Original Article

Quantitative analysis of the efficacy of Papanicolaou, acridine orange, and AgNOR in oral exfoliative smears smokers for detecting micronuclei – A cross-sectional comparative study

ABSTRACT

Context: Dentistry and its associated specialties play a crucial role in diagnosing and treating oral diseases due to deleterious oral habits. Oral health-care providers also conduct researches to identify the association between oral diseases resulting from these bad oral habits, such as oral squamous cell carcinoma.

Aim: The purpose of this quantitative cross-sectional study was to compare the efficacy of exfoliative Papanicolaou staining (PAP) stains, acridine orange (AO), and AgNOR for detecting micronucleus (MN) count in smokers' (individuals with the habit of smoking) oral mucosa.

Materials and Methods: Exfoliative cytology smears obtained from thirty smokers' oral mucosa were divided into three equal groups to evaluate the frequency of MN count after staining with PAP, AO, and AgNOR. Smears were collected from smokers' oral mucosa ranging in age from 30 to 70 years who visited the Department of Oral Medicine and Radiology and from the Department of Oral Pathology and Microbiology.

Results and Stats: This research revealed the slightly different and higher MN count in PAP stain in the mean count among all the three stains after analysis and evaluation along with considerably higher in AO stain compare to AgNOR stain. Data were analyzed using Statistical Package for the Social Sciences SPSS software (windows Version 22.0 Chicago, IL, USA).

Conclusion: In this quantitative, cross-sectional study, with limitations and pitfalls, the results showed higher proliferative activity in smokers' oral mucosa without any oral lesions and higher mean MN count in PAP stain followed by a mean range of AO stains.

Keywords: AgNOR, cytodiagnosis, exfoliative smears, fractal dimensions, oral mucosa, Papanicolaou

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INTRODUCTION

Oral cancer, the sixth most common cancer worldwide, is the most common cause of morbidity and mortality due to smoking (second most significant risk factor causing global death) high doses of benzopyrene and nicotine, which produces genetic adducts through methylation hydroxylation pathways, further leading to malignant transformation. Assessment of micronucleus (MN) count in exfoliative smears is a simple and reliable method for tracing the genotoxic exposures. This MN test method was first used by Stich *et al*. on exfoliated buccal mucosa cells for tracing the genotoxic exposure in humans.[1,2] MN are extracellular cytoplasmic bodies generated from chromosomal fragments and are a

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separated part of the nucleus during anaphase. Its test is an inexpensive, fast, efficient, and non-invasive method of screening, and it provides a quantitative analysis of reliable genotoxicity. Beedi/cigarettes/windpipe smokers often have poor oral hygiene and greater calculus accumulation, affecting individual oral and systemic health.[3] The carcinogenic and mutagenic agents and the cigarettes' nitrosamines are believed to be responsible for MN formation. Various studies were performed regarding MN, which showed the correlation of frequency of MN and severity of the genotoxic damage, and the severity of the condition can be measured in terms of grading the lesion. Analysis of MN in the oral exfoliated cells can indicate malignant transformation because any genetic damage such as MN formation seen in these basal cells is reflected in the exfoliated cells.[4,5] The most commonly used stains for MN are Papanicolaou (PAP), Feulgen stains, and Giemsa stains.[6] PAP can stain various layers differentially.[6] Thus, in this study, we compared the staining efficacy of acridine orange (AO), PAP, and AgNOR for MN in exfoliated smears of oral mucosal cells of smokers, and the fractal dimension of MN from each stain was calculated to analyze the morphological changes undergone by the epithelial cells to transform into the neoplastic cell.

MATERIALS AND METHODS

This study aims to compare PAP, AO, and AGNOR in detecting MN counts to evaluate MN frequency in smears of exfoliated cells obtained from smokers (individuals with the habit of smoking), and fractal dimension analysis was also done to analyze the morphological changes. Patients visiting the Department of Oral Pathology and Oral Medicine were recruited for the study. Thirty patients with a history of smoking cigarettes (excluding duration) were selected for this study and divided into three equal groups. The case history was recorded along with a thorough oral examination. The inclusion criteria for the study group were followed pertaining to the Fagerstrom index.[7,8] Patients with a Fagerstrom score of 5 and above were selected. Before the collection of samples, written consent was obtained from the individuals. The patients were asked to rinse their mouth with mouthwash (0.12% chlorhexidine mouthwash) thoroughly before sample collection to remove the oral debris. The buccal mucosa was scraped gently using a broad, moistened wooden spatula. The obtained cells from lesional tissue were immediately smeared on precleaned labeled slides, which were then fixed by placing labeled slides in Coplin jars containing 90% alcohol.

