

Original Research Article**Significance of Micronucleus in Buccal Mucosa as a Marker of Genotoxicity****Awani Jain¹, S.K. Nema², Sanjeev Narang³, Anjali Singh⁴, Nikhil Jain⁵, Neelambara Bidwai⁶**

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Abstract

Aim and Objectives: To assess the presence or increase of micronucleus in buccal mucosa smears of tobacco users and compare the micronucleus staining by PAP and Feulgen stain.

Methods: The study was conducted on 500 subjects (tobacco users and non-users), divided according to duration and form of tobacco use. Exfoliated buccal cells were obtained from each subjects, 2 slides were prepared, stained with PAP and Feulgen stain. Minimum 500 cells were examined from each slide and mean total number of MN and mean MN frequency was evaluated.

Study Design: Cross sectional study.

Results: There was statistically significant difference ($P < 0.05$) in the micronucleus assay of tobacco users as compared to non-users by PAP and Feulgen stain, where PAP stain was able to identify higher number of micronuclei.

In tobacco users, the mean total number of micronucleus with PAP and Feulgen stain was 17.5 ± 11.2 and 11.87 ± 6.71 respectively. In non-users, the mean total number of micronucleus with PAP and Feulgen stain was 5.70 ± 3.08 and 3.27 ± 1.82 respectively. In users, the mean micronucleus frequency with PAP and Feulgen stain was 1.79 ± 0.65 and 1.61 ± 0.49 respectively. In non-users, the mean micronucleus frequency with PAP and Feulgen stain was 1.18 ± 0.43 and 1.03 ± 0.13 respectively.

Duration and form of tobacco use also showed statistically significant difference with PAP stain.

Conclusions: All forms of tobacco can cause and increase micronuclei. Thus micronuclei assay can be used as a marker of genotoxicity.

Keywords: Micronucleus; Oral Cytology; PAP Stain; Feulgen Stain.

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Introduction

Oral cancer is a major problem in the Indian subcontinent where it ranks among the top three types of cancer in the country, it accounts for over 30% of all cancers in India. Almost 1 million people die of oral cancer in India. All forms of tobacco use are known risk factors

for oropharyngeal cancer. 57% of all men and 11% of women between 15–49 years of age use some form of tobacco in India. Oral cancer is of significant public health importance to India. It is diagnosed at advanced stages which result in considerable costs to the patients and low treatment outcomes. Early detection would improve cure rates as well as lower the morbidity and cost associated

with treatment. It is important that cost effective oral cancer screening be introduced in high like populations like India [1,2].

Oral habits of smoking or chewing tobacco damage the oral tissues. Tobacco contains several carcinogens. These material activate in different tissues, which cause the DNA adduct products. It is essential to have a reliable, relevant and minimally invasive biomarkers to improve the biomonitoring, diagnostics, and treatment of diseases caused by, or linked with, genotoxic damage. The MN assay is one such biomarker of genotoxicity [3,4,5].

Micronuclei

Micronuclei(MN) is a microscopically visible round or oval cytoplasmic, chromatin mass in the extranuclear vicinity, originated from aberrant mitosis. Micronuclei consists of eccentric chromosomes that have failed to reach spindle poles during mitosis and are used as biomarkers for assessment of genotoxic damage [6,7,8,9]. Genotoxic damage is the damage to genetic material (DNA) within a cell resulting in mutations, which may lead to cancer [10]. Micronuclei are characteristically induced in exfoliated cells of the buccal mucosa in precancerous and cancerous conditions .

Aim and Objectives

Aim: To study the significance of micronuclei in buccal mucosa as a marker of genotoxicity

Objectives:

- Analysis of buccal mucosa cells for micronuclei in tobacco users.
- Comparison of micronuclei staining by Pap stain and Feulgen stain

Materials and Method

- *Study Design:* Cross sectional study.
- *Study Population:* A total of 500 subjects between the age group of 18 to 80 years were included in study group after taking relevant history and local examination.
- *Place of Study:* Index Medical college Hospital and research centre.
- *Duration of study:* Two years
- *Ethical clearance:* Institutional ethics committee

Inclusion Criteria

1. Tobacco Users:
 - Tobacco powder chewer
 - Areca nut chewer

- Pan masala chewer
- Smokers(cigarette and beedi)

2. Age more than 18 years, both male and female.
3. Non exposure to radiography beam in recent 6 months.

Exclusion Criteria

1. Age below 18 years, both male and female.
2. Subjects with leukoplakia, erythroplakia, lichen planus, oral submucous fibrosis etc.
3. Subjects having buccal dysplasia.
4. Subjects having oral cancer.
5. Subjects with any other oral lesion.

