Micronucleus assay- an early diagnostic tool to assess genotoxic changes in tobacco and related habits

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Abstract

Objective: Micronuclei are induced in cells by a variety of substances, like UV radiation, infrared rays, X-radiations, and chemicals. Among them tobacco- specific nitrosamines have been reported to be potent mutagenic agents which are thought to be responsible for the induction of chromosomal aberrations resulting in production of micronuclei. The main aim of our study is to compare MN frequency among subjects, chewing tobacco only, chewing and smoking tobacco only, and chewing, smoking with alcohol, and to co-relate with control subjects.

Methods: Healthy subjects are included in the study and divided into four groups having 20 subjects in each group. Group-I is chewing only, group-II chewing and smoking, group-III chewing and smoking with alcohol, group-IV control. Smears were made from buccal exfoliated cells and stained with DNA specific stain Acridine orange. Frequency on MNC per 100 cells was assessed with, one way ANOVA & Tukey HSD Multiple Comparisons test with $p<0.05$.

Results: The mean number of MN was 2.3, 2.4, 3.6 in the group of chewing only, chewing & smoking, chewing, smoking & alcohol respectively. While assessing MN in the controls, out of 20 cases, 19 showed no MN among the cells examined while 1 patient showed 1 MN each per 100 cells examined.

Conclusion: The present study concludes that MN is a better surrogate biomarker to predict genotoxicity for tobacco related habits.

Key words: Acridine orange, Alcohol, Genotoxic, Micronuclei, Smoking, Tobacco.

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Introduction:

Cancer, a modern epidemic among the non communicable diseases is the second most common cause of mortality in developed countries and remains one of the ten most common causes of mortality in developing countries like India. The global incidence of cancers of the oral cavity, pharynx and larynx is about 500,000 cases per year with mortality of 270,000 cases per year (1). It is a complex disease with altered expression, abnormal growth and disruption of normal function of cells caused by genotoxic effects of chemical carcinogens or environmental pollutants resulting in genomic instability at an early stage of cancer, which is reflected often as potentially malignant lesions and conditions.

To evaluate the genotoxic effects/risks in tobacco users on buccal mucosa, DNA damages can be assessed by chromosomal aberrations, sister chromatid exchanges and micronucleus test. Out of these, micronucleus test is found to be most sensitive when compared to other tests as it is applicable to inter phase only; it is the best indicator of mitotic interference and chromosomal mutations or breakages. It is a non invasive and very economical procedure. It has

been used in the detection of oral squamous cell carcinoma and has been shown to have a sensitivity of 94%, specificity of 100%, and an accuracy of 95% (2).

A micronucleus (MN) is a small extra nucleus separated from the main one, generated during cellular division by late chromosomes or by chromosome fragments. It is a microscopically visible, round to oval cytoplasmic chromatin mass in the extra nuclear vicinity. They are induced in cells by numerous genotoxic agents that damage the chromosomes. The damaged chromosomes, in the form of a centric chromatids or chromosome fragments, lag behind in anaphase when centric elements move towards the spindle poles. The lagging elements are included in the daughter cells too, but a considerable proportion is transformed into one or several secondary nuclei which are as a rule much smaller than the principal nucleus and are therefore called micronuclei (3, 4).

In the literature there is no study till now which correlate the duration and frequency of tobacco related habits with micronuclei.

Aim and Objectives of the study are to determine the specificity and sensitivity and consequent early detection of dysplastic changes through MN assay in tobacco and related habits and to compare MN frequency among subjects, chewing tobacco only, chewing and smoking tobacco only and chewing, smoking with alcohol and to correlate with control subjects.

Methods:

Eighty Patients of either sex were selected at random, from patients reporting to the Department of Oral Medicine and Radiology, for routine diagnostic purposes. Selection criteria were related to the existence of deleterious adverse habits, without any apparent oral mucosal changes. The study content was explained to the patients, and their consent was duly obtained from each patient by taking signature on the consent form, and they were assured that their participation was voluntary and that all data was confidential.

Inclusion and exclusion criteria were met and were categorized based on the habits into four groups with 20 subjects in each group.

Group 1: Chewing tobacco and related products.

Group 2: Chewing tobacco and related products and smoking tobacco.

Group 3: Chewing tobacco and related products, smoking tobacco and alcohol.

Group 4: Control.

Ethical permission was taken before the start of the study from AJ Ethics Committee of A.J. Institute of Medical Sciences with review letter number AJEC/2009/61

Buccal smear was made form healthy patients visiting the department of Oral Medicine and Radiology, A.J. Institute of Dental Sciences, with habits of chewing, smoking tobacco and alcohol and those without any habit.

