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Nucleolar Organizer Region-Associated Proteins in Cytological Smears of Normal Oral Mucosa: The Effects of Smoking

[1]Maryam Seyedmajidi, [2]Kosar Dadgar, [3]Shahrooz Momeni and
[4]Mahmood Hajiahmadi
[1]Faculty of Dentists, Dental Materials Research Center,
Babol University of Medical Sciences, Babol, Iran
[2]Department of Prosthodontics, Faculty of Dentists,
Tehran University of Medical Sciences, Tehran, Iran
[3]Department of Oral and Maxillofacial Surgery, Faculty of Dentists,
Isfahan University of Medical Sciences, Isfahan, Iran
[4]Department of Biostatistic,
Babol University of Medical Sciences, Babol, Iran

Abstract: Oral cytology is a method that has been proposed for the early diagnosis of oral mucosal changes. Silver nitrate staining can be a valuable way to discover early cellular changes. Because an obvious cause and effect relationship exists between smoking and cancer, this study was conducted to evaluate the AgNORs in smears collected from normal oral mucosa of smokers and non-smokers. Cytological smears were collected from three anatomic sites of normal oral mucosa; floor of the mouth, border of the tongue and buccal mucosa. The smears were taken from two groups, smokers and non-smokers for the purpose of correlating smoking habits to possible cellular alterations. The AgNOR count in samples that were obtained from normal oral mucosa in smokers was higher when compared to the count in non-smokers ($p = 0.001$). There was a significant difference between smears that were obtained from the floor of the mouth in smokers when compared to that in non-smokers ($p = 0.004$). The mean number of nuclei with >5 AgNORs was higher in smokers and this difference in the floor of mouth and buccal mucosa was statistically significant ($p<0.0001$, $p = 0.019$, respectively). This study suggests that cigarette smoking is related to increased proliferative activity in oral mucosal cells.

Key words: Cytology, cigarettes, Nucleolar Organizer Regions (NORs), smokers, cellular changes, Iran

INTRODUCTION

Cancer is considered to be a public health problem throughout the world; it is currently the second cause of death in most countries. Cancer affects at least 9 million people and kills approximately 5 million people every year. If prevention and control measures are not taken, it is estimated that the incidence of cancer will double within the next 20 years (Cancado *et al*., 2001).

Squamous Cell Carcinoma (SCC) is the most common malignant tumor of the oral cavity and the eleventh most common cancer in humans. It is the 8th most common cancer in men and the 15th most common cancer in women (Shahrabi *et al*., 2005; Usta *et al*., 2008; Neville *et al*., 2009).

Tobacco is considered to be the most important etiologic factor in the development of oral cancer (Regezi *et al*., 2008; Neville *et al*., 2009). Despite the mouth being an easily accessible region for examination, delayed diagnosis of oral cancer still occurs approximately, half of diagnosed patients die because of the disease (Cancado *et al*., 2001). Early diagnosis is the 1st step to treat any disease (Hupp *et al*., 2008). Many studies have suggested using exfoliative cytology as an auxiliary resource for early diagnosis in prevention programs for oral cancer (Silverman *et al*., 1958; Sandler, 1964; Rovin, 1971; Folsom *et al*., 1972; Cowpe *et al*., 1985; Ogden *et al*., 1994; Sugerman and Savage, 1996; Tiecke and Blozis, 1996; Goral *et al*., 1999). Nucleolar Organizer Regions (NORs) represent loops of DNA that encode ribosomal RNA and are considered important for protein synthesis (Crocker and Nar, 1987; Freitas *et al*., 1993).

NORs are associated with acidic, argyrophilic, non-histonic proteins that are visualized as black or dark brown dots in the interphase nucleus by using the

Corresponding Author: Maryam Seyedmajidi, Department of Oral and Maxillofacial Pathology, Faculty of Dentists, Babol University of Medical Sciences, Babol, Iran

silver-staining technique in routine histological or cytological preparations (De Castro Sampaio *et al*., 1999; Salehinejad *et al*., 2007). The count of AgNORs is considered to be an index for measuring cellular mitotic potential (Salehinejad *et al*., 2007).

Previous studies have shown that quantitative and qualitative analyses of AgNORs are beneficial when determining the diagnosis and prognosis of many neoplasms (Khan *et al*., 2006; Shahrabi *et al*., 2005; Khan *et al*., 2007; Usta *et al*., 2008). It can be simple, economical and repeatable and also, it only requires one step (Khan *et al*., 2006; Shahrabi *et al*., 2005; Khan *et al*., 2007). Use of the AgNOR silver-soaking technique in exfoliative cytology is supported by many studies which include studies by Cardillo (1992), Sujathan *et al*. (1996), De Castro Sampaio *et al*. (1999) and Metze and Lorand-Metze (1999).

