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RESEARCH ARTICLE

EVALUATION OF CYTOGENIC DAMAGE IN ORAL EXFOLIATED BUCCAL CELLS OF TOBACCO USERS AND PATIENTS WITH POTENTIALLY MALIGNANT DISORDERS USING MICRONUCLEUS ASSAY - A PROSPECTIVE STUDY

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Abstract

Background: Cytogenic methods acts with complex type mutations, known as structural mutations (aberrations)and a special type of chromosomal damage, known as micronucleus. So, micronucleus (MN) assay has been used as a biomarker of genetic damage in buccal mucosa cells.

Aim and Objectives: To evaluate micronucleus in the exfoliated cells of buccal mucosa of individuals with potentially malignant disorders and different tobacco related habits and in control group. To observe the incidence of micronucleus count based on duration, frequency and type of tobacco usage and also auto immune condition of Lichen planus.

Materials and Method:
The study population is divided into 3 groups.
Group I : 15 healthy subjects (control),
Group II : 15 smokers or chewers with habits andno evidence of lesion
Group III : 30 Patients clinically diagnosed as having PMD of the oral cavity.
Smears stained with DNA specific Feulgen stain. Association of micronucleus between all groups and its mean and standard deviation was assessed with ANOVA with $p<0.05$ was considered as significant level.

Results: The mean MN revealed that individuals with potentially malignant disorders had 26.8±5.1, individuals with tobacco habit (smokers/chewers) had 11.0±5.5 and controls had 0.2±0.5. The ANOVA test for association and mean frequency of micronucleus between all groups revealed significant value of <0.001.

Conclusion: Hence, MN assay in oral exfoliated buccal cells can be conveniently used as a biomarker for screening of potentially malignant disorders of oral cavity. And also provides an insight into the importance of early detection and prevention of OPMDs.

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Introduction:-

Tobacco is one of the strongest carcinogens, for the development of different types of cancers.In developing countries like India, it is mainly consumed in two forms: smoked tobacco products and smokeless tobacco [(1)].

The Estimation of World Health Organization (WHO) reveals that tobacco causes nearly 6.4 million deaths and also economic damage of hundreds of billions of dollars will occur worldwide every year [(2)]. If this current scenario continues, by the year of 2030 tobacco will end the life of more than 8 million people worldwide each year, most of that will occur in developing countries with lower incomes [(3)].

Smoking tobacco specific nitrosamines, such as N-nitrosonor nicotine are potent carcinogens[(1)]. These chemicals cause extensive damage to deoxyribonucleic acid (DNA), contributing to malignant transformation[(4)].Smokeless tobacco is the major form of tobacco consumption in South-East Asia where over 80% of smokeless tobacco users live.Smokeless tobacco contains nearly, 25 powerful carcinogens, which can cause immense damage to deoxyribonucleic acid (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[(5,6)].

Oral potentially malignant disorders (OPMD) are chronic lesions or conditions which is characterized by a potential for malignant transformation. More precisely, "It is a group of disorders with different etiologies, mostly tobacco; marked by mutagen associated, spontaneous or hereditary alterations or mutations in the genetic material of oral epithelial cells with or without clinical and histo-morphological alterations[(7)]. Leukoplakia, lichen planus, oral lichenoid lesion, oral submucous fibrosis are among the most common of these lesions[(8)].Patients with Potentially malignant disorders, the average is around 50–69 years, which is 5 years before occurrence of oral cancer. Unfortunately, in recent years 5% of PMDs has been detected in persons under the age of 30 [(9)].

The micronucleus (MN) assay has been used as a biomarker of genetic damage in buccal mucosa cells, which are in direct contact with the chewing material[(10,11)].

Several investigators called micronucleus as an advancing marker of tumorogenesis.It is also used to educate and motivate people about the emerging risk of genotoxicity in tobacco users.These occurrence can be conveniently studied in the buccal mucosa, which is a simply approachable tissue for sampling cells in a non - invasive manner[(4)].

Materials and Method:-

This is a prospective, randomized, cross sectional study. The study is conducted in Meenakshi Ammal Dental college and hospital, Chennai. In the present study buccal smears of 60 individuals with different tobacco related habits were obtained, the smears were stained using Feulgen stain- deoxyribonucleic acid (DNA) specific staining method and then observed under 100X magnification in order to identify and quantify micronuclei in the exfoliated cells of buccal mucosa.

