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Assessment of Micronuclei Assay as a Screening Tool among Tobacco and Non Tobacco Users - A Cross Sectional Study.

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Abstract

Introduction: Oral cancer is the most common cancer in India. The reason for high prevalence of oral cancer in India is primarily because tobacco is consumed in the form of gutka, quid, snuff, misri and smoking. Such data make the oral cancer an important public health matter.[1] Micronuclei (MN) assay which is a part of Molecular biology is an area of increasingly intense activity and interest, combining molecular biology with epidemiologic methods (molecular epidemiology) that would strengthen the epidemiologic evidence. Hence the purpose of this study is to assess the micronuclei assay as a screening tool among tobacco and non-tobacco users.

Methodology: A total of 120 adult patients at the age group of 20-60 years who satisfy the inclusion and exclusion criteria are considered for the study. Informed consent has been taken from the participants before under taking the study. Mucosal cells are scraped from buccal mucosa using a slightly moistened wooden spatula. The cells are then smeared on pre cleaned slides and fixed with spray fixative. Cells from each sample have been focused under optic microscope and number of Micronucleated cells (MN) are counted by a single observer.

Results: The mean distribution of micronuclei using smokeless tobacco had highest mean number of micronuclei 20.43±3.59 followed by smoking 13.5 ± 4.55

whereas controls did not have any micronuclei. Statistically significant difference was found between the Tobacco smoking group, chewing tobacco group and control group. **(p=0.000*) ANOVA**

Conclusion: The present study showed that the significant increase in MN frequency in smokeless tobacco users as compared to smoking tobacco users. Significant difference was obtained between the micronuclei between the groups. Increased duration of tobacco usage caused increased micronuclei with a P value of 0.04.

Keywords: Exfoliated buccal cells, Micronuclei, Oral Cancer, Staining Procedures

Introduction

Oral cancer is one of the commonest cancers of morbidity and mortality today and constitutes a major health problem among the developing countries, representing as one of the leading causes of death. Globally about 5, 00,000 new oral and pharyngeal cancers are diagnosed annually and three-quarters of these are seen in India. Disproportionately higher prevalence of oral cancer is seen in India, it is found as one of the five leading cancers.[1,2,3]

The survival rate of patients with oral cancer is reduced drastically. Prognosis is considerably improved if oral cancer is detected early and the patient is referred to appropriate expertise. When detected early, the likelihood of surviving from oral cancer is significantly better than for most other cancers.[3]

Tobacco is most commonly smoked as cigarettes, both manufactured and hand-rolled. Pipes, cigars, bidis and other products. Effect of cigarette smoking is driven largely by the mutagenicity of various chemicals in the smoke, carcinogenic chemicals such as benzopyrene, radioactive polonium -210, nitrosamines (derivatives of nicotine, N –nitrosonornicotine , 1-1 butanone).[3,4,5] Smokeless tobacco is usually placed in the oral or nasal cavities against the mucosal sites that permit the absorption of nicotine and causes some changes in the oral mucosa, which will eventually give rise to clinically detectable lesions occurring in the oral mucosa like Leukoplakia, tobacco pouch keratosis, nicotine palatinus, oral submucous fibrosis, and oral cancer.

Early detection is being an area of interest to prevent the teratogenic effects of tobacco and its products. A number of techniques have been employed to supplement clinical examination in the diagnosis of these lesions. Various techniques, both invasive and non-invasive are available for confirmative diagnosis.

Invasive techniques cannot be used in certain conditions like diabetes, hypertension, bleeding disorders, etc., and non-invasive techniques like exfoliative cytology are used for the screening of malignancy in the large population. Papanicolaou and Traut (1941) developed cytological and staining techniques.[4]

The oral exfoliative cytology is a quick, simple, less technically demanding, painless, non-invasive, practical, reliable, cost-efficient and repeatable technique that can be used for multiple small or large suspected malignant and premalignant lesions. In oral exfoliative cytology, the false-negative dilemma may be attributed to various factors such as number of abnormal cells available for samples, may be limited by the keratinized nature of many oral lesions and high rate of epithelial turnover in the mouth.[6]

