Original Article

Comparative Study using Papanicolaou Stain and Silver-stained Nucleolar Organizer Region Counts in Exfoliative Smear of Oral Mucosa in Bidi Smokers and Nonsmokers

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ABSTRACT

Aim: The aim of this study is to compare the proliferative activity of exfoliated cells in bidi smoker's and nonsmoker's oral mucosa.

Materials and Methods: The oral mucosal exfoliate smears were prepared from 40 individuals (20 nonsmokers and 20 smokers) with the age group ranging from 25 to 70 years, in and around Akola (Maharashtra). The Papanicolaou (PAP) stain and silver-stained nucleolar organizer region (AgNOR) were used to prepare cytogenic smear to evaluate the presence of cytological alterations, suggestive of inflammation, dysplasia, keratinization, and proliferative activity of epithelial cells. The present study involves PAP Class I and Class II smears. The obtained data were tabulated and statistically analyzed using statistical software IBM SPSS IBM Corp., Statistics for Windows, Version 20.0. Armonk, NY, USA: IBM Corp., and using *t*-test.

Results: There was a significant difference in mean number of AgNORs/nucleus between nonsmokers (0.947 ± 0.2533) and smokers (3.021 ± 0.2256). There were 90% inflammatory changes observed in smokers whereas nonsmokers showed only 75% changes. PAP Class II changes, i.e., significant proliferative activity, were found between smokers and nonsmokers mucosa.

Conclusion: A significant difference of AgNORs/nucleus was found between nonsmokers and smokers.

Keywords: *Exfoliative smears, Papanicolaou staining, silver-stained nucleolar organizer region, smokers*

Introduction

Throughout South Asia and some parts of the Middle East, bidi smoking is one of the conventional methods of tobacco use. Today, bidi smoking is popular and inexpensive in India. Their consumption outpaces that of conventional cigarettes. Bidis accounted for 48% of Indian tobacco consumption in 2008. There is greater risk of oral cancers with bidi smoking than cigarette since tobacco-filled leaves deliver more than three times of carbon monoxide and nicotine.[1]

Currently, in the worldwide cancer statistical data, oral squamous cell carcinomas (OSCCs) hold six positions with 5-year survival rate (except the early stages).[2] Hence, early diagnosis of oral cancers is must. When the lesions become symptomatic in the late/advanced stages, biopsy can be considered as gold standard.

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All the diagnostic features and prognostic significance of cancer cannot be identified with routine histopathological examination and many problems encountered microscopically in the differentiation of malignant changes from the benign ones.[3] Hence, there is a need to develop the adjunct diagnostic methods to predict early malignant changes. Earlier studies have shown the correlation between nucleolar function, size, and cell doubling time in human cancer cell lines, which has shown the importance of the nucleus in tumor pathology.[4]

A cytomorphometric study conducted by Soumya *et al*. evaluated the effect of tobacco smoking on the buccal mucosa and alteration in smoking-related nuclear parameters.[5] Initial cellular alteration can be determined and monitored with exfoliative cytology which reflects the essential role of nucleus in the protein synthesis and control of proliferation. Silver-stained nucleolar organizer region (AgNOR) correlates with the rate of proliferation as it can be estimated by Ki-67 and the percentages of the S phase cells and the mitotic cells. Hence, the nucleolar organizer regions (NORs) are the loops of ribosomal DNA which occur in the nucleoli of the cells on the short arms of the acrocentric chromosomes, 13, 14, 15, 21, and 22.[6]

Using silver reaction, the interphasic NORs can be clearly identified at the light microscopical level which stains the acidic proteins of the NORs (RNA polymerase 1 upstream binding factor, topoisomerase 1, nucleolin, fibrillin, C23 protein, and B23 protein) on routinely prepared histopathological and cytological samples. The AgNORs can be visualized as black dots throughout the nucleolar area after silver staining. The AgNORs per nucleus suggest it as a marker of the proliferative activity of the cell. Rajput and Tupkari using Papanicolaou (PAP) stain and AgNOR method evaluated early oral cancer for population selected from Government Dental College, Aurangabad. They stated that this technique is easy and noninvasive method for the identification of cancer.[6]

Fontes *et al*. compared exfoliative cytology from the oral mucosa of smokers (30) and nonsmokers (30) using PAP staining and AgNOR quantification. The study participants were selected from Heart Institute's Patient Center and the Smoking Cessation Program of the University Hospital, University of São Paulo Medical School (InCor-HCFMUSP). They concluded that there was increased proliferative activity in smokers as compared to nonsmokers, even in the absence of clinically visible lesions.[7] Rao *et al*. suggested to use AgNOR analysis for the identification of quantitative marker of incipient cellular alterations.[8]

AgNOR is an alternative diagnostic method which presents several advantages, such as easy and rapid identification, low-cost, diagnostic safety, efficacy, and noninvasive, and can be reproducible properties. PAP method is widely used for screening. It is not diagnostic. It only identifies those at risk for dysplasia or cancer. Qualitatively (based on the shape, size, and pattern of distribution), AgNOR acts as a marker of premalignant or malignant change.[9] The present study was done to compare the exfoliated cells from the oral mucosa of bidi smokers and nonsmokers, with emphasis on proliferative activity.

