Original Article

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MICRONUCLEI ASSAY IN SMOKERS V/S NON SMOKERS - GAINING GENOMIC INSIGHT

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ABSTRACT

Introduction: A micronucleus (MN) is formed during the metaphase/anaphase transition of mitosis (cell division).It may arise from a whole lagging chromosome (aneugenic event leading to chromosome loss) or an accentric chromosome fragment detaching from a chromosome after breakage (clastogenic event) which do not integrate in the daughter nuclei. Carcinogenic and mutagenic compounds, including tobacco-specific nitrosamines are believed to be responsible for the induction of micronuclei. Hence, the micronucleus assay is one of the markers used to identify cellular changes of biological significance to carcinogenesis. **Materials and Methods**: This study was conducted on 20 patients with history of smoking > 5 years, using 20 patients free of habits as the control group. The samples were obtained from the buccal mucosa through exfoliative cytological technique and stained with PAP, Giemsa and Acridine Orange to provide a comparative analysis of micronuclei in the exfoliated buccal mucosal cells. **Results:** A statistically significant increase was observed in the buccal mucosal smears obtained from smokers as compared to non-smokers. The mean numbers of micronuclei observed were significantly higher with PAP in comparison with Geimsa and Acridine Orange stains. **Conclusion:** The micronuclei frequency was seen to significantly increase in people with cigarette smoking habits. Hence, the micronuclei assay detected by PAP stain is a useful biomarker to detect people at higher risk of developing malignancy.

KEYWORDS: Micronuclei; PAP; acridine orange; exfoliative cytology

INTRODUCTION

Out of the myriad of forms of tobacco consumption available, cigarette smoking is the major cause of oral cancer, accounting for around two-thirds of all reported cases of oral squamous cell carcinomas annually.[1] It constitutes a significant percentage of lifestyle oriented detrimental habits in the general population accounting for 1.1 billion people worldwide.[2,3] Cigarette smoke contains over 4000 identifiable chemical carcinogens, with 200 known carcinogens that show toxicity.[1,2,4] The tar content in tobacco filled cigarettes which are unfiltered or less filtered shows higher carcinogenic potential as compared to the filtered cigarettes consumed in developed countries.[1,4] In comparison to other anatomic sites of the body, the oral cavity shows an increased propensity for developing malignancy as it forms the first barrier in the route of inhalation or ingestion and can metabolize procarcinogens contained in tobacco smoke to reactive products. This increased susceptibility can also be attributed to the nature of the oral mucosa which is made of stratified non keratinized epithelia, thus allowing the superficial cells to maintain an intact structure which in turn facilitates colour absorption and easy observation of nuclear characteristics under the microscope.[5,6] Oral exfoliative cytology is a

Table 1: ANOVA test followed by Bon Ferroni correction

Group	Stains	N	Mean	SD	Std. Error	95% CI for Mean		Minimum	Maximum	P-Value	Sig. Diff	P-Value
						Lower	Upper					
Smokers	Van Giemsa Stain	15	15.33	4.64	1.20	12.76	17.90	9	22	<0.001*	P Vs V	<0.010*
	PAP	15	19.47	4.16	1.07	17.17	21.77	12	27		P Vs A	<0.001*
	Acridine Orange	15	2.27	0.80	0.21	1.82	2.71	1	4		V Vs A	<0.001*
Non-Smokers	Van Giemsa Stain	15	2.87	1.77	0.46	1.89	3.85	1	8	<0.001*	P Vs V	<0.001*
	PAP	15	4.87	1.36	0.35	4.12	5.62	3	8		P Vs A	<0.001*
	Acridine Orange	15	1.27	0.46	0.12	1.01	1.52	1	2		V Vs A	<0.005*

Table 2: Unpaired T test to compare the number of micronuclei using Geimsa stain between two study groups

Groups	N	Mean	SD	Std. Error Mean	95% CI of the Diff		Mean Diff	t	df	P-Value
					Lower	Upper				
Smokers	15	15.33	4.639	1.198	9.841	15.092	12.467	9.725	28	<0.001*
Non-Smokers	15	2.87	1.767	0.456	9.773	15.16				