In the 1970s the term micronucleus test was suggested by Boller & Schmidt.9 The Micronuclei assay (MN) is potentially an excellent candidate to serve as a biomarker. 9 Micronuclei (MN) are extra-nuclear small chromatin bodies that appear in the cytoplasm by the condensation of acrocentric chromosomal fragments or by whole chromosomes, lagging that are not included in the main daughter nuclei during nuclear division. Thus it is the only

biomarker that allows the simultaneous evaluation of both clastogenic and aneugenic effects in a wide range of cells and also aid as a marker in early-stage carcinogenesis. [1, 11, 6]

The method of smear collection produces a more than an adequate number of cells for examination but keratinized areas like the palate, gingiva require a firmer scrapping pressure than the non-keratinized areas like buccal mucosa. Histological changes of the buccal mucosa have been reported in humans and experimental animals in association with tobacco usage. These changes include hyperkeratosis, epithelial hyperplasia, acanthosis, and sub epithelial connective tissue hyalinization. In the present study, buccal mucosa was taken for the site of smear collection and stained.

The advantages of this staining over H&E staining is the fact that the dehydration and clearing solutions help in causing cellular transparency. This detects the overlapped cells and their individual morphology better, which otherwise would be confused for a giant cell, or bi or multinucleated cell. The second significant advantage is the differential staining for different degrees of differentiation, such as green-blue cytoplasm for basal cells and yellow-orange for a spinous or granular cell, the stability of stains over long periods, the stability of colour and better reproducibility of results.[8]

There are very few studies documented in the literature that have used micronuclei as a screening tool in detecting oral cancer. Hence the purpose of this study was to assess micronuclei assay as a screening tool among tobacco users and non-tobacco users.

Materials and Methods

A descriptive cross- sectional study was conducted to assess the micronuclei assay as a screening tool among tobacco and non-tobacco users attending outpatient department of dental college in Bangalore city. Convenience sampling technique was employed. Data was collected over a period of three months May 1st to July 31st 2017. Approval for the study was obtained from the college authorities & by the Head of the Dept. of Public Health Dentistry & Ethical Review Committee.

Before commencing the study, the training and calibration was done. The Kappa co-efficient value (*k*) 0.8. A pilot study was conducted to check for the feasibility and relevance of the prepared format.

The sample size is estimated based on the previous literature findings[7] and by consulting a Biostatistician. Sample is derived from the formula:

$$n = \left[\frac{r + Z_{1-\beta}}{\epsilon^2} \right]^2 \sigma^2$$

Thirty subjects each were taken in smoking tobacco and smokeless tobacco group and sixty age and sex matched adults were taken as control group. A total of 120 subjects, who satisfied the inclusion and exclusion criteria were included in the study.

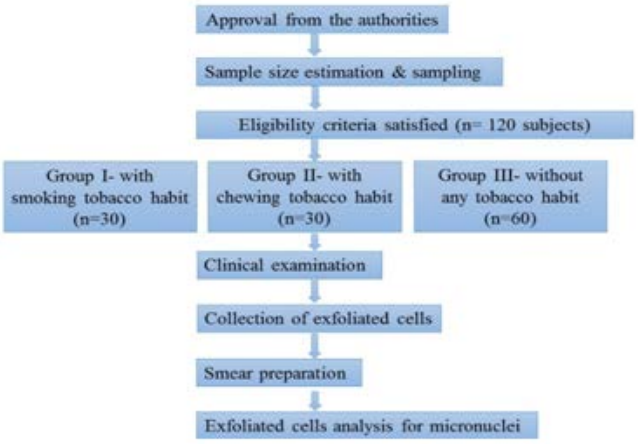
Subject's ≥ 20 years who are users of tobacco with tobacco habit ≥ 10 years**,** Subjects willing to participate in the study were included in the study. Subjects suffering from systemic diseases, on long term medication, use of antibiotics during the last 3 months, Subjects undergoing chemotherapy and/or radiotherapy, and who are medically compromised, Subjects who had a recent viral infection and Subjects who are alcoholic occasionally since 2 years were excluded.

Informed consent was obtained from the subjects. A structured questionnaire was prepared & filled. A single investigator carried out the oral examination for the study subjects. The clinical examination and recording took about 30- 45 minutes per study subject, 4-6 study subjects per day were examined.

During collection of samples, subjects were asked to sit in a relaxed position, buccal mucosal scrapes were collected using a wooden spatula. Cells were scraped using a gentle scraping motion, exerting little pressure, and were spread on the glass slide and fixed in 95% alcohol for 15 minutes followed by staining. The slides were transported in a closed air tight container to the lab for the staining procedure and analysis.

