

# Micronuclei Count in Oral Exfoliated Cells - A Tool for Biomonitoring DNA Damage in Tobacco Users

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## ABSTRACT

**Aim:** To compare and correlate the frequency of Micronuclei in individuals with tobacco habits but without any clinical lesion, patients clinically diagnosed with leukoplakia and healthy individuals without any tobacco habit. And also to compare papanicolau stained smears to that of Acridine orange for the estimation of micronuclei frequency.

**Materials and Methods:** Smears were made from 10 individuals without any tobacco habit, 10 individuals with tobacco habit but without any clinically identifiable oral changes and 10 individuals with clinically diagnosed leukoplakia. Cytological smears from the same patient were stained with both PAP and Acridine orange stain. Numbers of micronuclei in 2000 cells were counted in each patient.

**Statistical analysis used:** Comparison of number of micronuclei (papanicolau stained smears) among different groups was done using Kruskal Wallis test. While, comparison of number of micronuclei (papanicolau stained smears v/s acridine orange) among different groups was done using Mann Whitney U test.

**Results:** There was increase in micronuclei frequency from control to tobacco users to clinically diagnosed leukoplakia patients, but difference was not statistically significant. The micronuclei count was 4-5 times higher in Papanicolau stained slides as compared to acridine orange stain.

**Conclusion:** Genotoxic effect by carcinogen may manifest in various ways like apoptosis or nuclear degeneration and therefore, only counting micronuclei in exfoliated epithelial cells as biomarkers of exposure to lifestyle and occupation related genotoxic carcinogens warrants further evaluation. DNA specific dyes like Acridine orange should be preferred over other stains, for micronuclei estimation so as to reduce the overestimation.

**Keywords:** Micronuclei; tobacco; exfoliated cells; leukoplakia; papanicolau; acridine orange

## INTRODUCTION

Micronuclei are extra nuclear cytoplasmic bodies, induced in cells by numerous genotoxic agents that damage the chromosomes. [1] The frequency of occurrence of micronuclei is a measure of chromosome breakage in early cell divisions, and the number of micronuclei is known to increase with carcinogenic

stimuli, long before the development of clinical symptoms. Since, more than 90% of all human cancers are of epithelial origin, micronuclei assay with buccal epithelial cells can be a suitable bio-monitoring approach for the detection of increased cancer risk in humans. [2]

Oral squamous cell carcinoma (OSCC) is the third most common cancer in

India and reported to be the commonest type among the males. [3] India has highest number of OSCC cases in the world due to high prevalence of smokeless tobacco usage. [4]

OSCC due to its location in an accessible part of the body, i.e., oral mucosa, can be detected at an early stage. However, due to the lack of symptoms during an early stage, medical attention is not sought till the disease has advanced, leading to poor prognosis. It is therefore, imperative for all dental professionals to prepare themselves with the knowledge to detect this disease at the earliest possible stage. [5]

Oral carcinogenesis is a multistep process of accumulated genetic alterations, including chromosomal alterations, DNA changes and/or epigenetic alterations. Hence, DNA damage due to oral habits in potentially malignant disorders (PMDs) and malignancy patients may provide a wide array of useful diagnostic and prognostic information along with easy and reliable techniques for their early detection. [1] Of the carcinogens, tobacco is considered most common carcinogen related to OSCC, causing DNA damage in the oral epithelial cells. [6] 30–80% of OSCC cases arise from PMDs, of which leukoplakia is the most common. [7]

The purpose of present study was to assess the frequency of micronuclei in tobacco users and patients with leukoplakia, and possible role of micronuclei in detection of potentially malignant disorders even before the appearance of clinically visible lesion in the oral cavity using two different staining procedures i.e. papanicolau and acridine orange stain.