Table 3: Unpaired T-test used to compare the number of micronuclei using PAP stain between two study groups

Groups	N	Mean	SD	Std. Error Mean	95% CI of the Diff		Mean Diff	t	df	P-Value
					Lower	Upper				
Smokers	15	19.47	4.155	1.073	12.288	16.912	14.6	12.937	28	<0.001*
Non-Smokers	15	4.87	1.356	0.35	12.218	16.982				

Table 4: Unpaired T-test used to compare the number of micronuclei using Acridine Orange stain between two study groups

Groups	N	Mean	SD	Std. Error Mean	95% CI of the Diff		Mean Diff	t	df	P-Value
					Lower	Upper				
Smokers	15	2.27	0.799	0.206	0.513	1.487	1.00	4.207	28	<0.001*
Non-Smokers	15	1.27	0.458	0.118	0.507	1.493				

conventionally method that is easy to perform, non-invasive, relatively painless and does not cause undue stress to the patient.[6] This method is widely used to screen cellular alterations such as karyolysis, karyorrhexis, micronucleus formation, pyknosis, binucleation which indicates genotoxic damage.[6,7] Micronuclei are the erratic nuclei that originate from lagging chromosomes at anaphase or from accentric chromosome fragments. These fragments form their own membranes and on staining appear as Feulgen specific cytoplasmic chromatin masses with the appearance of a small nucleus and are subsequently exfoliated after maturation. Carcinogenic and mutagenic compounds, including tobacco-specific nitrosamines are believed to be responsible for the induction of micronuclei.[8,9] The presence of increased frequency of micronuclei within the cytoplasm of cells is indicative of chromosome loss or fragmentation, which is a sequelae of genotoxic damage. The Buccal Cell Micronucleus Assay was a novel method first proposed by Stitch et al in 1982 with the aim of biomonitoring the hazardous effects and potential carcinomatous transformation in population exposed to genotoxic agents. It implemented a technique involving analysis of micronuclei frequencies in obtained samples which act as 'endogenous

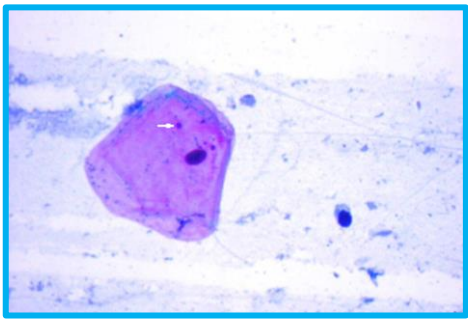

Fig. 1: PAP stained smear showing micronuclei in exfoliated buccal mucosal cells from smokers (observed under 1000X magnification)

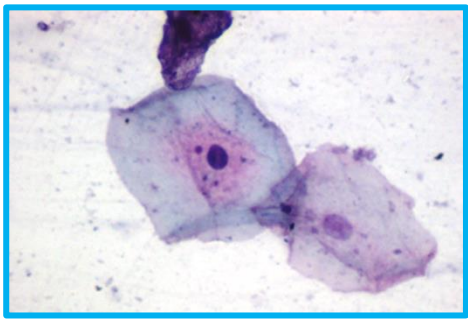

Fig. 2: PAP stained smear showing micronuclei in exfoliated buccal mucosal cells of non-smokers (observed under 1000X magnification)

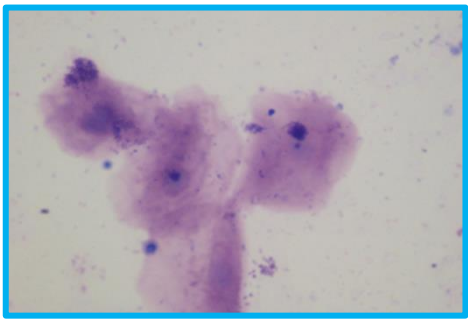

Fig. 3: Geimsa stained smear showing micronuclei in exfoliated buccal mucosal cells of smokers (Observed under 1000X magnification)

