Original Article

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Pathology Section

Utility of Acridine Orange Fluorescence Microscopy in Cytodiagnosis of Oral Lesions

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ABSTRACT

Introduction: Oral exfoliative cytology is a reasonably effective technique for rapid initial evaluation of suspicious lesions. Exfoliated cells obtained from saliva, lavage or scrapings are ideally stained with Papanicolaou stain. Acridine orange stain imparts nuclear and cytoplasmic fluorescence in malignant cells which have increased nuclear activity. This cytochemical property can be utilized for diagnosis of cancer.

Aim: The aim was to determine utility of Acridine Orange (AO) fluorescence microscopy as a rapid and easier method for oral cytodiagnosis as compared to Papanicolaou stain.

Materials and Methods: The study group included a total of 50 cases, 25 cases clinically suspicious of malignancy (Group I) and 25 non suspicious cases, e.g., inflammatory lesions etc., (Group II) in both sexes, aged > 40 years. The control group comprised 5 individuals in the age group of 20 years and above, without any clinically observable lesions. Scrapings were obtained from oral mucosa, stained with AO and Pap stains, visualised with fluorescence and light microscopy, respectively, and results were compared & analysed statistically.

Results: AO staining: Amongst 25 cases in group I, 18 were confirmed as Squamous Cell Carcinomas (SCC) on histopathology. Sixteen out of 18 confirmed cases (88.89%) showed greenish yellow nuclear fluorescence with brownish red cytoplasmic fluorescence. In group II, 18 cases showed too little fluorescence to be labelled as positive with limited nuclear details which were hence counted as negative for fluorescence. Papanicolaou staining: In group I, 13 out of the 18 confirmed cases (72.22%) were positive for malignancy. In group II, only one case was false positive for malignancy and the rest were negative. The differences between the two groups were compared using Chi-square test. A p value of less than 0.05 was considered statistically significant.

Conclusion: AO stain reliably demonstrated malignant cells based on differential fluorescence – a cytochemical criterion. Thus, AO proved to be more sensitive than Papanicolaou staining for diagnosing malignancies. Hence, it can be used for screening and early detection of potentially malignant lesions.

Keywords: Exfoliative cytology, Fluorescent stain, Oral cancer

INTRODUCTION

Oral mucosa exhibits a rapid turnover of cells and the exfoliated cells have a valuable role in diagnosis of certain local and systemic diseases. Oral cytopathology is the microscopic study of cell samples obtained from oral mucosal surfaces by exfoliative cytology via smears, scrapings or lavage. Exfoliative cytology is a diagnostic procedure which has been generally accepted and is growing rapidly in importance as a means of early diagnosis of cancer [1]. Oral cancers are part of a group of cancers commonly referred to as head and neck cancers. Oral cancers comprise about 85% of all the head and neck cancers. SCC are the most common (94%) type of oral cancers. Potentially Malignant Disorders given by the WHO include Leukoplakia, Erythroplakia, Oral lichen planus, Oral submucous fibrosis, Discoid lupus erythematosus, Palatal lesion of reverse cigar smoking etc., [2].

Acridine orange is a cell permeable stain which causes selective fluorescence with nucleic acids. It binds to DNA leading to an excitation maximum at 502 nm (cyan) and an emission maximum at 525 nm (green). When it binds with RNA, the excitation maximum shifts to 460 nm (blue) and the emission maximum shifts to 650 nm (red) [3]. The increased RNA of the malignant cell is reflected in an increased brilliance, so that the differential fluorescence of the malignant and normal cells allows a comparison of the total concentration of nucleic acids in the various cells in the preparation. In addition, the morphological features of the cell are clearly visualized [4]. Cytoplasmic RNA gives a brownish to red range of fluorescence, while nuclear DNA appears green to greenish yellow.

Pap stain is widely used to differentiate cells in gynaecological smears, FNAC samples and other body fluids like pleural fluid, synovial fluid, abdominal fluid, sputum, brushings, etc. It is a multichromatic staining cytological technique that provides a good differential stain and hence, is widely used for other routine cytology smears.