## MATERIALS & METHODS

Sample was collected from individuals visiting to the Department of Oral and Maxillofacial Pathology and divided in three groups: 10 healthy individuals without any tobacco habit, 10 individuals with tobacco habit but without any clinically identifiable oral lesion and 10

individuals with clinically diagnosed leukoplakia. Approval from the Ethical Committee was obtained and written consent was taken from all participants. After a thorough medical history and oral examination participants were asked to rinse the oral cavity. Using a wet wooden tongue depressor, exfoliated cells were collected from buccal mucosa in individuals without any lesion and from lesional site in patients with leukoplakia. Smears were immediately fixed by the Biofix spray (Bio Lab Diagnostics). Slides from the same patient were stained with both PAP (Rapid PAP, Biolab Diagnostics, Tarapur, Maharashtra) and Acridine orange stain (Research-Lab Fine Chem Industries, Mumbai). For Acridine orange (AO) staining, Modified Riva & Turner (1962) method was used. [8] Riva & Turner (1962, modified) method for AO stain:

- a) Agitate the fixed smears for 5 second in AO (0.025% AO in 2% acetic acid) solution,
- b) Differentiate in 2% ethyl alcohol in physiological saline for 2 second,
- c) Mount the smear with coverslip using physiological saline.

PAP stained smears were examined under light microscope while AO stained smears were examined under fluorescence microscope (Olympus, CX21i, with fluorescent attachments). Number of micronuclei in 2000 cells were counted in each participant.

### Criteria to assess micronuclei

Tolbert et al criteria were used for identifying micronucleus. The parameters were as follows:

- 1) Rounded smooth perimeter suggestive of a membrane
- 2) Less than a third the diameter of associated nucleus, but large enough to discern shape and color
- 3) Staining intensity similar to nucleus
- 4) Texture similar to nucleus
- 5) Same focal plane as nucleus
- 6) Absence of overlap with or bridge to nucleus

Cells with intact nuclei and cell boundaries were counted. Tolbert et al recommended the scoring of at least 1000 cells in a slide so that more precise results would be obtained with increasing number of cells. [1] Figure 1 showing PAP and AO stained smears with buccal epithelial cells showing micronuclei.

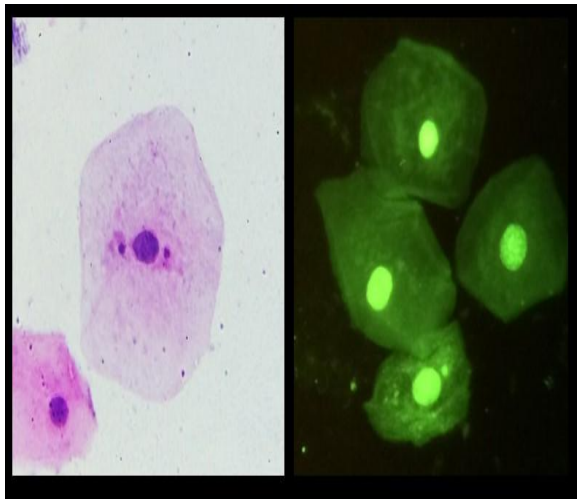


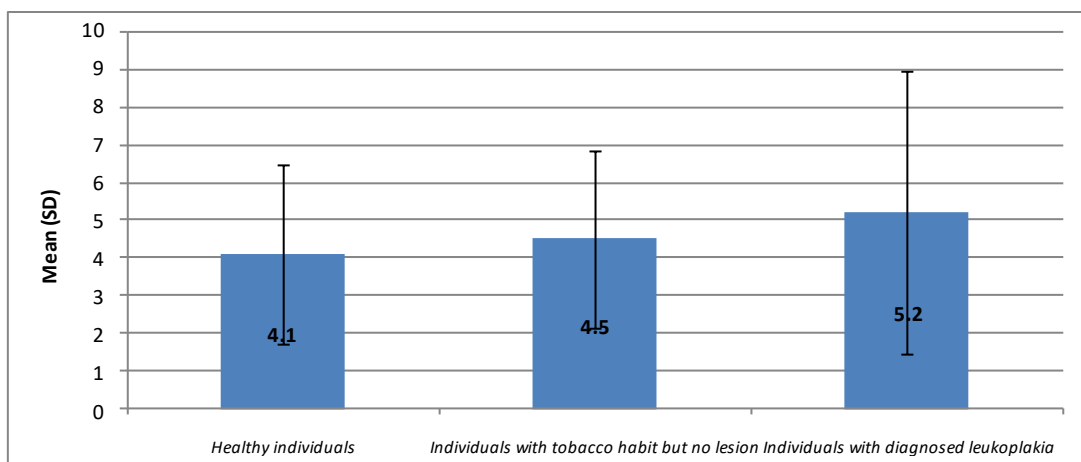
Figure 1: PAP (40X) stained smear showing two MN in buccal epithelial cell. AO (40X) stained smear showing one MN in buccal epithelial cell.

