ORIGINAL ARTICLE

Genotoxic Effects of Tobacco on Buccal Epithelium: Cell Nuclear Anomalies as Biomarker

SOHINI DAS BISWAS[1], BABLI GARAI[1], MOITREYEE BANERJEE[2,*]

[1] Post Graduate Department of Conservation Biology, Durgapur Government College, Durgapur, India
[2] Department of Zoology, Durgapur Government College, Durgapur, India

Abstract

Background: Tobacco use has toxic effects on different organs. This study was carried out to assess the effect of indigenous tobacco both in smoking (*bidi*) and smokeless (*gutkha, zarda and khaini*) forms on buccal cells at chromosomal level, through assessment of different nuclear anomalies as biomarker.
Methods: This study was done on people living in Durgapur and its adjacent areas, West Bengal, India during January to July 2011. The samples were collected from 50 smokers (case group), 50 smokeless tobacco consumers or chewers (case group) and 50 non-tobacco consumers (control group). Micronucleus assay was used to assess buccal cell nuclear changes. Buccal smears collected from study subjects were prepared on a grease free slide. Prepared slides were observed under light microscope and 2 to 5 fields were observed randomly for counting the different anomalies. In each field, the frequency of each anomaly was assessed in 100 cells and reported with percentage.
Results: Chewers had significantly the highest frequency of all nuclear anomalies compared to smokers and healthy controls (HCs). Smokers also had significantly more anomalies compared to HCs. Condensed chromatin (CC), karyolysis (KL) and bi-nucleation (BN) in chewers and CC, pyknosis and BN in smokers were the most frequent anomalies. KL was significantly more frequent in chewers compared to smokers (59.8 ± 6.4 vs. $24.2 \pm 12.4\%$, $P < 0.001$), however, the frequency of other nuclear anomalies were not significantly different in these two study groups. Presence of each nuclear anomaly was significantly greater in older ages in all study groups.
Conclusion: Tobacco can cause and increase the rate of nuclear anomalies in both smoking and smokeless forms compared to HCs. The genotoxic effects of tobacco on buccal cells are partly age-related. Cell nuclear anomalies in buccal tissue can be used as biomarker indicating the detrimental effects of tobacco.

Keywords: Micronucleus Tests; Mouth Mucosa; Smokeless Tobacco; Tobacco Products; Toxicogenetics

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INTRODUCTION

Tobacco has been considered as the greatest disease-producing chemical to humans. It is a common knowledge that cigarette smoking is the major cause of cancer and cardiovascular disease across the world contributing to hundreds and thousands of premature deaths each year (1). Tobacco use most commonly leads to diseases affecting the heart and lungs, with smoking being a major risk factor for cardiovascular diseases, myocardial infarction, cerebrovascular accidents, chronic obstructive pulmonary disease, emphysema, and various cancers (2). The likelihood of development of these effects depends upon the duration and the extent of tobacco use. Tobacco consumption is known to be associated with oral tissue neoplasia. Over 80% of oral cancer patients are tobacco users (3). Furthermore, the tar content in tobacco-filled cigarettes has a concerted role to increase the risk for these diseases. Cigarettes sold in developing nations usually contain higher tar content and are less likely to be filtered, which can potentially result in increase in tobacco-related diseases in these regions (4). According to the World Health Organization (WHO) appraisal, tobacco causes 5.4 million deaths a year worldwide (5,6). WHO also estimated that during the 20th century, 100 million deaths occurred due to tobacco that is likely to increase to 1 billion during the 21st century if the current trends continue (6). That is why the United States Centers for Disease Control and Prevention describes tobacco use as "the single most important preventable risk to human health and an important cause of premature death worldwide" (7). In the recent years, some developing countries have initiated preventive programs to reduce tobacco use and tobacco-related disease burden among adolescents and young adults (8,9).

In several developing countries, tribal people and people from lower income levels use different forms of tobacco which are much less refined than commercial cigarettes. In the present study, four such forms were considered including

*Correspondence to: Moitreyee Banerjee; MSc. Durgapur Government College, Jawaharlal Nehru Avenue, Durgapur, District- Burdwan 713214, India.
Tel: +91 94 3463 3285, E-mail: miotreyeebanerjee@gmail.com
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bidi, gutkha, zarda and *khaini*. *Bidi* is a thin, Indian cigarette filled with tobacco flake and wrapped in a leaf tied with a string at one end (10). *Gutkha* or *Gutka* is a preparation of crushed areca nut (also called betel nut), tobacco, catechu, paraffin, slaked lime and sweet or savory flavorings. *Gutkha* is consumed by placing a pinch of it between the gum and cheek and gently sucking and chewing. It is considered responsible for oral cancer and other severe negative health effects (11). *Zarda* or *jarda* tobacco is the flavoured tobacco, primarily used in betel leaves. It is prepared by blending tobacco leaves, perfumes, sweeteners, and other compounds unique to the manufacturers (12). *Khaini* ingredients are tobacco leaves, slaked lime paste and water. Scented *khaini* may be augmented by menthol, spices and betel nut (12).

