Cytomorphometric Analysis of Oral Premalignant and Malignant Lesions Using Feulgen Stain and Exfoliative Brush Cytology

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Abstract

Objective: Oral squamous cell carcinoma (OSCC) is the sixth most common cancer worldwide and accounting for 90% of cancers of oral cavity. Tobacco abuse has been proved to be the major risk factor in the development of OSCC. Despite advances in surgery, radiation and chemotherapy, the five year survival rate for oral cancer has not improved significantly over the past several decades and it remains at about 50 to 55%. 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.Our study was therefore carried out to analyze the cytomorphometric features of cells obtained by cytobrush and stained with Feulgen stain from oral premalignant and malignant lesions and to find out whether these features could be used to detect dysplasia and malignancy in their early stages. To analyze the cytomorphological features of cells in smears of oral premalignant and malignant lesions obtained from exfoliative brush cytology using Feulgen stain and to assess the efficacy of the same in detecting dysplasia and malignancy.

Materials and Methods: Our study comprised of clinically and histopathologically diagnosed sixty cases which were grouped into twenty cases each of tobacco users with lesions (Leukoplakia and Erythroplakia) (Group I); tobacco users without lesions (Group II); Oral squamous cell carcinoma (OSCC) lesions (Group III); and normal mucosa (Group IV). The epithelial cells from the lesion were collected with a cytobrush and smears were stained with Feulgen stain. The cells were measured using software for their nuclear area, nuclear diameter, cellular area, cellular diameter and nuclear to cellular area ratio (N:C).

Results: The exfoliated cells showed similar alterations as those occuring in histopathological sections of premalignant and malignant lesions. The N:C ratio, mean nuclear area and diameter value was highest in Group III and lowest in Group IV. The mean cellular area and diameter was highest in Group IV and lowest in Group III. Tukey-HSD formula for pairwise comparison showed a significant difference in mean values of nuclear and cellular area and diameter and N:C ratio between all the groups except in Group I and Group III.

Conclusions: Our study was able to differentiate dysplastic and malignant cells from normal ones using analysis based on nuclear and cellular parameters. We therefore conclude 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.

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the sixth most common cancer worldwide and accounting for 90% of cancers of oral cavity. Tobacco abuse has been proved to be the major risk factor in the development of OSCC. Despite advances in surgery, radiation and chemotherapy, the five year survival rate for oral cancer has not improved significantly over the past several decades and it remains at about 50 to 55% [1].

In its early stages, oral cancer may disguise itself and appear as an innocent lesion. Patients usually are unaware and report at a later stage at which point invasive treatment plan needs to be adopted. Early detection of such 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 [2].

Exfoliative cytology is a simple, non-invasive diagnostic technique which could provide as an adjunct in early diagnosis of oral premalignant and malignant lesions. Quantitative parameters like morphometry are objective and reproducible and may be important in cytological evaluations in these lesions [3]. 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 [4].

Our study was therefore carried out to analyze the cytomorphometric features of cells obtained by cytobrush and stained with Feulgen stain from oral premalignant and malignant lesions and to find out whether these features could be used to detect dysplasia and malignancy in their early stages.

METHODS

Our study sample comprised of a total of 80 patients. Twenty healthy individuals who were non smokers, non alcoholics and without lesions served as control and comprised Group IV, twenty cases who had the habit of chewing tobacco or smoking without any alteration of oral mucosa comprised Group II, and tobacco users with premalignant lesions (criteria set by Neville and Day, 2002 [1]) comprised Group I and twenty cases of clinically suspected OSCC comprised Group III (Table 1). Biopsy procedure was followed for cases in Group I and III to confirm the diagnosis. Informed written consent was obtained from all patients prior to obtaining a cytological smear and biopsy. Scrapings of the lesional mucosa in Group I

and III and buccal mucosa of Group II and IV were obtained using a cytobrush (Figure 1). The entire lesion was scraped and smeared on a glass slide and fixed immediately with commercial fixative spray [Yuccaspray; Yucca diagnostics, Kolhapur]. The modification of Feulgen staining protocol developed by Khandelwal et.al was followed for the staining of smears which included counterstaining with OG-6 and EA-50 [5].

