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QUANTITATIVE ASSESSMENT OF MICRONUCLEI IN ORAL EXFOLIATIVE BUCCAL SQUAMOUS CELLS IN SMOKERS, CHEWERS, LEUKOPLAKIA AND ORAL SQUAMOUS CELL CARCINOMA USING PAPANICOLAOUS STAIN AND GIEMSA STAIN

Dentistry

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ABSTRACT

Micronuclei (MN) are small, extranuclear bodies that serve as biomarkers of genotoxic damage and chromosomal instability in oral exfoliative cytology. Their presence in exfoliated oral epithelial cells has been associated with increased risk of malignant transformation. This study aims to evaluate and compare the frequency of micronuclei in buccal squamous cells among smokers, tobacco chewers, patients with leukoplakia, and those with oral squamous cell carcinoma (OSCC), using Papanicolaou (Pap) and Giemsa stains. **Materials And Methods:** A total of 120 individuals were divided into four groups: Group I (Tobacco smokers), Group II (Tobacco chewers), Group III (Leukoplakia), Group IV (Oral squamous cell carcinoma), with 30 subjects in each group. Buccal cell smears were collected using exfoliative cytology. The slides were stained using Pap and Giemsa stains. A total of 100 cells per subject were analyzed for the presence of micronuclei under a light microscope using established scoring criteria. **Results:** The frequency of micronuclei was significantly higher in Groups IV compared to the other group and it was statistically significant with $p < 0.001$. The highest mean MN count was observed in the OSCC group, followed by leukoplakia, chewers, and smokers. Both staining techniques successfully demonstrated micronuclei, with Giemsa showing slightly higher contrast in nuclear morphology, while Pap stain provided better cytoplasmic clarity. **Conclusion:** There is a progressive increase in MN frequency from smokers and chewers to leukoplakia and Oral Squamous cell carcinoma conditions, suggesting a strong correlation between tobacco-related habits and genotoxic damage. Micronucleus assay using Pap and Giemsa stains in exfoliated buccal cells can serve as a cost-effective, non-invasive tool for early detection and risk assessment of oral precancer and cancer.

KEYWORDS

Micronuclei, Leukoplakia, Oral squamous cell carcinoma, Papanicoulus, Giemsa stain

INTRODUCTION:

The main cause of oral cancer is consumption of tobacco in various forms such as chewing and smoking leads to oral potentially malignant disorders and oral cancer.[1] Different forms of tobacco, either chewing or smoking are widely used in India and South East Asian countries. The major smoking forms of tobacco that is used in the form of cigarettes, beedis, cigars and chutta.[2] Chewing tobacco in form of loose leaf or dry powdered tobacco is often mixed with various ingredients according to the local custom and consumed in various forms.[3]

Exfoliative cytology is microscopic examination of desquamated cells from the buccal mucosa. This procedure is noninvasive with higher conformity and an attractive technique for the early diagnosis of oral lesions. Micronucleus (MN) is defined as a microscopically visible, round or oval cytoplasmic chromatic mass next to the nucleus. It originates from aberrant mitosis, which consists of chromatic fragments and acentric chromosome[4] MN in oral exfoliated cells are a marker of chromosomal damage caused by genetic toxic agents from tobacco related substance, alcohol, etc. [5] MN in exfoliated cells is a promising tool for the study of epithelial carcinogens and can be used to detect chromosome breakage or mitotic interference, thought to be relevant to carcinogenesis[6]

The Papanicolaous and Giemsa stain is universal stain for exfoliative cytological preparations.[7] This special stain imparts a different color to the cytoplasm of the epithelial cells based on their degree of cellular differentiated. Increase in the number of MN gives most important criteria for diagnosing potentially malignant disorders, micronuclei (MN) assay provides a simple indicator of genotoxic damage. Hence the main aim of our study was quantitative assessment of micronuclei in oral exfoliative buccal squamous cells in smokers, chewers, Leukoplakia and Oral squamous cell carcinoma using Papanicolous stain and Giemsa stain.[8]

MATERIALS AND METHODOLOGY:

It is a prospective cross-sectional study among the individuals those come to Department of Oral Pathology with chief complaint of stains and calculus, burning sensation, clinically white patch or plaque and ulcero proliferative lesions was included for the period of three months. A total of 120 subjects with who were willing to participate the study with inclusion criteria were individuals with habits of tobacco consumption either in smoking or chewing for minimum of six months to 2 years, patients with clinically white patch or plaque and ulcer proliferative lesions. Individuals with no other known local or systemic disorders and Patients with long standing medication were excluded. The study was obtained clearance with Institutional Ethics Committee with Pr.421/IEC/SIBAR/2024. Based on inclusion and exclusion criteria the subjected were grouped as follows

- Group I: Tobacco smoking habit (n= 30)
- Group II: Tobacco chewing habit (n=30)
- Group III: Leukoplakia (n=30)
- Group IV: Oral squamous cell carcinoma (n=30)

Methodology:

Before starting we explain the study and procedure in detail with subjects and obtained informed consent. After obtained personal details of each individual such as age, gender, occupation and history of tobacco related habits such as chewing and smoking habits was be recorded. After taking detail history of duration of the habits we examined oral mucosa using diagnostic instruments.

Collection Of Samples:
Before collecting the sample, subjects were asked to rinse the mouth with normal saline and buccal mucosa was cleaned with the help of gauze piece. Using plastic agate spatula, the buccal mucosa just behind the angle of the mouth was gently scraped to collect smear from both healthy individuals and study group subjects.

Smear Preparation:
The Armamentarium for taking buccal smear in all the subjects and exfoliated cells scraping from each subject were smeared on to the center of plain dry microscopic glass slide. Two smears prepared on two slides and fixed with 95% alcohol then one slide was stained with Papanicolaous stain and the other slide with Giemsa stain. The stained smear was examined in a binocular microscope under 40X magnification. (Magnus MLXi TR plus LED Penta Head microscope, Uvsar India, Ghaziabad, Uttar Pradesh) In each slide100 cells without any overlap were counted for the presence of micronuclei. The cells were counted starting from one corner of the slide to the other end in an orderly zigzag pattern to avoid repeated counting of the same cells. The counting of micronuclei was done separately for both Papanicolaous (PAP) stain and Giemsa-stained slides and the results were tabulated. The counting of micronuclei was done with good cellular outline, well stained cytoplasm, single hyper chromatic nucleus with micronuclei, Binucleated cells, karyolysis, Karyohexis, and colonizing bacteria which look like micronuclei surrounding the nucleus circle were not taken for counting.

The identification and counting of micronuclei were done according to Tolbert etal., in 1991criteria, as follows
- Round smooth perimeter suggestive of membrane
- Less than a third the diameter of a associated nucleus, but large enough to discern shape and color
- Staining intensity similar to nucleus
- Texture similar to nucleus
- Same focal plane as nucleus
- Absence of overlap with or bridge to nucleus.[9]

Statistical Analysis:
Data was entered in Microsoft excel sheet and done stastical analysis using Stastical package for social studies (SPSS) version 17.0. 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.

RESULTS:
In the present study carried with 120 subjects was divided into four group in each group a total of 30 subjects was included. The mean number of micronuclei in all four groups was 266.15 ± 73. Among each group the maximum mean number of micronuclei were m in Group IV with 330.4 ± 35 and minimum in Group I with 201.4 ± 58. The mean number of micronuclei in PAP stain in all four groups was 233.3 ± 70, where as in Giemsa stain in all four groups showing 298.9 ±61. When comparison of mean micronuclei was higher in Giemsa when compared with PAP stain. Comparison with mean and SD of micronuclei using PAP stain with Giemsa stains in all four groups. Giemsa stain showed Group IV showed highest mean number of micronuclei i.e. 335.91 ± 40 where as in PAP stain it was 326.28 ± 28. Comparison of four groups in relation with mean number of micronuclei with two stains (PAP and Giemsa) using Two-way ANOVA showing statistically significant with $p \leq 0.05$. (Table 1)

Pair wise comparison of mean micronuclei using PAP and Giemsa stain with all four groups by Tukeys multiple posthoc procedures. Pair wise comparison of micronuclei intra and intra groups in all four groups i.e Group I compared with all three groups, Group II with Group I and Group IV, Group III with Group I , Group IV showed smokers highly stastically significant ($p \leq 0.0001$) Pair wise comparison of two stains (PAP and Giemsa) with mean number of Micronuclei by Tukeys multiple posthoc procedures showed Giemsa stain with mean number of micronuclei was higher than PAP and it was statistically highly significant ($p \leq 0.0001$) Pair wise comparison of four groups with PAP and Giemsa stain showed statistically highly significant ($p \leq 0.0001$).

