

# Micronuclei in Exfoliated Cells: A Biomarker of Genotoxicity in Tobacco Users

Meenakshi Upadhyay, Parul Verma<sup>1</sup>, Robin Sabharwal<sup>2</sup>, Santosh Kumar Subudhi<sup>3</sup>, Suruchi Jatol-Tekade<sup>4</sup>, Vihang Naphade<sup>5</sup>, Basanta Kumar Choudhury<sup>6</sup>, Pravudeva Devidutta Sahoo<sup>7</sup>

Department of Pedodontics and Preventive Dentistry, Saraswati Dental College, Lucknow, Uttar Pradesh, <sup>1</sup>Department of Conservative Dentistry and Endodontics, IDS, Sehora, Jammu, <sup>2</sup>Department of Oral Pathology and Microbiology, MMCD SR, Mullana, Ambala, Haryana, <sup>3</sup>Department of Oral and Maxillofacial Surgery, Bhubaneswar, Odisha, <sup>4</sup>Department of Orthodontics, Modern Dental College and Research Center, Gandhi Nagar, <sup>5</sup>Department of Oral Medicine and Radiology, Index Institute of Dental Sciences, Indore, Madhya Pradesh, <sup>6</sup>Department of Oral Medicine and Radiology, Institute of Dental Sciences, <sup>7</sup>Private Practitioner, Dental Surgeon, Bhubaneswar, Odisha, India

ABSTRACT

**Aims:** The aim of this study was to analyze the tobacco-related genotoxic effects in individual with habit of smoking and chewing tobacco. **Materials and Methods:** The present study sample consisted of 120 individuals attending the outpatient department of D. J. College of Dental Sciences and Research, Modinagar, Uttar Pradesh (UP). The sample was divided into four groups as follows: Group I (individuals with habit of smoking tobacco), Group II (individuals with habit of chewing tobacco), Group III (individuals with habit of smoking and chewing tobacco), and Group IV control group (nontobacco-exposed individuals). Patients were asked to rinse their mouth gently with water. The exfoliated cells were obtained by scraping the buccal mucosa of individuals with a wooden spatula. The scraped cells were placed on the precleaned slides. The smears were then stained with RAPID-PAP™ and analyzed under the microscope. Data were analyzed using SPSS statistical software. **Results:** In the present study, an arbitrary unit was obtained using frequency/day multiplied by the duration of years (risk multiplication factor [RMF]), a positive and significant correlation were observed between the RMF and the mean percentage of micronucleated cell count in smokers, chewers, and in individuals with both smoking and chewing habit, respectively. A weak positive and nonsignificant correlation were observed between age and mean percentage of micronucleated cells in smokers and smokers + chewers, respectively, while it was weak negative and nonsignificant in chewers. In control group, correlation between age and percentage of micronucleated cells was weak positive and nonsignificant at 5% level of significance. **Conclusion:** The micronuclei in exfoliated mucosal cells from buccal mucosa can be used as a biomarker of genotoxicity in predicting the effects of carcinogens.

**KEYWORDS:** Carcinogens, micronuclei, oral cancer, risk multiplication factor, tobacco

## INTRODUCTION

Oral cancer is the sixth most common form of cancer in the world. It is a well-established fact that oral cancer is frequently preceded by visible lesions which are termed as potentially malignant disorders because a significant number of these lesions transform into oral cancer.<sup>[1,2]</sup>

Micronucleus (MN) is a recently upgraded topic, especially in the field of oral cancer. Micronuclei originate from

chromosome fragments or whole chromosomes, which lag behind at anaphase during nuclear division. It could be argued that micronuclei in exfoliated oral epithelial cells represent a preferred target site for early genotoxic events induced by carcinogenic agents. Various studies

**Address for correspondence:** Dr. Robin Sabharwal, Department of Oral Pathology and Microbiology, MMCD SR, Mullana, Ambala, Haryana, India. E-mail: robin.sabharwal@yahoo.co.in

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have shown the correlation of frequency of micronuclei and severity of this genotoxic damage.<sup>[3]</sup> Micronuclei are characteristically seen in exfoliated epithelial cells such as buccal mucosa and urinary bladder wall during precancerous and cancerous conditions.<sup>[4]</sup>