In each slide 100 cells were counted at high power [40 x magnifications]. Only clearly defined cells were identified, uploaded in the software [Motic Images Plus 2 Image Analysis Software] and measured. For the nucleus and cell area the outline was traced and the software automatically gave the measurements in sq.µm. The nucleus and cell diameter were measured in two perpendicular planes and their mean was taken in µm. The N:C ratio was calculated by dividing the nuclear area by cell area.

One-way ANOVA was used for comparing the parameters for multiple groups. Comparison of the mean nuclear and cellular area and diameter values between groups was made using Tukey-HSD procedure. The P-value <0.05 was considered to be significant.

Figure 1. Scraping obtained with a cytobrush

Table 1. Distribution of cases.

Groups (n=20)	Cases	Sample size (cells counted)
Group I	Tobacco users with lesions	2000
Group II	Tobacco users without lesions	2000
Group III	Oral Squamous Cell Carcinoma	2000
Group IV	Normal mucosa	2000

RESULTS

In this study samples were collected from 80 patients who were divided into four Groups as shown in Table 1. We could identify the cell and nucleus outline very effectively by the modified staining procedure.

Group IV smears showed polygonal squamous cells with abundant eosinophilic cytoplasm and centrally placed nuclei. (Figure 2)

In Group I and II the cells showed a decrease in cell size and increase in nuclear size. There was also a decrease in the cell cytoplasm, ballooning degeneration and occasional multinucleation (Figure 3).

In Group III there were bizarre shapes of cells with scanty cytoplasm and vacuolization and inclusions in cytoplasm. There were irregular nuclear shapes with disproportionate enlargement of nucleus, hyperchromatic nuclei, large number of naked nuclei without cytoplasm and some nucleus showed fading and resorption (Figure 4).

There was a progressive decrease in cellular area and cellular diameter from Group IV to II to I and it was smallest in Group III. There was a progressive increase in a similar fashion in the nuclear area and diameter with Group IV having the smallest and Group III having the largest nuclear dimensions (Graph 1-4).

When multiple comparisons of cellular area and diameter and nuclear area and diameter was done using Tukey–HSD test, the different groups showed statistically significant difference from each other except for Premalignant lesions (Group I) and OSCC lesions (Group III) (Table 2 and 3).