Three slides were obtained from each subject of the study group. The three strains, namely, AO, AgNOR, and PAP, were prepared as follows:

Acridine orange fluorescence stain

50-mg AO, a cytochemical criterion,[9] is dissolved in 10 mL of distilled water to prepare a stock solution and stored in the refrigerator. 1 mL of AO stock solution and 0.5 mL of glacial acetic acid were added to 50 mL of distilled water to prepare a working solution.[1] The slide was put in the Coplin jar containing AO staining working solution (0.01%). After 2 min of staining, the slides was washed gently with water and dried, and then treated with xylene. The slides were then examined under a fluorescent microscope.

AgNOR

Composition of silver colloid developer included Solution A (silver nitrate: 50 g, deionized water: 100 mL) and Solution B (gelatin: 2 g, formic acid: 1 mL, deionized water: 100 mL). The working solution is Solution A: 2 part and Solution B: 1 part. The slides were stained with the freshly prepared silver colloidal solution (1 part by volume of 2% gelatin in 1% formic acid and two parts by volume of 50% aqueous silver nitrate solution)[10] in a closed Coplin jar at room temperature for 35 min. A dark atmosphere was maintained in the room throughout the reaction time. Then, the slides were washed with double-distilled ionized water. They were later treated with 5% sodium thiosulfate for 5 min and washed in double-distilled deionized water and dried.[9,10]

Papanicolaou

The slides were dipped in 1% acetic acid (10 dips) and then treated with Harris's hematoxylin,[10] and at preheated 60°C. They were washed in tap water and dipped in 1% acetic acid. They were then treated with OG-6, followed by 1% acetic acid (10 dips) and in EA-50. Again, they were dipped by 1% acetic acid, methanol (10 dips), and xylene (10 dips). The smokers' group slides were then subsequently stained with PAP, AO, and AgNOR stains.

From each slide, 100 exfoliated cells were viewed under × 400 magnifications under light microscope (PAP and AgNOR) and fluorescent microscope at 525-nm wavelength (AO). The count of MN in each of those cells was recorded, and later the photographs of the MN from each slide were collected and run through the ImageJ software (was created by Wayne Rasband at NIH in 1987) to calculate the fractal dimension value (D value).

Statistical analysis

Data were analyzed using the Statistical Package for the Social Sciences SPSS software (windows Version 22.0 Chicago, IL, USA). The recorded data were summarized in mean ± standard deviation and compared by one-way analysis of variance (ANOVA), and the significance of the mean difference between the groups was evaluated by Tukey's honestly significant difference *post hoc* test. A two-tailed ($\alpha = 2$) $P < 0.05$ was considered statistically significant.

RESULTS

Micronuclei count

The observed MN count of the three stains in exfoliated oral mucosal cells of smokers is summarized in Table 1 and depicted in Figure 1. The mean MN count in PAP was the highest, followed by AO and AgNOR the least (AgNOR < AO < PAP). Comparing the mean MN count of the three stains, ANOVA showed significantly different MN count among the stains ($F = 16.49$, $P < 0.001$) [Table 1]. Further, comparing the difference in the mean MN count between the stains, the Tukey's test showed a significant difference, and higher MN count in PAP stain as compared to both AO (26.07 ± 9.46 vs. 21.10 ± 6.07, diff. = 4.97, $q = 3.87$, $P < 0.05$) and AgNOR (26.07 ± 9.46 vs. 15.63 ± 4.74, diff. =10.44, $q = 8.12$, $P < 0.001$) stains [Table 2 and Figure 1]. Furthermore, it was also found to be significantly different and higher in AO stain than AgNOR stain (21.10 ± 6.07 vs. 15.63 ± 4.74, diff. =5.47, q = 4.25, P < 0.01) [Figure 2]. Moreover, the mean MN count in PAP stain was 19.1% and 40.0% higher than AO and AgNOR stains, respectively, and 25.9% higher in AO stain than AgNOR stain.

The fractal dimension of micronuclei count

The fractal dimension score of MN count in three stains is summarized in Table 3 and shown in Figure 3. The mean fractal dimension score of MN count in AO was the highest, followed by AgNOR and PAP the least (PAP < AgNOR < AO). Comparing the mean fractal dimension score of MN count of the three stains, ANOVA showed significantly different fractal dimension score of MN count among the stains (F = 20.71, $P < 0.001$) [Table 3]. Further, comparing the difference in the mean fractal dimension score of MN count between the stains, Tukey's test showed significant difference and higher fractal dimension score of MN count in AO stain as compared to both PAP (1.29 ± 0.12 vs. 1.11 ± 0.11, diff. =0.18, $q = 8.94$, $P < 0.001$) and AgNOR (1.29 ± 0.12 vs. 1.17 ± 0.10, diff. =0.12, $q = 5.96$, $P < 0.001$) stains [Table 4 and Figure 2]. However, it was found similar between PAP and AgNOR (1.11 ± 0.11 vs. 1.17 ± 0.10, diff. =0.06, $q = 2.98$, $P > 0.05$) stains, [Figure 4] that is, found to be statistically the same. Furthermore, the mean fractal dimension score of MN count in AO stain was 14.0% and 9.3% higher than PAP [Figure 5] and AgNOR stains, respectively, and 5.1% higher in AgNOR stain than PAP stain.