The aim of the study was to determine the utility of AO fluorescence microscopy for cytodiagnosis as a rapid and easier method for evaluation of cytological specimen. It also aimed at comparing AO fluorescence microscopy with the cytological technique using Pap stain and correlating with histology.

MATERIALS AND METHODS

We conducted a case control study comprising 50 cases of oral lesions in both males and females aged 40 years and above, along with 5 control cases lacking any oral lesions. The cases were divided into the following groups:

- Group I: 25 cases with oral lesions clinically suspicious of malignancy and
- Group II: 25 cases who had oral lesions, which did not suggest malignancy clinically.

The control group comprised 5 individuals in the age group of 20 years and above, without any clinically observable lesions. Oral brush cytology was done. Toothbrush was used for exfoliative

cytology to obtain a complete transepithelial biopsy. For every case in the study group, the most representative areas were selected by visual examination for obtaining the scrapings.

Material from the brush was spread on the middle third of two clean, dried glass slides. Smears were fixed immediately with Biofix Spray for Pap stain and with ether/alcohol for AO stain. Scrapings were then stained with AO and Pap stain.

Procedure for Pap Staining

1. Smear was fixed with Biofix Spray and was dried.
2. Smear was dipped in tap water for 1-3 minutes.
3. Excess water was blotted out from the slide with filter paper.
4. Slide was then dipped in haematoxylin solution for 45-60 seconds.
5. Slide was then dipped in Scotte's tap water (1ml Scotte's concentrate in 100ml tap water) for 30-45 seconds.
6. Slide was then blotted out and dipped in dehydrant for 30 seconds twice .
7. Slide then dipped in cytoplasmic stain (equal parts of orange G and EA) for 45 seconds.
8. Slide was then rinsed in tap water, then in Scotte's tap water for 20-30 seconds, dried and seen under the microscope.
9. Excess water was blotted out and slide dipped in dehydrant two times for 30 seconds each.
10. Slide was then dipped in Xylene for 20 seconds.
11. Slide was mounted with cover slip using a drop of D.P.X. Mountant.

Procedure for Acridine Orange Staining:

1. Smear was fixed in alcohol for 30 minutes.
2. The fixed smear was passed through descending grades of alcohol 80%, 70% and 50% for 10 seconds each and rinsed in distilled water.
3. It was then dipped in 1% acetic acid for 6 seconds.
4. It was then rinsed in distilled water twice.
5. Smear was then stained in 0.1% AO for 3 minutes.
6. It was then washed in M/15 phosphate buffer solution pH 6.0 for 1 minute.
7. It was then treated with 0.1M Calcium Chloride solution for 1 minute for differentiation.
8. Excess Calcium Chloride was removed by washing with phosphate buffer solution.
9. The slide was then mounted with cover slip in a drop of phosphate buffer solution.

Reddish brown fluorescence was observed by cytoplasmic RNA whereas that seen with nuclear DNA was greenish yellow in colour [5].

The cells exfoliated from the lesional tissue and from the normal buccal mucosa in the controls were stained and observed under fluorescence microscope with B-2A filter of excitation 450-490 nm and emission 520 nm wavelengths for AO in a dark room and under light microscope for Pap stain. Also, the findings of acridine orange staining were compared with Papanicolaou stain results. The results were compared and analysed statistically.

STATISTICAL ANALYSIS

Suitable statistical tests of comparison were done. Categorical variables were analysed with Chi-Square Test and Fisher-Exact Test. Statistical significance was taken as $p < 0.05$. The data was analysed using SPSS version 16 and Microsoft Excel 2010. The observations were depicted as follows.

RESULTS

The exfoliated epithelial cells from the normal buccal mucosa in control group, when seen under AO fluorescence microscopy which did not show fluorescence and with Pap stain, showed normal squamous epithelial cells with no evidence of malignancy. Information from the scrapings of lesional buccal mucosa in group I and II was obtained and observations compiled as follows:

The age group with maximum prediction for oral malignancy was found to be between 40 and 50 years, constituting 56% of the patients included in group I.