The Nuclear to Cellular area ratio (N:C) showed a progressive increase from Group IV to Group II to Group I and to Group III (Graph 5). Multiple comparisons showed a statistically significant difference from each other except for premalignant lesions (Group I) and OSCC lesions (Group III) (Table 4).
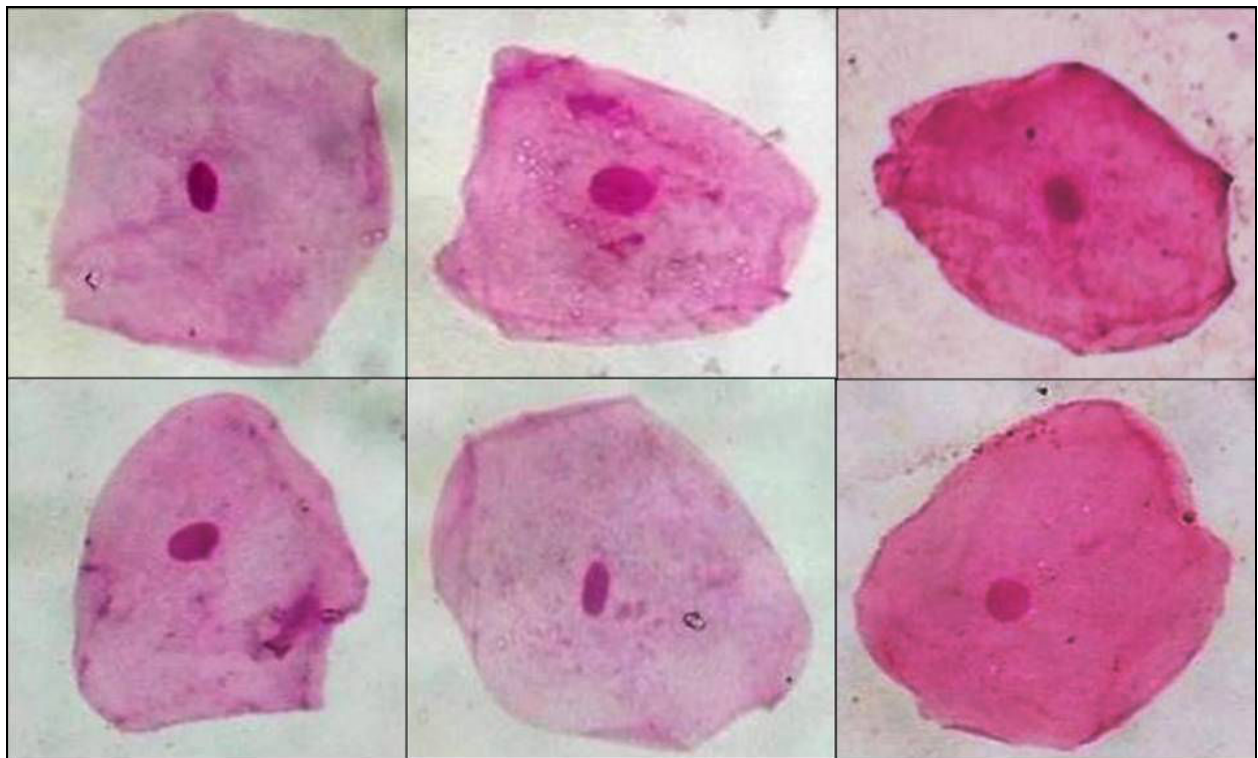
