Evaluation of Cytogenic Damage in the Form of Micronuclei in Oral Exfoliated Buccal Cells in Tobacco Users

Abstract

Background: Variety of substances such as tobacco, UV radiation, infrared rays, X-radiations, and chemicals on oral induction results in chromosomal aberrations and production of micronucleus (MN). Among them, tobacco-specific nitrosamines are potent mutagenic agents causing oral cancer. Objective: The objective of the study is to compare the genotoxicity in buccal mucosal cells, i.e. the MN count of all groups and to find the incidence of micronucleated cells (MNCs) in accordance to duration and frequency of tobacco usage and timing of contact of tobacco in the oral mucosa. Materials and Methods: Individuals without any oral diseases were divided into 3 groups having 25 in each group: smoking, chewing, and control. Smears were made from buccal exfoliated cells and stained with DNA-specific Feulgen stain. Frequency on MNC per 500 cells was assessed with one-way ANOVA and Tukey HSD multiple comparisons test and mean rank with Kruskal–Wallis test. Results: The mean micronucleus MN revealed that chewers had 8.00, smokers had 7.20 and controls had 0.4. The ANOVA test for mean frequency of micronucleated cell MNC revealed High significance (<0.001) for between groups comparison. The mean rank by Kruskal Wallis test revealed the MNC increases as the duration and frequency of habit increases. An increase in MNC in accordance to time of contact with buccal mucosa increases as the duration and time increases. Conclusion: Estimation of MN serve as an indicator of genetic damage and points that tobacco in chewing form induce genotoxic effect. This is studied in an easily accessible tissue- buccal mucosa in a non invasive manner.

Keywords: Buccal exfoliated cells, Feulgen stain, genotoxicity, micronucleus, tobacco

Introduction

Oral cavity is prone for a myriad of changes with advancing age as well as a result of the environmental- and lifestyle-related factors. Oral mucosal lesions can occur as a result of infections, local trauma or irritation, systemic diseases and excessive consumption of tobacco, betel quid and alcohol. Chewing and smoking of tobacco along with consumption of alcohol beverages have become common social habits in India.[1,2] Modern man lives in a hazardous environment and so continuously exposed to a large variety of natural and synthetic pollutants. These toxic pollutants may either cause mutation of germ cells resulting in accumulation of heritable abnormal genes or may lead to mutation of somatic cells leading to the formation of tumors.[3,4] United States Centre 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.”[5] Tobacco smoke is a complex, dynamic, and reactive mixture consisting of a gaseous phase and particulate phase and contains life-threatening chemicals such as benzopyrene and nitrosamines. These chemicals cause extensive damage to deoxyribonucleic acid (DNA), contributing to malignant transformation. About 250 million adults consume smokeless tobacco in the 11 countries of the WHO South East Asia region, which constitutes 90% of global smokeless tobacco users. Smokeless tobacco includes tobacco chewing, betel quid chewing, and snuffs. Smokeless tobacco has around 25 powerful carcinogens, which can cause extensive damage to DNA. Some of the potent carcinogens in smokeless tobacco include tobacco-specific nitrosamines, formaldehyde, and benzo(a)pyrene which possess cytotoxic, mutagenic, and genotoxic properties.[6-9] Genomic damage is produced by genotoxins, various medical procedures that include radiation and chemicals, lifestyle factors, and genetic factors such as inherited defects in DNA metabolism or repair.[9]
Cytogenetic damage can be studied by various methods such as chromosomal aberrations (CAs), sister chromatid exchanges (SCEs), and micronuclei test. The buccal cell micronucleus (MN) is defined as the microscopically visible, round, or oval cytoplasmic chromatin mass next to nucleus. MN 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 or whole chromosomes that lag behind at anaphase during nuclear division. Tobacco-specific nitrosamines are potent clastogenic and mutagenic agents responsible for the induction of chromatid/CAs resulting in the production of MN.[10,11]

The use of buccal exfoliated cells in the detection of genotoxic effects of tobacco and related products can be used as a mass screening procedure for the early detection of dysplastic changes. It can also be used to educate and motivate people regarding the potential risk of genotoxicity in tobacco users.[10] The quantitative estimation of MN may serve as a biomarker of genetic damage that has taken place. Genotoxic events can be conveniently studied in the buccal mucosa, which is an easily accessible tissue for sampling cells in a noninvasive manner.[12,13]

**Materials and Method**

This is a prospective, randomized, cross-sectional study conducted in the Department of Oral Medicine and Radiology. In the present study, buccal smears of 75 individuals with different tobacco habits were collected and the smears were stained using Feulgen stain (DNA-specific staining method) and then observed under microscope.

