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EVALUATION OF MICRONUCLEI USING GIEMSA AND ACRIDINE ORANGE STAIN IN EXFOLIATED BUCCAL CELLS OF SMOKERS AND NON SMOKERS - A COMPARATIVE STUDY

Dental Science

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ABSTRACT

Micronuclei are extranuclear cytoplasmic bodies induced in cells by numerous genotoxic agents that damage the chromosomes. The micronucleus test is used as an indicator of genotoxic exposition, since it is associated with chromosome aberrations. The buccal cell micronucleus assay provides information on the cytogenic damage in the tissues that are targets of human carcinogens and from which carcinomas can develop.

Aim and Objective: The present study aims to compare Giemsa and Acridine Orange stains in assessing the micronuclei in the exfoliated cells from the buccal scrapings in smokers and non smokers.

Material and Methods: 31 smears were taken from both smokers and nonsmokers and stained with Giemsa and Acridine Orange. The stained smears were evaluated for the presence of micronuclei. After the evaluation of the slides the results were compiled and subjected to statistical analysis.

Results: A significant increase in the frequency of micronuclei in smokers was noted compared to non smokers. Correlation analysis was performed using Pearson's correlation between Giemsa and Acridine Orange. Comparisons were made at the significance level of 0.01 (2-tailed). The values obtained after Pearson's correlation were found to be statistically significant in Giemsa than Acridine Orange.

Conclusion: The present study concludes that, the micronucleus assay in exfoliated buccal cells holds promise as a specific biomarker of genotoxicity, for screening of oral cancer and as prognostic indicator.

KEYWORDS

Smokers, Non smokers, Micronuclei, Acridine Orange, Giemsa.

INTRODUCTION

Oral cancer is one of the tenth most common cancer as stated by WHO and each year 75,000 new cases and 3,20,000 deaths occurs worldwide. Nearly 85% of all cancers have an environmental component and stressors and is therefore it is imperative and important to identify any potential genetic toxicity due to these environmental agents and to assess their biological impact on man.[1] Cigarette smoking, chewing of tobacco and their derivations are the major risk factors of oral cancer.[2]

Chronic exposure to tobacco carcinogens in the oral mucosa causes genetic changes in the epithelial cells. Cumulative genetic changes lead to genomic instability, development of premalignant lesions, and eventually invasive carcinoma.[3]

Tobacco induces proliferative activity through activation of the EGFR receptor and its downstream mechanisms. This activates cyclin D1, leading to greater proliferative activity and higher frequency of mutations, thus rendering the cell more susceptible to permanent genetic changes, that in turn may give rise to genomic instability and invasive carcinoma.[3]

The carcinogenic effect of cigarette smoking is driven largely by the mutagenicity of various chemicals in the smoke. Tobacco smoke induces an array of genetic aberrations, including gene mutations, chromosome aberrations, micronuclei, sister chromatin exchanges, DNA strand breaks, and oxidative DNA adducts in various models.[4]

Elaborate invasive as well as non invasive techniques have been discovered and at present used for early cancer detection and mass screening. One such reliable and sensitive diagnostic tool is exfoliative cytology. It is a useful screening method for detection of oral cancer.[5]

The study of DNA damage in exfoliated cells collected from the oral cavity holds great promise as a minimally invasive method for monitoring populations exposed to genotoxic agents. The presence of micronuclei (MN) and other nuclear anomalies within these cells has been shown to be associated with genetic defects in genome maintenance, accelerated ageing, exposure to genotoxic agents, oral cancer risk and neurodegenerative diseases and was also used in chemopreventive studies.[5]

Micronuclei are extranuclear cytoplasmic DNA bodies which are induced in cells by numerous genotoxic agents that damage chromosome. [6] Buccal cell micronuclei are a putative biomarker for oral cancer risk; evidence suggests that micronuclei are elevated in buccal mucosal cells of persons who harbor precancerous lesions and in cancer patients.[4]

The micronucleus (MN) assay in exfoliated buccal cells is a useful and minimally invasive method for monitoring genetic damage and serves as a tool for early detection of cancerous and precancerous lesions, which is really the need for the hour.[7]

Micronucleus assay is based on the recognition of extra nuclear cytoplasmic DNA bodies which needs accuracy. Various stains have been used for the same, but only little attention has been given to the effect of different staining procedures on the result of micronuclei assay.

