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Application of Fluorescent DAPI and Feulgen Reaction Assay to Evaluate Micronucleus Frequency in Oral Exfoliated Buccal Cells of Tobacco Chewers and Patients with Oral Squamous Cell Carcinoma

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ABSTRACT

Background: Micronucleus assay has been extensively used to assess chromosomal damage. DNA specific staining technique is best for assessing the morphological and morphometric aspect of cells. Hence, we designed a study to evaluate and compare the diagnostic efficacy of Feulgen and DAPI staining in detecting Chromosomal damage. Aim: To assess chromosomal damage in exfoliated cells collected from the surface of the lesional tissue in patients with OSCC. To compare efficacy and accuracy of assessing DNA damage using feulgen reaction and 4', 6-Diamidino-2-phenylindole (DAPI) as fluorescent marker. **Methods**: The study was conducted on 60 subjects. The Study group (a) constitute of 20 patients with OSCC, Group (b) constitute 20 Patients having history of tobacco consumption and Control group constituted 20 individuals. Oral exfoliated cells were obtained and 2 slides were prepared from each subject, stained with feulgen and DAPI stain respectively. Micronuclei frequency was scored according to criteria of Tolbert et al. **Results**: Micronucleus occurrence was significantly decreasing from study group (a) (OSCC) to study group (b) to control group (P=0.00001) .On comparison between staining reaction with feulgen and fluorescent DAPI ,it was found that Fluorescent staining showed significantly more micronucleated cells than feulgen staining in study groups and control group and this difference is statistically significant. **Conclusion**: The use of fluorescence dye in the screening of micronuclei enhances the demonstration of micronuclei as the fluorescent dye binds strongly to DNA. Use of Fluorescent micronucleus assay ensures a key role in the early and accurate evaluation of genotoxic damage.

Keywords: Squamous cell carcinoma, Micronucleus Assays, Cytology, Fluorescent Probes.

INTRODUCTION

Oral cancer is among the ten types of malignant neoplasia of highest incidence Worldwide and is particularly common in developing countries.[1] More than 95% of the carcinomas of the oral cavity are of squamous cell type in nature. They constitute a major health problem in developing countries, representing a leading cause of death. The survival index continues to be small (50%) as compared to the progress in diagnosis and treatment of other malignant tumors.[2] Since tissue homeostasis is the result of a subtle balance between proliferation and cell death, too little cell death by apoptosis can promote tumor formation as well as progression.[3] It is a dynamic process and is composed of three phases: elimination (i.e.,cancer immune surveillance), equilibrium and escape . Normal cells subjected to common oncogenic stimuli ultimately undergo transformation and generate pro-inflammatory danger signals that initiate cancer immune ending process.[4]

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Micronuclei are extra nuclear cytoplasmic bodies and they are induced in oral exfoliated cells by a variety of substances, including genotoxic agents and carcinogenic compound in tobacco, betel nut, and alcohol.[5] Micronuclei detection in oral squamous cell carcinoma has been shown to have a sensitivity of 94%, specificity of 100%, and an accuracy of 95%, Thus they are good prognostic indicators and occurrences of chromosomal damage in the oral epithelium can be evaluated using the micronucleus test, as suggested by Stich et al.[1] Therefore MN (Micronucleus) 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 centers. The advantage of micronuclei assay lies in its simplicity as scoring of MN is rapid, practical, and does not require much expertise.[6]

Early detection of premalignant or cancerous oral lesions promises to improve the survival and the morbidity of patients suffering from these conditions. Biopsy is an invasive technique with surgical implications and technique limitations for professionals and psychological implications for most patients. It also has limitations when the biopsy site is tricky to choose in case of large