Figure 2. Group IV: Photomicrographs of an oral brush biopsy specimen from normal mucosa (Feulgen stain, x40).
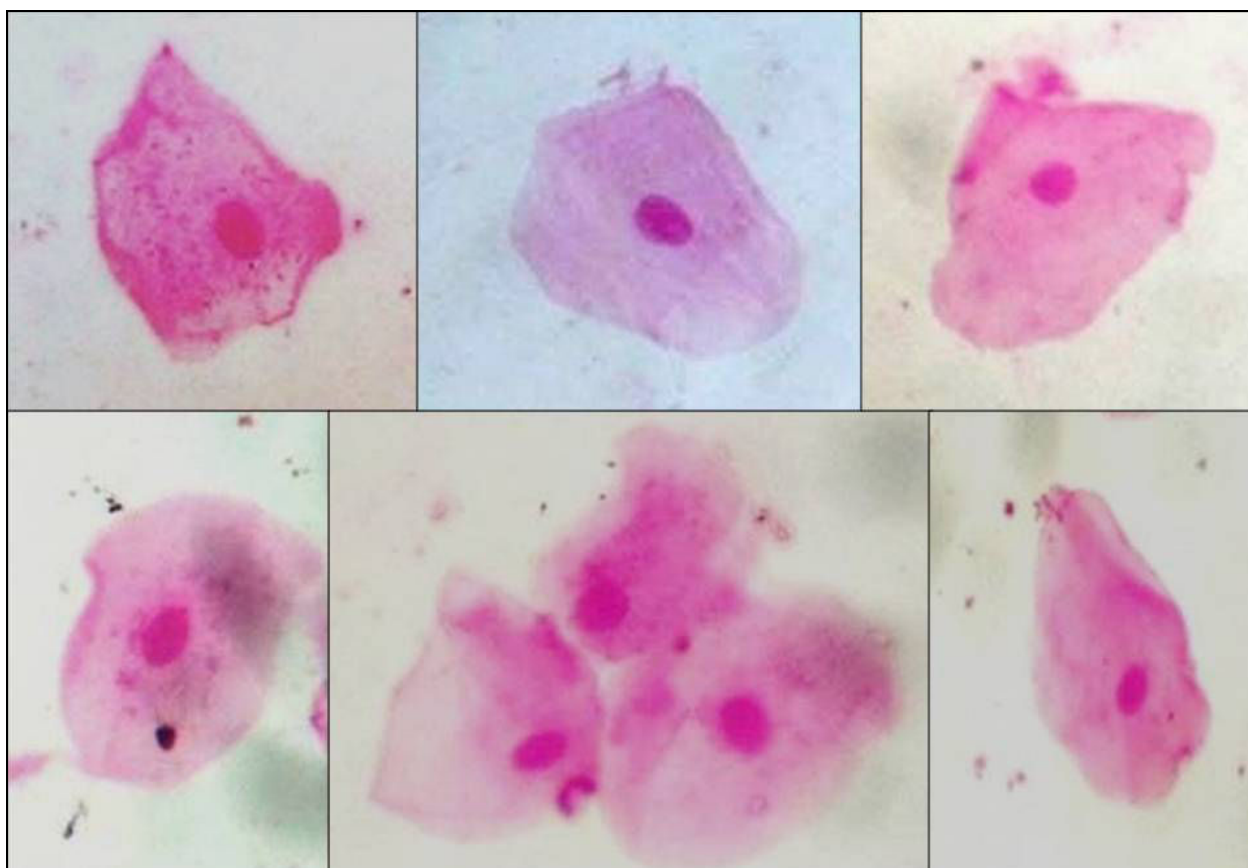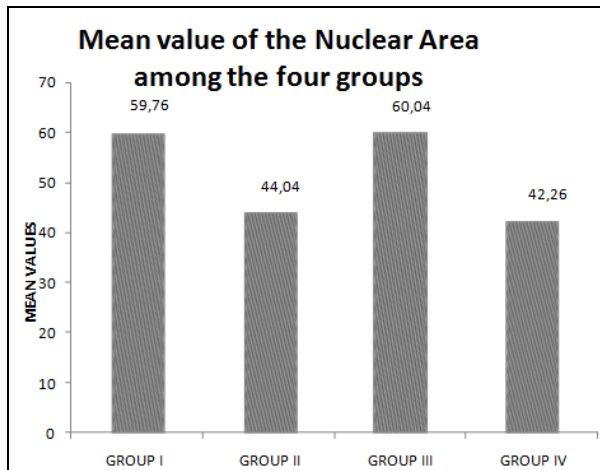
Figure 3. Group I: Photomicrographs of an oral brush biopsy specimen from premalignant lesions (Feulgen stain, x40).
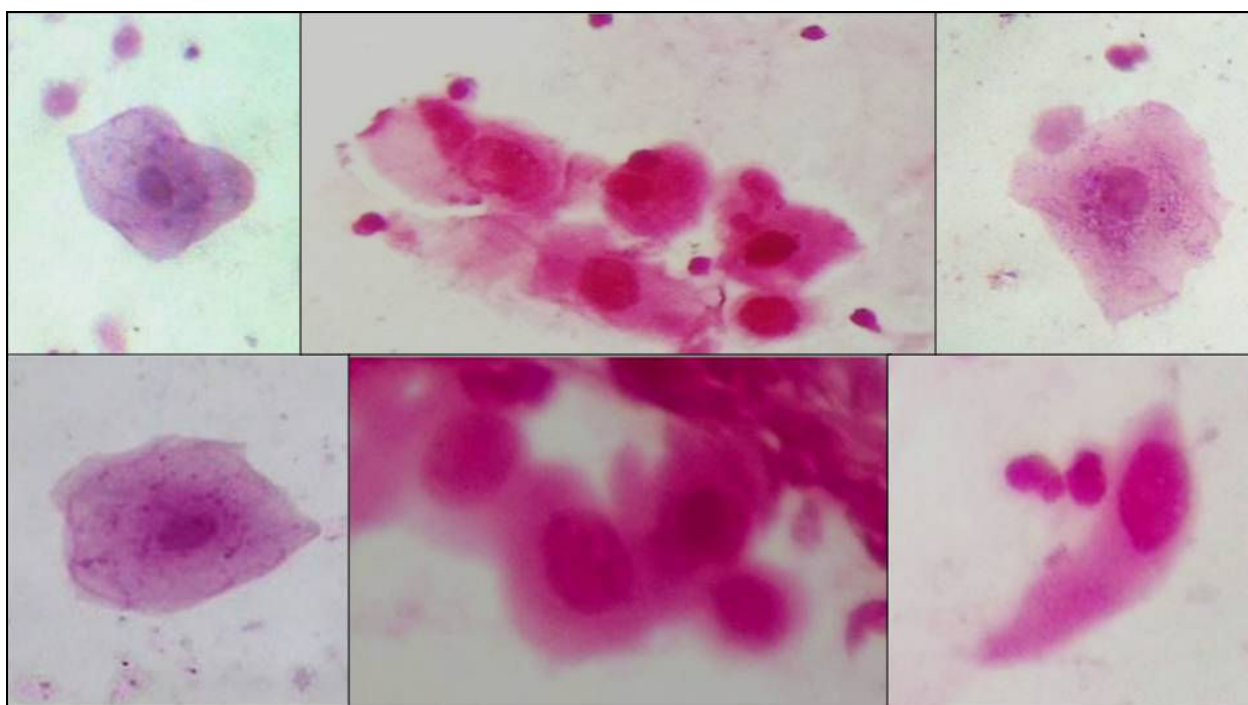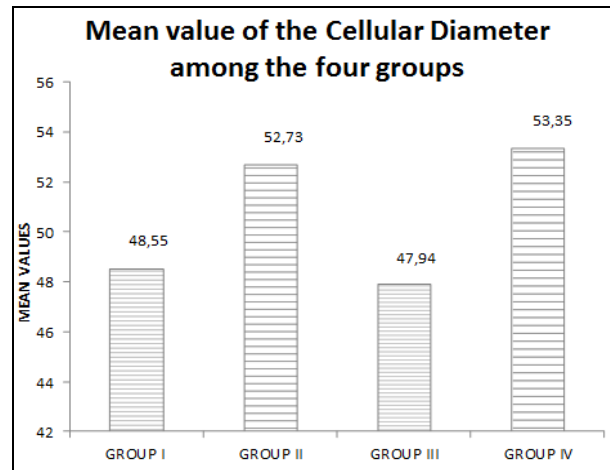

