munteanu and Didilescu, 2007). AgNOR staining technique is a simple, non-invasive, painless, inexpensive accurate method and valuable marker in cells (Bukhari, et al., 2007). In addition, it has been used to avoid the errors which are produced during the diagnosis of malignant and benign cells (Remmerbach, et al., 2003). It is not a complex method and it can be used as a monitor to follow up all changes in the oral cavity (Chattopadhyay, and Ray, 2008).
The secondary aim was to compare AgNORs parameters among smokers, nonsmokers and COPD patients and to establish prediction equations for the prediction of AgNORs using packs year, lung function tests and respiratory symptoms.

II. METHODS

A total of 879 volunteers participated in this study. These were generally white collar workers. The COPD patients were selected from Quefia Chest Hospital, Benghazi, Libya. All participants were from Benghazi city and their demographics were similar. Socioeconomic status was similar (Omar et al., 2010).

Exclusion criteria were: hypertension, diabetes, liver disease, hypercholesterolemia, drug and alcoholic addiction and oral cancer. Subjects who consumed alcohol were also excluded. The inclusion criteria were age greater than 30 years, no clinically visible oral lesions and no history of benign or malignant neoplasias.

Lung function testing (FVC, FEV1, PEFR and the ratio FEV1/ FVC %) was performed by a Vitalograph spirometer. The statistical package SPSS was used to obtain the regression equations and linear regression was used with FEV1, FVC, FEV1/FVC and PEFR with height (meter) and age as independent factors (Hardie, et al., 2002). Currently, there are no spirometric reference values for adults in Libya. This justified our approach which consisted in establishing reference values from the healthy sample of the population of Benghazi. The obtained predictive equations allowed us to calculate percentage from spirometric variables measured in smoker and in COPD patients.

The following procedure was used for AgNOR staining (Ploton et al., 1986): Volunteers rinsed their mouth with distilled water, samples were collected from human oral mucosa by cytobrush (scraping their cheeks). Air–dried smears from 5 to 10 min were taken at room temperature. The slides were then fixed in Carnoy's solution (1:3 (v/v) acetic acid: ethanol) for 5 minutes in a refrigerator at 4ºC. The slides were rehydrated progressively in alcohol (100%, 90%, and 70%). Followed by rinsing in distilled water (two changes). The staining process used AgNOR (1x2% (w/v) gelatin in 1 % (v/v) formic acid: 2x5 % (w/v) AgNOR). The slides were placed in an oven at 70ºC for 10 minutes and then rinsed in distilled water. A 5% (w/v) thiosulphate solution was used to wash out non-specific deposits and to prevent sensitivity of the slides to light). Finally, the slides rinsed again in distilled water and inspected with a microscope to count and examine the black dots' shape and size.

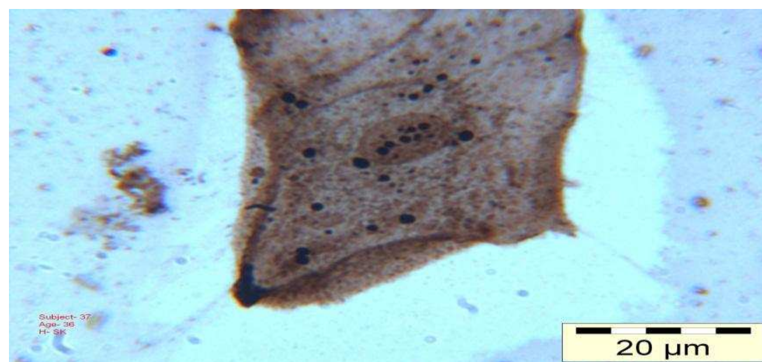

Figure 1. Oral mucosal cell in smokers including AgNOR count, AgNOR area and nuclear area (magnification, 100).

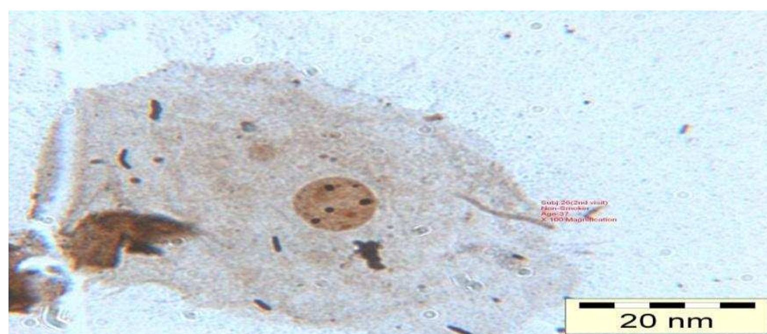

Figure 2, Oral mucosal cell in non-smokers including the AgNOR counts, AgNOR area and nuclear area (magnification, 100).