The present study was undertaken to assess micronucleus frequency in the smears taken from the exfoliated buccal mucosal cells of smokers and non smokers and further to investigate the effect of different stains on the results of micronuclei study.

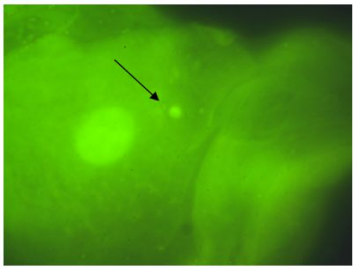
MATERIAL AND METHODS

31 Patients with a history of smoking were selected as the study group and 31 healthy patients without any habits were selected as control group depending upon the fulfillment of inclusion and exclusion criteria. Patients with existing oral mucosal lesions were excluded from the study.

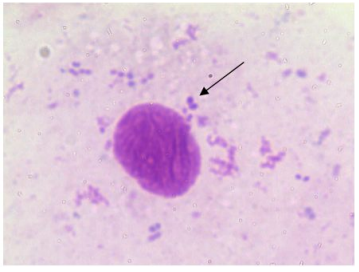
Collection of cells:

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

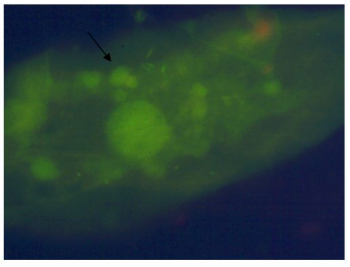
The fixed smears were stained with Acridine orange and Giemsa stains and the stained cells were focused under fluorescent microscope for Acridine Orange and under light microscope for Giemsa. (Photomicrograph 1,2,3,4)



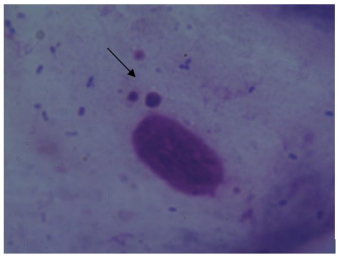
PHOTOMICROGRAPH 1: MICRONUCLEI IN NON SMOKER USING ACRIDINE ORANGE (100X)



PHOTOMICROGRAPH 2: MICRONUCLEI IN NON SMOKER USING GIEMSA (100X)



PHOTOMICROGRAPH 3: MICRONUCLEI IN SMOKER USING ACRIDINE ORANGE (100X)



PHOTOMICROGRAPH 4: MICRONUCLEI IN SMOKER USING GIEMSA (100X)

100 cells from each sample were focused under fluorescent microscope for Acridine Orange and under light microscope for Giemsa and the number of micronucleated cells (MN) were counted.

The scoring of micronuclei was done according to the criteria established by Countryman et al[8,9,10,11]

MN must

- Be less than 1/5th to 1/3rd diameter of the main nucleus
- Be on the same plane of focus with main nucleus
- Have the same colour, texture and refraction as the main nucleus
- Have smooth oval or round shape
- Be clearly separated from the main nucleus

Normal Values For MN Frequencies In Epithelial Cells

The average MN frequency reported in healthy population is 1-3 per 1000 cells, with no significant variation between different types of exfoliated cells.[12]