Materials

1. Wooden spatula – a dry and clean wooden spatula is used for scraping the buccal mucosa.
2. Gauge piece
3. Gloves
4. Marker pencil is used for numbering the slides.
5. Clean glass slides of 75 x 25 mm and thickness of 1.35 mm were used for taking the smears.
6. Fixative
7. Coplin jar- were used for the purpose of staining
8. PAP stain
9. Schiff's reagent
10. 1M HCl
11. Incubator
12. Distilled water
13. Slide tray
14. DPX solution
15. Cover slip(22mm X 22 mm)
16. Light microscope
17. Differential counter for calculating the cells

Methodology

Subjects were divided according to duration and form of tobacco use. Subjects were asked to rinse the oral cavity with water before taking the samples. The buccal mucosa were scrapped using wooden spatula and the exfoliated cells were spread over the glass slide to prepare the smears. Two slides for each subject were fixed and stained with PAP and Feulgen stain. Minimum 500 cells were examined from each slide and mean total number of MN and mean MN frequency was evaluated.

Measurement of micronuclei

Structures fulfilling below criteria by Tolbert et al. [11] were considered as Micronuclei:

- Diameter 1/3 to 2/3 of the main nucleus, but large enough to discern shape and colour;
- Staining intensity similar to, or slightly weaker than, that of the nucleus;
- Round-to-oval shape;
- Texture similar to that of the main nucleus;
- Close proximity but no actual contact with the nucleus;
- Plane of focus same as that of the main nucleus.
- ✓ Screening of slide was done in a zigzag manner.
- ✓ The average frequency of MN was further tabulated based on following formula:

$$\text{Average frequency of MN} = \frac{\text{Total number of MN}}{\text{Total number of cells with MN}}$$



Fig. 1: Photomicrograph showing normal buccal epithelial cells (PAP stain, 100X)

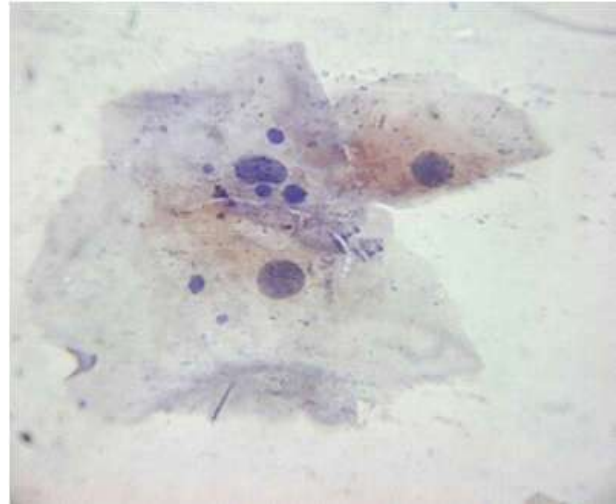


Fig. 2: Photomicrograph showing multiple micronuclei in buccal epithelial cells (PAP stain, 100X)