DISCUSSION:
Tobacco use is one of the most important risk factors for potentially malignant disorders and oral cancer.[1] Tobacco contributes to the pathogenesis of oral diseases through many pathways; some may have more impact on pathogenesis of diseases. Among many carcinogenesis present in the tobacco, the polycyclic aromatic hydrocarbons, aromatic amines and nitrosamines are very important components causes oral cancer. The effect of tobacco on the immune system plays on a major role in the development of periodontal diseases and impaired healing after treatment of the diseases.[2] Common form of tobacco smoking habits including cigarettes, beedis and chutta. Betel quid chewing with tobacco or chewing tobacco alone is the most common smokeless tobacco habits. The concept of micronuclei occurs due to exposure of any form of tobacco can be due to the nature of cellular response to stimuli from the end products of different types of tobacco usages.[10]

Oral cancer is the sixth most common type of cancer, with India contributing to almost one-third of the total burden, and the second country having the highest number of oral cancer cases.[11] Studying pre-neoplastic diseases and oral malignancies via exfoliative cytology is an economical, non-invasive and easy procedure for studying biomarkers of neoplastic changes in oral cells.[12] Biomarkers of genetic damage have immense potential to reveal the stage of the disease and are helpful in early diagnosis and progression of the disease. Among the various chairside investigations of lesions at an early stage, the micronuclei assay using Exfoliative cytology is a reliable procedure for diagnosing individuals who are at high risk of developing malignancy.[9] Buccal cells are the first barrier for carcinogens inhaled or ingested and are capable of metabolizing carcinogens into reactive products. These carcinogens that cause genetic damage to stem cells in the basal layer are referred to as micronuclei during nuclear division. These chromosomal fragments or whole chromosomes that did not reach the spindle pole thus lag behind anaphase during nuclear division and are not incorporated into the daughter nuclei in telophase but are covered by a nuclear membrane resembling a small nucleus. Thus, the cells were termed micronuclei. As the oral epithelium is constantly maintained by cell renewal, which occurs at the basal layer by mitosis, damaged cells with disrupted nuclear division migrate to the surface along with other cells and are collected via exfoliative cytology for evaluation.[13]

Palaskar et al., and Himanta et al. have shown in their study that the frequency of micronuclei is more in patients who are habituated to smokeless tobacco rather than the smoking form of tobacco.[14,15] This could be because of the micronucleus in oral buccal cells is considered to be a biomarker of chromosomal damage caused by genotoxic agents from substances related to tobacco, tobacco itself, and alcohol. The induction of micronucleated cells by carcinogens and mutagens is a sign of the genotoxic effect of such substances.

Our results, the micronucleated cell and micronuclei frequency in oral exfoliated cells was more in individuals with oral squamous cell carcinoma in comparison with smoking, chewing tobacco and leukoplakia indicating DNA damage. The multiple micronucleation in the target tissue indicates extensive genetic damage resulting in chromosomal instability, which is a hallmark of human tumors.[10] Our findings are in accordance with other studies carried out on oral mucosal cells.

CONCLUSIONS:
The present study concluding that number of micronuclei provides the evidence that consumption of oral squamous cell carcinoma in the form of chewing and smoking tobacco are high risk zone for developing oral cancer. In cellular levels these factors such as smokeless tobacco and smoking tobacco was effect on genetic components present in cells. These changes were more observed in smokeless tobacco use were more than that in smokers, thus indicating further carcinogenic potential of smokeless tobacco. Micronuclei assay can be used as biomarkers of genotoxicity and carcinogenesis progression. However to establish relationship between smokeless tobacco consumption influence on micronuclei has to perform more research to establish its relation as potential biomarkers for oral carcinogenesis.

Conflict Of Interest: There are no conflicts of interest

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Table 1: Comparison Of Four Groups And Two Stains (PAP and Giemsa) With Mean Number Of Micronuclei By Two Way ANOVA

Sources of variation	Sum of squares	Degrees of freedom	Mean sum of squares	F-value	p-value
Main effects					
Group	505061.18	3	168353.73	94.4248	0.0001*
Stain	258136.00	1	258136.00	144.7812	0.0001*
2-way interaction effects					
Group*Stain	121130.88	3	40376.96	22.6463	0.0001*
Error	413641.83	232	1782.94		
Total	1297969.90	239			

***p<0.05**

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