The buccal cell MN is defined as the microscopically visible, round or oval cytoplasmic chromatin mass next to nucleus. Micronuclei originate from aberrant mitosis and consist of acentric chromosomes, chromatid fragments, or whole chromosomes that have failed to be incorporated in the daughter nuclei during mitosis.<sup>[5]</sup>

Micronuclei are induced in oral epithelium by a variety of substances including genotoxic agents and carcinogenic compounds in tobacco, betel nut, and alcohol. These are also seen in various conditions such as chronic tonsillitis,<sup>[6]</sup> chronic renal diseases,<sup>[7]</sup> and rheumatoid arthritis.<sup>[8]</sup>

The genotoxic and carcinogenic chemicals released from betel nut and tobacco and also the calcium hydroxide content of lime present in the betel quid are thought to be responsible for the promotion of reactive oxygen species from areca nut extracts. These reactive oxygen species can, in turn, cause damage to the DNA.<sup>[9]</sup>

The micronuclei count is increased in potentially malignant disorders such as oral submucous fibrosis, leukoplakia, erythroplakia, lichen planus, and squamous cell carcinoma.<sup>[10,11]</sup>

Thus, the quantitative estimation of micronuclei may serve as an indicator of genetic damage that has taken place. Oral carcinogenesis is a multi-step process of accumulated genetic damage leading to cell dysregulation with disruption in cell signaling. These events can be conveniently studied in the buccal mucosa, which is an easily accessible tissue for sampling cells in a minimally invasive manner.<sup>[12]</sup>

Buccal cells are the first barrier for the inhalation or ingestion route and are capable of metabolizing proximate carcinogens to reactive products.<sup>[13]</sup>

The buccal mucosa provides a barrier to potential carcinogens that can be metabolized to generate potential reactive products. Exfoliated buccal cells have been used noninvasively to successfully show the genotoxic effects of lifestyle factors such as tobacco smoking, chewing of betel nuts and/or quid, medical treatments such as radiotherapy as well as occupational exposure to potentially mutagenic and/or carcinogenic chemicals, and for studies of chemoprevention of cancer.<sup>[14]</sup>

The present study was carried out to assess the effect of tobacco on the cells of buccal mucosa by comparing the micronucleated cell count in patients with a habit

of smoking tobacco (beedi or cigarette), chewing tobacco (gutkha), smoking and chewing tobacco, and in those not using tobacco in any form.

## MATERIALS AND METHODS

The present study was conducted in D. J College of Dental Sciences and Research, Modinagar, UP, from 2010 to 2013. This study was approved by the institutional reviewer board (Reference no. DJ/2010/EC/OP), and an informed consent was obtained from all the participants. The individuals attending the outpatient Department and those who smoked four cigarettes/day for 2 years and chewed at least two packs of gutkha/day for 2 years were included in the study sample. Most of the individuals were occasional drinkers who consumed alcohol once a week.

- The present study sample of 120 patients was divided into four groups with 30 patients in each group as follows:
  - a. Group I (individuals with habit of smoking tobacco)
  - b. Group II (individuals with habit of chewing tobacco)
  - c. Group III (individuals with habit of smoking and chewing tobacco)
  - d. Group IV Control group (nontobacco-exposed individuals).

### Exclusion criterion

Individuals using anabolic androgenic steroids, chlorhexidine mouthwash and those suffering from rheumatoid arthritis, chronic renal disease, and chronic tonsillitis were excluded from the study sample.

### Materials

- a. Pair of gloves
- b. Mouth mask
- c. Cotton wool, probe, mirror, and tweezers
- d. Wooden spatulas
- e. Microscopic slides
- f. Coplin jars
- g. Rapid papanicolaou (PAP) kit
- h. Diamond point glass marker
- i. Light Microscope.

### Reagents used

RAPID-PAP™ kit:-contents of RAPID-PAP™ kit which are as follows:

1. Nuclear stain (hematoxylin solution)
2. a) Cytoplasmic stain 2A (OG-6 solution)  
b) Cytoplasmic stain 2B (Light green-Eosin)
3. Biofix spray (Micro-anatomy fixative)
4. Dibutyl Phthalate Xylol (Glass mounting medium)
5. Dehydrant (Propanolol)
6. Xylene
7. Wash buffer.