The study population is divided into 3 groups.
Group I : Comprised of 15 healthy subjects without habits and clinically no evidence of lesion
Group II : 15 Smokers or chewers without any oral lesions
Group III : 30 Patients clinically diagnosed as having Potentially malignant disorder (PMD) of the oral cavity.

Potentially malignant disorders such as Oral submucous fibrosis, Oral Leukoplakia, Oral Lichen planus were included in the study. Study population included males, with an age range from 20 to 55 years.

Clinical inclusion criteria:

1. Individuals willing to participate in the study.
2. Patient with habit of chewing tobacco or smoking.
3. Patient with clinically diagnosed PMD of the oral cavity.

Clinical exclusion criteria:

1. Patients under radiation therapy or recent exposure to radiographs.

2. Patients with major systemic illness such as rheumatoid arthritis
3. Patients under steroid therapy
4. Patients with recent viral infection
5. Patient with Oral mucosal lesions occurred as a result of infections, local trauma or irritation.

Inclusion criteria for total cell count:
Collection of smear with no debris.
Tolbert et al. criteria for identifying MN:
• No overlap with adjacent cells
• Round smooth perimeter characteristic of a membrane
• Cytoplasm intact and lying relatively flat
• Nucleus normal and intact with nuclear perimeter smooth and well-defined
• Staining intensity of micronuclei was close to nucleus
• Micronuclei on same focal plane like nucleus.

Collection Of Exfoliated Cells:
All patients were detailly explained about this study, and an informed consent was acquired in their native languages to prevent language bias and later was subjected to collection of buccal smears.
First,patients were allowed to rinse their mouth gently with water. Mucosal cells were scraped from buccal mucosa by a sterile dry polypropylene cotton swab. The cells are directly smeared on precleaned microscopic slides. Just before drying, the smears are fixed with 80% ethanol. Then slides will be coded to make sure of observer blindness.

Staining procedure:
Feulgen staining
1. Rinsing briefly with cold 1N hydrochloric acid
2. Then place into prewarmed hydrochloric acid for the appropriate time at 60°C
3. Rinse the slides briefly with cold 1N hydrochloric acid
4. Rinse briefly with distilled water
5. Then place into Schiff's reagent for 30–60 min at room temperature
6. Wash well with water
7. Counterstain with light green for 1 min
8. Dehydrate with ethanol and mount.

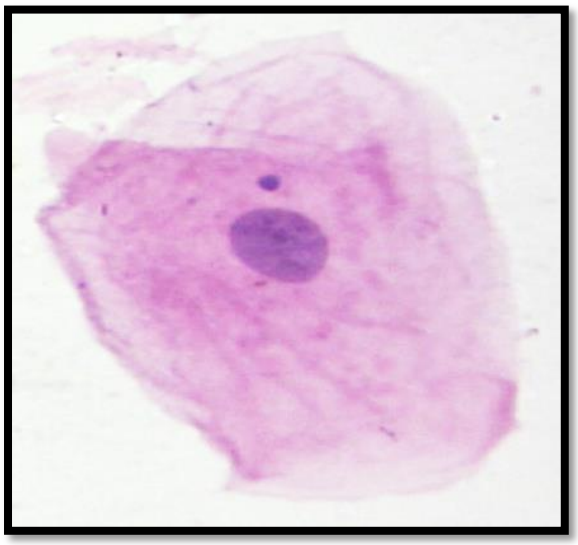


Figure 1:- Single Micronucleus.

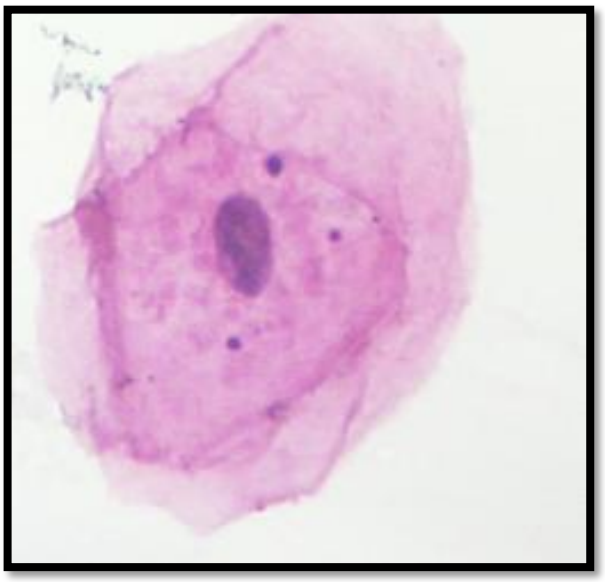


Figure 2:- Multiple Micronucleus

The swabs will be observed under 100X magnification, an eyepiece grid is used and 500 cells per slide are considered for micronuclei (Figure 1 and Figure 2). MN identification and scoring is performed.