Over 60 chemical carcinogens have been identified in cigarette smoke (13). Carcinogenic pyrolytic compounds in cigarette smoke bind to DNA and cause genetic mutations (14). Oropharyngeal, pulmonary, renal, urothelial, hepatic, esophagus, gastric and pancreatic cancers has been strongly linked to tobacco use while there is also convincing evidence of the effect of tobacco on other types of cancers (15-17). Oral cancer affects as many as 274,000 people worldwide annually, and the frequency of oral cancer around the world is often indicative of the patterns of tobacco products use (15-17). It has been established that there is a dose-response relationship between the amount of tobacco product used and the development of oral cancer (15,16,18,19). All parts of the oral cavity are susceptible to carcinogenic effects of tobacco use, either by smoking or chewing, including the lips, tongue, palate, gum, and cheek (18,19). Mouth is the only body site that allows easy observation of the ravages of smoking and smokeless (chewable) forms of tobacco with naked eyes. Today, there exists a need to identify biomarkers which can act as predictors of oral cancers and their association with tobacco (20). The most important biomarkers used as an indicator of structural and numerical chromosomal aberrations are micronuclei and other nuclear anomalies which are also indicators of disease progression (21). Micronuclei assay is considered to be an effective and innovative technique to assess the cytological effects of carcinogenic mixtures (22). Buccal cell changes are associated with tobacco use. When compared with other body sites, the mouth offers a unique opportunity for defining biomarkers because the mouth permits noninvasive, repetitive examinations in longitudinal studies of tobacco-associated acute and chronic diseases (23).

This study was carried out to assess the effect of indigenous tobacco both in smoking (*bidi)* and smokeless (*gutkha, zarda and khaini)* forms on buccal cells at chromosomal level, through assessment of different nuclear anomalies as biomarker.

METHODS

Subjects

This study was done on people living in Durgapur and its adjacent areas, West Bengal, India during January to July 2011. The samples were collected from 50 smokers (case group), 50 smokeless tobacco consumers or chewers (case group) and 50 non-tobacco consumers (control group). All study subjects (cases and controls) were men. Chewers used indigenous tobacco forms called *gutkha, zarda or khaini* in India. Smokers, rather than commercial cigarettes, used *bidi* that is an indigenous form of tobacco filled cigarettes. The control group consisted of age-matched healthy individuals without history of tobacco use in any forms. Age of the study subjects was categorized to 20-29, 30-39, 40-49, 50-59 and over 60 years. Informed consent was taken from all volunteers and all parameters according to Declaration of Helsinki were considered during the sampling.

Pathologic assessment

In this study, micronucleus assay was used to assess buccal cell nuclear changes (22). Prior to collection of buccal smear samples, mouth of the volunteers was washed carefully. Buccal smear was collected with a pre-moistened spatula and a smooth smear was prepared on a grease free slide. The slides were air dried and fixed with absolute methanol within 24 hours of collection. Each slide was coded according to the study groups and related age group. Finally the slides were stained with Giemsa stain. Giemsa staining technique has been shown to give the best result for micronuclei and other biomarkers (24). Prepared slides were observed under light microscope (10×40, Olympus Medical Systems India Pvt. Ltd., Kolkata, India). Two to five fields were observed randomly for counting the different anomalies. Anomalies studied were as follow:

a) Bi-nucleation (BN): Presence of 2 nuclei (of equal size and stain) within a cell.

b) Condensed chromatin (CC): Enlarged nuclei with chromatin materials appeared to be aggregated or vesicular.

c) Pyknosis (PK): Deep stained shrunken nuclei.

d) Broken egg (BE): Nuclei are appeared clinched.

e) Karyohexis (KH): Nuclear disintegration involving loss of integrity of the nuclei.

f) Karyolysis (KL): Nuclear dissolution with a ghost like appearance.

g) Micronucleus (MN): Smaller in size than the parent nucleus with almost same staining intensity.

Statistical Analysis

In each field, the frequency of each anomaly was assessed in 100 cells and reported with percentage. The findings are presented with mean and standard deviation (SD). The mean difference between two groups were analysed by Students t-test. To analyse the mean difference of nuclear anomalies among different age groups, one-way analysis of variance (ANOVA) was performed. P values of less than 0.05 were considered statistically significant. All statistical analysis was carried out using SPSS version 20 (IBM Corp., Armonk, USA).