Working cytoplasmic stain was prepared by mixing an equal volume of cytoplasmic stain 2A (OG-6 solution) and 2B (Light green-Eosin). Working Scott's tap water buffer was prepared by adding 1 ml of RAPID-PAP™ wash buffer to 100 ml of tap water.

The patients were asked to rinse their mouth gently with water and the exfoliated cells were obtained by scraping the buccal mucosa of individuals with a wooden spatula [Figure 1]. The scraped cells were placed on the precleaned slides [Figure 2], and then, fixed and stained with Biofix spray and RAPID-PAP, respectively.

### Scoring procedure

The most commonly used method, i.e., the zigzag method, was followed for the screening of slides. The counting was started from the lower right corner of the slide going across the shorter dimension of the slides, then the field of vision was moved slightly laterally and screening was done vertically downward along the slide. In this way, screening was done along vertical columns until 100 intact exfoliated cells were counted [Figure 3].

### Scoring criteria

Criteria for the inclusion of total cell count which are as follows:<sup>[15]</sup>

1. Cytoplasm intact and lying relatively flat
2. Little or no overlap with adjacent cells
3. Little or no debris/stain residue
4. Nucleus normal and intact, nuclear perimeter smooth, and distinct.

In order for a cell to be considered a micronucleated cell should satisfy the above criteria regarding inclusion in total cell count and the suggested criteria for identifying micronuclei which are as follows:

1. Rounded, smooth perimeter suggestive of membrane
2. Less than a third the diameter of the associated nucleus but large enough to discern shape and color
3. Feulgen positive (i.e., pink in bright field illumination)
4. Staining intensity similar to that of nucleus
5. Texture similar to that of nucleus
6. Same focal plane as nucleus
7. The absence of overlap or bridge to nucleus.

## RESULTS

The present study was carried out to assess the effect of tobacco on the cells of the buccal mucosa by comparing the micronucleated cell count in patients with a habit of smoking tobacco (beedi or cigarette), chewing tobacco (gutkha), smoking and chewing tobacco both, and in those not using tobacco in any form. This study sample of 120 individuals was divided into four



Figure 1: Sample collection

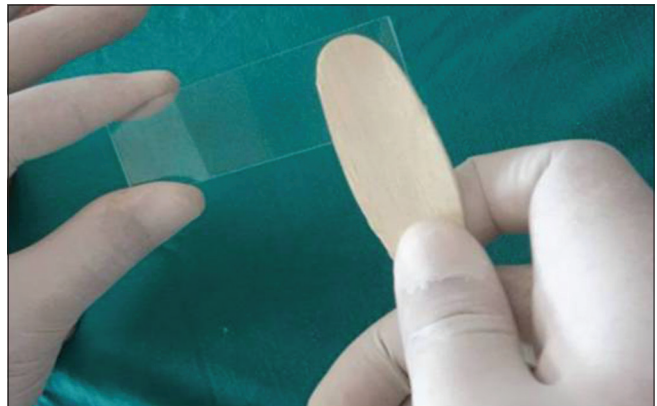


Figure 2: Smear preparation

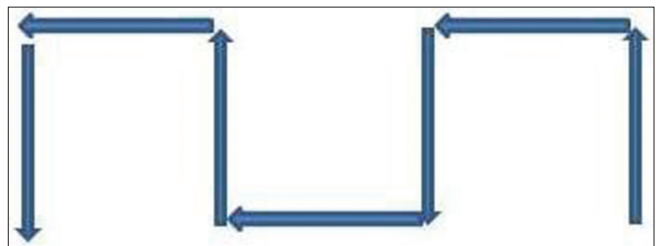


Figure 3: Scoring procedure (zigzag method)

groups with 30 patients in each group as follows: Group I (individuals with a habit of smoking tobacco), Group II (individuals with a habit of chewing tobacco), Group III (individuals with a habit of smoking and chewing tobacco), and Group IV (nontobacco-exposed individuals).