The study population is divided into 3 groups, Group I – having a habit of smoking, Group II – chewing tobacco habits, and Group III – without any habit. The study population included males, with an age range 25–55 years.

**Clinical inclusion criteria**

1. Patient without any clinical lesion in oral cavity.
2. Patient with habit of chewing tobacco or smoking
3. Good oral hygiene
4. Patients willing to participate in the study.

**Clinical exclusion criteria**

1. Under radiation therapy or recent exposure to radiographs
2. Major systemic illness such as diabetes mellitus, rheumatoid arthritis, and patients under steroid therapy
3. Recent viral infection.

**Inclusion criteria for total cell count**

Smear collection with no debris.

Tolbert et al. criteria for identifying MN:

- No overlap with adjacent cells
- Round smooth perimeter suggestive of a membrane
- Cytoplasm intact and lying relatively flat
- Nucleus normal and intact with nuclear perimeter smooth and distinct
- Staining intensity of micronuclei similar to nucleus
- Micronuclei in same focal plane as nucleus.

**Method of collection of data**

All patients were explained in detail about this study, and an informed consent was obtained in their native languages to prevent language bias and later was subjected to collection of buccal smears. Patients were asked to rinse their mouth gently with water. Mucosal cells were scraped from buccal mucosa using a sterile dry polypropylene cotton swab. The cells are immediately smeared on precleaned microscopic slides. Just before drying, the smears are fixed with 80% ethanol. Then slides will be coded to ensure observer blindness.

**Feulgen staining procedure**

1. Slides were rinsed briefly with cold 1N hydrochloric acid
2. Then placed into prewarmed hydrochloric acid for the appropriate time at 60°C
3. Slides were rinsed briefly with cold 1N hydrochloric acid
4. Rinsed briefly with distilled water
5. Then placed into Schiff’s reagent for 30–60 min at room temperature
6. Washed well with water
7. Counterstain light green added and kept for 1 min
8. Dehydrated with ethanol and mounted.

The smears were observed under ×100 magnification, an eyepiece grid was used, and 500 cells per slide are counted for micronuclei [Figures 1 and 2] MN identification and scoring is performed.

**Results and Statistical analysis**

The exfoliated epithelial cells were collected from 75 patients in different groups, sex-matched individuals (males). Their ages ranged from 25 to 55 years (mean: 36.06). The collected data of micronucleated cells and MN were analyzed with SPSS 17 version. In all the statistical tools, the $P = 0.005$ was considered as statistically significant.

All the three study groups showed positive expression of micronucleated cells. The mean value of micronucleated cells (MNCs) revealed that chewers had increased count than smokers and controls [Table 1]. The mean MN revealed that chewers had increased count than smokers and controls. The ANOVA test [Table 2] for mean square and frequency of MNC revealed significance value for between-groups comparison and duration of the habit in both smokers and chewers. The multiple comparison by
Tukey HSD post hoc tests [Table 3] revealed significance value for between-groups comparison. The mean rank by Kruskal–Wallis test [Table 4] revealed increased mean value of MNC count in smokers compared to chewers and controls and the mean values of MNC in accordance with duration and frequency of habit in smokers revealed increase in mean value as duration and frequency increases. An increase in MNC in accordance with duration of habit and time of contact with buccal mucosa in chewers increases as the duration and time increases. The Chi-square test [Table 5] for MNC values were 0.001.

Graph 1 showing Comparison of total number of micronucleated cells in three study groups, Graph 2 showing Total number of micronucleus – comparison between three groups, Graph 3 showing Total number of micronucleated cells with duration of habit; Graph 4 showing Total number of micronucleated cells with frequency per day; Graph 5 showing Total number of micronucleated cells with time of contact with buccal mucosa in chewers.

Hence, from the above-obtained results, it is evident that estimation of MN may serve as an indicator of genetic damage that has taken place and also points to the fact that tobacco in any form can induce genotoxic effect which is marked by the presence of micronuclei. These events can be conveniently studied in the buccal mucosa of tobacco-related habit individuals even before the dysplastic changes occurs, which is an easily accessible tissue for sampling cells in a minimally invasive manner. The cell counts also helped us to counsel and educate the patient to quit their habit, thereby insisting the need of micronuclear assay as a potential biomarker to determine the disease progression.