Healthy patient without any clinical lesion in oral cavity and having history of chewing, smoking tobacco and alcoholism was included in the study.

Cell counting was done by taking care that there was no debris, no overlap with adjacent cells, Cytoplasm intact and lying relatively flat. Cells with normal and intact nucleus with nuclear perimeter smooth and distinct were counted during the study.

Patients under vitamins and antioxidants supplementation and was or under radiation therapy was the excluded from the study.

Subjects were asked to rinse their mouth gently with water. Mucosal cells were scraped from buccal mucosa using a slightly moistened wooden spatula. The cells were immediately smeared on pre-cleaned microscopic slides. Just prior to drying; the smears were fixed with commercially available spray fixative (available as BIOFIX). Then slides were coded to ensure observer blindness and were fixed in 100% alcohol.

Staining procedure

Acridine orange solution

A pH of 6.0 is required for the differential staining of RNA and DNA. Formalin- fixed material does not stain satisfactorily, neither does tissue fixed in Bouin's solution; so, alcohol was the fixative of choice.

0.1% aqueous acridine orange was prepared and before use one part of the stain was diluted with 10 part of phosphate 0.06M buffer at pH 6.0 to give a 0.01% solution.

BUFFER

pH 6.0 phosphate buffer was prepared by mixing 88 grams of NaH_2PO_4. $2H_2O$ and 12 grams of KH_2PO_4.

Differentiator

0.1 M calcium chloride (11.099g calcium chloride in 100 ml distilled water).

Technique

Alcohol-fixed smear was taken to distilled water then rinsed in 1% acetic acid for a few seconds and in two changes of distilled water over 1 minute then smear was stained in the diluted acridine orange solution at pH 6.0 for 3 minutes after 3 minutes smear was rinsed in pH 6.0 buffer for 1 minute after that smear was differentiated in the 0.1 M calcium chloride solution for ½-1 minute then washed in phosphate buffer and mount in the same.

The frequency of micronuclei in buccal cells were evaluated by scoring 100 buccal epithelial cells per sample under fluorescent microscope (figures 1, 2), by Pathologist in the Department of Pathology, A.J. Institute of Medical Sciences, Mangalore. The scoring of micronuclei was done according to the criteria established by Countryman *et al*.

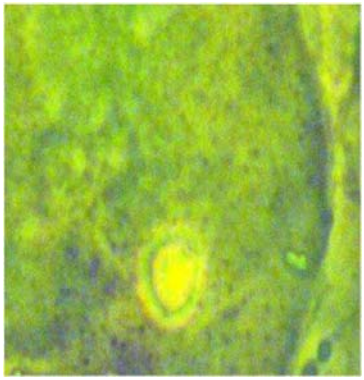

Figure 1- micronuclei under fluorescent microscope

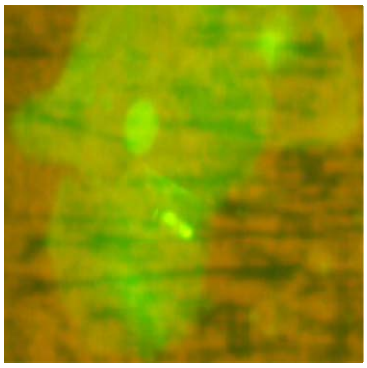

Figure 2- micronuclei under fluorescent microscope

Different statistical tests such as one way ANOVA & Tukey HSD multiple Comparisons test was done.

Results:

Results obtained were analyzed using different statistical tests such as one way ANOVA & Tukey HSD Multiple Comparisons test. $p< 0.05$ was considered to be statistically significant.

The types of tobacco related habits which were found to be highly prevalent among the chewing group was betel quid with tobacco, and among smoking was beady and among drinking alcohol is local packed drink. In the group of patients having chewing habit, the age range was from 23 to 70 years with the mean being 44.7 years and standard deviation 13.84 years. In the group of smoking and chewing the age range was 23 to 59 years with mean of 39.65 years and standard deviation 11.54 years. While in groups of chewing, smoking, drinking and control the age range was 23 to 87 (mean 55.9, standard deviation 15.07) and 18 to 45 years (mean 23.7, standard deviation 6.06) respectively.

With regards to the sex distribution, out of 20 subjects from the chewing group 5 were males and 15 were females, and in chewing and smoking group and chewing, smoking with alcohol all were male subjects, where as in control group 8 were male and 12 were females.