Smears were taken, stained with Rapid Pap stain and analyzed with the help of binocular light microscope and manual cell counter. A total of 300 cells were examined from each slide for the presence of micronuclei.

Data were subjected to statistical analysis using unpaired *t*-test and Karl Pearson's correlation coefficient. Comparison of mean percentage of micronucleated cells in different groups was made using unpaired *t*-test



**Table 1: Mean and standard deviation of age, risk multiplication factor and percentage micronucleated cells in the different groups**

Groups	Mean±SD		
	Age	RMF	Percentage of micro nucleated cells
Smokers	48.166±13.901	169.966±130.593	7.589±5.672
Chewers	33.566±11.542	54.033±45.094	10.413±3.865
Smokers + chewers	33.33±11.7	171.56±138.275	21.996±9.916
Control	34.9±10.456	0±0	1.033±1.265

RMF: Risk multiplication factor, SD: Standard deviation

**Table 2: Comparison of mean percentage of micronucleated cell count in different groups by unpaired t-test**

Groups	Probability of unpaired t-test	P/significance
Smokers and chewers	0.0281	<0.05 significant
Smokers and smokers + chewers	0.0000	<0.05 significant
Smokers and control	0.0000	<0.05 significant
Chewers and smokers + chewers	0.0000	<0.05 significant
Chewers and control	0.0000	<0.05 significant
Smokers + chewers and control	0.0000	<0.05 significant

**Table 3: Correlation of age, risk multiplication factor and percentage of micronucleated cells in smokers (Karl-Pearsons correlation)**

	Age	RMF	Percentage of micronucleated cells
Age	1		
RMF	0.347503766	1	
Percentage of micronucleated cells	0.258221645	0.64874403 (significant)	1

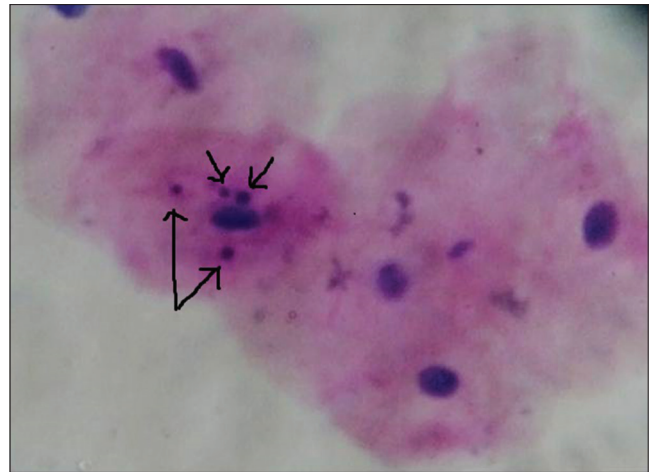
RMF: Risk multiplication factor

**Table 4: Correlation of age, risk multiplication factor and percentage of micronucleated cells in chewers (Karl-Pearsons correlation)**

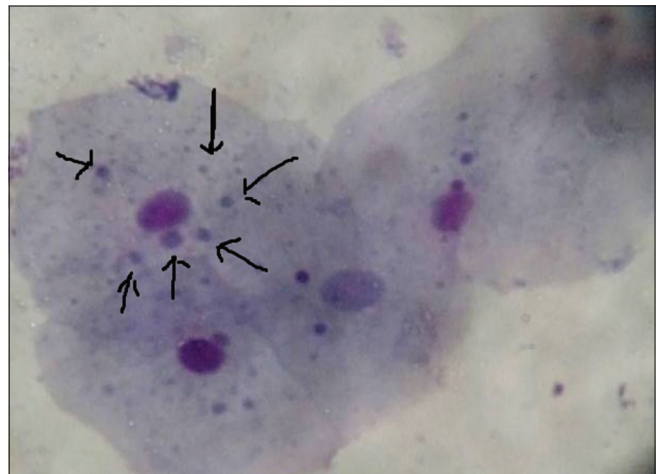
	Age	RMF	Percentage of micronucleated cells
Age	1		
RMF	-0.031237507	1	
Percentage of micronucleated cells	-0.068809698	0.831414245 (significant)	1