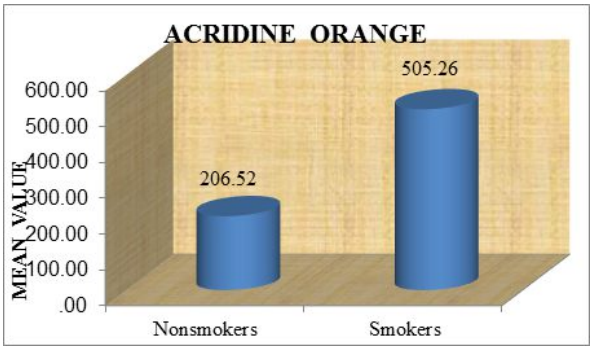
Results and Observation

The results obtained were compiled using MS Excel Worksheet and analyzed using statistical software SPSS version 17.

The mean value of the micronuclei frequency in smokers after staining with Acridine Orange is 505.26 and the mean value of the micronuclei frequency in non smokers is 206.52. The micronuclei frequencies scored are significantly higher in smokers than in non smokers. Standard deviation in smokers is 33.59 and that in non smokers is 28.14. The mean percentage of micronuclei frequency in smokers is 505.26± 33.59 and that in non smokers is 206.52± 28.14. t- Test was done for the given values and a result of 37.96 was obtained. The values were found to be statistically significant ($p < 0.001$). (Table 1,Graph 1)

Group	N	Mean	Standard Deviation	T
Smokers	31	505.26	33.59	37.96
Non smokers	31	206.52	28.14	P < 0.001

Table 1: Table showing the mean values of micronuclei in smokers and non smokers using Acridine Orange stain.

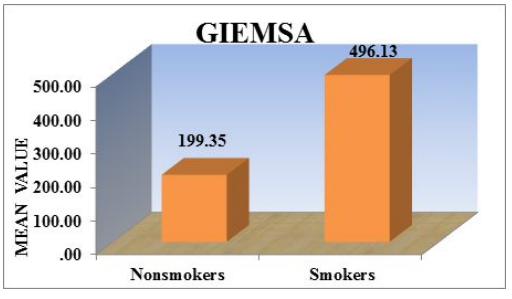


GRAPH 1: MEAN VALUES OF MICRONUCLEI IN SMOKERS AND NON SMOKERS USING ACRIDINE ORANGE STAIN.

The mean value of the micronuclei frequency in smokers after staining with Giemsa is 496.13 and the mean value of the micronuclei frequency in non smokers is 119.35. The micronuclei frequencies scored are significantly higher in smokers than in non smokers. Standard deviation in smokers is 32.98 and that in non smokers is 25.51. The mean percentage of micronuclei frequency in smokers is 496.13 ± 32.98 and that in non smokers is 119.35± 25.51. t- Test was done for the given values and a result of 39.63 was obtained The values were found to be statistically significant ($p < 0.001$). (Table 2, Graph 2)

Group	N	Mean	Standard Deviation	T
Smokers	31	496.13	32.98	39.63 P < 0.001
Non smokers	31	119.35	25.51	

Table 2: Table showing the mean values of smokers and non smokers using Giemsa stain.



GRAPH 2: MEAN VALUES OF MICRONUCLEI IN SMOKERS AND NON SMOKERS USING GIEMSA STAIN.

Correlation between Acridine Orange and Giemsa stain was done to evaluate the effect of different staining procedures on the result of micronuclei study.

Non Smokers

		Giemsa
Acridine Orange	Pearson's correlation	0.963 < 0.001
	Sig [2-tailed]	
	N	31

Table 5: Pearson's correlation between Giemsa and Acridine Orange in non smokers

Smokers

		Giemsa
Acridine Orange	Pearson's correlation	0.993** 0.000
	Sig [2-tailed]	
	N	31

** Correlation is significant at the 0.01 level (2-tailed).
Table 6: Pearson's correlation between Giemsa and Acridine Orange in smokers

Correlation analysis was performed using Pearson's correlation. Comparisons were made at the significance level of 0.01(2-tailed). The values obtained after Pearson's correlation were found to be statistically significant in Giemsa than Acridine Orange.