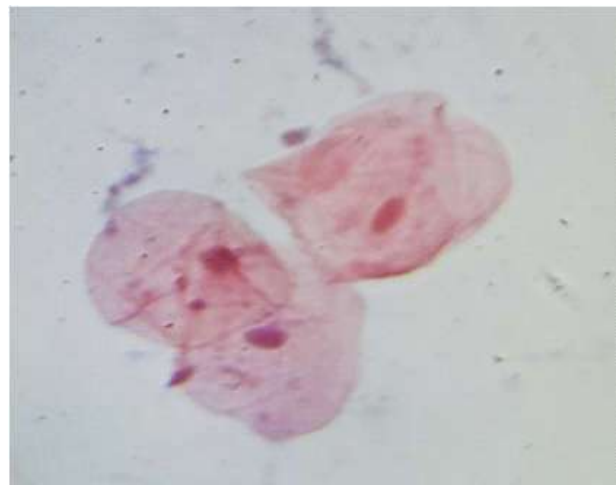
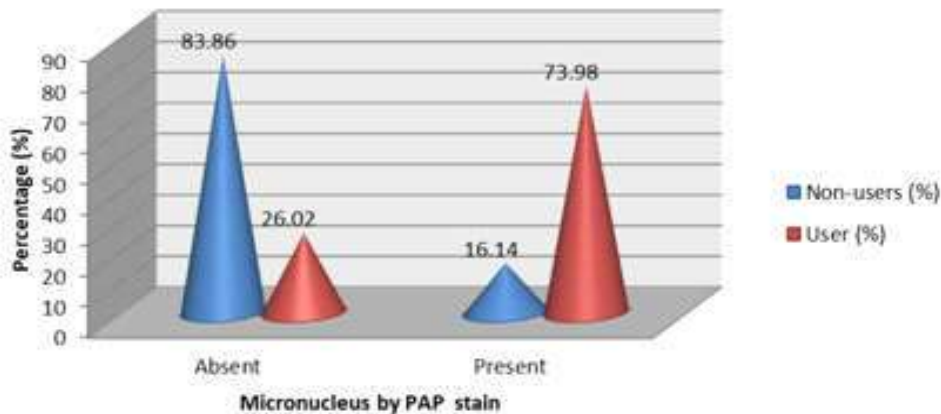
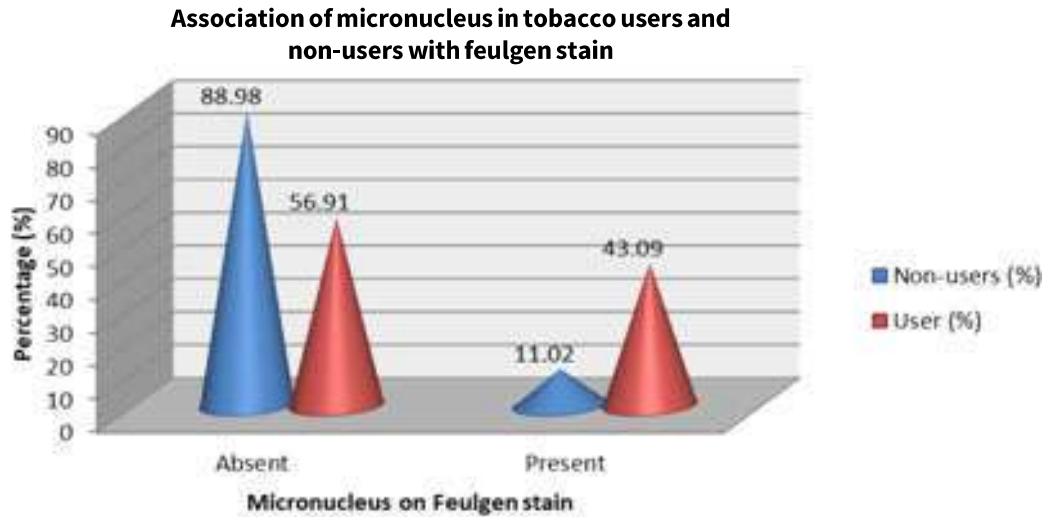


Fig. 3: Photomicrograph showing multiple micronuclei in buccal epithelial cells (Feulgen stain, 100X)

Association of micronucleus in tobacco users and non-users with pap stain



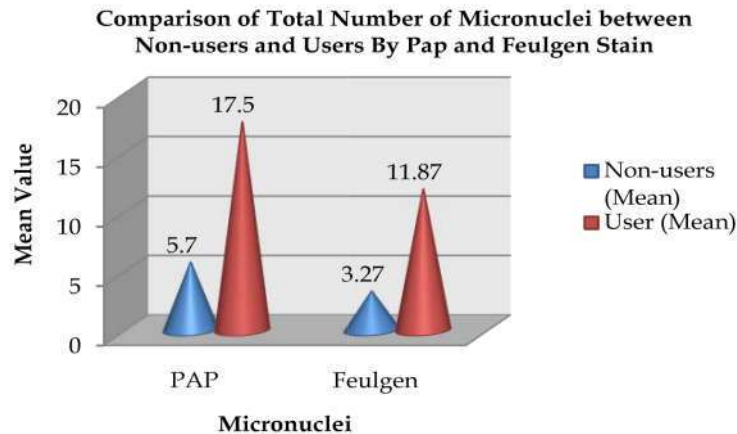
Graph 1: Cone diagram showing distribution of micronuclei in tobacco users and non-users with PAP stain



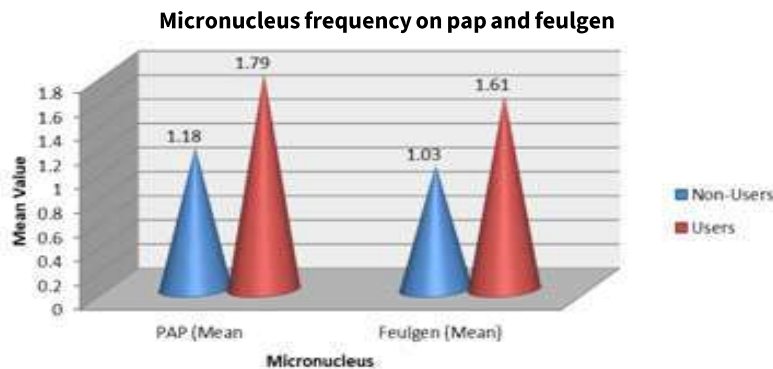
Graph 2: Cone diagram showing distribution of micronuclei in tobacco users and non-users with Feulgen stain

Cone diagram showing distribution of micronuclei in tobacco users and non-users with PAP stain and Feulgen stain. There was statistically significant difference ($P < 0.05$) in the micronuclei assay of tobacco users as compared to non-users by PAP and Feulgen stain, where PAP stain was able to identify higher number of micronucleated cells.