RESULTS

The samples were mostly collected from bus drivers and conductors, local road side vendors and factory labors. Mean percentage of the seven nuclear anomalies in each study group is shown in table 1. As can be seen, chewers had significantly the highest frequency of all nuclear anomalies compared to smokers and healthy controls (HCs).

Smokers also had significantly more anomalies compared to HCs. The most common nuclear anomaly in all three study groups was CC, while MN had the lowest frequency in smokers and chewers and KH had the lowest frequency in HCs. The percentages of the majority of nuclear anomalies in smokers and chewers were significantly higher than HCs, except for the CC. With the exception of MN and PK, the percentage of other nuclear anomalies was higher in chewers compared to smokers. However, except for the KL that was significantly more frequent in chewers compared to smokers (59.8 ± 6.4 vs. 24.2 ± 12.4, P < 0.001), the frequency of other nuclear anomalies were not significantly different in these two study groups.

The frequency of each nuclear anomaly according to age-groups is shown in table 2. Using ANOVA test, it was revealed that percentages of nuclear anomalies were significantly different among the age groups in all 3 study groups (P < 0.001). In general, presence of each nuclear anomaly was greater in older ages as the duration of exposure to tobacco increased in both smokers and chewers. The same pattern existed for HCs. The frequency of nuclear anomalies in total showed an upward linear trend according to age groups (Figure 1).

DISCUSSION

Tobacco use (either by smoking or chewing) has harmful effects on buccal tissue (25). The major toxic components of tobacco are nicotine, tar and polycyclic hydrocarbons. Reactive oxygen species has also been suspected to be a major cause of tobacco toxicity (26). Assessment of tobacco toxicity with various forms of cigarettes showed that tar and nicotine content has genotoxic effects on buccal cells manifesting with different nuclear anomalies which can be used as biomarkers (27).

In this study, we showed that tobacco in both smoking and smokeless forms has significant effects on the buccal cells causing several nuclear anomalies. We also found that chewers of tobacco were more affected than smokers. Besides, the nuclear anomalies seems to be related to age as we observed more anomalies in older ages compared to younger ages in each study group. Nevertheless, we showed that tobacco use in each age group could significantly increase the frequency of anomalies in smokers compared to HCs and in chewers compared to smokers and HCs (Table 2, Figure 1). Similar to our findings, Kuasar et al and Sharma et al showed that cytogenetic damages in exfoliated buccal cells of tobacco chewers were higher than smokers and HCs (28,29). This finding can be explained by the fact that smokeless forms of tobacco contain other ingredients than nicotine that are highly toxic for various organs (30). Furthermore, more direct contacts of tobacco preparations to buccal cells in chewing compared to smoking might be another reason for the difference in chewers and smokers (31).

The present study was carried out on a particular group of the society which mainly included low income people. These individuals were either illiterate or less literate and consumed raw and indigenous forms of tobacco (*gutkha*, *khaini*, *zarda* and *bidi*), as they cannot afford the refined forms. Although, it can be assumed that refined forms of tobacco preparations and cigarettes may have less toxic impacts, this is beyond the design of the present study to be evaluated, as we only included subjects using indigenous non-refined forms. In this study, seven most common biomarkers of chromosomal change associated with pre-neoplastic stage of oral cancer as well as non-neoplastic oral diseases due to tobacco use were evaluated (31,32). These nuclear anomalies can be used as biomarker for studying the effects of tobacco on exfoliated buccal epithelial cells. In the current study, we found that CC, KL and BN in chewers and CC, PK and BN in smokers were the most frequent anomalies. Kauser et al similarly showed higher frequency of PK and KL in chewers and PK and BN in smokers (28). Likewise, in the study by Sharma et al which the same seven anomalies as ours were studied, KL, PK and MN in chewers and smokers were the most frequent anomalies (29). However, being MN a frequent anomaly in the study

Table 1. Compassion of the frequency of nuclear anomalies in buccal cells of tobacco users (smokers and chewers) and healthy controls