The goal of this study was to compare the cell count of AgNORs collected from normal oral mucosa by exfoliative cytology in smokers and non-smokers.

MATERIALS AND METHODS

A total of 44 patients, 22 smokers (mean age 40.82±9.6 years) and 22 non-smokers (mean age 41.95±8.55 years) were selected for the study. None of the patients who participated in the study had any oral lesions, systemic disease or consumed alcohol. All smokers had smoked at least 20 cigarettes a day for a minimum of 10 years (Newman *et al*., 2006).

The collection of cells was performed on both groups through the exfoliative technique from three anatomic sites; border of the tongue, floor of the mouth and buccal mucosa. First, the patient was required to rinse his mouth with 0.9% saline solution. The sample was then taken using a disposable sterile cytobrush. The obtained material was spread over a dry microscopic slide which was identified by the patient's clinical chart number. The slide was fixed using a pathofix spray (containing 95% ethanol).

Fixed smears were subjected to the silver-staining method for AgNOR proteins according to Ploton's method (De Castro Sampaio *et al*., 1999; Usta *et al*., 2008). 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 the dark with prepared silver solution for 30 min at 45°C.

The AgNOR count was established in 100 cells for each cytologic smear. The cells were examined at 1000×magnification under immersion oil with an optical microscope (Olympus BX41, Japan) by an oral and maxillofacial pathologist who was not aware of the study groups or the locations from which the smears were taken. All slides were evaluated horizontally from left to right.

In each slide, the number of AgNORs present in 1st 100 cells was counted using the Crocker method which counts the black dots inside the well-defined nuclei; the black aggregates (overlapped or merged black dots) count as one structure (Crocker *et al*., 1989). To calibrate the examiner, the 1st ten slides were counted three times in anon-consecutive way.

The mean AgNORs count and the mean percentage of nuclei that totaled to >5 AgNORs were calculated. Data were analyzed using SPSS software, the Mann-Whitney test and the t-test.

RESULTS

The sample comprised 44 individuals that were divided into two groups smokers and non-smokers. There were a total of 22 individuals in each group. In the smoker group, the mean number of AgNORs per nucleus was 4.07±0.62. The mean number of AgNORs/nucleus in the smears collected from the border of the tongue was 3.72±1.54 in those collected from the floor of the mouth and buccal mucosa, the means were 4.49±1.55 and 4±1.74, respectively.

In the non-smoker group, the mean number of AgNORs per nucleus was 3.27±1.54. The mean number of AgNOR/nucleus in the smears collected from border of the tongue was 3.48±0.94 in those collected from the floor of the mouth and buccal mucosa, the means were 3.07±0.7 and 3.24±1.04, respectively.

The only significant difference in the mean number of AgNORs/nucleus between smokers and non-smokers was observed in samples from the floor of the mouth with higher counts in the smoking group ($p = 0.004$) (Table 1). The mean percentage of nuclei with >5 AgNORs was 25.27±21.56% in the smoking group and 9.27±9.42% in the non-smoking group and there was a significant difference between them ($p<0.0001$). It was 20.41±20.23% on the border of the tongue in smoking group while the means the floor of the mouth and buccal mucosa were found to

Table 1: Mean of AgNOR count in cytological smear of normal mucosa in smokers and nonsmokers according to location

Study groups	No. of cases	Location	AgNORs count mean±SD
Non-smokers	22	Buccal mucosa	3.24±1.04
		Floor of the mouth	3.07±0.70
		Border of the tongue	3.48±0.94
Smokers	22	Buccal mucosa	4.00±1.74
		Floor of the mouth	4.49±1.55
		Border of the tongue	3.72±1.54

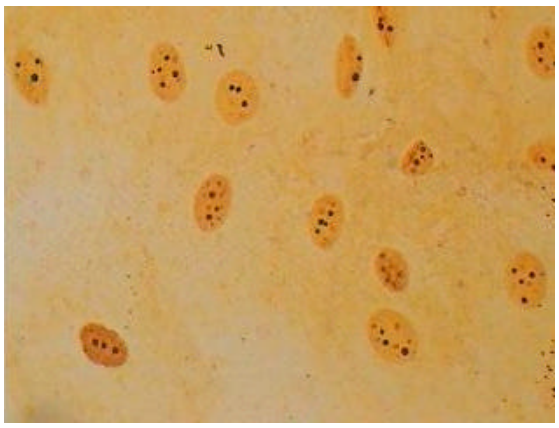

Fig. 1: AgNORs in normal oral mucosa in non-smoker (AgNOR stain 1000x)