Armamentarium used are sterile glass slide, wooden spatula & Bio fix. Olympus Pentahead microscope (BX43), Digital camera (Cannon 13x43) with photomicrography adaptor was used for image capture & Q capture pro-6 software was used for image analysis

Smears were observed under light microscope and fifty non-overlapping cells with well-defined borders were randomly selected. All the images of the cells were captured with a 40x achromatic objective. Images thus captured were stored on the computer and analysis was done.



The data was entered in Microsoft Excel & processed and analysed using the, SPSS software version 23. Results of continuous measurements are presented as mean ± SD (Min-Max) and results of categorical measurements are presented as number (%). Significance is assessed at 5 % level of significance at 95% confident interval. Tests used to assess the statistical significant difference are Chi Square test, ANOVA.

Results

A total of 120 subjects, who satisfied the inclusion and exclusion criteria were included in the study. The data thus obtained from the study was complied, tabulated and subjected to statistical analysis.

Figure 1 depicts the distribution of study participants according to the gender. Among a total of 120 subjects, the study subjects consisted of 53.3% males while 46.7% of them were females. Distribution of participants based on Occupation. Among the Smoking group 30% of them were semi-skilled workers followed by another 30% who were skilled workers. 20% of them had arithmetic skill jobs while 16.7% of them were unskilled workers and 3.3% of them were unemployed. Among the smokeless tobacco group there were only 50% of each semi-skilled workers and workers with arithmetic skill jobs. Among the controls 5% were skilled workers and 28.3% had arithmetic skill jobs. Also 50% of them were semi-professional workers & 16.7% of them were professional.(Figure 2)

Figure 3 shows the distribution of study participants based on Education. Among the Smoking group 50% were graduates, 26.7% had high school certificate and 23.3% had an intermediate education. In the smokeless tobacco group 26.7% were unemployed, 46.7% had high- school certificate & 26.7% had an intermediate education. Among the controls, all of them had a post graduate or professional degree. Figure 4 shows the distribution based on the no .of cigarettes smoking. The study participants based on the no. of cigarettes smoking. 86.75 of them shows that they smoke tobacco 10-20 per day and 13.3% of them smoke tobacco more than 20per day.

Figure 5 shows the study participants based on the no. of pouches they use. Majority 60% of them shows that they

chew tobacco 6-10 per day were as 40% of them chew tobacco less than 5per day. The mean distribution of micronuclei among the study participants. Out of all the three groups, participants using smokeless tobacco had highest mean number of micronuclei 20.43±3.59 followed by smoking 13.5 ± 4.55 whereas controls did not have any micronuclei.(table-1) Table 3 shows the Pearson correlation to find out the correlation among the study variables, there was a positive correlation(r=0.378) for duration of smoking with micronuclei which was found to be statistically significant (p=0.04). Table 2 shows the results based on one way ANOVA to compare mean micronuclei among all the three groups, statistically significant difference was found between the Tobacco smoking group, chewing tobacco group and control group. **(p=0.000*)**