Figure 1 shows that oral mucosal cell has large AgNORs dots, more AgNOR counts and large nuclear area compared with non-smokers. Figure 2 shows AgNOR count, diameter of AgNOR and the nuclear area were the lowest in non-smokers compared with other two groups.

Overlapped, single and cluster black dots were counted as one structure Crocker, and Murray, (2003). Nuclear area (nucleus spherical in shape = $\pi*r2$ and nucleus oval in shape= $\pi*a*b$) and nuclear volume (nucleus spherical in shape = $4/3 * \pi *r 3$ and nucleus oval in shape = $4/3 * \pi *a*b2$) were measured. Images were captured from the slides by light microscope with colour camera. Two images were taken for each participant. The data was used to build a linear discriminant model (Fens et al 2009).

III. RESULTS

Table 1 shows, there were no significant differences in age (P= 0.710) and height (P=0.999) between smokers and non-smokers group. Age was higher in COPD patients than smoker and non-smokers. Weight was higher in non-smokers than smokers (87.30kg vs 83.87kg) and it was the lowest in COPD patients (68.35kg) compared with these two groups. However, there was no significant difference in height between smokers and non-smokers groups. Height was higher in smokers and non-smokers than COPD patients. Body mass index (BMI) was higher in non-smokers than smokers (28.57 vs 27.42) and it was the lowest in patients group (24.74).

Table 1 Physical Measurements of the participants (mean ± S.D)

Variables	Non- smokers (n= 404)	Smokers (n= 403)	Patients (n= 72)
Age (yr)	40.95 (7.33)***	40.48 (8.68)	63.15 (11.79)
Height (cm)	174.82 (6.49)***	174.80 (7.02)	165.88 (7.14)
Weight (kg)	87.30 (15.23)***	83.87 (17.26)	68.35 (17.05)
BMI	28.57 (4.77)***	27.42 (5.23)	24.74 (5.65)

(***) P ≤ 0.001

Table 2 AgNOR parameters (mean (±S.D))

Variables	**Non-smokers (n= 404)**	**Smokers (n= 403)**	**Patients (n=72)**
No. of AgNOR per nucleus (AgNOR count)	3.27 (0.35)***	5.20 (0.41)	6.77 (0.58)
Nuclear area (um^3)	55.65 (7.62)***	74.66 (8.70)	110.42 (7.04)
Nuclear volume (um^3)	304.01 (67.70)***	462.67 (69.76)	813.26(81.92)
AgNOR ≥ 5	14.75 (10.14)***	73.93 (11.98)	96.97 (4.10)
AgNOR area	1.25 (0.29)***	2.46 (0.51)	3.82 (0.56)
AgNOR volume	0.67 (0.20)***	1.43 (0.39)	2.30 (0.43)
Proportion of nuclear area occupied by AgNOR %	2.25 (0.51)***	3.28 (0.61)	3.47 (0.51)
Proportion of nuclear volume occupied by AgNOR%	0.23 (0.07)***	0.31 (0.11)	0.29 (0.59)

(***) P ≤ 0.001

Table 3. Classification accuracy percentages for discrimination analysis using individual AgNOR variables and all AgNOR variables combined

Analysis	Non-smoker(%)	Smoker(%)	COPD patient(%)	Overall%(%)
1. AgNOR area	97.8	80.4	91.7	89.3
2. AgNOR volume	96.5	75.4	84.7	85.9
3. AgNOR count	99.3	96.0	93.1	97.3
4. Combined (1-3)	99.0	93.8	98.6	98.6

A linear regression model was built to predict the heavy smokers who are at risk of developing COPD and give an indication of changes observed in the oral mucosal cells. In this study, the equation was designed to help us to estimate these changes, depending on the pack years and FEV1 (% predict). These variables allow us to apply this equation to identify smokers who at risk of developing COPD depending on the results from actual AgNOR slide and the predicted results from the equation. This equation was tested on the subjects and the results were compared with the outcome from the AgNOR slides. In this study, all AgNOR parameters such as nuclear area and nuclear volume have been used to construct the best equation which can be used to predict the

smokers at risk of development COPD. For this reason, several models using different variables were constructed.