Discussion

DNA damage caused due to the use of tobacco and related products assessed by MN test is found to be most sensitive when compared with other tests as it neither requires tedious procedures such as cell culture and metaphase preparation nor it requires any specific DNA stains. MN is a microscopically visible round or oval cytoplasmic chromatin mass in the extranuclear vicinity. They originate from mitosis and consist of eccentric chromosomes, chromatid fragments, or whole chromosomes, which failed to reach spindle poles during mitosis. As the focus is to detect early genotoxic damage, the MN test provides a simple, noninvasive, yet reliable screening technique for assessing early genotoxic damage much before any clinical or histological signs of cancer is evident. Thus, in our study, we have included all individuals with habit of chewing or smoking tobacco, and clinically no changes of potentially malignant lesions/disorders are evident in the oral cavity.

According to Proia et al. (2006),[17] buccal cells are useful not only for characterizing the molecular mechanism underlying tobacco-associated oral cancers but also express diverse changes that offer promise as candidate biomarker for the early detection of oral cancer. This roused our interest to collect smears from buccal cells as it is easily accessible and noninvasive and patients readily allowed us. In the present study, all the samples were men who were in the age group of 25–55 years with mean age of 36.05yrs. The age and sex of the individuals are being reported as the major contributors to differences in micronuclei frequency, and so in the present study, all the samples were men. The present study detected that there is no relation between age and the increase of micronuclei mean count. This conforms to the findings of other studies by Sellapa et al.[5] and Farha et al.,[18] that the age of the individuals did not affect the number of micronuclei of both exposed and control individuals.

Of the types of tobacco habits, chewing came out to be more dangerous with respect to genotoxicity, as the MN was maximum in that group with mean value of (8.00 ± 7.906) when compared to smokers (7.20 ± 7.083) and controls (0.4 ± 1.2). This could be because of the increased production of superoxide ions led to increased lipid peroxidation, DNA fragmentation, and triggered apoptotic cell death leading to increase in nuclear cell division causing increase number of MN.[6,19] This observation was similar to Palaskar et al., Patel et al. (2009),[20] and Bansal et al. (2012) who analyzed...
tobacco-related genotoxic effects in chewers monitoring MN and chromosome aberrations and mean MN was increased in chewers compared with smokers and control as proven by our study.

Smoking also has equal genotoxic effects as frequency of occurring MNC and MN almost equal to chewers, and this could be because tobacco smoke contains more than 4,000 substances of which 200 are toxic to human being and more than 50 substances have carcinogenic action that includes polycyclic hydrocarbons and specific nitrosamines. Few enzymes metabolize hydrocarbons of tobacco and convert them into powerful carcinogens such as aryl hydrocarbon hydroxylase which increases carcinogenic potential of benzopyrene present in tobacco smoke along with combustion products released during smoking. Haveric et al. (2010) assessed the genotoxicity of cigarette consumption in young smokers and correlated the results of cytogenetic analysis in peripheral blood lymphocytes and exfoliated buccal cells. Significantly higher frequencies of MN were revealed in buccal cells of smokers. According to Kamath et al. (2014), there is significant increase in total number of micronuclei with increase in duration and frequency of smoking, as the duration and frequency of tobacco-related habits increased, the additive effects of the carcinogens led to increases genotoxicity in the individuals which was manifested as increase in MN count. There were no literature reports that showed any similar study conducted to correlate the effect of frequency and duration of habits, i.e., in both chewing and smoking, as in our study, there is near positive correlation of increase in MN in smokers accordance with duration and frequency, but no correlation of frequency of habit intake in chewers. This may be because of no patients in the chewers group with frequency of intake of habit more than 7 times a day.

All the studies Noushin et al., Anagha Motgi et al., Amrin Sheik et al., Bansal et al., Venkatesh et al. and Gupta et al. have shown that smoking and chewing increases the mean number of micronuclei in buccal mucosa cells. In our study, the role of time in increasing the mean number of the cell’s MN in both chewers and smokers has