	Study groups			P value		
	Smoker (n = 50)	Chewer (n = 50)	Control (n = 50)	Smokers vs. controls	Chewers vs. controls	Smokers vs. chewers
Age (year); mean ± SD	45.1 ± 15.8	46.5 ± 16.2	44.0 ± 14.9	0.890	0.561	0.788
Nuclear anomalies						
Bi-nucleation (%); mean ± SD	29.2 ± 8.5	46.8 ± 16.8	8.2 ± 4.7	0.002	0.002	0.098
Condensed chromatin (%); mean ± SD	53.8 ± 19.1	73.8 ± 15.2	48.0 ± 24.4	0.710	0.117	0.139
Pyknosis (%); mean ± SD	38.0 ± 13.1	36.6 ± 11.5	7.6 ± 7.3	0.004	0.003	0.876
Broken egg (%); mean ± SD	14.2 ± 9.8	19.2 ± 7.5	3.2 ± 4.4	0.070	0.006	0.440
Karyohexis (%); mean ± SD	13.8 ± 7.5	23.6 ± 13.39	0.8 ± 1.1	0.009	0.009	0.278
Karyolysis (%); mean ± SD	24.2 ± 12.4	59.8 ± 6.4	4.6 ± 4.3	0.018	< 0.001	< 0.001
Micronucleus (%); mean ± SD	11.0 ± 6.6	5.0 ± 3.9	1.4 ± 1.1	0.021	0.126	0.156
Percentage of nuclear anomalies in total (%); mean ± SD	26.3 ± 14.4	37.8 ± 22.3	10.5 ± 8.5	0.025	0.001	0.115

Table 2. Frequency of nuclear anomalies in buccal tissue of study subjects according to age group

Study group	Anomalies	Age groups (year)				
		20-29	30-39	40-49	50-59	>60
Smoker	Mean duration of tobacco use (year)	5	10	15	20	>20
	Bi-nucleation (%); mean ± SD	24.9 ± 2.9	35.0 ± 2.0	20.0 ± 3.0	23.0 ± 4.3	42.9 ± 8.3
	Condensed chromatin (%); mean ± SD	43.0 ± 4.6	56.0 ± 5.9	33.0 ± 5.9	48.0 ± 7.6	89.0 ± 6.4
	Pyknosis (%); mean ± SD	15.0 ± 6.4	52.0 ± 9.3	35.0 ± 2.1	39.0 ± 8.3	49.0 ±15.2
	Broken egg (%); mean ± SD	6.0 ± 3.6	32.0 ± 13.5	10.0 ± 4.5	6.0 ± 4.6	17.0 ± 4.4
	Karyohexis (%); mean ± SD	10 ± 5.9	28.0 ± 6.9	8.0 ± 5.3	8.0 ± 2.9	15.0 ± 1.9
	Karyolysis (%); mean ± SD	4.0 ± 4.2	32.0 ± 9.9	21.0 ± 2.0	23.0 ± 7.8	41.0 ± 6.6
	Micronucleus (%); mean ± SD	8.0 ± 6.3	17.0 ± 3.9	0.0 ± 0.0	12.0 ± 2.9	18.0 ± 4.3
	Percentage of nuclear anomalies in total (%); mean ± SD	15.8 ± 12.8	36.0 ± 12.6	18.1 ± 12.0	22.7 ± 14.7	38.8 ± 24.3
Chewer	Mean duration of tobacco use (year)	5	10	15	20	>20
	Bi-nucleation (%); mean ± SD	14.0 ± 2.4	50.0 ± 2.8	54.0 ± 4.9	55.0 ± 3.4	61.0 ± 6.4
	Condensed chromatin (%); mean ± SD	48.0 ± 7.8	67.0 ± 1.1	80.0 ± 5.8	82.0 ± 2.7	92.0 ± 3.8
	Pyknosis (%); mean ± SD	38.0 ± 7.1	35.0 ± 7.8	34.0 ± 9.8	20.0 ± 4.4	56.0 ± 2.4
	Broken egg (%); mean ± SD	8.0 ± 3.4	13.0 ± 3.7	22.0 ±3.0	25.0 ± 2.4	28.0 ± 6.4
	Karyohexis (%); mean ± SD	12.0 ± 4.5	15.0 ± 6.1	28.0 ± 8.1	15.0 ± 5.1	48.0 ± 3.5
	Karyolysis (%); mean ± SD	52.0 ± 5.7	54.0 ± 4.3	61.0 ± 8.6	62.0 ± 9.4	70.0 ± 3.9
	Micronucleus (%); mean ± SD	0.0 ± 0.0	3.0 ± 2.0	8.0 ± 4.3	3.0 ± 2.5	11.0 ± 3.1
	Percentage of nuclear anomalies in total (%); mean ± SD	25.6 ± 19.4	33.8 ± 22.4	41.0 ± 23.1	37.4 ± 26.8	52.3 ± 24.8
Controls	Bi-nucleation (%); mean ± SD	3.0 ±1.5	4.0 ± 2.3	8.0 ± 4.6	10.0 ± 2.2	16.0 ± 5.1
	Condensed chromatin (%); mean ± SD	23.0 ± 2.5	28.0 ± 7.8	45.0 ± 16.5	52.0 ± 14.4	92.0 ± 3.3
	Pyknosis (%); mean ± SD	8.0 ± 4.9	0.0 ± 0.0	2.0 ± 1.7	6.0 ± 1.9	22.0 ± 8.6
	Broken egg (%); mean ± SD	2.0 ± 1.3	2.0 ±0.9	0.0 ± 0.0	0.0 ± 0.0	12.0 ± 1.5
	Karyohexis (%); mean ± SD	0.0 ± 0.0	0.0 ± 0.0	1.0 ± 0.3	0.0 ± 0.0	3.0 ± 2.7
	Karyolysis (%); mean ± SD	0.0 ± 0.0	0.0 ± 0.0	3.0 ± 1.9	8.0 ± 3.9	12.0 ± 3.2
	Micronucleus (%); mean ± SD	1.0 ± 0.8	0.0 ± 0.0	0.0 ± 0.0	2.0 ± 1.8	4.0 ± 2.5
	Percentage of nuclear anomalies in total (%); mean ± SD	5.3 ± 3.6	4.8 ± 4.5	8.4 ± 5.1	11.1 ± 7.1	23.0 ± 18.2