Results And Statistical Analysis:-

The exfoliated epithelial cells were collected from 60 patients. Study population included males, with an age range from 20 to 55 years.The collected data of micronucleus was analyzed with SPSS software. The probability value less than 0.05 was considered as statistically significant level.

All the three study groups showed positive expression of Micronucleus. The mean value of Micronucleus by ANOVA revealed that patients with potentially malignant disorder had increased count than patients with habit either smoking or chewing tobacco and controls with p value <0.001* (Table - 1).Comparison of number of micronucleus based on type of tobacco by Chi- square test revealed that, it is not significant with p value 0.566 (Table – 2). Comparison of number of micronucleus based on duration and frequency of tobacco consumption was done by pearson correlation and Linear regression analysis, which shows that increase in duration and frequency of use of tobacco, there will be 3 times more increase in number of micronucleus and this was found to statistically significant with p value 0.001*. The R square value is 0.833 which means that both duration and frequency has a strong effect (0.833) on number of micronucleus (Table – 3,4).Frequency of micronucleus was compared among different potentially malignant disorders with p value 0.03* (Table – 5).

Incidence of micronucleus in group I, group II and group III (Graph – 1,2,3). Comparison of micronucleus in all three groups (Graph - 4). Hence from the above obtained results, it is evident that estimation of Micronucleus 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 non- invasive manner. The cell counts also helped us to counsel and educate the patient to quit their habit, thereby insisting the need of micronucleus assay as a potential biomarker to determine the disease progression.

Table 1:- ANOVA.

GROUPS	N	Mean±SD	P-VALUE
I	15	0.2±0.5	<0.001***
II	15	11.0±5.5	<0.001***
III	30	26.8±5.1	<0.001***

Table 2:- Chi Square Test
Incidence of Micronucleus in accordance to type of tobacco usage.

Brand	Micronucleus				
	No. 01 to 05	No. 06 to 10	No. 11 to 15	No. 16 to 20	
Cigarette	0	3	1	2	0.566
Beedi	0	0	1	1	
Pan masala	1	2	0	0	
Mawa	1	0	0	0	
Betel quid	0	0	1	1	
Hans	0	1	0	0	

Table 3:- Pearson Correlation of duration and frequency with Micronucleus in Group II (smokers or chewers).

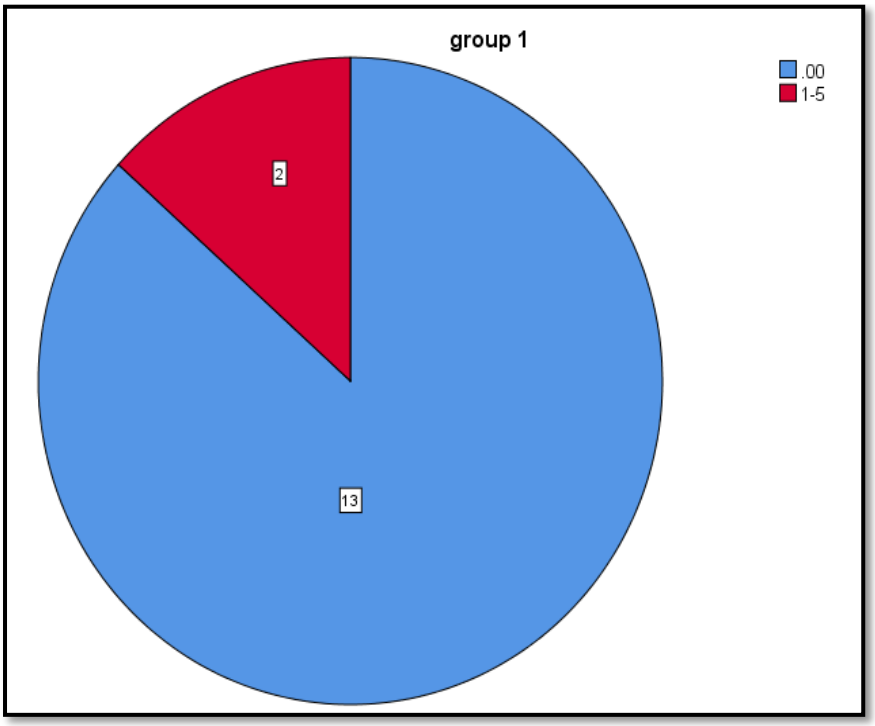
	No. of micronucleus	P-value
Duration	0.85	<0.001*
Frequency	0.55	0.01*