| Variable | n  | Mean±SD | SE  |
|----------|----|---------|-----|
| Age      | 75 | 36.05±9.339 | 1.078 |
| Number of MNC |     |         |     |
| Smokers  | 25 | 1.44±1.417 | 0.283 |
| Chewers  | 25 | 1.64±1.551 | 0.310 |
| Control  | 25 | 0.12±0.332 | 0.066 |
| Number of MN |     |         |     |
| Smokers  | 25 | 7.20±7.083 | 1.487 |
| Chewers  | 25 | 8.00±7.906 | 1.581 |
| Control  | 25 | 0.4±1.2    | 0.078 |
| MNC in smokers-duration of habit |     |         |     |
| ≤5 years | 8  | 0.50±0.756 | 0.267 |
| 6-10 years | 9 | 2.00±1.414 | 0.500 |
| >10 years | 9  | 2.33±1.732 | 0.377 |
| MNC in smokers-frequency/day |     |         |     |
| 1-3 times | 7 | 1.85±1.952 | 0.738 |
| 4-6 times | 9 | 1.11±1.616 | 0.539 |
| 7-10 times | 8 | 2.00±1.195 | 0.423 |
| MNC in chewers-duration of habit |     |         |     |
| ≤5 years | 11 | 0.55±0.820 | 0.247 |
| 6-10 years | 9 | 1.78±0.394 | 0.465 |
| >10 years | 5  | 2.80±0.304 | 0.583 |
| MNC in chewers-frequency/day |     |         |     |
| 1-3 times | 5 | 1.00±1.000 | 0.577 |
| 4-6 times | 20 | 1.65±1.461 | 0.327 |
| 7-10 times | 0 | 0.00±0.000 | 0.000 |
| Time of contact with buccal mucosa for chewers |     |         |     |
| ≤5 min | 5  | 1.60±1.517 | 0.678 |
| 5-10 min | 13 | 1.62±1.387 | 0.385 |
| >10 min | 7  | 1.00±1.528 | 0.577 |

MNC=Micronucleated cells, MN=Micronucleus, SD=Standard deviation
Table 2: ANOVA-for mean square, frequency and significance of micronucleated cells

| Variables                                  | Sum of squares | df | Mean square | F   | Significant |
|--------------------------------------------|----------------|----|-------------|-----|-------------|
| Age (between groups)                       | 419.227        | 2  | 209.613     | 2.501| 0.08        |
| MNC (between groups)                       | 34.107         | 2  | 17.053      | 11.310| 0.000       |
| Smoking duration (between groups)          | 15.760         | 2  | 7.880       | 4.128| 0.003       |
| Smoking frequency (between groups)         | 4.014          | 3  | 1.338       | 0.523| 0.671       |
| Chewing duration (between groups)          | 19.077         | 2  | 9.539       | 7.216| 0.004       |
| Chewing frequency (between groups)         | 5.610          | 2  | 2.805       | 1.450| 0.256       |
| Time of contact with buccal mucosa (between groups) | 1.833 | 2  | 0.942       | 0.448| 0.645       |

MN= Micronucleated cells

Table 3: Multiple comparisons-Tukey honest significant difference for micronucleated cells

| Group (I) | Group (J) | Mean difference (I-J) | SE   | Significant |
|-----------|-----------|-----------------------|------|-------------|
| Smokers   | Chewers   | 0.200                 | 0.347| 0.833       |
| Control   | Smokers   | 1.520                 | 0.347| 0.000       |
| Chewers   | Smokers   | −0.200                | 0.347| 0.833       |
| Control   | Smokers   | −1.520                | 0.347| 0.000       |
| Control   | Chewers   | −1.320                | 0.347| 0.001       |

SE= Standard deviation

been evaluated for the first time. The mean number of MN of buccal mucosal cells in smokers and chewers with habit of >10 years showed higher in comparison with habit of less than ≤5 years, 6 to 10 years. The percentage of the cells with MN in smokers and chewers in accordance with frequency and duration of the habit who smoked and chewed was statistically significant.

MN frequency is increased in the oral mucosa of individuals with known carcinogenic exposure, such as the site of tobacco quid placement as compared with grossly normal-appearing mucosa. The time of contact of the tobacco with the buccal mucosa is considered as a major factor because MN increases as the time duration increases as proven by our study.

Evans et al. (1959) made first attempt to use micronuclei as a monitor of cytogenetic damage induced by gamma rays and neutrons in plant material. Chakraborty et al. (2006) conducted a study on 45 arsenic-exposed individuals from West Bengal revealed 3.34 fold increases in MN in buccal mucosal cells. This method is increasingly used in epidemiological studies for investigating the impact of nutrition, lifestyle factors, to genotoxic substances, medical procedures (radiation and chemicals), micronutrient deficiency (folic acid), lifestyle (alcohol, smoking, drugs, and stress), urban pollution, chronic contact with arsenic and chromium and genetic factors, such as defects in metabolism and/or in the repair of DNA chromosomal mal-segregation and cell death (Anagha Motgi et al., 2014 and Guruprasath et al., 2014).