In tobacco users, the mean total number of micronuclei with PAP and Feulgen stain was 17.5 ± 11.2 and 11.87 ± 6.71 respectively. PAP was able to identify higher total no. of Micronuclei in tobacco users. In non-users, the mean total number of micronuclei with PAP and Feulgen stain was 5.70 ± 3.08 and 3.27 ± 1.82 respectively.



Graph 3: Cone diagram showing comparison of total number of Micronuclei



Graph 4: Cone diagram showing comparison of micronuclei frequency on PAP and Feulgen stain

In users, the mean micronuclei frequency with PAP and Feulgen stain was 1.79 ± 0.65 and 1.61 ± 0.49 respectively. In non-users, the mean micronuclei frequency with PAP and Feulgen stain was 1.18 ± 0.43 and 1.03 ± 0.13 respectively.

Duration and form of tobacco use also showed statistically significant difference with PAP stain.

Discussion

In our study, the mean total number of MN and mean frequency of MN in tobacco users and non-users were evaluated.

The study also observed the effect of duration of tobacco use, different forms of tobacco like cigarette, beedi, tobacco powder and different stains like Pap and Feulgen on the MN assay.

When micronuclei association was studied in tobacco users and non-users. The results were statistically significant ($P < 0.05$) showing micronuclei is associated with tobacco use. Also, higher percentage of MN were seen with Pap stain as compared to Feulgen. This is due to the fact that Feulgen is DNA specific stain and stains only nuclear material while Pap being DNA non-specific cannot distinguish true MN from keratohyalin granules, bacterial clumps or stain deposits. Kayal et al. (1993) also reported in his studies significantly higher frequencies of micronucleated cells in exfoliated oral mucosal cells in tobacco chewers [12].

When the association of MN was studied in relation to duration of tobacco use, the highly significant ($P < 0.05$) difference was observed suggesting that MN is dependent on duration of tobacco use.

When the association of MN was seen in relation to form of tobacco, the result were statistically significant ($P < 0.05$). Further, strongest association of MN was observed in combined users followed by tobacco chewers and smokers.

Values were higher with Pap as compared to Feulgen stain as Pap is more sensitive but Feulgen is more specific stain.

A highly significant difference ($P < 0.05$) was observed in mean total number of MN in tobacco users based on duration of tobacco use.

Naderi et al. [13] also reported significant ($P < 0.05$) results in micronuclei number based on duration of tobacco.

When Mean MN number was studied in relation to form of tobacco in our study, the mean total number of MN was lowest in the smokers, while it was highest in combined users.

Bansal (2012) and Ozkul (1997) also reported higher mean number of micronuclei in smokeless tobacco as compared with smokers and non-users [14,15].

To summarize the results, MN can be seen in normal mucosal cells. But the number and frequency of MN is higher in tobacco users as compared to control groups irrespective of form of tobacco or duration of tobacco users. Also, Feulgen is gold standard for MN, but certain limitations were observed during the procedure such as time consumption and technique sensitive procedure. So, Pap stain can be used for routine screening purpose in high risk populations. Suspicious patients with high number of MN found with Pap stain can further be substantiated with Feulgen for confirmation.

Table 1: Association of micronuclei in relation to tobacco use

Stain	User		Non-User	
	MN Present	MN Absent	MN Present	MN Absent
PAP	73.98%	26.02%	16.14%	83.86%
Feulgen	43.09%	56.91%	11.02%	88.98%

Table 2: Association of micronuclei on Pap and Feulgen stain in relation to duration and form of tobacco

Stain	Duration			Form of Tobacco		
	≤ 5 years	6-10 years	> 10 years	Beedi/ Cigarette	Tobacco Powder	Both
PAP	53.52%	80%	83.48%	86%	70%	88%
Feulgen	12.68%	28.33%	69.57%	50%	72%	76%

Table 3: Mean total number of micronuclei in relation to duration and form of tobacco

Stain	User	Non user	Duration			Form of Tobacco		
			< 5 yrs	6-10 yrs	> 10 yrs	Beedi/ Cigarette	Tobacco	Both
PAP	17.5	5.70	10.45	12.37	22.94	12.81	17.04	25.91
Feulgen	11.87	3.27	5.50	7.11	13.78	10.38	11.88	13.05

Conclusion

My study aims at emphasizing that genotoxic effects of tobacco cause chromosomal damage in buccal epithelial cells and these are reflected as increase in micronuclei in tobacco users. This is present even in the absence of clinically evident changes. This observation is vital in utilization of the micronuclei detection in smears as a prognostic, educational and interventional tool in the management of patients with tobacco habits.

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