In our study we observed MN in the exfoliated cells of all the study groups. The frequency of occurrence of MN in exfoliated cells of oral epithelium was estimated in different study groups and compared the results with that of control group. The comparison of MN frequency was done using the one way ANOVA. The mean number of MN was 2.3, 2.4, 3.6 in the group of chewing only, chewing & smoking, chewing, smoking & alcohol respectively. While assessing MN in the controls, out of 20 cases, 19 showed no MN among the cells examined while 1 patient showed 1 MN each per 100 cells examined (Table 1).

Table 1- Micronuclei with mean and standard deviation

Group	Mean (SD)	p-value
1	2.30 (1.17)	< 0.05
2	2.45 (1.19)	< 0.05
3	3.65 (0.87)	< 0.05
4	0.50 (0.22)	> 0.05

The results of the Tukey HSD multiple comparison tests showed that chewing, smoking with alcohol was the most harmful habit group practiced by the subjects followed by chewing and smoking and finally chewing only, which was in accordance of the results obtained with the MN frequency using ANOVA.

Correlation of duration of habit with micronuclei frequency was done using the one way ANOVA. The mean of micronuclei in group-I was 1.13 in 0 to 10 years duration, 2.83 in 11 to 20 years of duration, 3.00 in 21 to 30 years and 4.00 in 31 to 40 years.

The mean micronuclei in group-II were 1.50 in 0 to 10 years duration, 3.25 in 11 to 20 years of duration, 3.35 in 21 to 30 years and 3.67 in 31 to 40 years. The mean micronuclei in group-III were 2.33 in 0 to 15 years duration, 3.42 in 16 to 30 years of duration, 4.12 in 31 to 45 years and 4.50 in 46 to 60 year.

Correlation of frequency of habit with micronuclei frequency was done using the one way Analysis of Variance (ANOVA). The mean of micronuclei in group-I was 2.09 in 1 to 5 times daily, 2.43 in 6 to 10 times daily. The mean of micronuclei in group-II was 2.35 in 1 to 5 times daily, 3.00 in 6 to 10 times daily.

The mean of micronuclei in group-III was 3.67 in 1 to 5 times daily, 3.80 in 6 to 10 times daily (Table 2).

It was seen that as the frequency and duration of habit increased in each sub group, the number of MN also increased, thus establishing a positive correlation between the frequency and duration of habit and genotoxic effect.

Table 2- Micronuclei in relation to frequency of habit

Freq. of habit (Per days)	Mean of MN per group	p-value
1-5	2.09 in group-1 2.35 in group-2 3.67 in group-3	< 0.05
6-10	2.43in group-1 3.00 in group-2 3.80 in group-3	< 0.05

Discussion:

Oral cancer is one of the most debilitating diseases afflicting mankind. In spite of the best efforts of researchers and clinicians, the global incidence of cancer is on a high today. Even though it is an established fact that tobacco and related products are one of the leading causative agents for oral cancer, their use is still very prevalent. As the use of tobacco products are not being banned totally due to various reasons our focus shifts to finding methods to detect early genotoxic damage, and hence an early diagnosis of oral cancers.

Micronucleus formation has been shown to be a sensitive biomarker for cytogenetic damage due to the use of tobacco products, increased frequencies of micronucleated cells reflects the exposure with clastogenic and aneugenic modes of action. The possible genetic effect from tobacco and related habits, by observing micronucleus occurrence in the buccal epithelial cells, has been assessed in our study.

Advantages: (5)

Cell to cell approach, possible co-detection of apoptosis/necrosis, applicable on many cell types, rapidity, cheap, simplicity, valuable for mass screening purposes, potential for automation, statistical power

Disadvantages: (5)

Does not detect all structural chromosome aberrations (only a centric fragments), requires cell division for expression of MN

Characteristics of the MN-Test: (5)

Biomarker: relevant for risk assessment of cancer, endpoint: identification of chromosome + genome mutation, expression of MN requires cell division and MN contain either a whole chromosome or an a centric fragment

Advances in knowledge:

To detect early genotoxic damages, to compare the compare the genotoxic potential of the different tobacco products, to correlate the effect of frequency and duration of tobacco related habits to genotoxicity observed as micronuclei count, in this study we used DNA specific Acridine orange stain which is very accurate for the counting of micronuclei as compared to other stains.

The present study was designed to determine the specificity and sensitivity and consequent early detection of dysplastic changes through MN assay in tobacco and related habits and to compare MN frequency among subjects, chewing tobacco only, chewing and smoking tobacco only, and chewing, smoking with alcohol, and to co-relate with control subjects.

Our study group subjects were divided into four groups with 20 subjects in each group. Group-I chewing tobacco only, group-II chewing and smoking tobacco, group-III chewing, smoking along with alcohol consumption and group-IV was control group i.e. without any habit. From our study, we could observe that minimum age at which the habits of chewing tobacco, smoking or alcohol started was 20-23 years. This observation of our study infers that deleterious habits of using of commercial tobacco products are acquired at a relatively younger age, subsequently increasing the cumulative genotoxic effects.