Figure 4. Group III: Photomicrographs of an oral brush biopsy specimen from OSCC lesions (Feulgen stain. x40).
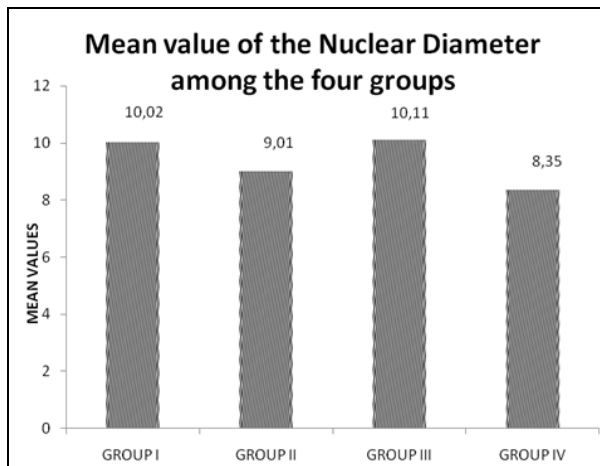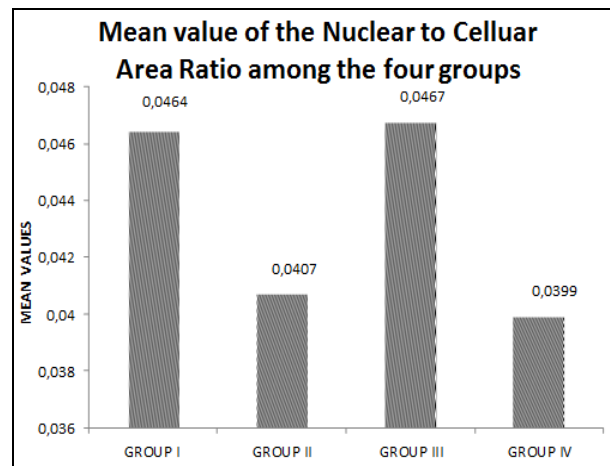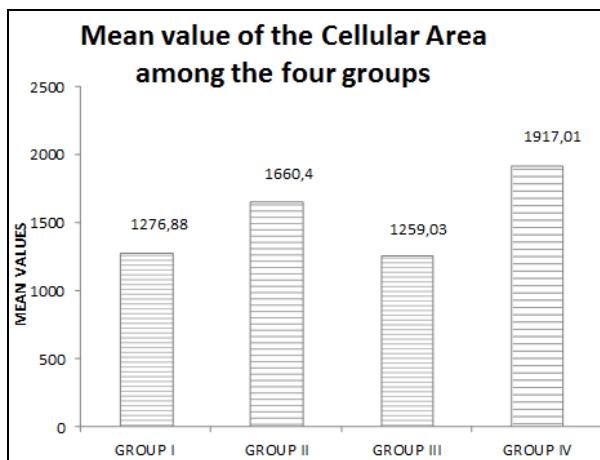
Graph 1. Nuclear Area: Distribution of mean among the four groups.