The present study showed that the frequency of micronuclei is increased in smokers when compared to non smokers, implicating that smoking causes genotoxic damage to epithelial cells.

The present study also showed that the non specific stain, Giemsa is a better stain for the evaluation of micronuclei than DNA specific stain Acridine Orange.

DISCUSSION

Majority of oral cancers are caused by habits like tobacco smoking, tobacco chewing and alcohol consumption. It has been established that there is a dose-response relationship between the amount of tobacco product used and the development of oral cancer.[13]

Oral squamous cell carcinomas are characterized by complex karyotypes that involve many chromosomal deletions, translocations and structural abnormalities. Cells from these types of tumors often have errors in chromosome segregation that lead to the formation of a lagging chromosome or chromosome parts that become lost during the anaphase stage of cell separation and are excluded from the reforming nuclei. The laggards are observed in the cytoplasm as micronuclei.[14]

The study of DNA damage in exfoliated cells collected from the oral cavity holds great promise as a minimally invasive method for monitoring populations exposed to genotoxic agents. The presence of micronuclei (MN) and other nuclear anomalies within these cells has been shown to be associated with genetic defects in genome maintenance, accelerated ageing, exposure to genotoxic agents, oral cancer risk and neurodegenerative diseases.[15]

MN are small fragments of extranuclear DNA formed during cell division, which provide a nonspecific but quantifiable marker of DNA damage, so it is used to identify cellular damage caused by carcinogenic agents. Smoking is a well-known source of carcinogenic influence in humans. More than 4 000 chemical substances, such as acetone, benzene, benzopyrene, cyanamide, methane, which are found in cigarette smoke, are carcinogenic.[16]

The buccal cell micronucleus (MN) assay was first proposed in 1983 and it continues to gain popularity as a biomarker of genetic damage in numerous applications. MN assays provide information on the cytogenetic damage in the tissues, that are targets of human carcinogens and from which carcinomas can develop.[14]

Various studies have reported that the micronuclei is also seen in blood lymphocytes and urinary bladder epithelial cells for measuring the clastogenic effect as stated by Majer BJ et al.[17] In the present study the analysis of micronuclei is done exclusively by taking the buccal mucosal scrapings.

The major advantage of exfoliative cytology is the noninvasive character of the technique, which allows a simple and pain-free collection of intact cells from different layers in the epithelium for microscopic examination and quantitative evaluation.[18]

Moore et al said that in his work the buccal cells were scraped by the help of a wood tongue depressor.[19] According to Ahmer EL et al the buccal cells also can be scraped by using a cotton swab.[20] Cells were also scraped by using a short bristle cytobrush in a study by Besarati Nia et al[21] and also with the help of a tooth brush in a study by Marchand LL et al.[22] In the present study buccal scrapings were taken by the help of wooden spatula.

An evaluation of the literature shows that a variety of different stains is used in micronuclei studies. Among the DNA-specific stains, the ones which are most widely used are Feulgen and Acridine Orange; in some experiments, 4, 6-diamidino-2-phenylindole (DAPI) and propidium iodide were also used. About 30% of the studies on epithelial cells were conducted by using nonspecific stains (PAP, H & E, Giemsa, May-Grunwald's Giemsa, and less frequently, Orcein), but till date little attention has been given on the effect of different staining procedures on the results of micronuclei assays .[14,23]

The aim of the present study was to use micronucleus assay as a genotoxic marker in smokers and non smokers and also further to investigate the effect of different stains on the results of micronuclei study.

The present study showed that the frequency of micronucleus was significantly higher in smokers than in non smokers and the mean difference between the two was statistically significant. (Table 1,2)

In a study by Suhas et al on buccal cell changes which are associated with smoking by using the micronucleus assay, there was found to be a significant correlation between the habit of smoking and the frequency of the micronucleated oral mucosal cells.[24] The results of present study are in accordance with this study.