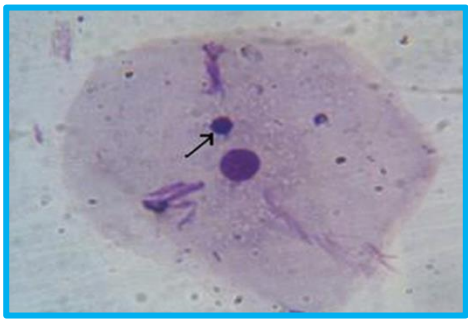

Fig. 4: Geimsa stained smear showing micronuclei in exfoliated buccal mucosal cells of non smokers (observed under 1000X magnification)

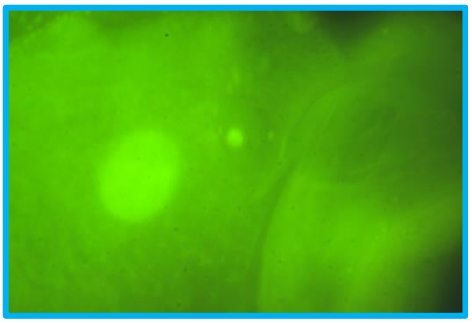

Fig. 5: Acridine orange stained smear showing micronuclei in exfoliated buccal mucosal cells of smokers (observed under fluorescent microscope.1000X magnification)

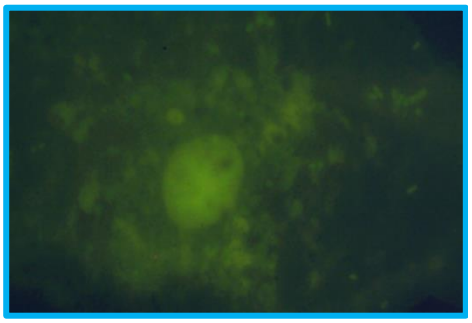

Fig. 6: Acridine orange stained smear showing micronuclei in exfoliated buccal mucosal cells of non smokers (observed under fluorescent microscope 1000X magnification)

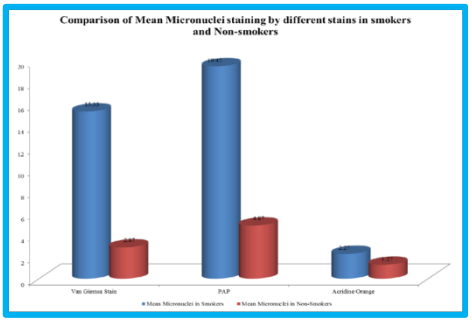

Fig. 7: Graphical representation of mean micronuclei in smokers and non smokers using pap, acridine orange and giemsa stains

dosimeters' of chromosomal alterations.[7,8] The present study aims to provide a comparative analysis of micronuclei in exfoliated buccal mucosal cells of smokers and non smokers using DNA specific stains such as Acridine Orange and non-DNA specific stains such as PAP and Geimsa, using microscopic and statistical analysis.

MATERIALS AND METHODS

The purpose of this study was to evaluate the frequency of micronuclei in smears of exfoliated cells obtained from two groups i.e., smokers and non - smokers. We have also attempted to investigate the comparative results of staining by PAP, Geimsa and Acridine Orange on detection of micronuclei in these smears. The sample and control groups for the study were obtained from the Out Patient Department of our institution. 20

patients with history of smoking cigarettes for five years or more were selected as the study group and 20 healthy individuals with no history of habits were selected as the control group. Individuals with medically compromising conditions were excluded from the study. Before collection of samples, written consent was obtained from the individuals to be included in the study. The patients were asked to rinse their mouth with mouthwash thoroughly prior to sample collection to remove all oral debris. A wide edged, moistened wooden spatula was scraped with gentle pressure against the buccal mucosa of the patients and the cells obtained were immediately smeared on pre-cleaned labeled slides and fixed by placing in 80% alcohol contained in Coplin jars. Three slides were obtained from each subject of the study group. The slides were subsequently stained with PAP, Geimsa and Acridine Orange stains. From each slide, 1000 cells were examined under the microscope using 400X magnification and on location of MN cells, 1000X magnification was used under oil immersion .The slides stained with Acridine Orange were observed under fluorescent microscope at 525 nm wavelength of radiation input. The inclusion criteria for the study were followed pertaining to Tolbert *et al*. On microscopic analysis, smears with debris and other proteinaceous substances that may mask the micronuclei and folded or overlapping epithelial cells were not taken into consideration for MN assay. The slide stained with PAP obtained from smokers showed more frequency of micronuclei in comparison to the one obtained from the sample obtained from non smokers (Fig. 1 & Fig. 2). Similar results were obtained with Giemsa stain (Fig. 3 & Fig. 4) and Acridine Orange stains (Fig. 5 & Fig. 6).