FVC (forced vital capacity), FEV1 (forced expiratory volume in the first second), FEV1/FVC Ratio (forced expiratory ratio), and PEFR (peak expiratory flow rate), SaO_2 (percentage saturation of haemoglobin with oxygen). AgNOR ≥ 5 (percentage of cells with 5 or more AgNOR per nucleus).

In the non-smokers group (control group), FVC, FEV1, PEFR and FEV1/ FVC respectively were within normal values (4.98, 3.90, 505.57 and 78.07). AgNOR count, AgNOR ≥ 5%, AgNOR area, AgNOR volume, proportion of nuclear area occupied by AgNOR% and proportion of nuclear volume occupied by AgNOR% were low in this group compared with smokers and COPD patients. In the smokers group, FVC, FEV1, FEV1/ FVC and PEFR respectively were lower than normal (4.34, 3.28, 75.52 and 407.91) and AgNOR count was higher in the oral mucosal cells in smokers compared with non-smokers (5.20 vs 3.27). Nuclear area (74.66 vs 55.65) , nuclear volume (462.67 vs 304.01), AgNOR ≥ 5 (73.93 vs 14.75), AgNOR area (2.46 vs 1.25), AgNOR volume (1.43 vs 0.67), proportion of nuclear area occupied by AgNOR percentage (3.28 vs 2.25) and proportion of nuclear volume occupied by AgNOR percentage (0.31 vs 0.23) were higher in this group compared with non-smokers group. In COPD patients, FVC, FEV1, FEV1/ FVC and PEFR respectively were the lowest (1.76, 0.96, 52.64 and 144.30) compared with normal smokers and non-smokers respectively. In addition, Saturation of oxygen was also the lowest in this group (92.28) and AgNOR count (6.77), AgNOR ≥ 5 (96.97), nuclear area (110.42), nuclear volume (813.26) and proportion of nuclear area occupied by AgNOR% also were the highest values (3.47) compared with others groups.

Figure 4 provides a box and whiskers plot of the data showing the contrasts between the three groups.

The discriminant analysis model was built using cross-validation in order to correctly classify (predict) the test cases into one of three categories (smoker, non-smoker or COPD patient). Table three shows the results, each classifier is shown with the internal breakdown for each of the three classes and the overall accuracy.

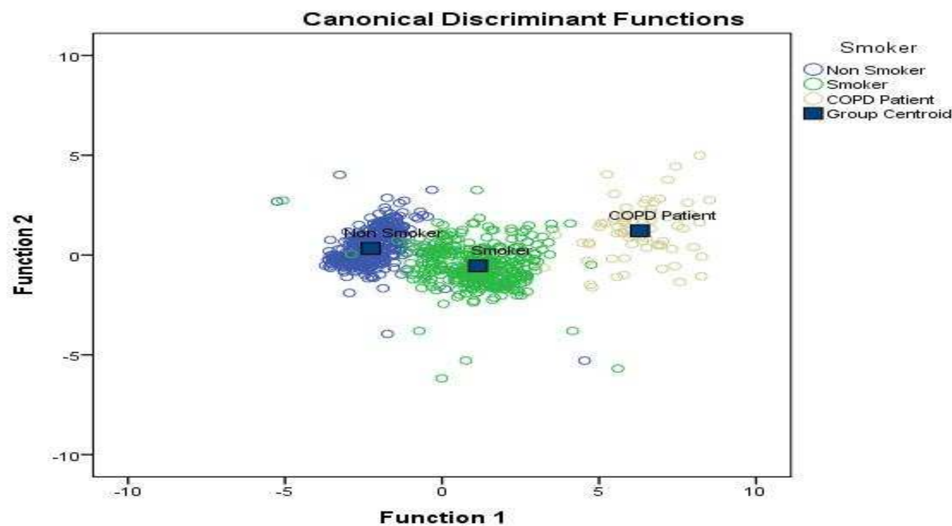

Figure 3 Showing discrimination between smokers, non-smokers and COPD patients

The most accurate cross-validated model used all three AgNOR variables for a combined accuracy of 98.6%. Figure 3 shows the discriminant functions which clearly reveals the segregated natured of the three classes.