## RESULTS

The mean age of control group was 32.4 years while that of tobacco users was 39.8 years and in leukoplakia patients it was 49.8 years. No correlation was found between age and the frequency of micronuclei. Also, no significant correlation was seen between sex of individual and micronuclei frequency. Comparison of number of micronuclei (papanicolau stained smears) among different groups was done using Kruskal Wallis test. While, comparison of number of micronuclei (papanicolau stained smears v/s acridine orange) among different groups was done using Mann Whitney U test. On examination of papanicolau stained slides, the frequency of micronuclei was found to be higher in leukoplakia patients ( $5.20 \pm 3.736$ ) as compared to tobacco users ( $4.50 \pm 2.369$ ) and the control group ( $4.10 \pm 2.378$ ) (Table 1, Graph 1).

Table 1: Comparison of number of micronuclei (papanicolau stained smears) in terms of {Mean (SD)} among different groups using Kruskal Wallis test

Group	N	Mean	Std. Deviation	Chi square value	P value
Healthy individuals	10	4.10	2.378	0.245	0.885
Individuals with tobacco habit but no lesion	10	4.50	2.369		
Individuals with diagnosed leukoplakia	10	5.20	3.736		
Total	30	4.60	2.836		

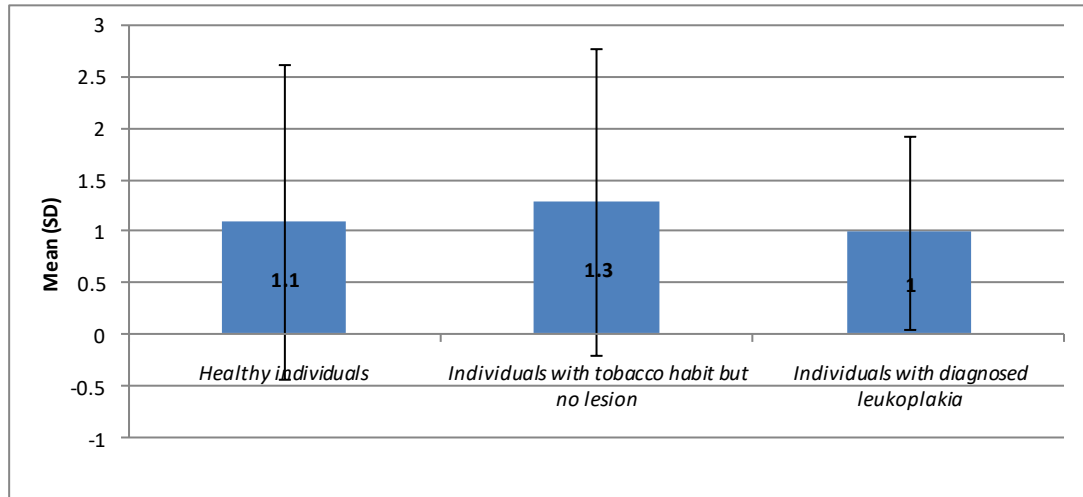


Graph 1: Comparison of number of micronuclei (papanicolau stained smears) in terms of {Mean (SD)} among different groups using Kruskal Wallis test

While with Acridine orange stained slides, the micronuclei frequency was higher in tobacco users ( $1.30 \pm 1.494$ ) as compared to control ( $1.10 \pm 1.524$ ) and leukoplakia patients ( $1.0 \pm 0.943$ ) (Table 2, Graph 2).

Table 2: Comparison of number of micronuclei (Acridine orange) in terms of {Mean (SD)} among different groups using Kruskal Wallis test

Group	N	Mean	Std. Deviation	Chi square value	P value
Healthy individuals	10	1.10	1.524	0.173	0.917
Individuals with tobacco habit but no lesion	10	1.30	1.494		
Individuals with diagnosed leukoplakia	10	1.00	0.943		
Total	30	1.13	1.306		