Graph 2. Nuclear Diameter: Distribution of mean among the four groups.

Graph 3. Cellular Area: Distribution of mean among the four groups.

Graph 4. Cellular Diameter: Distribution of mean among the four groups

Graph 5. Nuclear to Cellular Area ratio: Distribution of mean among the four groups

Table 2. Multiple comparisons of the mean values of the Nuclear Area and Nuclear Diameter.

Groups compared		Nuclear area		Nuclear diameter	
		Mean difference	p	Mean difference	p
Group I	Group II	15.72	0.001*	1.01	0.001*
	Group III	0.28	0.0589	- 0.09	0.921
	Group IV	17.50	0.001*	1.67	0.001*
Group II	Group III	14.98	0.001*	- 1.10	0.001*
	Group IV	1.78	0.0338	0.66	0.001*
Group III	Group IV	16.76	0.001*	1.76	0.001*

* Tukey HSD formula, p-value-significant

Table 3. Multiple comparisons of the mean values of the Cellular Area and Cellular Diameter:

Groups compared		Cellular area		Cellular diameter	
		Mean difference	p	Mean difference	p
Group I	Group II	-383.52	0.001*	-4.18	0.001*
	Group III	17.85	0.753	0.61	0.652
	Group IV	-640.13	0.001*	-4.80	0.001*
Group II	Group III	401.37	0.001*	4.79	0.001*
	Group IV	-256.61	0.001*	-0.62	0.001*
Group III	Group IV	-657.979	0.001*	-5.41	0.001*

* Tukey HSD formula, p-value-significant

Table 4. Multiple comparisons of the mean values of the Nuclear to Cellular Area Ratio:

Groups Compared		Mean difference in nuclear/cell area ratio	p
Group I	Group II	0.0059	0.001*
	Group III	0.0012	0.541
	Group IV	0.0061	0.001*
Group II	Group III	0.0042	0.001*
	Group IV	0.0049	0.001*
Group III	Group IV	0.0037	0.001*

* Tukey HSD formula: p-value-significant.

DISCUSSION

In our study we used a novel technique combining exfoliative brush cytology, modified Feulgen staining, and cytomorphometry. We could therefore collect large number of individual cells and easily identify and map the cell and nuclear outline.

Malignant cells show a significant increase in the nuclear area and diameter due to the increase in nuclear content required for replication and in such cells with increase activity the ability of the cell to form cytoplasm decreases. The amount of cytoplasm a cell makes is decreased as compared to the amount of nucleoplasm in malignant cells, and therefore their nuclear dimensions increase and the cellular dimensions decrease [6, 7]. Accordingly we found that there was a decrease in the cellular parameters and an increase in the nuclear parameters.

Exfoliative brush cytology

Babshet et.al; in their study compared oral brush cytology and punch biopsy in premalignant and malignant lesions. They showed no statistically significant difference between histopathology and brush cytology in assessing both the lesions. They concluded that brush cytology is reliable and easily performed with less cost and discomfort to patient and can be used when patient refuses to perform a biopsy or

in medically compromised patients [8]. In a study from Sudan, oral scrape smear cytological analysis has been proposed as a useful early diagnostic method for epithelial atypia and for malignant oral lesions where nearly 5% of clinically benign appearing mucosal lesions were sampled by this technique and later confirmed by typical scalpel biopsy to represent dysplastic epithelial changes or invasive cancer [9].