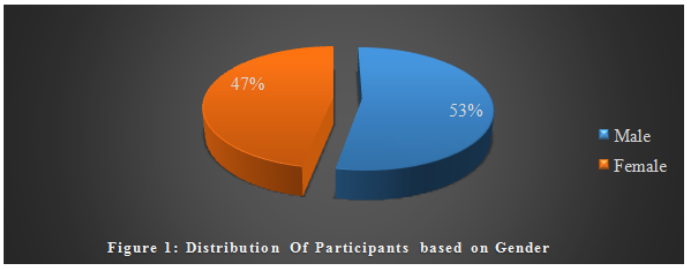


Figure 1: Distribution Of Participants based on Gender

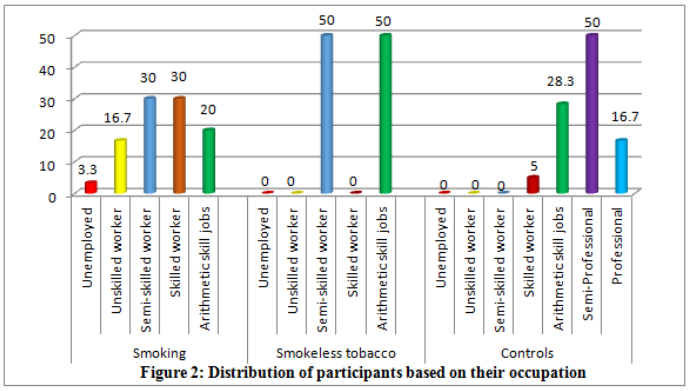


Figure 2: Distribution of participants based on their occupation

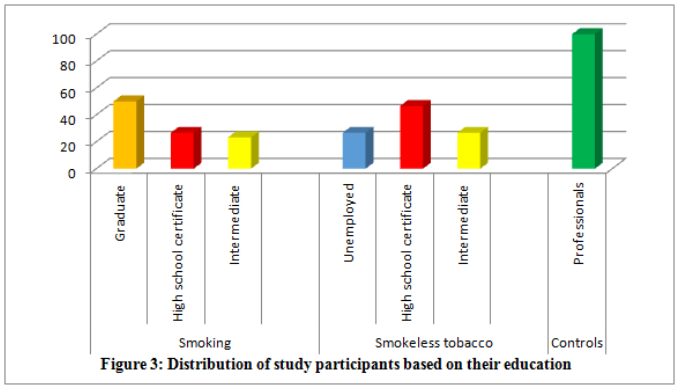


Figure 3: Distribution of study participants based on their education

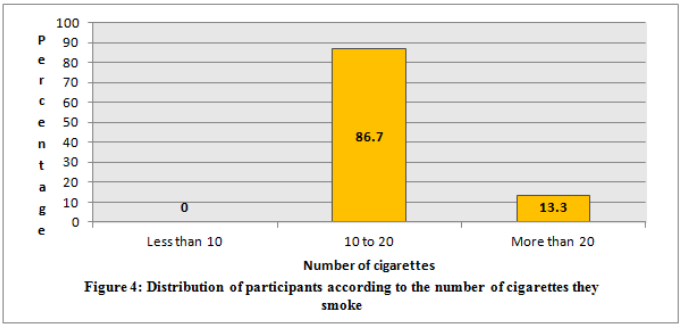


Figure 4: Distribution of participants according to the number of cigarettes they smoke

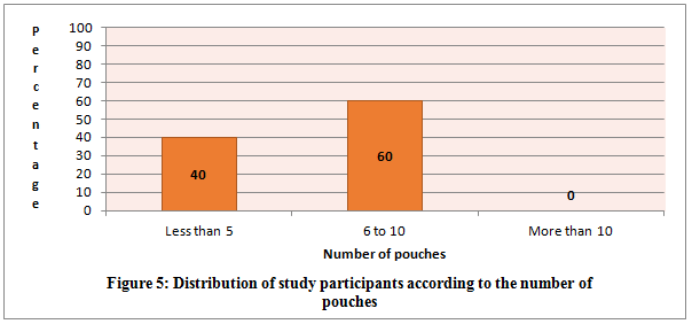


Figure 5: Distribution of study participants according to the number of pouches

Table 1: Mean Distribution of the Micronuclei

Groups	Minimum	Maximum	Mean	Std. Deviation
Smoking	4.0	20.0	13.500	4.5543
Smokeless Tobacco	14.0	26.0	20.433	3.5977
Controls	.0	.0	.000	.0000

Table 2: Comparison of the Micronuclei Among The Groups

Groups	Sum of squares	Df	Mean square	F	Significance
Between groups	9357.100	2	4678.550	560.353	.000*

Within groups	976.867	117	8.349		
Total	10333.967	119			

Table 2(a): One way –ANOVA – Post Hoc tests

Groups	Mean Difference	Significance	Confidence Interval	
			Lower Bound	Upper
I V/S II	-6.9333	.000*	-8.7455	-5.1212
I V/S III	13.5000	.000*	11.9307	15.0693
II V/S III	20.4333	.000*	18.8640	22.0027

Table- 3: Correlation of The Frequency, Number And Duration Of The Tobacco Habit With Micronuclei In Smoking And Smokeless Tobacco Groups

Pearsons Correlation		r value	p value
Smoking tobacco	Frequency	-.016	.931
	Duration	.378	.040*
	Number	-.195	.302
Smokeless tobacco	Frequency	-.255	.174
	Duration	-.087	.648
	Number	-.213	.259

