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Evaluation of Micronucleus Frequency in Exfoliated Buccal Cells of Tobacco Users with and Without Oral Lesions using Methyl Green-Pyronin Staining

Vegendla Swathi[1], Kiran Kumar Katttappagari[2], Ruttala Sai Praveena[3], Sudheera Perupaga[4], Manem Bala Murali Krishna[5], Poosarla Chandrashekar[6], Baddam Venkat Ramana Reddy[7]

[1]Lotus Dental Clinic, Dilshuknagar, Hyderabad
Email: swathiperni07[at]gmail.com

[2]Professor & HOD, Department of Oral Pathology, Sibar Institute of Dental Sciences, Guntur
Email: *drkirankumark123[at]gmail.com*

[3]Senior Resident, Government Dental College & Hospital, Vijayawada.
Email: *saipraveena1304[at]gmail.com*

[4]Postgraduate, Department of Oral Pathology, Sibar Institute of Dental Sciences, Guntur
Email: sudheeraperupaga[at]gmail.com

[5]Postgraduate, Department of Oral Pathology, Sibar Institute of Dental Sciences, Guntur.
Email: *manembalamurali45[at]gmail.com*

[6]Professor & HOD, Department of Oral Pathology, Sibar Institute of Dental Sciences, Guntur
Email: *chandu1481[at]gmail.com*

[7]Principal, Sibar Institute of Dental Sciences, Guntur
Email: *drramanabv[at]gmail.com*

Abstract: ***Background**: Tobacco consumption in the form of smoking and smokeless is a major etiological factor for mucosal changes and transformation of dysplasia. Genotoxic damage induced by tobacco can be detected by the micronucleus (MN) assay, a simple, non-invasive, and reliable biomarker for assessing chromosomal instability. The Methyl Green–Pyronin (MGP) stain provides excellent nuclear and facilitating accurate identification of micronuclei in exfoliated buccal epithelial cells. Aim: To evaluate and compare the frequency of micronuclei in exfoliated buccal epithelial cells of tobacco users with and without oral lesions using Methyl Green–Pyronin staining. **Materials and Methods**: A cross-sectional comparative study was conducted among 60 participants divided into four groups and each group consists of 15 samples. Exfoliated buccal mucosal cells were collected using a sterile steel spatula, fixed in 95% ethanol, and stained with Methyl Green–Pyronin stain. Micronuclei were identified according to Tolbert's criteria, and 100 epithelial cells per participant were examined under a light microscope. The mean micronucleus frequency was compared using one-way ANOVA followed by post hoc analysis, with a significance level of $p < 0.05$. **Results**: The mean number of micronuclei were high in Group IV. The comparison of four groups with mean micronuclei by one way ANOVA test showed statistically significant with $p \leq 0.05$. The mean number of micronuclei were higher in Group IV using Methyl green Pyronin and showed statistically significant $p \leq 0.05$. **Conclusion**: The micronucleus assay using Methyl Green–Pyronin staining is a simple, economical, and effective cytogenetic technique for detecting early genotoxic changes in the oral mucosa of tobacco users. Increased micronucleus frequency in tobacco users, particularly those with oral lesions, highlights its potential as a valuable biomarker for early screening, risk assessment, and monitoring of individuals at increased risk for malignant transformation.*

Keywords: Micronucleus, Methyl Green–Pyronin stain, Buccal exfoliative cytology, Tobacco smoking, Smokeless tobacco, Genotoxic effect

1. Introduction

Oral cancer is the 6th most common cancer, in India oral cancer accounts 60- 70%. It is most debilitating diseases affecting mankind. The etiological factors for oral cancer is tobacco in the form of chewing or smoking.[1] Tobacco has grabbed millions of people over the world and across various social barriers. Tobacco available in the forms such as smoking or chewing. [2] The word pre cancer coined suggesting that benign it may develop into invasion and finally it leads to malignancies for prolong time.[3] According to WHO proposed the term "Precancerous lesion and pre-cancerous condition later the WHO merged as potentially malignant disorders.[4] Exfoliative cytology is the examination of exfoliated squamous cells from buccal mucosa under microscope. This exfoliative cytology is non-invasive and early detection of potentially malignant disorders.[5]