Table 4:- Linear regression analysis of duration and frequency on number of micronucleus in Group III (Patients with Potentially malignant disorders).

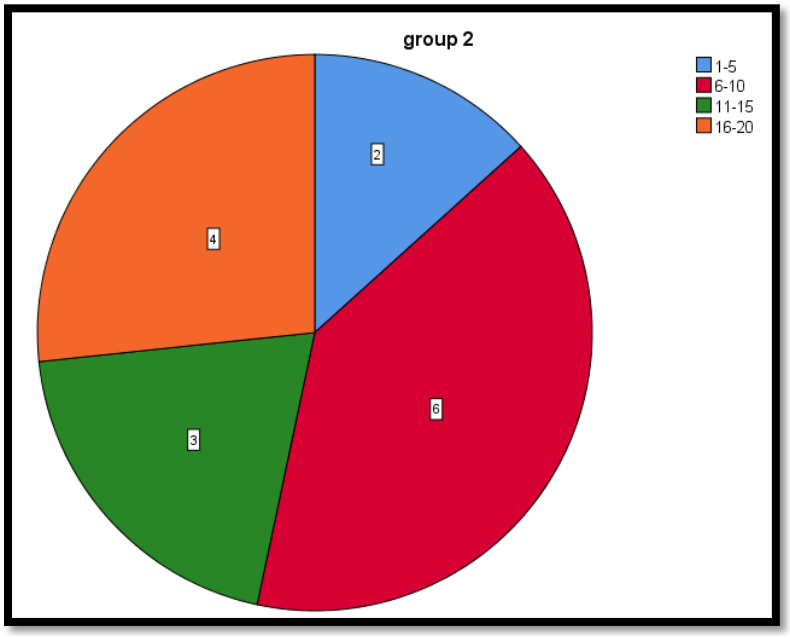
	No. of micronucleus- or (95% CI)	P-value	R- square
Duration	3.0 (1.93-4.06)	<0.001*	0.83*
Frequency	2.0 (0.74-3.26)	0.005	

Table 5:- Frequency of Micronucleus in Group III (Patients with Potentially malignant disorders).

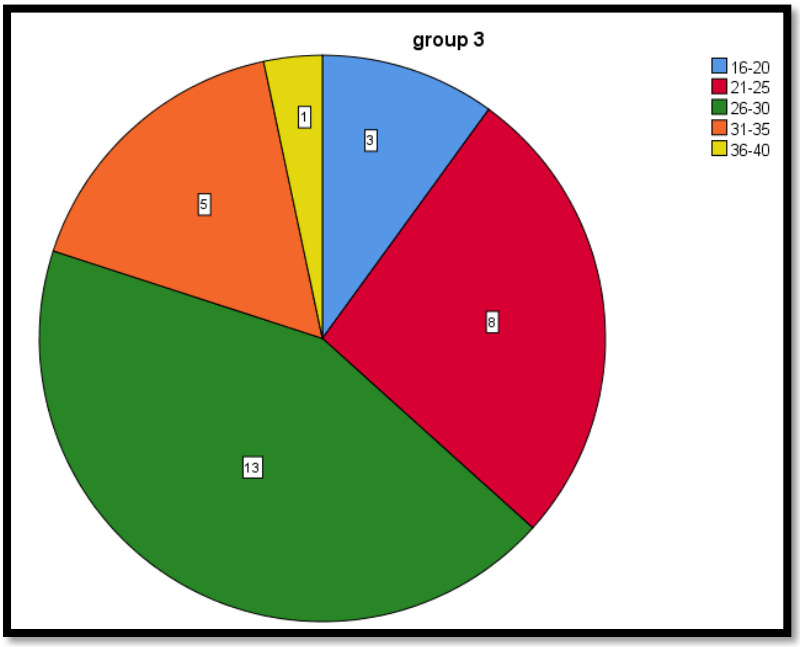
Frequency of Micronucleus in Group III		
	Frequency	Percent
Leukoplakia	11	36.7
Oral Lichen planus	9	30.0
Oral submucous fibrosis	10	33.3