RESULTS

ASSESSMENT OF MICRONUCLEI

The micronuclei were counted in each slide and the frequency was analysed by means of ANOVA, the one way analysis of variance test followed by Bon Ferroni correction method to analysis the staining properties of the three stains used. Multiple unpaired T-test comparisons were done amongst the different groups to count mean value score of micronuclei. The test results obtained on statistical analysis in (Table 1) showed mean values of 15.33, 19.47 and 2.27 in the smokers group for Geimsa, PAP and Acridine Orange stains respectively. The corresponding values obtained for the control group were 2.87, 4.87 and 1.27 for Geimsa, PAP and Acridine Orange stains respectively. A significant p value of <0.001 was obtained for both study groups indicating a higher mean number of micronuclei among smokers as compared to non smokers. Also, the value was the highest for PAP as compared to Geimsa and Acridine Orange stains. On multiple unpaired T-test comparisons to compare the number of micronuclei in both categories using PAP (Table 2), Acridine Orange (Table 3) and Geimsa staining (Table 4)), a significant P value of <0.001 was found in all three comparative studies with the mean value being higher for smokers in comparison to non-smokers. A significantly higher mean micronuclei count of 29.47 was seen with PAP stained sections in comparison to values obtained for Giemsa and Acridine Orange stain (Fig. 7). Hence, we can conclude that there is significant difference in the staining property of the micronuclei by the different stains in the smokers group [P<0.001]. Also, among the three stains, PAP showed increased staining of the micronuclei with mean of 19.47 +/- 4.16 compared to Van Giemsa stain [mean 15.33 +/- 4.64] at P<0.010 and Acridine Orange [mean 2.27 +/- 0.80] at P<0.001. Thus, PAP stain exhibits better staining property of the micronuclei in the smokers group as compared to the other two stains.

DISCUSSION

Carcinogenic pyrolytic products of tobacco smoke such as polycyclic hydrocarbons and nitrosamines are metabolized by enzymes which increase the carcinogenic potential of compounds such as benzopyrene and benzanthracene by forming epoxides on subsequent oxidative combustion.[4] Micronuclei are small, additional nuclei seen as cytoplasmic chromatin masses formed by the exclusion of chromosomal fragments or whole chromosomes lagging at anaphase stage of mitosis.[10] The term micronucleus was first proposed in early 1970's by Schimdt, Boller and Heddle who demonstrated it as a reliable indicator of genotoxic potential of mutagens after in vivo exposure using bone marrow erythrocytes in animal subjected studies.[8] Tolbert *et al*., in 2001 recommended the