In group I (patients with oral lesions suspicious of malignancy), out of 25 cases 19 were males and 6 were females with male to female ratio of 3.2:1. Thus, malignant lesions were encountered more commonly in males.

Maximum of 28 cases i.e., 56% (15 in group I and 13 in group II) had a history of less than 6 months. Only 8% cases showed bilaterality. Malignancies were most commonly seen on the buccal mucosa and mostly as masses (52%) and non-suspicious lesions were also seen mostly on buccal mucosa followed by tongue and mostly as ulcers (44%).

Main risk factor for oral lesions was tobacco intake seen in 58%, smoking in 32% and alcohol intake in 32% cases. In 25 suspicious cases, 88% patients had one or more of these three habits.

In group I, among 25 patients, 80% cases were of suspected malignancy and 20% were of suspected carcinoma recurrence. In group II, maximum cases were of leukoplakia.

Amongst the suspicious cases, 72% (n=18) were positive for malignancy with Pap stain because of altered nucleo-cytoplasmic ratio, hyperchromatism and pleiomorphism. In the non suspicious category, there was 4% positivity for malignancy and 96% were negative. Amongst the suspicious cases, 84% (n=21) showed fluorescence with altered nucleo-cytoplasmic ratio and were positive for malignancy using fluorescent AO method. In the non suspicious category, 28% showed fluorescence and 72% did not show fluorescence and were negative for malignancy. Eighteen out of 25 suspicious cases were followed up and were biopsied.

Malignant disease was diagnosed as SCC by biopsy in all the 18 cases (100%) of the lesions. Six out of 25 patients were positive for metastasis on FNAC. Seven cases came out to be positive for metastasis on histopathology. With Pap stain, out of 18 confirmed SCC cases on histopathology, 72.22% were reported positive for malignancy and 27.78% of the lesions were thus reported as "false negative" when compared with biopsy reports. With AO stain, out of 18 confirmed SCC cases on histopathology, 88.89% were reported positive for fluorescence and 11.11% were thus reported as "false negative" when compared with biopsy reports. The sensitivity of AO stain was found to be 88.89% as against 72.22% sensitivity of Pap stain [Table/Fig-1-3].

DISCUSSION

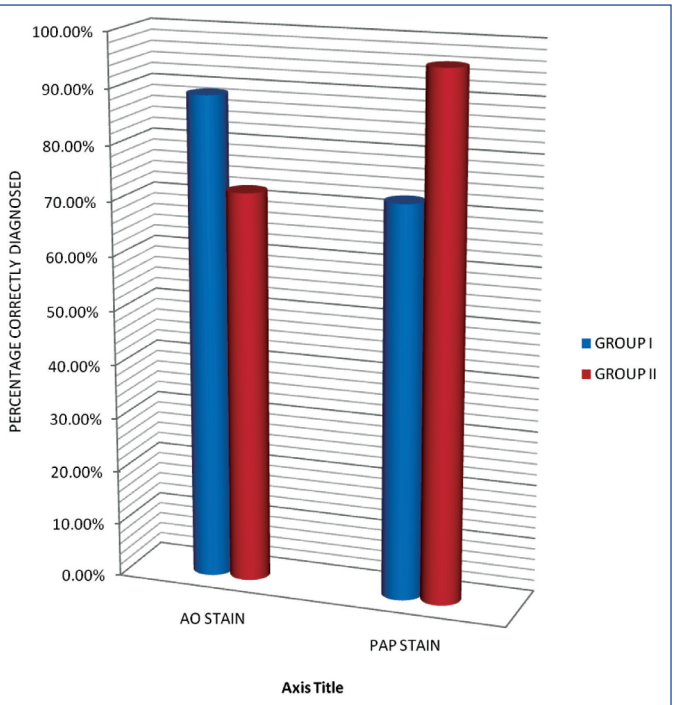
Exfoliative cytology is a simple and non-invasive diagnostic technique which could provide as an adjunct in early diagnosis of oral

	AO Stain	Pap Stain	AO Stain	Pap Stain
	Group I		Group II	
Total No. of Cases	25	25	25	25
True Positives	16	13	0	0
True Negatives	2	2	18	24
False Positives	5	5	7	1
False Negatives	2	5	0	0
% Correctly Diagnosed	88.89%	72.22%	72%	96%

[Table/Fig-1]: Results of Acridine orange and Pap stain.