Table 1: Micronuclei count in three different stains

Stains	*n*	Mean±SD	*F*	*P*
PAP	30	26.07±9.46	16.49	<0.001
AO	30	21.10±6.07		
AgNOR	30	15.63±4.74		

ANOVA: Analysis of variance, *F*: ANOVA *F* value, SD: Standard deviation, PAP: Papanicolaou, AO: Acridine orange, AgNOR: Argyrophilic nucleolar organiser region

Table 2: Comparison of the difference in mean micronuclei count between stains by Tukey's test

Comparison	Mean difference	*Q*	*P*	95% CI of difference
PAP versus AO	4.97	3.87	<0.05	0.62-9.32
PAP versus AgNOR	10.44	8.12	<0.001	6.09-14.79
AO versus AgNOR	5.47	4.25	<0.01	1.12 -9.82

Q: Tukey's test value, CI: Confidence interval, PAP: Papanicolaou, AO: Acridine orange, AgNOR: Argyrophilic nucleolar organiser region

Table 3: Fractal dimension score of micronuclei count in three different stains

Stains	*n*	Mean±SD	*F*	*P*
PAP	30	1.11±0.11	20.71	<0.001
AO	30	1.29±0.12		
AgNOR	30	1.17±0.10		

ANOVA: Analysis of variance, *F*: ANOVA *F* value, SD: Standard deviation, PAP: Papanicolaou, AO: Acridine orange, AgNOR: Argyrophilic nucleolar organiser region

Table 4: Comparison of the difference in mean fractal dimension score of micronuclei count between stains by Tukey's test

Comparison	Mean difference	*Q*	*P*	95% CI of difference
PAP versus AO	0.18	8.94	<0.001	0.11-0.25
PAP versus AgNOR	0.06	2.98	>0.05	0.01-0.13
AO versus AgNOR	0.12	5.96	<0.001	0.05-0.19

Q: Tukey's test value, CI: Confidence interval, PAP: Papanicolaou, AO: Acridine orange, AgNOR: Argyrophilic nucleolar organiser region