Micronuclei are genotoxic due to external factors. It is visible in oral exfoliative cytology cells next to nucleus. This originates from aberrant mitosis which causes chromatid fragments and acentric chromosomes.[6] In recent year the study of micronuclei increased in the field of oral cancer. Micronuclei is a biomarker to assess the chromosomal fragmentation which leads to anaphase. Micronuclei in exfoliated buccal squamous cells represents early genotoxic changes due to carcinogenic agents such as tobacco consumption.[7] Micronuclei microscopically observed as

small round chromatin mass present in the cytoplasm next to nucleus. This Micronuclei formed due to aberrant mitosis and formed as acentric chromatic fragments that is not going to formed or incorporated into the daughter nuclei during mitosis. [8] The biproduct of arca nut and tobacco increases reactive oxygen species and it will cause damage to the DNA with in the nucleus and causes aberrations with in the nucleus. [9]

Methyl green-pyronin (MGP) is a special staining technique consists of cationic dyes which is differentiation of DNA and RNA. This stain will go and bind specific for phosphate radicals in double helix of DNA taken up green blue color. The other component called as Pyronin is not affinity to bind the negatively charged RNA staining appears as red color.[10] Methyl Green pyronin (MGP) is simple and low cost – effective histological stain. It is a cationic dye as it has two positive charges. In the oral cavity squamous cells are the first cells will come and contact with the carcinogenic biproducts to reactive products.[11] Oral exfoliative cytology for identification effect of genotoxic as micronuclei as one of the tools for identifications of changes in the nucleus.[12] The aim of the study is to assess the effect of tobacco on the squamous cells by comparing the micronucleus count individuals with a habit of tobacco consumption with or without oral lesions.

2. Materials and Methodology

The present study was carried out in the Department of Oral and Maxillofacial pathology and Microbiology. This study was approved from Institutional Ethics committee (Pr No:224/IEC/SIBAR/2023) and the sample calculation was done using G* power 3.1.9.7 software with effect size 0.718, α error 0.01 and power 0.95. Total sample was 60 in all the four groups. Those willing to participation for this study was obtained written consent and collected personal details such as age, gender, occupation, history of tobacco related habits such as chewing or smoking habits with or without clinical evidence of oral mucosal changes. The total samples were divided into four groups as follows

- Group I – Smokers without oral mucosal lesions (n=15)
- Group II: Chewers without oral mucosal lesions(n=15)
- Group III: Smokers with clinical evidence of oral mucosal lesions (n=15)
- Group IV: Chewers with clinical evidence of oral mucosal lesions(n=15).

The oral exfoliative buccal cells were collected with following inclusion and exclusion criteria. Individuals with habits of smoking for the past one year without clinical evidence of mucosal lesions and Individuals with habits of smoking for the past one year shows clinical evidence of mucosal lesions and Individuals with habits of chewing for the past one year without clinical evidence of mucosal lesions and Individuals with habits of chewing for the past one year shows clinical evidence of mucosal lesions was included and Patients with an anemia or any other systemic diseases, Patients with malignancy were excluded. Before buccal smear collection, participants were instructed to rinse their mouths with water, and the buccal mucosa was gently cleaned using sterile gauze. Exfoliated buccal cells were then collected by scraping the buccal mucosa posterior to the angle of the mouth with a sterile stainless-steel spatula. The collected exfoliated buccal cells were evenly smeared onto the centre of a clean, dry glass slide, with one slide prepared per participant. The smears were immediately fixed in 95% ethanol for 20 minutes to prevent air drying. After fixation, the slides were stained using the Methyl Green–Pyronin staining technique [13] and examined under a binocular light microscope (MAGUS, Japan) at 40× magnification. Each stained slide was examined under high-power magnification, and 10 non-overlapping high-power fields (HPFs) were evaluated for the presence of micronuclei. The slides were screened in a systematic zigzag pattern from one end to the other to avoid repeated counting of the same cells. Micronuclei were counted separately for tobacco smokers, tobacco chewers, and tobacco users with oral lesions. The assessment of micronuclei was done using **Tolbert et al., criteria** 1991.[14]

Statistical analysis:
Data was entered in Microsoft excel sheet and done statical analysis using Statical package for social studies (SPSS) version 20.0 the data between smokers and chewers with control using ANOVA test, comparison of groups with stains using two-way ANOVA test and the individual p value of different groups was obtained. Mean number of micronuclei with three groups was done using Tukeys multiple prosthoc procedures and the individual p value of different groups was obtained. A p value was considered 0.05 significant level.