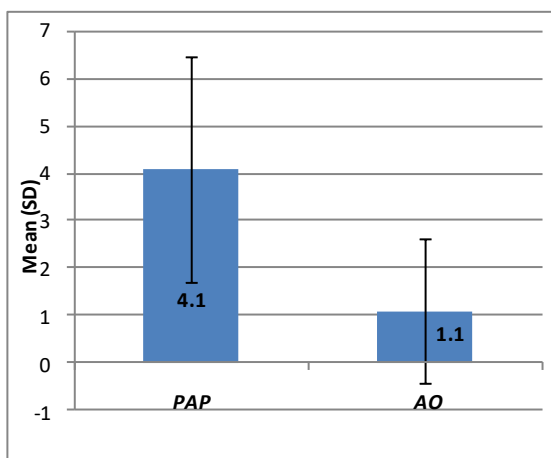
Graph 2: Comparison of number of micronuclei (Acridine orange) in terms of {Mean (SD)} among different groups using Kruskal Wallis test

However, the difference was not statistically significant on either papanicolau or acridine orange staining. Micronuclei count was 4-5 times higher when stained with papanicolau stain as compared to acridine Orange. Also, there was statistically significant difference in micronuclei count on comparison between the two stains (Table 3, Graph 3).

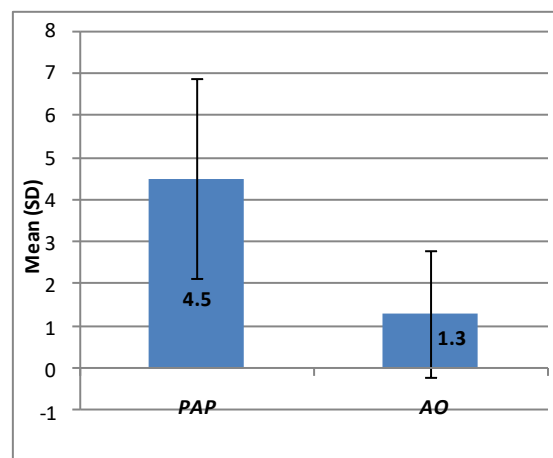
Table 3: Comparison of number of micronuclei (papanicolau stained smears v/s Acridine orange) in terms of {Mean (SD)} among different groups using Mann Whitney U test

Group	N	Mean	Std. Deviation	Z value	P value
Healthy individuals	PAP	4.10	2.378	2.709	0.007*
	AO	1.10	1.524		
Individuals with tobacco habit but no lesion	PAP	4.50	2.369	2.808	0.005*
	AO	1.30	1.494		
Individuals with diagnosed leukoplakia	PAP	5.20	3.736	3.579	<0.001**
	AO	1.00	0.943		

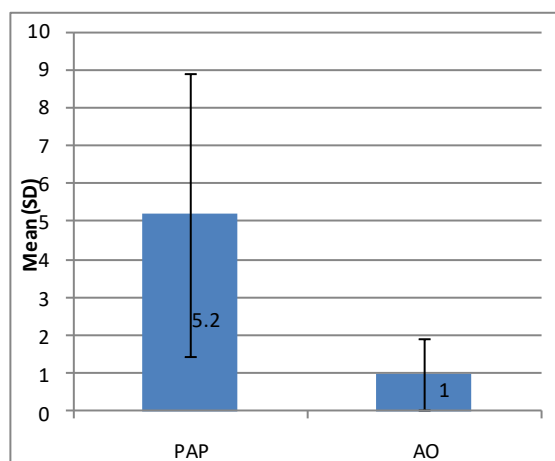
(p < 0.05 - Significant\*, p < 0.001 - Highly significant\*\*)



(Healthy individuals)



(Individuals with tobacco habit but no lesion)



(Individuals with diagnosed leukoplakia)  
Graph 3: Comparison of number of micronuclei (papanicolau stained smears v/s Acridine orange) in terms of {Mean (SD)} among different groups using Mann Whitney U test

## DISCUSSION

Micronuclei (MN) in oral epithelial cells can be induced by various factors including genotoxic agents like, medical procedures (e.g., radiation and chemicals), carcinogenic components in tobacco, betel nut and alcohol, and genetic factors such as inherited defects in DNA metabolism and/or repair. [9]

There are two predominant mechanisms leading to the MN in a mitotic cell: [10]

- Chromosomal breakage by clastogens
- Dysfunction of the mitotic apparatus by aneugenic agents