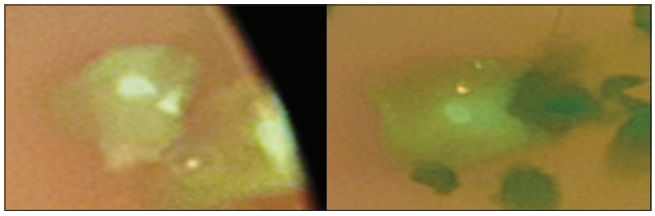

Figure 1: Micronuclei in acridine orange stain

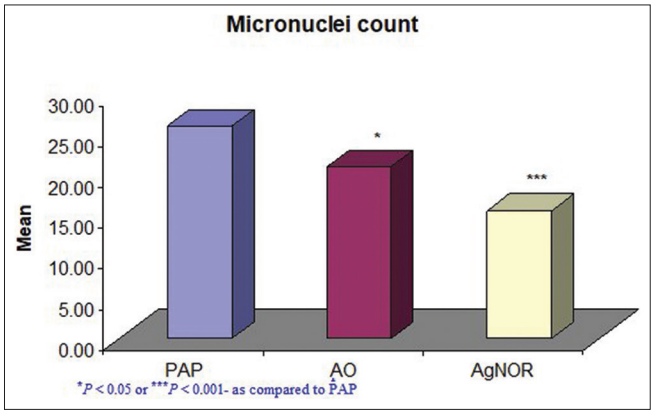

Figure 2: Number of MicroNuclei (MN) counts in all three stains

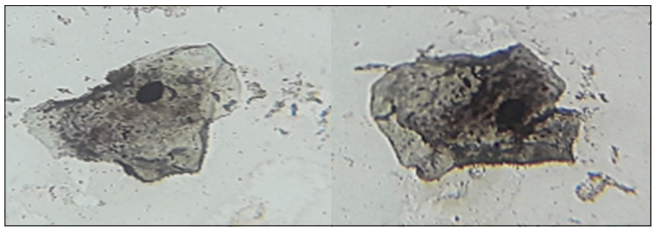

Figure 3: Micronuclei in AgNOR stain

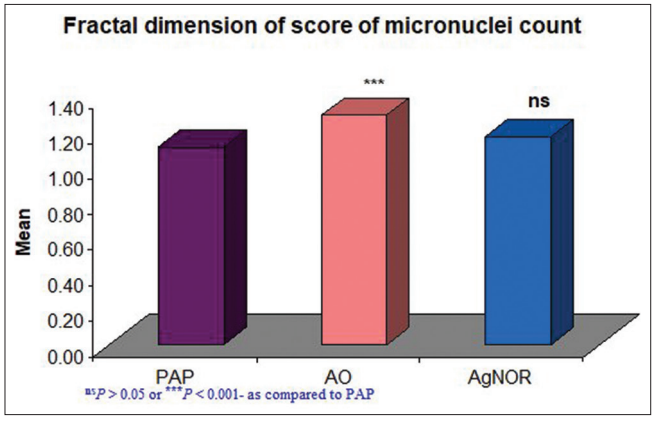

Figure 4: Fractal dimension score of MicroNuclei (MN) count

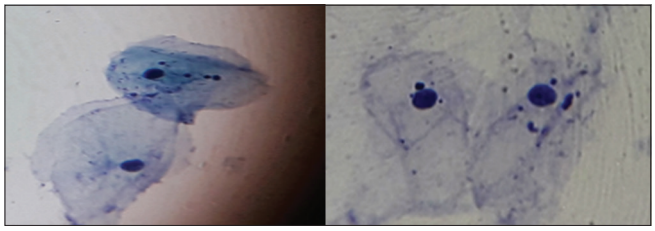

Figure 5: Micronuclei in Papanicolaou stain

DISCUSSION

With 1/3rd diameter of that primary nuclei, MN can be detected in oral exfoliated cells by various substances, including genetic agents such as carcinogenic components found in betel nut, tobacco, and alcohol.[9] The reactive cigarette products are carcinogenic, such as polycyclic hydrocarbons and nitrosamines, which are metabolized and result in epoxide and oxidative combustion production. The MN are fragments of chromosomes or whole chromosomes lost during cell mitosis, especially the anaphase stage because of a clastogenic event, causing chromosomal breakage aneugenic event – fragmentation of spindle fibers. The term micronucleus was first proposed in the early 1970s by Schmidt, Boller, and Heddle.[10,11] They demonstrated it as a reliable indicator of mutagens' genotoxic potential after *in vivo* exposure using bone marrow erythrocytes in animal-subject studies. These MN are formed due to the genotoxic and carcinogenic components present in tobacco, alcohol, betel nut, etc., primarily consumed by the general population. According to a study, a significant correlation was observed between increased frequencies of MN and cigarette smoking. These MN can be considered indicators of genotoxic damage and chromosomal aberrations, which can detect early-stage carcinogenesis.

Kashyap and Reddy[12] have proposed distinct advantages of the MN assay over other cytological techniques that account for the readily accessible site of sample collection from multiple areas of a particular site in the same patient, noninvasive technique, and feasibility in conducting longitudinal epidemiological studies. Another study by Suhas *et al*.[13] showed a significant correlation between smoking and MN. Kazanowska *et al*.[14] in 2014 published a paper on the role and application of exfoliative cytology in the diagnosis of oral mucosal pathologies and gave their opinion that exfoliative cytology could be a beneficial diagnosis technique in detecting of oral carcinoma with the help of microscopic evaluation of epithelial cells. Kalim *et al*., in 2019,[15] done a study of MN assay in oral exfoliated cells of tannery workers using PAP stains similar results were found in few more other studies[14] done on PAP stain and concluded that PAP stain could be used as a specific marker for MN assessment.[15] Palaskar and Jindal[16] compared PAP and Giemsa staining techniques to detect MN in exfoliated buccal mucosal cells and concluded that PAP is a better stain over Giemsa for MN detection.

CONCLUSION

MN formation indicates genotoxic and cytotoxic changes in the oral mucosa, a standard dysplastic change seen in oral cancerous cells. The presence of MN in smokers; exfoliated cells can be attributed as an early dysplastic marker for disease onset and progression. When comparing PAP, AO, and AgNOR, it was found that all the stains showed some differences in their quality of detecting a MN. This study also includes the fractal dimension; it gives even more details and quality of the stains so that the appropriate stain can be fixed for MN.

Limitations in the present study

We have not compared these three stains in nonsmoker patients, and even we have not analyzed using demographic data such as age. The morphological descriptions obtained using all these three staining methods are typically not sufficient. We lack research on oral cytodiagnosis to determine the most significant stains in PAP, AO, and AgNOR stains.

Ethical committee approval

Patients involved in this research read and signed the consent form. Ethical Committee approval was taken from the Institutional Human Ethics Committee (No. 559/IHEC/3-19).

What's new

In this analysis, MN frequency through exfoliated smears in smoking individuals was observed with three different types of stains.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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