RMF: Risk multiplication factor

and using the statistics package (Statistical Package for Scientific Studies) for Windows SPSS 20, IBM, Armonk,



**Figure 4:** Oral exfoliated cells showing micronuclei in oral mucosa of smokers. RAPID-PAP™ Stain (×40)



**Figure 5:** Oral exfoliated cell showing micronuclei in oral mucosa of chewers. RAPID-PAP™ Stain (×40)



**Figure 6:** Oral exfoliated cell showing micronuclei in oral mucosa of individuals with smoking and chewing habit. RAPID-PAP™ Stain (×40)

NY, USA. A value of  $P = 0.05$  or less was considered for statistical significance. Karl Pearson's correlation coefficient was used to compare age, risk multiplication

**Table 5: Correlation of age, risk multiplication factor and percentage of micronucleated cells in (smokers + chewers) (Karl-Pearsons correlation)**

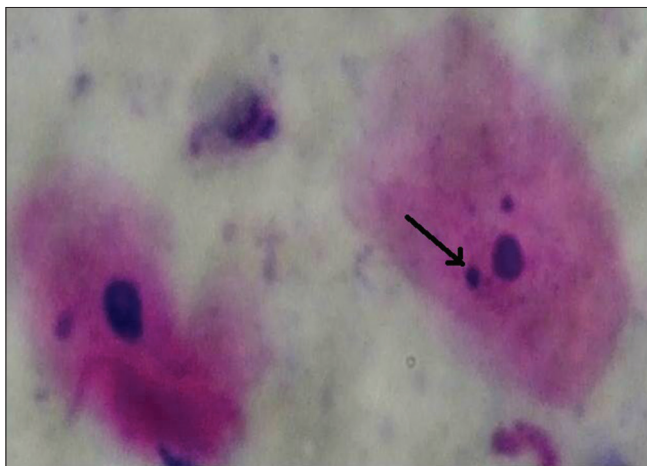
	Age	RMF	Percentage of micronucleated cells
Age	1		
RMF	0.09197654	1	
Percentage of micronucleated cells	0.000969449	0.945532725 (significant)	1

RMF: Risk multiplication factor

**Table 6: Karl-Pearson correlation coefficient between age and percentage of micronucleated cells in control group**

Group	Correlation coefficient	P/significance
Control (age and percentage of micronucleated cells)	0.1704	0.0987 >0.05 (NS)

NS: Not significant



**Figure 7:** Oral exfoliated cell showing micronuclei in non-tobacco exposed subjects. RAPID-PAP™ Stain (×40)

factor (RMF), and percentage of micronucleated cells in different groups.

All the values of age, RMF, and mean percentage of micro nucleated cells in smokers, chewers, smokers + chewers, and control groups were expressed as mean and standard deviation, respectively [Table 1]. The unpaired “*t*” test revealed a significant difference between different pairs of groups for mean percentage of micronucleated cells [Table 2]. However, Karl Pearson’s correlation coefficient revealed a positive and significant correlation between RMF and mean percentage of micronucleated cells in each experimental group, respectively [Tables 3-5]. Further, a weak positive and nonsignificant correlation were observed between age and mean percentage of micronucleated cells in smokers

and smokers + chewers, respectively, while it was weak negative and nonsignificant in chewers. In control group, correlation between age and percentage of micronucleated cells was weak positive and nonsignificant at 5% level of significance [Table 6].

## DISCUSSION

Oral cancer is considered as the sixth most common malignancy, and is a major cause of cancer morbidity and mortality worldwide. Early detection of precancerous or premalignant oral lesions would improve the survival to a great extent.<sup>[12]</sup> Oral cancer arises as a result of gradual accumulations of mutations over time, but there is a more profound event termed “chromothripsis” that represents a catastrophic change of genome structure, with widespread damage and simultaneous acquisition of multiple mutations. This latter process involves a cluster of chromosomal rearrangements.<sup>[16]</sup>

This study aimed to explore a link between the tobacco-related genotoxic effects in individuals and increased frequency of micronuclei.