Material and Method

In the present study, participants (Group I: 20 with history of bidi smoking and Group II: 20 nonsmokers) were obtained from the Outpatient Department of Oral medicine. The sample size for this pilot study was selected from 2145 population at 95% confidence level and confidence interval of 125.35. Informed consent was obtained from all participants, and institutional ethical approval was obtained (No. Res. SMBT 052/15). Exfoliative smears were taken from the buccal mucosa using endocervical brush in 40 individuals (20 bidi smokers and 20 nonsmokers) with age ranging from 20 to 70 years, in and around Akola (Maharashtra). Group I was further categorized into Groups A, B, and C depending on number of bidi packs smoking habit per year (Group A, 20–39 pack-years; Group B, 40–59 pack-years; Group C, 60–79 pack-years). The study includes the following inclusion criteria: no alcohol addiction, absence of any oral lesion, and no history of benign or malignant oral neoplasms. Smokers were defined as those individuals who smoked over 10 bidis or more per day for at least 15 years. Cigarette, pipe smokers, or consumers of tobacco in other ways were not included. Nonsmokers were defined as people who have never smoked. The cytologic smears were stained using PAP stain and AgNOR and observed at ×40 and ×100, respectively.

Silver-stained nucleolar organizer region staining procedure

In 95% absolute ethanol, alcohol-fixed smears were immersed followed by rehydration and washing in distilled water. AgNOR staining was done according to the one-step method of Ploton *et al*. with slight modifications suggested by Lindner.[10,11]

Using oil immersion method, AgNOR counting was performed under ×100. The AgNORs were identified as brown-black discrete dots of different sizes within the nuclei [Figure 1]. AgNOR in 50 cells was counted.

Papanicolaou staining

The PAP test has been widely applied all over the world in the diagnosis of precancerous and cancer lesions. Identification of PAP-stained smears was performed at ×40.

The epithelial atypia can be considered with the presence of one or more of following features: hyperchromatism, increased nuclear-cytoplasmic ratio with nuclear enlargement, chromatin clumping with moderately prominent nucleolation and irregular nuclear borders, bi- or multi-nucleation, increased keratinization and scantiness of the cytoplasm, and variations in size and/or shape of the cells and nuclei.

The obtained data were tabulated and statistically evaluated using statistical software IBM SPSS IBM Corp, Statistics for Windows, Version 20.0. Armonk, NY, USA: IBM Corp, and using *t*-test.

Results

The average age of the participants was 54 years for Group I (bidi smokers) and 33 years for Group II (nonsmokers). In Group 1 as reported by patients, 27 years was the mean duration of smoking and the mean number of cigarettes smoked per day was 14. AgNORs showed a significant difference between nonsmokers (0.947 ± 0.2533 AgNORs/nucleus) and smokers (3.021 ± 0.2256 AgNORs/nucleus) [Table 1].

Patients with a history of smoking were divided into three groups according to the number of pack per years to assess the role of the smoking on the number of AgNORs, (pack-years = number of bidis × number of years smoked/number of bidis in one bundle): Group A, 20–39 pack-years; Group B, 40–59 pack-years; Group C, 60–79 pack-years. Table 2 and Graph 1 indicate the mean AgNOR number and respective standard deviation in each group. There was no significant difference in the mean number of AgNORs per nucleus between Group A and Group B ($t = 0.8607$, $P > 0.01$); Group B and Group C ($t = 0.8802$, $P > 0.01$); and Group A and Group C ($t = 0.8715$, $P > 0.01$).

With PAP staining method in the smoking group, 18 slides (90% of the sample) were classified as PAP Class II, whereas the two remaining slides were Class I. In the nonsmoking group, 15 (75%) slides were categorized as Class II and 5 (15%) as Class I.

The number of AgNORs was determined in smokers and nonsmokers according to the PAPANICOLAOU classification [Table 3].

The Papanicolaou classification[12]

- Class I – Atypical or abnormal cell absence
- Class II – Atypical cytology with no evidence of malignancy
- Class III – Cytology suggestive of, but not conclusive for malignancy
- Class IV – Cytology strongly suggestive of malignancy
- Class V – Cytology conclusive for malignancy.