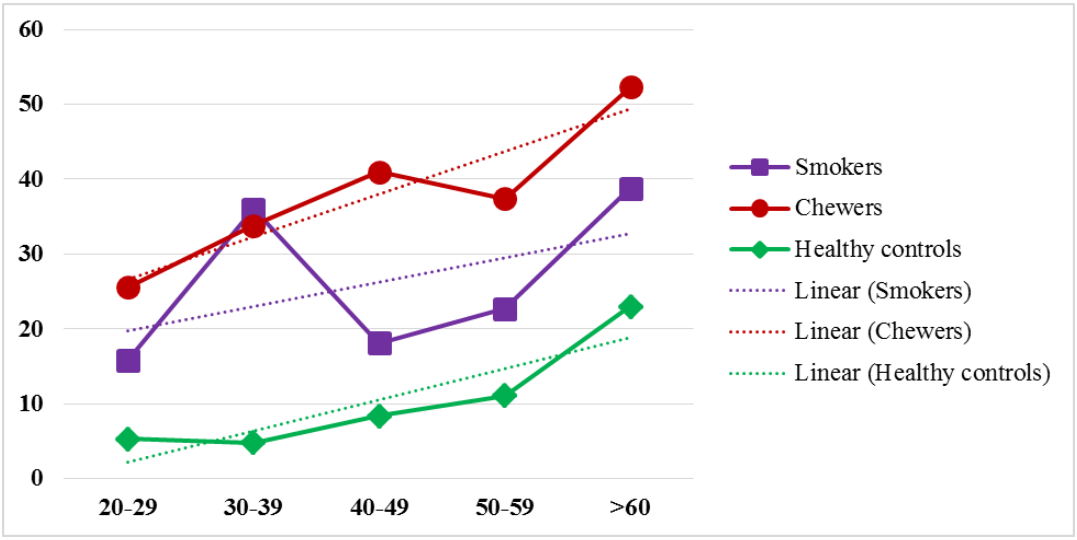

Figure 1. Trends in mean percentage of nuclear anomalies (in total) according to age

by Sharma et al is contrary to our findings that showed MN had the lowest frequency in smokers and chewers. CC in the present study was also a frequent anomaly in HCs. Hence, it can be inferred that this anomaly was associated with normal degradation of the cells and tobacco in both forms was capable of accelerating this process.

In this study, we found that, in all three study groups, the frequency of nuclear anomalies increased with age. Saranya and Sudha similarly showed an age-dependent progressive increase in the cytomorphologic damages of buccal cells in tobacco chewers (33). This can be explained by the fact that in tobacco users the duration of exposure to this toxic agent increases as they become older. On the other hand, ageing itself can make buccal tissues more vulnerable to toxic exposures and mutagenic lesions, which is due to decreased thickness, integrity and regenerative capacity of buccal epithelium in older ages (34). In this regard, Thomas et al showed a clear association between ageing and increased number of nuclear anomalies (the biomarkers for DNA damage) in buccal tissue (35).

CONCLUSION

Tobacco can cause and increase the rate of nuclear anomalies in both smoking and smokeless forms compared to HCs. The genotoxic effects of tobacco on buccal cells are partly age-related. Cell nuclear anomalies in buccal tissue can be used as biomarker indicating the toxic and detrimental effects of tobacco. Conducting a comparative study on the effects of tobacco between users of processed refined tobacco products and indigenous forms is recommended.

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