Various scientific studies in literature provide evidence that MN frequency in peripheral blood lymphocytes is a predictive biomarker of cancer risk within a population of healthy subjects. Buccal cells in oral cavity can prove to be a better site for MN estimation owing to its non-invasive sampling, short turnover time, less DNA repair capacity of the cells and direct correlation with cancer progression. [7]

Katarkar et al (2014) observed that MN assay in buccal cells and the comet assay in peripheral blood cells have similar accuracy and precision for detecting DNA damage. [7]

Ceppi et al (2010) reported a strong correlation of MN frequency in buccal exfoliated cells with MN frequency in lymphocytes. This correlation suggests that

systemic genotoxic effects within the blood stream may also impact on and be detectable in buccal cells. [11]

Melkundi et al (2013) observed significantly elevated frequencies of micronucleated cells (MNC) from normal (n=20) (1.95%), tobacco habit (n=32) (4.26%) and leukoplakia groups (n=19) (5.05%). Exfoliated buccal mucosal cells were stained with freshly prepared giemsa stain. [12] Although the present study showed increased MN count in leukoplakia patients but the difference was not statistically significant. This could be attributed to the small sample size used for the study.

Palaskar and Jindal found that Papanicolau was better stain for counting as MN were easily seen in clear cytoplasm in regard to other stains like Giemsa stain. [13] Variability in the MN assay in buccal cells arises due to differences in scoring by observers, inter-individual variability and type of stain used. [7]

Nersesyan et al (2006) found that the results of MN assays in exfoliated oral mucosa cells of smokers and nonsmokers depend strongly on the staining methods. The micronuclei frequencies in oral mucosa cells of heavy smokers (n = 20) and nonsmokers (n = 10) were evaluated with nonspecific (Giemsa, MGG) and DNA-specific (DAPI, Feulgen, acridine orange) stains. With Giemsa-based stains, the frequencies of micronuclei in smokers were significantly (4- to 5-fold) higher in the smokers group, no significant increase was observed with any of the DNA-specific stains. These comparisons indicate that micronuclei formation in epithelial cells may be overestimated when non-DNA-specific stains are used. [2] Also, in the present study AO stained samples showed no significant difference in the MN frequency of different groups.

One of the possible confounding factors in MN studies can be the formation of keratin granules, found in degenerated cells with nuclear anomalies. These round cytoplasmic bodies, which are formed as a consequence of cell injury, do not contain

DNA and may be classified as micronuclei with the nonspecific stains. [2]

The increased risks of oral cancer in smokers may be due to acute toxic effects and inflammation that are not associated with micronuclei formation. Therefore, the use of micronuclei in exfoliated epithelial cells as biomarkers of exposure to lifestyle and occupation related genotoxic carcinogens warrants further evaluation. Genotoxic effect by carcinogen may manifest by various ways like apoptosis, nuclear degeneration and only counting micronuclei cannot be considered as marker for genotoxicity. [2]

Although MN assay has been used since the 1980s to demonstrate cytogenetic effects of environmental and occupational exposures, lifestyle factors, dietary deficiencies and different diseases, important knowledge gaps remain about the characteristics of MN and other nuclear abnormalities, the basic biology explaining the appearance of various cell types in buccal mucosa samples and effects of diverse staining procedures and scoring criteria in laboratories around the world and their relationship to disease states and outcomes. To address these uncertainties, the HUMN (HUMANMicroNuclei) project coordinating group initiated a new international validation project for the buccal cell MN assay in 2007, similar to the project previously performed using human lymphocytes. [14] To distinguish it from the lymphocyte project, this project was given the acronym HUMNxL, i.e. human MN assay in exfoliated buccal cells. [15]

## CONCLUSION

Although many studies have shown a significant increase in the buccal cell MN frequency in human populations exposed to genotoxic agents, the magnitudes of the changes are usually relatively small. Different confounding factors influencing the buccal cell MN assay such as gender, age and lifestyle habits have to be considered. Genotoxic effect by carcinogen may manifest in various ways like apoptosis

or nuclear degeneration and therefore, only counting micronuclei in exfoliated epithelial cells as biomarkers of exposure to lifestyle and occupation related genotoxic carcinogens warrants further evaluation. DNA specific dyes like acridine orange should be preferred over other stains, for micronuclei estimation so as to reduce the overestimation.

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