3. Results

A total of 60 participants were enrolled in the study, with 15 individuals allocated to each of the four groups (Groups I–IV). The gender distribution varied across the groups, with a higher proportion of males observed in Groups I and IV. Comparison of the mean age among the four groups using one-way ANOVA showed that Group III had the highest mean age (35.60 ± 5.91 years). However, the differences in mean age between the groups were not statistically significant ($p \geq 0.05$). The mean number of micronuclei stained with Methyl Green–Pyronin was compared among the four groups. Group IV exhibited the highest mean micronuclei count (70.98 ± 7.33) compared with the other groups. One-way ANOVA demonstrated a statistically significant difference in the mean number of micronuclei among the four groups ($p \leq 0.05$). Pairwise comparisons were performed using Tukey's multiple post hoc test (Graph 1). The mean micronuclei count of Group II versus Group I, Group III versus Group I, and Group IV versus Group I showed statistically significant differences ($p \leq 0.05$). In contrast, the comparison between Group IV and Group III was not statistically significant ($p \geq 0.05$).

4. Discussion

Oral cavity is considered one of the important susceptible to countless changes take place in related to environmental and lifestyle such as habits and other factors. Habits such as tobacco in the form of chewing tobacco or smoking tobacco were significantly increased in young age group. These habits may lead to changes in the oral mucosal buccal cells. The oral buccal cells changes can evident microscopically using special stains such as Papanicolaous stain and Methyl Green pyronin stains will give evidence in relation with cellular,

nuclear and cytoplasmic alterations. These stains can be used for identification or diagnosis for malignancy.[15]

Oral cancer is the sixth most common malignancy worldwide and the second most common cancer affecting the head and neck region. It represents a significant public health burden due to its high morbidity and mortality. The incidence of oral cancer continues to rise, particularly in populations with widespread tobacco use in both smokeless (chewing) and smoked forms. Tobacco consumption remains the most important modifiable risk factor, substantially contributing to the development and progression of oral malignancies.[16] The global burden of oral cancer continues to increase, with approximately 500,000 new cases diagnosed annually. Despite advances in diagnostic and therapeutic approaches, oral cancer remains associated with substantial morbidity and mortality, largely due to delayed diagnosis and presentation at advanced stages. In India, tobacco consumption in both smoked and smokeless forms is a major public health concern and is responsible for a significant proportion of cancer-related deaths. According to the World Health Organization (WHO), tobacco use is one of the leading preventable causes of mortality, with tobacco-related diseases accounting for a considerable disease burden in the country. Early detection and timely intervention are therefore essential to improve survival rates and reduce the overall burden of oral cancer.[17,18]