Discussion

The majority of the oral cancers are preceded by the potentially malignant lesions and conditions mostly related to the use of Tobacco. The direct correlation between the micronuclei formation and genomic damage makes the micronuclei assay an efficient screening tool.[4]

The study subjects were selected according to the history of habit which compared Tobacco users with Healthy controls. This was done to see the difference in the number of micronuclei cells among the Two Groups this was in accordance to the studies conducted by Bansal et al, Palaskar *et al*.,Ozkul *et al*,and Patel *et al.*

Age and Gender of the Study Population:

In the present study, figure-1 comprised of males 53.3% and 46.7% females with in an age range of 20- 60 years. this age group was selected to consider for better compliance and exposure to tobacco for considerable amount of time as oral carcinogenesis is a multi-step process of accumulated genetic damage which occurs over a period of time. In the present study there was no significant difference between the no. of MN when age and gender wise comparison was made between the control study groups. This was in accordance with most of the studies conducted by Noushin Jalayer Naderi et al , and Patel *et al,* Naderi et al, Nair et al, Stich et al. Akanksha Gupta et al.

Smokers V/S smokeless tobacco users

The present study comprised smokeless tobacco users, smokers (more than 10 years) and healthy adults who were not habituated to any form of tobacco consumption, and compared to the no. of exfoliated Micronuclei cells in the buccal mucosa. This was done because many studies have shown that all the habit groups had significantly higher frequency of micronucleated exfoliated oral mucosal cells. Fareed et al, Himanta et al Naderi et al

Matching

The 60 study subjects were age and gender matched to 60 healthy individuals with no habit to find out of the effect of tobacco on the no of MN cells of buccal mucosa. This facilated to remove the effect of confounding factors in the present study and detect the effect only of exposure. This was in accordance with the studies conducted by Palaskar *et al*.,Ozkul *et al,* and Patel *et al.*

Relationship of tobacco users with micronuclei cells

In the present study, on assessing the distribution of study participants based on the duration of tobacco habit among smoking group majority of them 36.7% was smoking for more than 15 years, 23.3% were smoking for 11-15 years, another set of 23.3% were smoking for 1-5 years

followed by 16.7 % of the subjects for 6-10 years. In case of smokeless tobacco 30% of the subjects were using for 6-10 years, 26.7% for 1-5 years, 23.3 % for more than 15 years, 20% for 11-15 years.

In the present study, on evaluation of the no. of micronuclei, among tobacco users showed significantly higher frequency of MN when compared to the control group. Similar results were found in Bansal et al, Palaskar *et al*.,Ozkul *et al,* and Patel *et al*

In the present study on evaluation of distribution of micronuclei among subjects having habit of smoking, chewing tobacco and among controls highest number of micronuclei was observed in subjects having tobacco chewing habit with a mean of 20.43 ± 3.59, followed by 13.5 ± 4.55 tobacco smoking whereas micronuclei was absent in controls, the difference was statistically significant with a P value of 0.00*.

Bansal et al did a study where the results showed that the overall level of mean number of micronuclei in smokeless tobacco were higher (24.13 ± 10.68) as compared with smokers (11.96 ± 4.23) and controls (4.17 ± 2.99). This observation was similar to those reported by Palaskar *et al*., Ozkul *et al*, and Patel *et al*, when all the groups were further compared with each other for the mean difference, the result was highly statistically significant ($P < 0.05$), In the present study smokeless tobacco had greater frequency of MN cells when compared to smokers, which was in accordance with the previous studies by Palaskar *et al*. and Patel *et al*., the reason being chronic use of tobacco is linked to abnormal epithelial growth in the mouth due to the presence of various carcinogens indicating more genotoxic effect of smokeless tobacco as compared with smoking. Whereas Ozkul *et al* found no difference between the mean percentages of MN cells for the groups considered ($P > 0.05$). Hence, more research is requires to establish micronuclei assay as a potential biomarker for oral carcinogens

Frequency and quantity of Tobacco use

Considering the number of cigarette smoking by the participants 86.75% smoked around 10-20 per day and 13.3% of them smoke around 20 per day whereas based on the number of pouches of tobacco consumption 60% of them chewed around 6-10 per day and 40% of them used around 5 per day.