scoring of at least 1000 intact epithelial cells for micronuclei. The mean prevalence of micronuclei was given as 0.0 to 0.9 %.[10,11] Micronuclei are induced in exfoliated oral epithelial cells by genotoxic and carcinoma causing agents in tobacco, betel nut and alcohol which are consumed on a large scale by the general population, especially in underdeveloped and developing countries.[7,11] They measure between one-fifth and one-third of the size of the main nucleus, are observed in the same plane as the nucleus and show similar staining properties and distribution of chromatin material.[10] A correlation has been observed in increased frequencies of micronuclei and cigarette smoking. Thus, these micronuclei function as "internal dosimeters" or indicators of genotoxic damage and chromosomal aberrations which can indicate early stage-carcinogenesis.[9] The Micronucleus assay was first proposed by Stitch *et al*., in 1983, who proposed that micronuclei in exfoliated buccal cells served as biomarkers to define exposure to carcinogenic agents, show their biological effect on tissues and to provide information about individual's susceptibility to development of oral cancer.[9,11] This method is one of the most widely used short term tests implemented by authorities worldwide to evaluate genotoxicity and mutagenicity.[13] Kashyap *et al*., have proposed distinct advantages of the micronuclei assay over other cytological techniques such as readily accessible site of sample collection from multiple areas of a particular site in the same patient, non-invasive technique and feasibility in conducting longitudinal epidemiological studies for the purpose of drug discovery and assessment. According to a study conducted by Suhas et al on buccal cell changes which are associated with smoking by using the micronucleus assay, a significant correlation between the habit of smoking and the frequency of the micronucleated oral mucosal cells was noted. Palaskar S and Jindal C compared PAP and Giemsa staining techniques to detect micronuclei in exfoliated buccal mucosal cells in individuals with different tobacco habits, concluded that PAP is a better stain over Giemsa for micronucleus assay screening of the buccal cells. Nersesyan A *et al*., in evaluated the impact of smoking on the frequencies of micronuclei and other nuclear abnormalities in exfoliated oral cells and concluded that the nicotine and tar contents of cigarettes affect the DNA stability in cells. Naderi et al evaluated the micronuclei frequency in the exfoliated buccal cells in smokers. The micronuclei frequency in smokers was found to be significantly high in smokers compared to nonsmokers in epithelial oral cells. In the present study conducted, micronuclei were observed and scored using three different staining procedures using PAP, Acridine Orange and Geimsa stain. PAP is a multichromatic stain used principally on exfoliated cytologic specimens. It provides great transparency and delicacy of detail and is commonly used in micronuclei assay, due to its widespread use as a cytological stain and also due to superior background clarity in smears, free of debris and other proteinaceous material. Giemsa stain is specific for the phosphate groups of DNA and attaches itself to regions of DNA where there are high amounts of adenine-thymine bonding. In oral smears, cytoplasm appears pink with nucleus and nuclear material stained blue. The accurate detection micronucleus is often marred by presence of proteinaceous debris and particulate matter in the background. Acridine Orange (AO) is a slightly cationic, lipophilic, weak base, capable of permeating cell and organelle membrane structure due to its metachromatic properties and is commonly used in fluorescence microscopy. Due to its selectivity for nucleic acids the scoring of micronuclei becomes easy with less possibility of error.[13] The results of the study conducted concluded that smokers show an overall increase in frequency of micronuclei when compared to non-smokers; also PAP was found to give superior results in detecting micronuclei with relative accuracy in comparison with Geimsa and Acridine Orange stains. It should also be taken into consideration that Acridine Orange, inspite of showing statistically lower frequencies of micronuclei has fewer chances of false positive results as compared to the DNA non specific stains. According to Grover *et al*, misinterpretation of the following factors as micronuclei can lead to false positive results with DNA non-specific stains.[8]

1. Nuclear anomalies such as binucleated cells, broken eggs, condensed chromatin, karyorrhexis, karyolysis, pyknosis or

2. karyorrhexis, karyolysis, pyknosis or shrunken nuclei etc
3. Keratin granules presenting as round, cytoplasmic bodies as a result of cell injury
4. Bacterial contaminants
5. Dye granules

CONCLUSION

The micronucleus assay provides an efficient method to detect and subsequently score micronuclei present in buccal mucosal cells, which indicates an early onset of carcinogenesis manifested by evidence of genotoxic damage and chromosomal aberrations. A significant correlation was established between cigarette smoking and micronuclei frequencies. Both DNA specific and non-DNA specific stains can be used for this purpose with the former giving significantly higher values, keeping in mind their tendency to give false positive results. Hence, this technique should be further developed by combining cytological methods with molecular aids to provide accurate results for better prognosis and meticulous treatment planning protocol.

CONFLICT OF INTEREST & SOURCE OF FUNDING

The author declares that there is no source offunding and there is no conflict of interest among all authors.

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