Cancer develops through a multistep process driven by the progressive accumulation of genetic alterations that disrupt normal cellular homeostasis. DNA damage and genomic instability play pivotal roles in carcinogenesis by promoting mutations in oncogenes, tumor suppressor genes, and DNA repair genes. In addition to genetic mutations, epigenetic modifications and phenotypic alterations contribute significantly to the initiation, progression, and malignant transformation of oral potentially malignant disorders into invasive oral squamous cell carcinoma.[19] Histopathological examination of a biopsy specimen remains the gold standard for the definitive diagnosis of oral cancer. However, the invasive nature of biopsy limits its utility as a routine screening tool for large populations. Consequently, several non-invasive diagnostic techniques have been developed for the early detection of oral cancer and oral potentially malignant disorders. Among these, oral exfoliative cytology has emerged as a simple, safe, cost-effective, and minimally invasive screening method. The assessment of cytogenetic biomarkers, particularly micronuclei, in exfoliated buccal epithelial cells has gained considerable attention as a reliable indicator of chromosomal damage and genomic instability, facilitating the early identification of premalignant and malignant changes.[20] Tobacco use is the most significant modifiable risk factor for the development of oral potentially malignant disorders (OPMDs) and oral cancer. It contributes to oral carcinogenesis through multiple molecular and cellular mechanisms, including the induction of DNA damage, oxidative stress, chronic inflammation, and immune dysregulation. Tobacco contains numerous carcinogenic compounds, among which polycyclic aromatic hydrocarbons (PAHs), tobacco-specific nitrosamines (TSNAs), and aromatic amines are considered the principal agents responsible for initiating and promoting malignant transformation of the oral epithelium. In addition to its carcinogenic effects, tobacco adversely affects the host immune response, increasing susceptibility to periodontal diseases, delaying wound healing, and impairing tissue repair following periodontal and surgical treatment. Collectively, these effects contribute significantly to the initiation, progression, and poor prognosis of oral diseases, including oral squamous cell carcinoma.[1] The most common forms of smoked tobacco include cigarettes, bidis, and chutta, whereas smokeless tobacco is predominantly consumed as chewing tobacco alone or in combination with betel quid. Both smoked and smokeless forms of tobacco expose the oral mucosa to a wide range of carcinogens capable of inducing genetic and cytological damage. One of the earliest manifestations of such genotoxic injury is the formation of micronuclei, which arise from chromosomal breakage or mitotic spindle dysfunction during cell division. The frequency of micronuclei in exfoliated oral epithelial cells reflects the cellular response to chronic exposure to tobacco-derived carcinogens and serves as a sensitive biomarker of chromosomal damage, genomic instability, and the early stages of oral carcinogenesis.[21]

Early detection of oral potentially malignant disorders (OPMDs) and premalignant changes in the oral mucosa is essential for preventing their progression to invasive oral cancer. Timely diagnosis and appropriate intervention can facilitate early treatment, improve patient survival rates, reduce disease-related morbidity, and significantly enhance the quality of life of affected individuals.[22] Although biopsy followed by histopathological examination remains the gold standard for the diagnosis of oral potentially malignant disorders (OPMDs), its invasive nature may result in patient apprehension, discomfort, and psychological stress, limiting its acceptance as a routine screening procedure. Consequently, oral exfoliative cytology has emerged as a valuable preliminary diagnostic adjunct for the early detection of cellular and nuclear alterations associated with premalignant and malignant transformation. As a non-invasive, simple, rapid, and cost-effective technique, oral exfoliative cytology can be performed easily in routine clinical and chairside settings without causing significant trauma to the oral mucosa. These advantages make it a useful screening tool for identifying individuals who require further diagnostic evaluation by biopsy and histopathological examination.[23]

Micronuclei are small, extranuclear cytoplasmic bodies that originate from acentric chromosomal fragments or whole chromosomes that fail to be incorporated into the daughter nuclei during mitosis. Their formation is a well-established indicator of chromosomal damage and genomic instability induced by genotoxic agents, including tobacco in both smoked and smokeless forms. The assessment of micronuclei in exfoliated oral epithelial cells has emerged as a reliable and non-invasive biomarker for the early detection of nuclear abnormalities associated with oral potentially malignant disorders and oral carcinogenesis.[24] Micronuclei are readily identified in exfoliated oral epithelial cells obtained by oral exfoliative cytology. The assessment of micronucleus frequency in buccal mucosal cells has emerged as a valuable, non-invasive biomarker for detecting early carcinogenic changes in the oral epithelium. Micronuclei arise as a consequence of chromosomal breakage (clastogenic events) or chromosome loss resulting from mitotic spindle dysfunction (aneugenic events), thereby reflecting

chromosomal instability and genomic damage. Their increased frequency is directly associated with genotoxic exposure and genetic damage, making the micronucleus assay a sensitive indicator of early carcinogenesis and the malignant transformation of oral potentially malignant disorders.[5]