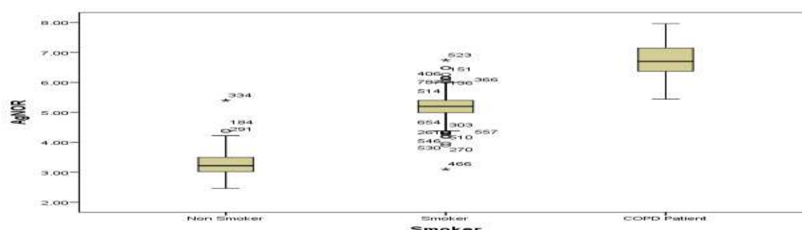

Figure 4, Boxplot of the distribution of AgNOR counts in smokers, non-smokers and COPD patients.

IV. DISCUSSION

Main Findings

The AgNOR staining technique has demonstrated that there is a strong relationship between silver nitrate and the proliferative cellular activity which appears as black dots in oral mucosal cells (cheeks). Increase of AgNOR count in the oral mucosal cells indicates an increase in the proliferative cellular activity through the formation of protein, to repair the damage resulted from the effect of tobacco smoking. An increase in the proliferative activity leads to a strong chance of mutation in the mitosis cycle which finally could cause development of malignancy cells in the oral mucosa. Furthermore, the technique is simple, painless, efficient and inexpensive compared to immunohistochemistry and molecular biology techniques (De Sousa et al., 2009).

Strengths and Limitations

This is the first study looking at the association between histological changes in the buccal mucosa related to environmental exposure and heavy smoking, respiratory symptoms and decline in pulmonary function in order to identify those at risk of developing COPD. COPD is not widespread in Benghazi Libya and this has been the major limiting factor during this study. In order to give a realistic indication of the situation, it is important to monitor patients for at least one month, especially when the symptoms initially appear. However, the limited number of patients did not allow direct comparison between COPD patients, smokers and non-smokers. In the future, it would be beneficial to use a larger number of patients, although this might require a much longer period of investigation, as only 200 patients (in Benghazi) are registered as suffering from COPD, but they are not regularly monitored. Some of these patients go to hospital only when they present severe symptoms.

Interpretation of findings in relation to previously published work

A study by Cancado, et al., (2004) showed that there is relationship between AgNOR count and the number of cigarette smoked per day. An increase in AgNOR counts in the oral mucosal cells in smokers who smoke more than 20 cigarettes per day (Ray, et al., 2003).The work of Usta, et al., (2008) discovered that the average AgNOR count in non-smokers was 3.47 ± 0.30 and in smokers was 4.20 ± 0.40. In our study, the mean of AgNOR count per nucleus in smokers was 5.20 (±0.41), in non-smokers was 3.27 (±0.35) and in COPD patients 6.77 (±0.58).

Implications for future research, policy and practice

Future work will investigate the effect of cigarette smoking on oral mucosal cells and the airway epithelial cells of smokers. Comparison of this effect and investigating the relationship between them is needed as well as investigating different severity groups of COPD patients. Comparisons will be made between smokers and COPD patients with, emphasis on AgNOR counts in the oral mucosal cells and the neutrophils count in sputum. We suggest that AgNOR staining can be used as a tool in cytology and histopathology laboratories to follow, estimate and diagnose COPD disease and other diseases such as oral carcinoma that result from the changes in oral epithelial cells. Furthermore, the method can be used to evaluate these changes before they appear in oral mucosa.

V. CONCLUSIONS

We have analysed the relationship between cigarette smoking and cellular proliferative activity of buccal mucosa in smokers and in chronic obstructive pulmonary disease (COPD) patients. The study has also been able to predict smokers who are at risk of developing COPD and to detect early cellular proliferative activity in buccal mucosa. The variation in AgNOR counts in this project reflects the rate of the proliferative activity and the degree of cytotoxic damage in the oral mucosal cells which results from exposure to chronic tobacco smoking. Moreover, it leads to a decline in the lung function which results from the destruction of the epithelial cells which line the respiratory airways and an increase in the number of goblet cells (increase mucus secretion) which stimulate the production of coughing and sputum as well as inflammatory process that lead to the obstructive disease. This study has successfully used the AgNOR method to analyse oxygenic stress in COPD patients and smokers. The results show that heavy smokers and COPD patients have high levels of cellular proliferative activities in the oral mucosal epithelial cells, which reflects the level of hyperplasia existing in COPD patients. Tobacco smoking is responsible for the changes in the nuclear diameter and genetic structure. There was a strong association between AgNOR counts and histological changes in the oral mucosal cells and it can be used as a marker for the proliferative activity in the oral epithelial cells.