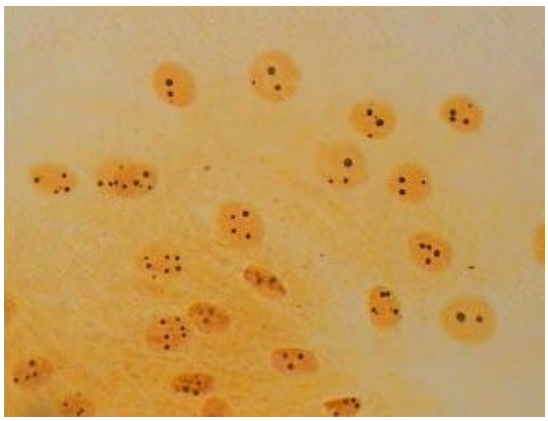

Fig. 2: AgNORs in normal oral mucosa in smoker (AgNOR stain 1000x)

be 30.18±20.73 and 25.5±22.30%, respectively. Among the non-smokers, the average percentage of nuclei with >5 AgNORs on smears collected from the border of the tongue was 12.23±10.23% while smears collected from the floor of the mouth and buccal mucosa had a mean of 6.5±5.49 and 9.9±11.07%, respectively. The results did not show a significant difference in the mean percentages of nuclei with >5 AgNORs from the three anatomic areas in the smoker group. The same result was found in the non-smoker group and the differences among the three anatomic sites were not significant. There were significant differences when comparing the mean percentage of nuclei with >5 AgNORs in smokers to the mean percentage in non-smokers based on anatomical areas that were higher in the smoker group (for floor of the mouth, $p<0.0001$ and for buccal mucosa, $p = 0.019$). AgNOR dots were located strictly within the nuclei and clearly visible as black dots. Compared with non-smokers, the number of AgNORs found in the smoker group was higher and their sizes were smaller (Fig. 1 and 2).

DISCUSSION

The silver-staining technique used to identify NORs has been recently introduced and it is commonly used for formalin-fixed, paraffin-embedded samples (Crocker and Nar, 1987). This method is used for showing the relationship between the number of AgNORs in the nucleus and the proliferation activity of various lesions (Goodpasture and Bloom, 1975; Fakan and Hernandez-Verdun, 1986; Cabrini *et al*., 1992; Mourad *et al*., 1994; Sujathan *et al*., 1996). Malignant tumor cells are small in size have a scattered distribution and irregular shape of AgNORs and show higher number of AgNORs per nucleus; benign tumor cells are round and large in size with a clustered distribution and have a small number of AgNORs per nucleus (Salehinejad *et al*., 2007). The study successfully used the AgNOR technique for collecting cytologic smears of oral mucosa.

The findings support the effective use of the AgNOR method for cytological material as mentioned by Morgan *et al*. (1988), Cardillo (1992), Sujathan *et al*. (1996), De Castro Sampaio *et al*. (1999) and Metze and Lorand-Metze (1999).

Crocker and Nar (1987), De Castro Sampaio *et al*. (1999) and Sujathan (1996) propose that the study of AgNORs for cytological material is more precise than evaluation of histological specimens because the entire nucleolus can be analyzed by using smears; this is in contrast to the evaluation of histological specimens where only part of the nucleolus can be analyzed at a time (Crocker *et al*., 1989; Sujathan *et al*., 1996; De Castro Sampaio *et al*., 1999).

The results of the present study suggest that smoking is related to increased proliferative activity in oral mucosal cells. Tobacco and smoking are the most important etiologic factors in oral squamous cell carcinoma (Regezi *et al*., 2008; Neville *et al*., 2009). An effective method of preventing cancer is monitoring high-risk lesions. The exfoliative cytology method helps to screen long-term changes in oral epithelium (Cancado *et al*., 2001). Sugerman and Savage (1996) has suggested exfoliative cytology as a rapid, non-invasive method to evaluate changes in dysplastic oral epithelium. The efficiency of the use of quantitative techniques in cytology such as DNA analysis, cytokeratin expression analysis and the use of markers like PCNA and Ki-67 has been reported by many researchers including Bongers *et al*. (1996), Ogden *et al*. (1997) and Cancado *et al*. (2004). In this study, the silver-staining technique was successfully used to evaluate AgNORs in the smears collected from normal oral mucosa in smokers and non-smokers from