Oral exfoliative cytology is a well-established, non-invasive diagnostic adjunct for the early detection of oral potentially malignant disorders (OPMDs) and oral cancer. This technique enables the evaluation of exfoliated epithelial cells for a wide range of cytomorphological alterations associated with malignant transformation. Characteristic cellular and nuclear abnormalities observed in exfoliative cytology include karyorrhexis, karyolysis, pyknosis, binucleation, and micronucleus formation. These cytological changes reflect underlying genetic damage, cellular injury, and genomic instability, making oral exfoliative cytology a valuable screening tool for the early identification of premalignant and malignant lesions.[25]

The findings of Piyathilake C.J. et al. in 1995 suggest that micronucleus analysis in exfoliated buccal cells is a useful biomarker for assessing tobacco-induced genotoxic damage. The study demonstrated that the micronucleus assay is a feasible and non-invasive approach for the early detection of cellular alterations associated with oral carcinogenesis. Furthermore, the absence of a significant gender-based difference indicates that micronucleus frequency is more closely associated with tobacco exposure than with sex.[26] In the present study, the proportion of male participants was higher than that of female participants. The higher representation of males may be attributed to the greater prevalence of tobacco consumption among men, although gender differences were not found to significantly influence the assessment of micronucleus frequency.

Ravi Mehrotra et al. et al., 2006 they observed suggesting that age may influence the clinical presentation and progression of different oral potentially malignant disorders.[27] Raman R.K. et al. et al., 2019 evaluated the efficacy of Methyl Green–Pyronin Y (MGP) staining in oral exfoliated cells from patients with oral leukoplakia and oral squamous cell carcinoma using cytomorphometric analysis they observed that cytomorphometric assessment demonstrated significant differences in nuclear morphology, including micronucleus frequency, nuclear-cytoplasmic ratio, cellular diameter, and nuclear contour index among the study groups.[28]

Dosi T. et al. 2016 evaluated micronucleus frequency in individuals with oral precancerous lesions (OPLs) and habit-matched controls using the Methyl Green- Pyronin staining technique they observed that micronucleus assay is a simple, non-invasive, and cost-effective screening tool with considerable potential for large-scale oral cancer screening and the early detection of tobacco-induced genotoxic damage.[29] Vaid N. et al. in 2019 did a study to observe frequency of micronuclei in tobacco chewers and individuals with oral potentially malignant disorders (OPMDs) to evaluate micronuclei as a biomarker of genotoxic damage. The study concluded that micronucleus assessment is a sensitive and reliable indicator of early nuclear damage associated with tobacco exposure and oral carcinogenesis.[30] Similarly, in the present study, the mean micronucleus frequency assessed using Methyl Green–Pyronin staining showed statistically significant differences among three of the study groups ($p \leq 0.005$), corroborating the findings of the previous study.