Piyathilake CJ et al in 1995, in their study showed that smokers are three times more likely to have micronucleated buccal mucosal cells.[25]

Naderi et al in 2013 evaluated the micronuclei frequency in the exfoliated buccal cells in smokers. The micronuclei frequency in smokers was found to be significantly high in smokers compared to nonsmokers.[2] The findings are in agreement with the present study.

Wu et al have reported the positive relation between micronuclei frequency and smoking intensity. The micronuclei frequency in buccal cells was higher in heavy smokers.[26]

Various workers have used different stains to study the micronuclei. In a study by Armen Nersesyan et al stated that the micronuclei frequencies found in heavy smokers and non smokers varied with different staining procedures. The micronuclei frequencies scored with Giemsa were significantly (5 fold) higher in smokers than in non smokers, also with May Grunwald-Giemsa the number of micronuclei was substantially (4.5 fold) higher. On the contrary no significant effects were observed with DNA specific stains. With Acridine Orange the micronuclei frequencies were 90% higher in smokers with DAPI and Feulgen, the differences were 30% and 120% respectively. When Feulgen stained slides were evaluated under fluorescence an 89% increase was observed.[23]

In the present study, Giemsa stain was found to be giving a better result in evaluating the micronuclei frequency in both smokers and non smokers than Acridine Orange.'

Ying Tian et al compared conventional Giemsa staining and the DNA-specific DAPI staining in preimplantation embryo induced by maternal exposure to chlorpyrifos to evaluate the accuracy of micronuclei. Compared with DAPI staining, the sensitivity of Giemsa staining was 85.0%, 86.0% and 90.9% for control, 40 mg/kg, and 80 mg/kg chlorpyrifos treated group, respectively. The specificity was 97.9%, 91.4% and 96.5% for control, 40 mg/kg, and 80 mg/kg chlorpyrifos treated group, respectively and recommend that Giemsa staining technique is a standard staining method in detecting micronuclei.[27] in the present study the sensitivity of Giemsa staining in smokers was 99.3% and in non smokers was 96.3%

The findings of the present study indicate that Giemsa staining method provides a reliable data in evaluating micronuclei compared to Acridine Orange.

A few studies reported the possibility that cellular structures, such as keratohyalin granules or bacteria, resembling MN, can lead to false-positive results. MN scoring can be interfered by the bacteria that are commonly found in the mouth. Bacteria can be differentiated from MN by their characteristic shape, smaller size, color, staining intensity, and their presence upon and between buccal cells on the slide. Small dye granules may sometimes resemble MN but usually have a slightly different refractility and color intensity. [23,28,29,30,31]

Limitations of the present study were the presence of stain granules and varied staining intensity. Acridine orange stained slides gave better results when viewed immediately following staining.

The present goal in many research laboratories is to develop screening strategies indicating individual cancers with certain biomarkers. Biomarkers are instruments of individual tumor prevention and help to detect high-risk patients. Early detection of a premalignant or cancerous oral lesion would improve the survival to a greater extent and also will reduce the morbidity associated with the treatment to a considerable extent.

From the present study it can be inferred that individual cancer risk can be predicted on the basis of increased percentage of micronuclei in oral epithelial cells and helps in identifying individuals with high risk of developing oral cancer.

Thus, micronucleus assay is valuable in showing genotoxic damage even before phenotypic changes are attributed to the healthy mucosa of people with high risk of developing oral cancer, but it cannot predict when such transformations will occur.

Interpretation and conclusion:

The study concluded that there is significant increase in the micronuclei frequency in smokers when compared to non smokers and also that Giemsa is a better stain than Acridine Orange for the evaluation of the micronuclei. The present study concludes that, the micronucleus assay in exfoliated buccal cells holds promise as a specific biomarker of genotoxicity, for screening of oral cancer and as prognostic indicator.

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