Table 1: Statistical analysis of silver-stained nucleolar organizer region dots in normal buccal mucosa cytology of smokers and nonsmokers

Study group	Number of cases	Mean of AgNOR±SD	Mean percentage of cells with 3 or >3 AgNOR dots
Nonsmokers	20	0.947±0.2533	4.4
Smokers	20	3.021±0.2256	73.6

Test used: *t*-test, significance: $P<0.05$. SD=Standard deviation, AgNOR=Silver-stained nucleolar organizer region

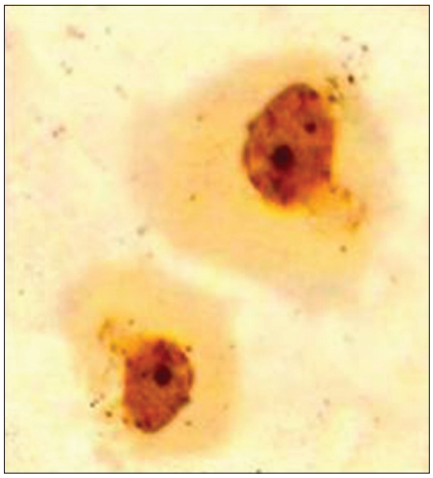
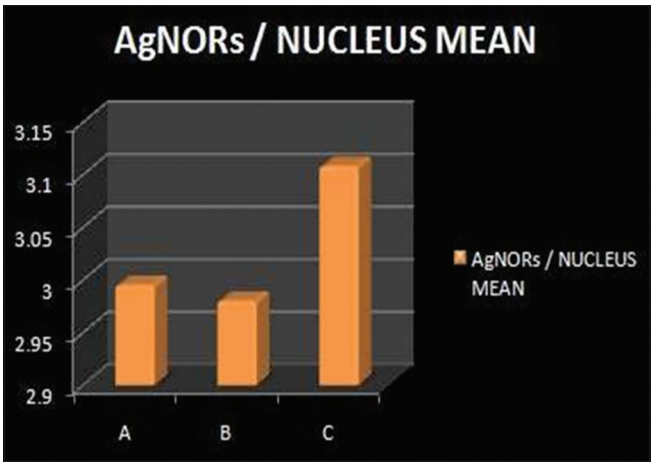


Figure 1: Section showing <3 silver-stained nucleolar organizer region dots in each of the squamous cells of normal mucosa in smokers (AgNOR, ×100)



Graph 1: Mean of silver-stained nucleolar organizer regions per nucleus in 50 cells in 20 groups of smokers versus pack-year

Table 2: Mean±standard deviation of silver-stained nucleolar organizer regions per nucleus in 50 cell in 20 groups of smokers divided according to the pack-years

Group	Pack-years	Patients (20)	AgNORs/nucleus mean±SD
A	20–39	12	2.995±0.2142
B	40–59	3	2.98±0.0721
C	60–79	5	3.108±0.1977

P<0.05. SD=Standard deviation, AgNOR=Silver-stained nucleolar organizer region

Table 3: Number of silver-stained nucleolar organizer regions per nucleus in smears from smoking and nonsmoking patients divided according to the cytological evaluation

Patients	Mean number of AgNOR/nucleus±SD	
	PAP Class I	PAP Class II
Smokers	2.82±0.22 (*n*=2)	3.043±0.21423 (*n*=18)
Nonsmokers	0.732±0.126965 (*n*=5)	1.01733±0.242825 (*n*=15)

P<0.05. SD=Standard deviation, AgNOR=Silver-stained nucleolar organizer region, PAP=Papanicolaou

A significant difference in proliferative activity was observed between smokers and nonsmokers classified as PAP Class II.

Discussion

Heat and the chemical products in tobacco increase the proliferative capability of oral mucosal epithelial cells. Thus, smokers have higher potential to develop OSCC. This proliferation is observable with AgNOR staining before any clinical symptoms appear. Our findings indicate a significant correlation between bidi smokers and the mean number of AgNOR/nucleus. AgNOR technique has been successfully used in various studies that included cigarette smokers without any oral lesions as in our study that included bidi smokers showing higher cellular proliferation as compared to nonsmokers. Fontes *et al*. studied AgNOR count of the tongue in cigarette smokers and nonsmokers on the basis of number of cigarettes consumed per day and the duration of smoking. Tobacco smokers were found to be at major risk to develop premalignant lesions.[7]

Gowhar evaluated the cellular alterations in smokers and nonsmokers using the AgNOR and PAP-stained smears from tongue and buccal mucosa and concluded that the proliferative activity was enhanced in smokers as compared to nonsmokers. Gowhar compared the exfoliative cytology of tongue among smokers and nonsmokers using PAP stain and AgNOR counts. They concluded that proliferative activity was enhanced in smokers compared to nonsmokers.[13]