The results showed that the oral cells are highly susceptible to cigarette smoking and this was indicated by an increase in the AgNOR counts inside the nucleus, which resulted from the increase of the proliferative activity.

Acknowledgements : The authors wish to thank Dr Awad Ben-Dardaf of the Quefia hospital in Benghazi.
Conflicts of interest The authors declare that they have no conflicts of interest in relation to this article.
Contributorship GASO planned the study and collected the data, KM conducted the statistical analysis, MS supervised the project. All authors contributed to the manuscript. The data can be made available upon request to the corresponding author.
Funding This study was funded by the Libyan Embassy in London, UK.

REFERENCES

[1]. Bukhari, M. H., Niazi, S., Hashmi, I., Abro, S. A-K., Tayyab, M., and Chaudhry, N. A. (2007). Use of AgNOR index in grading and differential diagnosis of astrocytic lesions of brain. Pakistan journal of medical sciences, 23 (2), 206 - 210.

[2]. Cancado, R. P., Yurgel, L. S., and Filho, M. S. (2004). Comparative analyses between the smoking habit frequency and the nucleolar organizer region associated proteins in exfoliative cytology of smoker’s normal buccal mucosa. Tobacco induced diseases. **2** (1), 43 - 49

[3]. Chattopadhyay, A. and Ray, J. G. (2008). AgNOR cut- point to distinguish mild and moderate epithelial dysplasia. J. Oral Pathol. Med., 37, 78 - 82.

[4]. De Sousa, F-A-C-G., Paradella, T-C., Carvalho, Y-R. and Rosa, L-E-B. (2009). Comparative analysis of cell proliferation ratio in oral lichen planus, epithelial dysplasia and oral squamous cell carcinoma. Med.Oral Pathol. Oral Cri. Buccal,14 (11), 563 - 367.

[5]. Fens, N., Zwinderman, A, van der Schee, M .P, S B de Nijs, Dijkers E., Roldaan A, Cheung D, Bel E, Sterk PJ. (2009), American Journal of Respiratory and Critical Care Medicine, 180, 1076-1082.

[6]. Kadivar, M., and Attar, M. (2008). Argyrophilic nucleolar organizer region counts in exfoliative cytology of buccal mucosal from opium addicts, smokers and non-smokers. Anayticall Quantitative Cytoogy Histology, 30 (5), 274 - 878.

[7]. Munteanu, I., and Didilescu, C. (2007). Chemistry and toxicology of cigarette smoke in the lungs. Pneumologia, 56 (1) pp 43 - 46.

[8]. Omar, G., McGarry, K.,and Shamssain, M, ,Association between lung function, respiratory symptoms and the argyrophilic nuclear organizer regions from oral mucosa in smokers, Thematic Poster Session in the European Respiratory Society Conference, Barcelona, Sept 18th-22nd 2010.

[9]. Ray, J. G., Chattopadhyay, A., and Caplan, D. J. (2003). Usefulness of AgNOR counts in diagnosing epithelial dysplasia. J. Oral Pathol. Med., 32, 71 - 76.

[10]. Remmerbach, T. W., Weidenbach, H., Muller, C., Hemprich, A., Pomjanski, N., Buckstegge, B., and Bocking, A. (2003). Diagnostic value of nucleolar organizer regions (AgNORs) in brush biopsies of suspicious lesions of the oral cavity. Analytical cellular pathology, **25**,139 - 146

[11]. Schwint, AE, Savino, T. M., Lanfranchi, H. E., Marschoff, E., Cabrini, R. L., and Itoniz, M. E. (1994). Nucleolar organizer regions in lining epithelium adjacent to squamous cell carcinoma of human oral mucosa. Cancer, **73** (11), 2674 - 2679.

[12]. Sirri, V., Roussel, P., and Hemandez-Verdun, D. (2000). The AgNOR proteins: qualitative and quantitative changes during the cell cycle. Micron, **31**,121 - 126.

[13]. Usta, U., Berberoglu, U., Helvaci, E., Altaner, S., Sut, N. and Ozdemir, C. (2008). Evaluation of cytological alterations in normal appearing oral mucosal epithelia of smokers and non-smokers via AgNOR counts and nuclear morphology. Trakya Univ. Tip Fak Derg., 25 (2), 110 - 116.