With regards to the sex distribution, males were predominant among the group of subjects of chewing and smoking, and chewing, smoking along with alcohol group and whereas females predominated in the chewing group. These results indicate that smoking and the use of

alcohol were predominantly male centric habits, while females prefer self prepared traditional betel quid chewing.

These results points to the fact that tobacco in any form can induce genotoxic effect which is marked by the presence of micronuclei. While assessing MN in the controls, out of 20 cases, 19 showed no MN among the 100 cells examined, while 1 patient showed 1 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 like environmental pollutants, radiations or chemicals etc.

Out of all the groups, group-III i.e. chewing, smoking with alcohol is found to be more dangerous with respect to genotoxicity, as the presence of micronuclei were highest in this group. This could be because of synergistic effect of alcohol with smoking and chewing. It was observed that a slight lower frequency of micronuclei in exfoliated oral epithelial cells of chewing group, as compared with chewing along with smoking group. This could be because of additive effect of chewing and smoking. Earlier some similar studies had shown the positive results includes Stich *et al.* (1982) (6), Sellappa *et al*. (2009) (2), Kausar *et al*. (2009) (**7**).

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 (8).

Parvathi devi *et al*. (2011) assessed the micronuclei frequency in potentially malignant and malignant disorders with a mean percentage of micronuclei in precancerous group was 0.12% and in malignant group was 0.06% and concluded that the micronucleus assay can be used as a prognostic indicator in potentially malignant and malignant disorders (9).

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 (10).

Patel *et al*. (2009) conducted a study to analyze tobacco related genotoxic effects in chewers monitoring MN and chromosome aberrations, they concluded that MN is a better surrogate biomarker to predict genotoxicity than chromosome aberrations (11).

Chatterjee *et al*. (2009) applied MN test to oral mucosal cells. Their analysis revealed that MN frequencies in cancer and precancerous cases were 4 fold elevated and 3.87 fold elevated for other non-malignant pathologies (12).

Chakraborty *et al*. (2006) conducted a study on 45 arsenic exposed individuals from west Bengal revealed 3.34 fold increases in MN in buccal mucosa cells (13).

Nersesyan *et al*. (2006) conducted a study in which MN frequencies in oral mucosa cells of heavy smokers and non smokers were evaluated with non specific and DNA specific stains. They concluded that the DNA specific stains give a much more reliable indicator of MN frequency than non specific stains (14).

Proia *et al*. (2006) conducted a literature review and concluded that the literature has established that buccal cells 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 (15).

Faldu Harikrishna *et al.* (2011) concluded that in tobacco chewing individuals, micronuclei formation was significantly higher along with an increase in the size of the nucleus (16).

Bansal *et al*. (2012) concluded that a positive correlation is found between increased micronucleus frequencies in tobacco using habits (17).

Adhikari *et al*. (2013) concluded that micronuclei percentage was higher in subjects

with betel quid chewing habit (18).

Kamath *et al*. (2014) concluded that there is significant increase in total number of micronuclei wit increase in duration and frequency of smoking (19).

The results of the Tukey HSD multiple comparison tests showed that chewing smoking with alcohol was the most harmful habit group used by the subjects followed by chewing smoking and finally chewing only, which was in accordance of the results obtained with the MN frequency using ANOVA. There were no studies that compared the genotoxic potential of chewing only, chewing with smoking and chewing, smoking with alcohol, using the MN assay using Acridine orange a specific DNA stain.

To check whether the frequency and duration of the habits had any effect on the number of MN Spearman's correlation test was done. It was seen that as the frequency and duration of habit increased in each sub group, the number of MN also increased, thus establishing a positive correlation between the frequency and duration of habit and genotoxic effect. 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 tobacco related habits to genotoxicity observed as MN.

Conclusion:

In this study we undertook the assessment and comparison of genotoxic potential of different forms of tobacco and related habits using the MN assay and we concluded:

1. Analysis of the data obtained showed that there was a positive MN count and increased in frequency of MN in all the three habit related study groups from buccal exfoliated cells of the subjects with chewing, smoking with alcohol (group-III) followed by subjects with chewing and smoking (group-II) and finally those subjects with chewing habit only (group-I) as compared to control group.
2. So, as the severity of habit increased the count of micro nucleated cells also increased. DNA specific stain Acridine orange provides a more accurate detection of micronuclei, especially when micronuclei are small.
3. We also concluded conclude that micronuclei can be used to detect early genotoxic changes.

Conflict of Interest: "None Declared"

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