lesions.[7] Exfoliative cytology is a simple, non-invasive diagnostic technique which could act as an Adjunct in early diagnosis of oral premalignant and malignant lesions. Quantitative Introduction parameters like morphometry are objective and reproducible and may be important in cytological evaluation in these lesions.[8]
Cytobrush sampling is more frequently used nowadays for exfoliative cytology, since it maximizes the number of cells obtained, and facilitates their uniform distribution onto the microscope slide, thus probably improving sensitivity. Previous studies have shown false-positive results in micronucleus frequency when using Romanowsky type stains resulting inaccurate data interpretation. Studies have shown that the sensitivity of DNA specific dyes is almost twice as compared to nonspecific dyes.[9]
One of the unique features of the staining technique using DAPI (4, 6 Di Amidino-2- Phenyl Indole di hydrochloride) is that DNA material fluoresces when viewed under fluorescent microscope with DAPI band filter (emission wavelength 425 nm).[10]
The best application of using feulgen and DAPI staining technique is for assessing the morphological and morphometric aspect of cells and chromosome damage and apoptosis.
Hence, we designed a study to evaluate and compare the diagnostic efficacy of Feulgen and DAPI staining in detecting Chromosomal damage i.e. Micronuclei in exfoliative cytological smears of patients having history of tobacco consumption in any forms and patients with clinically suspected lesions of OSCC.

MATERIALS AND METHODS

The present study was conducted in the Department of Oral Pathology and Microbiology of the institution. The Study constituted group (a) of 20 patients with clinically suspected lesions of OSCC, Excluding patients undergoing chemotherapy and radiotherapy and Group (b) of 20 Patients without oral lesions but having history of tobacco consumption in any forms.
Control group constituted 20 individuals without history of tobacco consumption in any form. Selection of patients was done randomly from amongst those attending OPD of the institution. After taking informed written consent detailed case history and clinical examination was done. Examination of the oral cavity was carried out under adequate illumination with the help of tongue depressor, mouth mirror and gauze sponge. Any changes in color, texture of the mucous membrane, inflammatory areas, indurations and ulcerative growth in the oral mucosa were identified.
The lesional tissue of study group "a", buccal mucosa of study group "b" and Control group was selected for acquiring two cytological smears. Before taking the sample, patient was asked to rinse his/her mouth with water. Smears were obtained with the help of cytobrush [Figure 1]. The entire selected site was scraped and transferred to a clean glass slide and spread cautiously to avoid overlapping of cells. The smears were then immediately fixed with Clarke"s fluid for 16-18 minutes. After Fixation, one smear was stained with feulgen reaction and the other with DAPI fluorescent stain.
Solutions used for feulgen staining were Schiffs Reagent (100 ml Yucca Diagnostics.YD092), 1 N Hydrochloric acid (Conc. HCl 36% and 11.65 M used to prepare 1 N HCL), Distilled Water, Counter Stain Orange G (Yucca Diagnostics) and DPX mounting media.
Solutions Used for DAPI staining were DAPI dihydrochloride [(MW 350.3) Invitrogen, Life technologies], Phosphate buffer saline (Ph 7.4), Deionized water (dH20) and DPX mounting media.
The slides were analyzed at 1000X magnification using Motic BA400 Trinocular Compound Microscope.
The criteria which used for counting the MN was developed by Tolbert et al.[11]

The Criteria for designating extra nuclear body as "micronuclei" were as follows:

1. Diameter less than 1/3rd of the main nucleus.
2. Staining intensity similar to or slightly weaker than that of the nucleus.
3. Round to oval shape.
4. Texture same as that of the main nucleus.
5. Close proximity but no actual contact with the nucleus.
6. Plane of focus same as that of the main nucleus.

Only those structures fulfilling the above mentioned criteria were regarded as micronuclei.

Statistical analysis:

Ethical clearance was taken by the institute before commencing the study.
All calculations were performed using Microsoft 2007 version for windows for Excel. The data Obtained and expressed as means with standard deviation. It was statistically analyzed with the help of Kruskal Wallis ANOVA, Mann-Whitney U tests. Chi-square test was used for correlation Data.

RESULTS

The present study was undertaken for accurate assessment of chromosomal damage in OSCC and patients having history of tobacco consumption using feulgen reaction and fluorescent DAPI assay. In study group (a) (OSCC) , study group (b) and control group equal number of patients were in age group I (21-40 years) and group II (41-60 years) i.e. N=25(41.66%); while 10(16.66%) were from age group III (61-80 years) [Table 1].

Of total 60 patients, 45(75%) were males and 15(25%) were females hence male to female ratio was 3:1.