anatomic areas (floor of the mouth, border of the tongue and buccal mucosa). This method is an effective technique in evaluating ploidy and proliferation activity of epithelial cells which was reported by Hernandez, Kacerovska, Ploton, Carbini and Vandiest (Cancado *et al*., 2001). Usta *et al*. (2008)'s study also reported that the number of AgNORs in a tissue section from a paraffin block results approximately, two times lower when compared to the number of AgNORs in a cytology sample. In the obtained smears from the present study, AgNORs were observed as black dots in the nucleus. The dots in the nuclei of cells had a round and regular shape, except in some smokers (who have had cumulative distribution despite who had benign clinical features of normal mucosa). It seems that a relationship exists between the number and size of AgNORs; the mucosal cells of smokers, when compared to the cells of non-smokers had more dots with smaller sizes. These findings were also confirmed by Cancado *et al*. (2001), Sujathan *et al*. (1996) and Cardillo (1992). A significant difference was found in the mean counts of AgNORs between smokers and non-smokers.

The results show that smoking can cause changes in cell activity and increase the proliferation of cells that other studies have shown similar results. The results of this study were similar to Usta *et al*. (2008)'s study that found the mean number of AgNORs to be 4.22 and 3.47 in smokers and non-smokers, respectively.

In the present study, the AgNORs count found in both smokers and non-smokers is higher than that found in many studies (De Castro Sampaio *et al*., 1999; Cancado *et al*., 2001, 2004; Orellana-Bustos *et al*., 2004; Paiva *et al*., 2004). One reason for this difference might be related to the technique used for collecting the cytology samples. Instead of using a spatula or wooden swab, the collection of samples was performed in this study by using a cytobrush. Orellana-Bustos *et al*. (2004) showed that a higher percentage of cells are obtained from deep layers of epithelium when using a brush, only superficial cells without nuclei are collected when using a spatula or wooden swab to collect samples.

The slide preparation time is different in different studies; 30 min in De Castro Sampaio *et al*. (1999)'s study, 20 min in Cancado *et al*. (2001)'s study and 25 min in Orellana-Bustos *et al*. (2004)'s study.

In the present study, staining was performed for 30 min. Leyva-Huerta *et al*. (2008) showed that staining time with silver nitrate had no effect on the AgNOR count. There are other factors that explain the differences in the mean counts of AgNORs such as differences in the quantity and duration of smoking. In the study by Cancado *et al*. (2001), the duration of smoking ranged from 10-50 years using 10-20 cigarettes day^{-1}. De Castro Sampaio *et al*. (1999) studied patients who used at least 20 cigarettes day^{-1} for at least 15 years. In the study, the selected patients used at least 20 cigarettes day^{-1} for a minimum of 10 years. Cancado *et al*. (2001) showed that the number of cigarettes consumed and the number of years one smoked were not related to the AgNOR count.

However, Zimmerman, Khan and Kapczinski showed that the rate of cellular change was associated with the number of cigarettes consumed per day(Cancado *et al*., 2001; Khan *et al*., 2007). Salehinejad *et al*. (2007) and Usta *et al*. (2008) collected samples from only one anatomical area whereas, samples were collected from three anatomic areas in the present study. No significant difference was found when comparing the AgNOR counts according to anatomical area in the non-smoker group and this was confirmed by the studies of Cancado *et al*. (2001) and Shahrabi *et al*. (2005). In the smoker group, the difference was not significant, although the mean number of AgNORs from the floor of the mouth was higher than from the buccal mucosa and border of the tongue. There was a significant difference that was found when comparing the mean AgNOR counts between the two groups from the floor of the mouth. The reason might be due to a higher sensitivity to the effects of smoking on the thin and non-keratinized mucosal surfaces of the floor of the mouth when compared to other areas that can be thick and keratinized. The percentage of nuclei with >5 AgNORs in smokers was higher than in non-smokers, this may be due to the higher rate of cell proliferation.

CONCLUSION

It seems that a higher number of AgNORs found in the normal oral mucosa of smokers when compared to that in non-smokers can be attributed to their high proliferative activity. Consequently, the risk of error in cell division and amplification increases; subsequently, the risk of pre-cancerous changes and cancer increases as well.

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