Cytology has now been widely accepted as a tool in the early diagnosis of cancer. Oral exfoliative cytology can reveal various cellular alterations that include karyorrhexis, karyolysis, MN formation, pyknosis, and binucleation.<sup>[15]</sup> Micronucleated cells in buccal mucosa seem to reveal the occurrence of genotoxic, and by implication, carcinogenic agents within the oral cavity. They seem to represent an internal dosimeter in tissue exposed to genotoxic and/or carcinogenic agents.<sup>[17]</sup> MN is formed in the cell by numerous genotoxic agents that damage the chromosomes during the metaphase/anaphase transition of mitosis cell division. Micronuclei form from lagging whole chromosomes that are not incorporated into the main nucleus following anaphase.<sup>[18]</sup>

Cigarette smoking and the use of smokeless chewing tobacco have been closely related to the development of micronuclei in cells of oral mucosa, The most well-characterized chemicals found in tobacco and tobacco smoke are polycyclic aromatic hydrocarbons, such as benzopyrene, and the highly addictive alkaloid, nicotine, and its metabolites that result in DNA damage/mutation.<sup>[19]</sup>

The cytological smears were obtained from the buccal mucosa of thirty individuals from each group and were stained with RAPID PAP method [Figures 4-7].

In the present study, the mean percentage of micronucleated cells was  $7.589 \pm 5.672$  in smokers,  $10.413 \pm 3.865$  in chewers,  $21.996 \pm 9.916$  in smokers and chewers and  $1.033 \pm 1.2658$  in control group. Decreased percentage of micronucleated cells was observed in the buccal mucosa of

the control group; however, the highest mean percentage was observed in group III individuals, which was similar to the findings reported by Sudha Sellappa *et al.*<sup>[20]</sup> who observed a gradual increase in the mean percentage of micronucleated cells from control, chewers and in chewers with smoking habits. In a similar study conducted by Kohli *et al.* concluded that there was a significant increase in the micronuclei count during the transition of normal mucosa to Oral premalignant disorders.<sup>[21]</sup>

de Geus *et al.* conducted a systematic review of clinical studies to evaluate the frequency of micronuclei in the oral mucosa of smokers and non-smokers in adult patients and concluded that there was a higher frequency of micronuclei exfoliated cells in smokers compared to non-smokers.<sup>[22]</sup>

Derici Eker *et al.* conducted a study to determine genotoxic effects of hookah smoking by using MN test and chromosome aberration methods and concluded that a significant statistical difference was observed in individuals who smoked hookah and those who did not in terms of fragment, gap, MN, and binucleus parameters, suggesting that smoking a hookah may cause genotoxic effects.<sup>[23]</sup>

A significant ( $P < 0.05$ ) increase in frequency of micronucleated cells was observed in three groups (Group I, II, III) when compared to control (Group IV) which was in accordance to the findings of Kamel Ahmad Jaber Saleh<sup>[24]</sup> who also observed significant increase of micronucleated frequency in three groups (smokers but not khat chewers, khat chewers but not smokers, both smokers, and khat chewers) when compared to control (nonsmokers and non khat chewers) and concluded that the combination of chewing and smoking almost duplicate the genotoxicity effect. Kayal *et al.*<sup>[25]</sup> observed significantly higher frequencies of micronucleated cells in those with “no habit” healthy individuals compared to chewers using either areca nut alone, mava, tamol, tobacco with lime, dry snuff, or masher.

The mean percentage of micronucleated cells in our study was found to be significantly higher in chewers ( $10.4133 \pm 3.8657$ ) as compared to smokers ( $7.5897 \pm 5.6722$ ) and this was in accordance with the findings of Palaskar and Jindal<sup>[26]</sup> who observed significant increase in mean percentage of micronucleated cells in smokeless tobacco users ( $4.5 \pm 0.61$ ) as compared to smokers ( $2.20 \pm 0.5$ ).