Graph 1:- Incidence of Micronucleus in Group 1

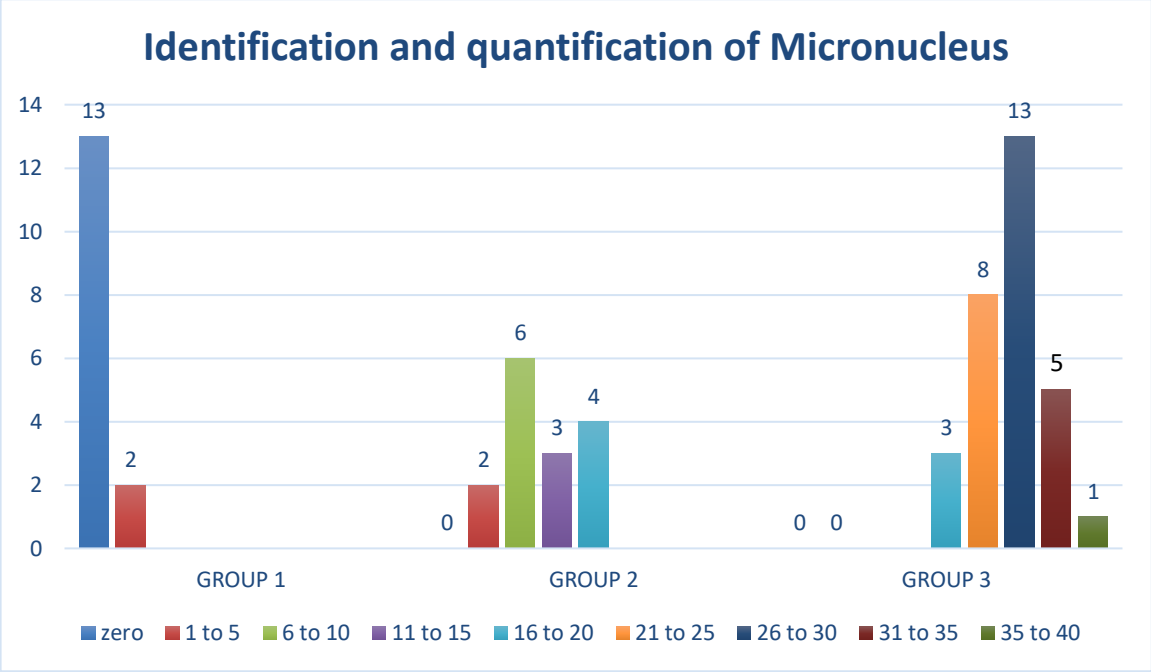


Graph 2:- Incidence of Micronucleus in Group 2.



Graph 3:- Incidence of Micronucleus in Group 3.

Graph 4:- Comparison of Micronucleus in all three groups.



Discussion:-

Micronucleus (MN) is a small, additional nuclei formed by the exclusion of chromosome fragments or whole chromosomes lagging at mitosis. Therefore, MN rates indirectly reflects chromosome breakage or impairment of the mitotic apparatus. The quantitative detection of MN is widely used for analysis of cytogenic damage. About 30 years ago, Stich et al.[12]illuminated a procedure for MN assays with exfoliated buccal mucosa cells, which was widely used in occupational and lifestyle studies. It was repeatedly emphasized that this non invasive 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. The level of baseline chromosome damage in untreated cancer patients and also in various PMDs is much higher than in cancer-free controls. Therefore, MN 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[13,14].

Evans et al[15]. in 1959 made first attempt to monitor cytogenetic damage induced by gamma rays and neutrons in plant material.

In a study conducted among 45 arsenic exposed individuals from west bengal by Chakraborty et al[16]. in 2006 observed that 3.34-fold increase in MN in buccal cells.

Arora P in 2014[17], estimated the potential genotoxic effect of routinely used panoramic radiation exposure in exfoliated epithelial cells as measured by the formation of micronuclei and compared the genotoxicity of X-rays on keratinized epithelial gingival cells and the nonkeratinized buccal epithelial cells and noted significant increase in the MN frequency in buccal epithelial cells after exposure to panoramic radiation.