In study group (b), 9 (45%) patients had history of tobacco consumption in smoked form; while 11 (55%) patients had habit of using smokeless tobacco. All the tobacco smokers had duration of habit since 1-10 years and 7(77.77%) patients had frequency of tobacco smoking 4-6 times per day. Amongst all smokeless tobacco users 7(63.63%) had duration of habit since 1-10 years; while 4(36.36%) had habit since 11-20 years [Table 2].

Feulgen and DAPI stained cytological smears were screened for 100 intact epithelial cells per slide for micronuclei as per criteria mentioned earlier. The MN cells observed are shown in [Figure 2].

Feulgen Stained smear shows, frequency of micronucleated cells was 6 in control group, 16 in study group (b) and 34 in study group (a) (OSCC). Mean frequency of micronucleated cells varied from 0.3 in control group to 0.8 in study group (b) and 1.3 in study group (a) (OSCC) and this result is highly significant statistically. (P= 0.00001). Micronucleus occurrence was significantly decreasing from study group (a) (OSCC) to study group (b) to control group (P=0.00001) [Table 3]. However, by using DAPI staining Frequency of micronucleated cells was found to be 7 in control group, 23 in study group (b) and 41 in study group (a) (OSCC) .Mean frequency of micronucleated cells varied from 0.4 in control group to 1.2 in study group (b) and 2.1 in study group (a) (OSCC) and this difference is highly significant statistically (P=0.00001).Micronucleus occurrence was significantly decreasing from study group (a) (OSCC) to study group (b) to control group [Table 3]. On comparison between staining reaction with feulgen and fluorescent DAPI it was found that variation existed in frequency of mean micronucleated cells in different groups. Fluorescent staining showed significantly more micronucleated cells than feulgen staining in study groups and control group and this difference is statistically significant (p=0.08978) when level of significance is 0.10 [Table 3]

Figure 1: Cytosmear sampling from the buccal mucosa using cytobrush.

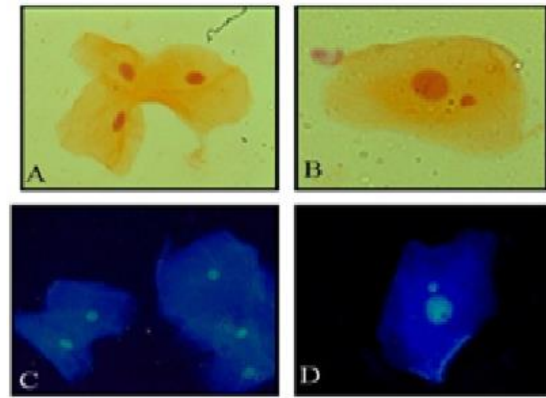

Figure 2: A Showing feulgen stained normal exfoliated cell (400x). C Showing DAPI stained Normal exfoliated cells (400x). B and D revealing cells with Micronucleus (1000x) by Feulgen and DAPI stains respectively.

Table 1: Age and Gender distribution in Study groups and control group.

Group (N=20)	Demographic distribution					
	Group 1 21-40		Group II 41-60		Group III 61-80	
	M	F	M	F	M	F
Study group (a)	3	1	8	2	5	1
Study group (b)	9	1	5	3	2	0
Control	6	5	5	2	2	0
Total	18	7	18	7	9	1
%	41.66%		41.66%		16.66%	

Table 2: Frequency, duration and form of tobacco chewing habit.

Form of tobacco	Smoking (N=9)		Smokeless (N=11)	
Frequency per day	1-3 times	2	1-3	9
	4-6 times	7	4-6	2
Duration (Years)	1-10	9	1-10	7
	11-20	0	11-20	4

Table 3: Comparison of mean number of micronucleated cells amongst various groups with feulgen and DAPI staining by Kruskal Wallis ANOVA

Groups (N=20)	Miconuclei			
	Feulgen		DAPI	
	Mean	SD	Mean	SD
Control	0.3	0.5	0.4	0.5
Study group (b)	0.8	0.7	1.2	0.7
Study group (a)	1.7	0.8	2.1	0.8
Total	0.9	0.9	1.2	0.9
H-value	25.8246		32.6568	
p-value	0.00001*		0.00001*	
Pair wise comparison by Mann-Whitney U test				
Control vs Study group (b)	P=0.0326*		P=0.0011*	
Control vs Study group (a)	P=0.00001*		P=0.00001*	
Study group (b) vs Study group (a)	P=0.0020*		P=0.0018*	

DISCUSSION

Squamous cell carcinoma of the oral mucosa accounts for 90% to 95% of all oral malignancies.