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Figures and Tables

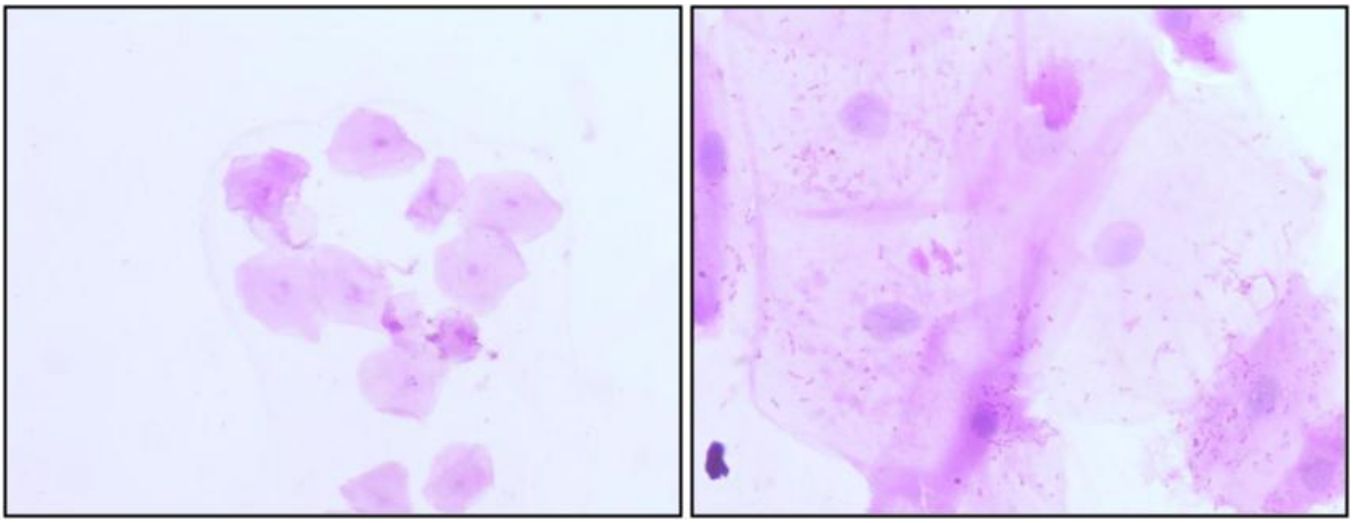
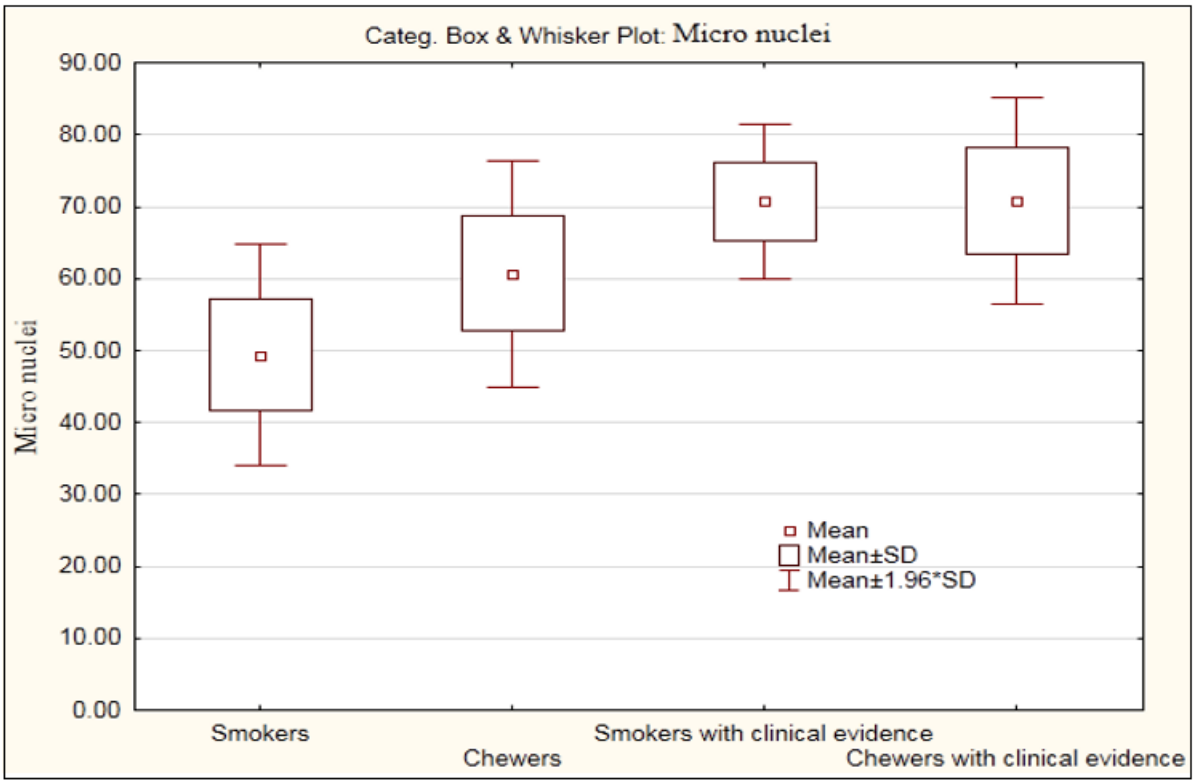


Figure 1and 2: Photomicrograph of methyl green pyronin stained cytological exfoliative buccal cells shows micronuclei in chewers with clinical evidence of oral mucosal lesions



Graph 1: Pair wise comparison of four groups with mean micronuclei using Methyl green Pyronin stain by Box & Whisker plot procedures