This method is increasingly used in epidemiological studies for investigating the impact of nutrition, lifestyle factors (alcohol, smoking, drugs, stress), medical procedures (radiation and chemicals), micronutrient deficiency, environmental pollution, chronic contact with arsenic and chromium and also genetic factors such as defects in metabolism or in the repair of DNA and cell death.

According to Joshi M.S., et al.[18] 2011, Nuclear anomalies were compared among chewers, non-chewers and OSMF subjects and related with factors such as consumption, frequency and duration of quid's. MN cells were seennotably

higher among chewers and OSMF subjects as compared to non-chewers. This results was in accordance with our study.

In our study, we included potentially malignant disorders such as Oral submucous fibrosis, leukoplakia and oral lichen planus and compared the MN frequency with healthy individuals. Similar group comparison was done by Grover et al.[19] in 2012.

In our study, we observed a significant increase in total number of micronuclei with increase in duration and frequency of tobacco habit. This similar observation was noted in the study done by Kamath et al.[20] in 2014.

In our study, MN count was not statistically significant in individuals with different tobacco related habits. This observation was negatively correlated with the study done by MR Pradeep et al.[21] in 2014.

The reliability of Feulgen stain for MN assay over Papaincolau (PAP) stain was compared by Kumar et al.[22] in 2016 and observed that high number of MN in Papaincolau stain can be used to detect theunusual cytological changes but not to score MN. Hence, they stated the DNA specific Feulgen stain can be used as a definitive stain to evaluate Mn over the non- specific DNA stain. In our study, we used DNA specific Feulgen stain to interpret MN, because several studies suggested that Feulgen staining method has higher accuracy in examining the MN as compared with nonspecific DNA staining.

In our study, we compared the frequency of MN between individuals with potentially malignant disorders and with habits and healthy individuals. The mean obtained in control group was 0.2±0.5 and habit group (smokers / chewers) 11.0±5.5 which was comparable with study done by Gopal, K. S., & Padma, M.[4] in 2018 i.e., control group was 0.4±1.2 and smokers 7.20±7.083 and chewers 8.00±7.906.

In our study mean of MN frequency were increasing from control to potentially malignant patients, this was in accordance with the study conducted by Sangle, Varsha Ajit et al.[23] in 2016.

The age and gender of the subjects are being reported as the major contributors to MN frequency. So, in our study only males were included. This was in accordance to the study done by Gopal, K. S., & Padma, M.[4] in 2018.

Dave, Gunjan T., et al.[24] in 2019 Compared two groups between controls and histologically proven potentially malignant patients and mean of MN frequency was 6.47+2.240. This was less and not in accordance with our study.

In our study higher frequency of micronucleus was noted in patients with leukoplakia (36.7%) than oral submucous fibrosis (33.3%) and least frequency was seen in oral lichen planus (30.0%). But in the study conducted by Dave, Gunjan T., et al.[24] in 2019, he concluded that occurrence of MN was highest in patients of oral submucous fibrosis followed by leukoplakia and lichen planus patients.

While assessing the incidence of MN in the control group, out of 15 cases, 13 showed no MN among the 500 cells examined, while 2 patients showed a total of 3 MN. Because of the fact that MN formation is not a phenomenon specifically related to exposure to tobacco, it may also reflect the effect of genotoxic agents like environmental pollutants, radiations or chemicals etc. The biomarkers of exposure and effect and clinical disease can all largely be influenced by susceptible 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[4].

Conclusion:-

The observations of this study showed tobacco in any form is genotoxic especially patients with potentially malignant disorders are with higher micronucleus count. So, it can be used as a simple non-invasive, reliable marker for genotoxic evaluation. These genotoxic and carcinogenic chemicals have been described as a potent clastogenic and mutagenic agents which may be responsible for the induction of chromatid/chromosomal aberrations resulting in the production of micronuclei. By observing the results of various studies, it is decided that the gradual increase in micronucleus (MN) counts from normal oral mucosa compared with potentially malignant disorders suggested as a biomarker with neoplastic progression. MN scoring would be highly promising method for screening potentially malignant disorders and also as a biomarkerfor high-risk population oforal cancer. Therefore, it can be used as a screening prognostic and educational aid in community centresfor potentially malignant disorders and risk of oral

cancer.Further Research studies can be employed in patients with histologically proven potentially malignant disorders to signify micronucleus count according to the histological stage.

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Conflicts of interest:
There are no conflicts of interest.

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