The improved accuracy of oral cytology is attributed to the ease in obtaining full transepithelial cellular samples and the evaluation of smears with an image analysis system. Full-thickness sampling is essential if histomorphological, evaluation of the collected cells is to yield representative findings [10]. In accordance with the above studies we therefore used a cytobrush for obtaining smears so that the entire epithelial thickness could be evaluated.

Feulgen Staining

Static cytometry permits the quantification of DNA content in cells obtained by exfoliative cytology. However, routine Haematoxylin-Eosin staining is inadequate for this purpose, and special techniques are required to ensure that staining intensity is in proportion to DNA content. The Feulgen reaction is a stoichiometric procedure; each fixed molecule of Schiff's reagent corresponds to a constant and equivalent portion of the DNA molecule [11].

Feulgen stained malignant cells display an elevation in nuclear area corresponding to the abnormality in the DNA profiles that is not always evident in Papanicoloau stained smears [12, 13]. The biological activity of any cell is best reflected in the nucleus and the functional activity in the cytoplasm. Therefore, Khandelwal et.al, in their study used a combination of Feulgen stain (nuclear stain) and OG-6 and EA-50 (cytoplasmic differentiation stain) to derive optimal information on the cellular events taking place in the keratinocytes [5]. We used a similar procedure for staining the smears so as to view both the cytoplasmic and nuclear features.

Cytomorphometry

Ogden et al. suggested that quantitative techniques, based on the evaluation of parameters such as nuclear area (NA), cytoplasmic area (CA), and nucleus-to-cytoplasm area ratio (NA/CA), may increase the sensitivity of exfoliative cytology for early diagnosis of oral cancers, since these techniques are precise, objective and reproducible [14]. Cowpe et al. demonstrated that exfoliative cytology is capable of detecting malignant changes, through estimation of NA/CA using the planimeter method in Papanicoloau-stained smears [10]. Cowpe et al. found that tissues undergoing malignant transformation typically show a

reduction in CA before the reduction in NA. They also suggested that samples of healthy mucosa from the same patient provide the best control [15].

Ramaesh et.al, used cytomorphometric techniques to assess nuclear diameter and cellular diameter in normal oral mucosa, in dysplastic lesions, and in oral squamous cell carcinoma. They found that cellular diameter was highest in normal mucosa, lower in dysplastic lesions and lowest in oral squamous cell carcinoma and nuclear diameter was lowest in normal mucosa, higher in dysplastic lesions, and highest in oral squamous cell carcinoma [16].

Einstein TB and Sivapathasundharam B, in their study reported cytomorphologic alterations in the form of reduction in cellular diameter and increase in nuclear diameter in buccal squames of tobacco users in the south Indian population [17]. According to Frost JK [7], in actively proliferating cells, there is a decrease in the cellular diameter and increase in the nuclear size. All the above studies suggested that reduced cell size and increased nuclear size are useful early indicators of malignant transformation, and thus exfoliative cytology is of value for monitoring clinically suspect lesions and for early detection of malignancy.

In this present study, the cellular area and cellular diameter was highest in normal mucosa, lower in premalignant lesions and lowest in OSCC lesions. The nuclear area and diameter and nuclear to cellular area ratio was lowest in normal mucosa, higher in premalignant lesions and highest in OSCC lesions. However there was no statistically significant difference (p>0.05) between premalignant and OSCC lesions in any of the cellular or nuclear parameters. This indicated that there was an impending transformation of the premalignant cells and in such lesions there is a need for biopsy to rule out malignancy. Our study results are comparable with results of similar previous studies and were able to differentiate between cells of normal mucosa and premalignant and malignant lesions.

In conclusion, exfoliative brush cytology is reliable and is easily performed with less cost and discomfort to the patients. It can be used for screening suspicious oral lesions when biopsy is not advisable. In conclusion, we can state that Cytomorphometric analysis coupled with Feulgen staining in exfoliative brush cytology gives an added advantage in monitoring clinically suspect lesions and for early detection of malignancy.

Future directives:

Large scale studies can be carried out to further confirm the efficacy of this procedure in detection of malignant lesions. The combined use of exfoliative brush cytology, Feulgen staining and morphometric

analysis can be used to increase the diagnostic value of Oral cytology. This method can also be utilized for routine screening procedure of patients to detect any changes from the normal mucosa.

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