The reason for increased micronucleated cell count in chewers when compared to smokers was because chewing tobacco remains in contact with the oral mucosa for longer period of time. According to the Centre for Disease

Control, chewing tobacco used 7–8 times a day may be equivalent to smoking 30–40 cigarettes per day. Other factors such as the use of slaked lime and continuous contact with the oral mucosa, led to more absorption of nicotine through smokeless tobacco use. In addition, in contrast to the smokers who absorbed nicotine primarily through the pulmonary vasculature, chewing tobacco users were found to absorb nicotine through the buccal mucosa and the gastrointestinal tract mucosa.<sup>[27]</sup>

There was a significant increase in the mean percentage of micronucleated cells in individuals with both smoking and chewing habit ( $21.9967 \pm 9.9164$ ) when compared to smokers ( $7.5897 \pm 5.6722$ ) and chewers ( $10.4133 \pm 3.8657$ ), respectively, which was in contrast to the study conducted by Sudha Sellappa *et al.*<sup>[25]</sup> in which nonsignificant results were observed between the mean percentage of micronucleated cells in chewers ( $1.90 \pm 1.03$ ) and in chewers with smoking habit ( $2.00 \pm 1.12$ ).

The increased mean percentage of micronucleated cells in individuals with both smoking and chewing habit when compared to individuals with smoking and chewing habit alone in our study was due to the synergistic effect of more than one habit.

In our study, an arbitrary unit was obtained using frequency/day multiplied by duration of years (RMF), a positive and significant correlation was observed between the RMF and the mean percentage of micronucleated cell count in smokers, chewers and in individuals with both smoking and chewing habit respectively. This indicates that with increase in tobacco exposure the mean percentage of micronucleated cells also increases. These findings were by the study conducted by Patel *et al.*<sup>[28]</sup> in which there was a significant increase in micronuclei count with an increase in lifetime tobacco exposure in tobacco chewers.

The significant positive correlation was observed between the duration and frequency of tobacco consumption and the frequency of micronuclei in buccal mucosa as reported in earlier studies by Sarto *et al.*<sup>[29]</sup> and Chadha and Yadav<sup>[30]</sup> that is mainly due to increased exposure to the carcinogens to buccal mucosa present in tobacco products.

In the present study, a weak positive and nonsignificant correlation was observed between the age of individuals and mean percentage of micronucleated cell count in three groups (Group I, Group III and Group IV), while in Group II, a weak negative and nonsignificant correlation was observed. These results were in accordance with the study conducted by Tolbert *et al.*,<sup>[31]</sup> Armen Nersesyan *et al.*<sup>[32]</sup> that showed a weak positive nonsignificant correlation between these two parameters, however some



earlier studies conducted by Kayal *et al.*,<sup>[25]</sup> Chadha and Yadav<sup>[30]</sup> showed a significant positive correlation between the age of individuals and mean percentage of micronucleated cell count.

### Limitations

The weak positive correlation was observed in our study in Group I, III and IV probably due to longer period of exposure in older individuals, but weak negative correlation in Group II were due to the negative correlation of RMF with mean percentage of micronucleated cell count in these individuals. The nonsignificant association between the age and the micronucleated cell count in our study was because the number of the individuals in the specific strata was unstable and could be due to narrow age range that is  $48.16 \pm 13.90$  years in smokers,  $33.56 \pm 11.54$  years in chewers,  $33.33 \pm 11.7$  years in smokers and chewers and  $34.9 \pm 10.45$  years in control.

### CONCLUSION

The following conclusions were drawn from the present study:

1. There is a significant increase in the frequency of micronucleated cell count in individuals with habit of smoking, chewing and both smoking as well as chewing tobacco when compared to the nontobacco exposed patients normal counterparts, indicating strong genetic damage secondary to genotoxic and carcinogenic agents released by tobacco
2. The significant increase in micronucleated cell count in chewers as compared to smokers indicated the greater genotoxic effect of chewing tobacco
3. The synergistic effect of smoking and chewing tobacco was greater than the smoking or chewing tobacco alone indicated by a significant increase of micronucleated cell count in former
4. A positive and significant correlation was observed for the risk of tobacco exposure and the frequency of micronucleated cell count
5. No significant correlation was observed between the age of the individuals and the frequency of micronucleated cell count.

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Nil.

### Conflicts of interest

There are no conflicts of interest.

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