Oral exfoliative cytology has been used extensively for screening cellular alteration in oral squamous cell carcinoma cases. An accuracy of 95% and a reliability of more than 96% in detection of squamous cell carcinoma in mass screening have been reported in the literature.[5] Micronucleus is a potential biomarker to screen genotoxicity, biomonitoring of various diseases, detection of malignancies and preneoplastic conditions and also a lot of other diseases.[12] The MN assay has been reported to correlate well with the histological grading of oral squamous cell carcinoma and leukoplakia. Incidence of micronuclei has been analyzed by various studies in normal patients, oral premalignancies, and oral squamous cell carcinomas.[5] Present study was undertaken for accurate assessment of chromosomal damage and apoptosis in patients having history of tobacco consumption in any form without oral lesions and OSCC and also to compare the relative advantage of fluorescent staining (DAPI) over the conventional (Feulgen) one in micronucleus and apoptosis detection.

As stated by Prabhu and Daftary oral cancer like most cancers affects the individuals in higher age group, mostly above 40 year.[13] But peak incidence in India is 5th-6th decade of life as quoted in IARC data base.[14] In the current study, patients were in the age range of 24-80 years. 4 (20%) patients of OSCC were below 40 years which shows decline of demographic incidence data. This is in accordance with National Cancer Institute's Surveillance, Epidemiology and End Results (SEER) database which provide evidence for an increase in percentage of the oral carcinomas that occur in adults younger than 40 years, from 3% in 1973 to approximately 6% in 1993. These rising trends have been attributed to increased smokeless tobacco use in younger individuals.[15]

Out of 20 patients of OSCC only 4(20%) were females. However this is not representative of the true incidence of OSCC in Indian women because of lesser sample size. In contrast to the study done by Jones et al female patients with carcinoma of tongue out numbered males by almost 2:1 in patients under 40 years.[16]

For the purpose of collection of exfoliated buccal cells various methods have been proposed. Studies have been done utilizing wooden tongue depressor, cotton swab and short bristle cytobrush and tooth brush to obtain cells for study in cytology. Stephanie et al developed a buccal cell collection method suitable for use in large-scale, school-based studies of children Using Water and a Toothbrush. They recommend the toothbrush-rinse method for large scale epidemiological studies as this method yields a large amount of high-quality DNA for PCR assays.[17] Obwald et al used the swish and spit method to obtained buccal cells. In this method the samples contained two cell types buccal and polymorphonuclear leukocytes, but the viability of polymorphonuclear leukocytes was good.[18] Joshi and Kaijkar analyzed the cytomorphological features of cells in smears of oral premalignant and malignant lesions obtained from exfoliative brush cytology using Feulgen stain. They have shown that cytomorphometric analysis using exfoliative brush cytology can be of great value for monitoring and follow up of suspicious lesions and can provide an excellent additional diagnostic test for detecting early oral malignancy.[7] We used the same method as suggested by Joshi and Kaijkar to our advantage.

Micronucleus is the nucleus that expresses the genotypic alterations caused in the process of malignancy. It is characteristically seen in exfoliated epithelial cells like buccal Mucosa and urinary bladder wall during pre-cancerous and cancerous conditions in less and large proportions respectively. Micronucleus, a microscopically visible, round or oval cytoplasmic chromatin mass in the extra nuclear vicinity, originates from aberrant mitosis. It consists of eccentric chromosomes, chromate fragments or whole chromosomes which have failed to reach spindle poles during mitosis.