Study done by Akanksha Gupta et al (2016) showed micronuclei frequency in the oral exfoliated cells in the control group was observed to be in range of 0% to 0.4% with a mean micronucleus frequency of 0.21%.Study done by Palve et al (2008) and Kassie et al (2001) where micronuclei frequency was 0.21% and 0.20% respectively. According to Akanksha Gupta et al micronuclei in the potentially malignant disorder group was observed to be in range of 0.50 % to 1.70 % with a mean micronucleus frequency of 1.08%, Bloching et al (2000), Grover et al (2012) with 1.90% and 1.68% respectively. Micronuclei frequency in the oral exfoliated cells in the malignant disorder group was observed to be in range of 2.10 % to 2.90 % with a mean micronucleus frequency of 2.53%, which was quite similar with those reported by Bloching et al (2000) and Palve et al (2008) with 2.05% and 1.86% respectively. Similar results were found by Casartelli et al (2000) , Bloching et al (2000), Halder et al (2004), Palve et al (2008) , Devi et al (2011) , Dindgire et al (2012) , which showed a gradual increase in micronuclei frequency from normal to precancerous to cancerous lesions.

Similar results were observed by study done by M Fareed et al were MN frequencies in oral buccal region of tobacco chewers (3.56 ± 0.719) is significant in comparison to control (0.75 ± 0.171). Study done by Sivasankari.et al showed increased amounts of micronuclei present in both alcohol and smoking and betel nut chewers. Gabriel et al

conducted a study were increased micronuclei frequency was observed in exfoliated buccal mucosal cells (EBMC) of tobacco (i.e., cigarettes) smokers. Similarly study done by Suhas et al showed increased micronuclei frequency in smokers compared to chewing tobacco. According to the recent studies of Sellapa et al , Patel et al, Gandhi et al, Ramakrishnan et al where the MN count in smokeless tobacco users were higher than that in the control group. Continuous exposure to mutagens/carcinogens is probably responsible for the damage to oral mucosal cells.

On correlating the frequency, number and duration of the tobacco habit with micro nuclei among smoking group and smokeless tobacco groups in the present study statistically significant correlation was found with a P value of 0.04 which showed increased duration of the habit causes increased micro-nucleus.

Nair et al, Stich et al showed exposure related increase in the incidence of micronuclei. S. Bonassai et al showed the association between increased MN frequency and heavy cigarette smoking was found only in subjects who were not exposed to occupational carcinogens or mutagens. The reason could be that occupational exposure to genotoxins may have stimulated the expression of DNA repair genes or detoxification mechanisms that are also important in attenuating the genotoxic effects of chemicals in cigarette smoke.

According to study done by Wu et al reported positive relation between micronuclei frequency and smoking intensity. Whereas study done by Naderi et al correlation between pack-years smoking quantum with the percentage of cells containing micronucleus and average of micronucleus number were not significant.

Detection of micronuclei and their assay is an upcoming research domain in the field of cancer prevention and therapeutics. The presence and frequency of MN represent genomic damage. The frequency of increase in MNC's from normal mucosa to subjects having habit of tobacco can suggest a link of this biomarker with malignant neoplastic progression.

Tobacco users are at a risk of developing different types of oral cancer, it becomes necessary to screen the population for it possible risk. Therefore, MN assay in exfoliated cells holds promise as a specific biomarker for exposure to various carcinogens, and can also be used as a screening test in oral health centres. Further study of large group of cases and controls will strengthen the findings of the present study.

Limitation

The limitation of this study was the sample size which could have been larger. Micronuclei scoring can be interfered by the bacteria that are commonly found in the mouth which can be differentiated by their characteristic shape**.** Small dye granules may sometime resemble MN, but usually have a slightly different refractivity and colour intensity**.** Other cellular structures such as keratohyalin granules resembling MN can lead to false positive results.

Conclusion

Based on the results obtained the study can be concluded as:-

1- Highest number of micronuclei was observed in subjects having tobacco chewing habit with a mean of 20.43 ± 3.59, followed by 13.5 ± 4.55 tobacco smoking whereas micronuclei was absent in controls, the difference was statistically significant with a P value of 0.00*. The present study showed that the significant increase in MN frequency in smokeless tobacco users as compared to smoking tobacco users.

2- Significant difference was obtained between the micronuclei between the groups.

3- Increased duration of tobacco usage caused increased micronuclei with a P value of 0.04.

Scope for further research

Due to the more false positive results MN frequency in combination with genetic polymorphism in DNA repair may serve as a better predicator of risk.

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