Ahmed *et al*. using the AgNOR and PAP methods evaluated the cytological atypical changes in healthy oral mucosa exposed to smoking, alcohol, hot meals, and pepper. We observed a greater mean number of AgNORs per nucleus in smokers (3.68) followed by (2.82) alcohol consumers, compared to the habitual peppers and hot meal consumers (2.28) and the nonexposed group (2.00) which is statistically significant, whereas in case of PAP method, increased keratinization was detected among 45% of the smokers, 32.7% of the pepper and hot meals consumers, 11.8% of the alcohol consumers, and among 3.7% of the nonexposed group.[14]

The present study includes only bidi smokers as opposed to cigarette smokers in previous studies. In our study, a mean number of AgNORs per nucleus in 50 cells was observed between bidi smokers (3.21 ± 0.225573) and nonsmokers (0.946 ± 0.253338), which was statistically significant. The mean number of AgNORs per nucleus was highest in Group C (3.8), including bidi smokers with the largest number of pack-years ranging from 60 to 79. Almost equal number of AgNORs per nucleus was observed in Group A (2.995) including bidi smokers which had a range of 20–39 pack-years compared to Group B (2.98) with a range of 40–59 pack-years. Although there was a trend for increase in AgNORs with increase in bidi pack/years, it was not statistically significant in the present study. There was no significant difference on comparison of the mean number of AgNORs per nucleus according to the smoking duration.

Hashemipour *et al*. evaluated the keratinization, nuclear and cytoplasmic changes of oral epithelial cells in smokers, nonsmokers using exfoliative cytology method. They observed reduction in cellular diameter as well as an increase in nuclear diameter and nuclear/cytoplasmic ratio in smears taken from both smokers and opium addicts compared to nonsmokers.[15]

Malgaonkar *et al*. compared gutkha chewers and nonchewers with AgNOR staining method and concluded that AgNOR method effectively detected early molecular changes and AgNOR count was more in gutkha chewers.[16] Jindal *et al*. compared the alteration in apparently normal buccal mucosal cells due to effect of alcohol and tobacco by assessing AgNORs and micronuclei. They concluded that carcinomatous changes can be due to tobacco and alcohol consumption with alteration in buccal mucosal cells.[17]

In our study, the study participants did not have habit of alcohol consumption and there were neither clinical oral lesions nor history of malignant or benign lesions.

Cytological alterations (increased nuclear-cytoplasmic ratio, hyperchromatism, chromatin clumping with moderately prominent nucleolation and irregular nuclear borders, bi- or multi-nucleation, increased keratinization

and scantiness of the cytoplasm, and variations in size and/or shape of the cells and nuclei) were observed both in bidi smokers and nonsmokers, even when there are no clinical manifestations, and may indicate epithelial modifications in response to a physiochemical environment caused by the substances present in bidis.

Farhadi *et al*. conducted a similar study evaluating the effect of smoking on the buccal epithelial cells in smokers and nonsmokers and concluded that nuclear changes such as micronuclei formation and karyolysis frequency were higher in smokers as compared to nonsmokers.[18]

Roy *et al*. conducted a study in which quantitative DNA estimation (DNA ploidy), AgNORs, and koilocytotic changes were assessed in oral leukoplakia for risk categorization. They concluded that DNA ploidy and AgNOR count may serve as a prognostic marker for cancer risk prediction in cases of oral leukoplakia.[19]

Use of PAP classification alone is not sufficient, but it can be used along with AgNOR quantification for better diagnosis. Evaluation of the mean number of AgNORs per nucleus in smears classified as Class II PAP smears showed a significant difference between smokers and nonsmokers.

The effect of smoking was demonstrated by a significantly larger number of AgNORs per nucleus in mucosal cells of the buccal mucosa, indicating a higher proliferative activity of these cells.[20] Sood *et al*. observed that AgNOR is useful in estimating nuclear proliferative activity.[21]

The findings of our study showed that bidi smoking can result in modification in the mechanisms of cell growth. Tobacco can be considered as an initiating factor in oral carcinogenic condition. Oral mucosa is susceptible to cellular changes and increased cellular proliferation due to bidi smoking which corroborates similar findings in cigarette smokers.

The results of this study indicate higher proliferative activity in bidi smokers compared to nonsmokers, even in the absence of clinical lesions. PAP stain and AgNOR method are helpful in the identification of early cancer detection.

Bidi smokers show higher cellular proliferation as compared to nonsmokers, which is similar to results obtained for cigarette smokers. Limitation of the present study is smaller sample size, and there is no clear information whether bidi smokers are at a greater risk of malignant transformation to OSCC than cigarette smokers.

Further research is required to evaluate the identification of oral cancer using PAP stain and AgNOR method on larger sample size.

Conclusion

Our study concluded that PAP staining alone is not sufficient to find the effects of smoking on oral mucosa. PAP staining in combination with the AgNOR technique produced the required results. Other proliferative indices such as Ki-67 may be used for comparison with the AgNOR method with as well.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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