Various workers have used different stains to study the micronuclei. A study by Armen 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 nonsmokers. 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 Felugen, the differences were 30% and 120% respectively and in flouresent feulgen increase of 89% were observed.[19]

Joshi et al found significantly higher micronucleated cells ($p<0.0001$) among tobacco chewers and oral submucous fibrosis subjects when compared to non-chewers. Nuclear buds was significantly higher ($p<0.0001$) in oral submucous fibrosis subjects as compared to chewers as well as non-chewers.[20] Kausar et al have reported significant increase in the frequency of micronucleus in sadagura chewers (0.48%, $p<0.01$), smokers (0.46%, $p<0.01$), betel quid with sadagura chewers (0.91%, p, 0.001) and smokers also chewing betel quid with sadagura (0.53%, $p<0.001$) as compared to unexposed control group 0.07%.[21] Agrawal et al showed that The mean no. of micronucleated cells in the tobacco exposed group was found to be significantly higher as compared to the control group ($p<0.05$).[22] Stich et al showed an elevated frequency of MN in buccal cells of smoking and khaini tobacoo chewers (exposed) as compared with healthy control

subjects (unexposed).[23] Celik et al studied the micronucleus in buccal mucosal cells of 60 painters in which 30 were smokers and 30 were nonsmokers in comparison to healthy control subjects. Their results showed a statistically significant increase in the frequency of micronucleus in buccal epithelial cells of the exposed groups $p<0.05$ when compared with the control group.[24]

Our results are similar to the result of the study done by Joshi MS et al., Kausar et al, Stich et al and Celik et al Where the micronucleus frequency was of significantly higher value in tobacco exposed group when compared with the unexposed group ($p=0.0001$).

Sivasankari et al in their study observed that the MN index was four folds higher in malignant lesions when compared with the control group by using May Grunwald and Giemsa stain. The comparision of mean MN index between malignant and control group was significant with $p<0.05$.[25] Kiran et al observed that the frequency of MN was three to four times higher in patients with OSCC as compared to patients in the control group ($p<0.05$) by using rapid Papanicolaou stain.[26] Lavınia et al observed higher frequency of micronuclei in exfoliated cells from malignant lesions than control group ($p<0.001$).[1] Palve et al have done micronuclei analysis by using rapid Papanicolaou technique instead of fluorescent dyes for staining since it was very simple to use ,less time consuming and economical. They have shown that the mean micronucleus frequency in oral exfoliated cells was significantly increased in oral squamous cell carcinoma (OSCC) group as compared to the control group ($p<0.001$).[5] Our results are similar to the results of the above mentioned studies. The micronucleus showed higher value in malignant group when compared with the control group.

Using Fluorescent staining Methods Kumar et al have shown that the levels of MN were significantly increased in OSCC group as compared to Control group ($p<0.005$). They followed a fluorescent acridine orange stain and the analysis was done under flouresence microscope, which increases the specificity to identify DNA containing structures.[27] Kamboj et al studied micronucleus frequency using Acridine orange and feulgen stains and according to their study the frequency of micronucleated cells increased significantly in leukoplakia and squamous cell carcinoma compared to controls. They also stated that fluorescent staining was found to be more sensitive than the conventional one for micronucleus detection.[28]

Similar results were obtained in our study, Fluorescent staining with DAPI showed significantly more micronucleated cells than feulgen staining in study groups and control group and this difference is statistically significant ($p=0.08978$) when level of significance is 0.10. The use of fluorescence dye in the screening of micronuclei enhances the demonstration of nuclei and micronuclei as the fluorescent dye binds strongly to DNA.

Micronucleus count can be a useful biomarker and it can be used as a screening test for patients with habit of tobacco consumption and patients with manifestations of oral lesion including pre malignant and malignant conditions. The mean micronucleus frequency in oral exfoliated cells was significantly increased from control group to tobacco chewer group to oral squamous cell carcinoma (OSCC) group. The sensitivity of fluorescent DAPI was found to be higher as compared to feulgen staining.

Future scope of the study is to consider the fact that oral cancer develops after a long latency period, and hence, it is difficult to determine the clinical outcome. Use of Fluorescent micronucleus assay ensures a key role in the early evaluation of genotoxic damage and primary prevention in the future, which will help in survival to a greater extent and also will reduce the morbidity.

CONCLUSION

The use of fluorescence dye in the screening of micronuclei enhances the demonstration of micronuclei as the fluorescent dye binds strongly to DNA. Use of Fluorescent micronucleus assay ensures a key role in the early and accurate evaluation of genotoxic damage.

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