Different addictions (chewing/smoking) are also responsible in many cases for developing cancer of oral cavity, pharynx, and esophagus (Proia NK et al., 2006 and Ali Shafi et al., 2015). Due to chewing and smoking habits, the buccal cells are directly exposed to the harmful chemicals which are having mutagenic or carcinogenic potential. In the present study, patients who were exposed for X-rays for the past 3-month duration were not included as the frequency of buccal cell micronuclei was found to increase following radiography compared with the frequency of micronuclei before radiological exposure, suggesting X-rays are potent mutagenic agents capable of inducing both gene mutations and CAs as proven by Piyathilake et al., 1995[28] Sandhu M et al., 2015 [29]

While assessing MN in the controls, out of 25 cases, 17 showed no MN among the 500 cells examined, while three patients showed a total of nine MN. This could be because MN formation is not a phenomenon exclusively related to exposure to tobacco, it could also reflect the effect of multitude of genotoxic agents such as environmental pollutants, radiations, or chemicals. The biomarkers of exposure and effect and clinical disease can all largely be influenced by susceptibility factors, which include polymorphisms that alter the activity of relevant DNA repair, carcinogen metabolism, and apoptotic pathway genes, as well as dietary factors that alter the activity of such genes.

Our findings collaborate with those of Stich HF, and Rosin MP who conducted a study in which the MN test was applied to exfoliated cells of the buccal mucosa in alcoholics also and observed that an elevated frequency of micronucleated buccal mucosa cells were seen only in
Gopal and Padma: Genotoxicity in tobacco users

Table 4: Kruskal-Wallis test-mean rank

| Group          | n  | Mean rank |
|----------------|----|-----------|
| MNC            |    |           |
| Smokers        | 25 | 47.56     |
| Chewers        | 25 | 44.32     |
| Control        | 25 | 22.12     |
| Smokers-duration (years) |          |          |
| ≤5            | 8  | 6.88      |
| 6-10          | 8  | 15.63     |
| >10           | 9  | 16.11     |
| Smokers-frequency (time) |        |          |
| 1-3           | 7  | 13.07     |
| 4-6           | 9  | 9.33      |
| 7-10          | 8  | 15.56     |
| Chewers-duration |        |          |
| ≤5            | 11 | 8.45      |
| 6-10          | 9  | 14.89     |
| >10           | 5  | 19.60     |
| Chewers-frequency (time) |        |          |
| 1-3           | 3  | 11.33     |
| 4-6           | 20 | 14.05     |
| 7-10          | 0  | 0.00      |
| Time of contact with buccal mucosa for chewers |        |          |
| <5 min        | 10 | 10.43     |
| 5-10 min      | 9  | 13.80     |
| >10 min       | 6  | 14.08     |

MNC=Micronucleated cells

Table 5: Chi-square tests

|               | Value | df | Asymptotic significant (two-sided) |
|---------------|-------|----|-----------------------------------|
| Pearson χ²    | 31.868 | 12 | 0.001                             |
| Likelihood ratio | 37.008 | 12 | 0.000                             |
| Linear-by-linear association | 14.980 | 1  | 0.000                             |
| Number of valid cases | 75 |    |                                    |

*High significant

apparatus dysfunctions, aneuploidy, and genetic instability. Hence, as a biomarker of genomic damage, micronuclei have been proved to be an important upcoming marker of tumorogenesis. There was stepwise increase found in the percentage of micronucleated cells and micronuclei from control to precancer patients, and from precancer to cancer patients in a study by Saran et al.[34] It is evident that our findings agree with those of Casartelli et al.[30] a gradual increase in micronuclei frequency from normal to precancerous to cancerous lesions. They concluded that the gradual increase in micronuclei counts from normal mucosal to precancerous lesions to carcinoma suggested a link of this biomarker with neoplastic progression.[57]

It was repeatedly emphasized that this noninvasive method might be a suitable biomonitoring approach for the detection of increased cancer risk in humans because more than 90% of all human cancers are of epithelial origin.[30] The level of baseline chromosome damage in untreated cancer patients and also in various potentially malignant disorders is much higher than in cancer-free controls. Therefore, micronuclei scoring can be used as a biomarker to identify different preneoplastic conditions much earlier than the manifestations of clinical features and might specifically be utilized in the screening of high-risk population for a specific cancer.[39]

However, in our study, we have emphasized on the tobacco cessation counseling for nonhabit associated patients, and in our future perspectives, we would like to identify the micronuclei counts in potentially malignant disorder patients also. In this study, Feulgen staining (DNA specific staining) has been used to represent the MN. Nersesyan et al.[26] have shown that Feulgen staining method has higher accuracy in examining the MN as compared with nonspecific DNA staining such as Giemsa stain.

**Conclusion**

The observations of this study showed that chewing of gutka/mawa/pan masala resulted in increased MN in buccal cells and also suggest that increase in duration, frequency, and time of chewing habit increases the MN which reflects
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Conflicts of interest

There are no conflicts of interest.

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