	True Positive	False Positive	True Negative	False Negative	N	Sensitivity (%)	Specificity (%)	Positive Predictive Value (%)	Negative Predictive Value (%)	Accuracy (%)
Group I AO	16	5	2	2	25	88.89	28.57	76.19	50.00	72.00
Group I PAP	13	5	2	5	25	72.22	28.57	72.22	28.57	60.00
Group II AO	0	7	18	0	25	-	72.00	0.00	100.00	72.00
Group II PAP	0	1	24	0	25	-	96.00	0.00	100.00	96.00

[Table/Fig-2]: Showing comparison between acridine orange and pap stain.



[Table/Fig-3]: Diagrammatic representation of comparison of AO & PAP stain.

premalignant and malignant lesions. Statistics in the USA for 2003–2007 showed that the median age at diagnosis for cancer of the oral cavity and pharynx was 62 years [6]. Similar studies showed age group involved in oral cancer to be around 40-50 years. Male to female ratio was increased for oral cancers in accordance with Shenoi et al., [7] and Padmakumary et al. Clinical presentation was also in similar to our study. According to the study by Mishra et al., the most common clinical presentation of patients with oral cavity lesions in both retrospective study and prospective study was ulceration, growth in cases of squamous cell papilloma and carcinoma & white patch in leukoplakia [8]. Sanghvi et al., observed that the risk ratio for oral cancers were four-fold in tobacco chewers, two-fold in smokers and four-fold in chewers and smokers both [9]. They also found a synergistic effect of consumption of tobacco and alcohol in the causation of oral cancers. According to Silverman S [10], leukoplakia, the most common pre-cancerous condition of the oral cavity, quite often the subject of research investigations affects from 0.2% to 11.7% of the population. In another study by Da Silva SD, the rates of OSCC recurrence vary from 18 to 76% for patients who underwent standard treatment and it is considered the major cause of poor survival rates [11].

In a study by Prakash N, in the Pap stained smears obtained from study group I (suspicious cases), 14 out of 20 cases (70%) were positive and rest 6 (30%) were negative [12]. Pap stained smears from study group II (non suspicious cases) stained did not exhibit malignant cytological features. In study by Reddy SP [13], 13 out of 15 known carcinoma cases (87%) were correctly diagnosed by Pap stain. 14 smears (93%) out of 15 normal patients were correctly diagnosed by Pap stain. Thus, Pap stain provides a good differential stain and as a result, is widely used for oral cytology.

In the study by Prakash N, using fluorescent AO method, in study group I, 17 out of 20 cases (85%) were positive and rest 3 (15%) were negative. In study group II, 9 of the 20 cases (45%) were positive and the remaining 11 (55%) cases were negative. In the study by Reddy SP, 14 out of 15 known carcinoma cases (93%) were correctly diagnosed by AO confocal microscopy. Fourteen smears (93%) out of 15 normal patients were correctly diagnosed by AO method. This differential fluorescence of AO in malignant and normal cells allows a comparison of total concentration of nucleic acids in the various cells in the preparation.

Prakash N reported 6 (30%) of the lesions in group I as "false negative" by the Pap stain, when compared with biopsy reports. Caulder reported 3 (18%) of the 16 malignant lesions as "false negative" by the conventional Papanicolaou method, when compared with the biopsy findings. Prakash N reported 3 (15%) of the lesions in group I as "false negative" by the fluorescent AO method, when compared with biopsy reports. Caulder reported 4 (13%) of the lesions in group I as "false positive" by the fluorescent AO method, when compared with biopsy reports [14]. There were no "false-negative" reports with this technique. Hence, we conclude with the finding that since AO has a strong affinity for nucleic acids; it binds with DNA and RNA of malignant cells giving higher positivity for fluorescence in neoplastic lesions than the non-neoplastic lesions.

Regarding the technique sensitivity of AO, there are no established standards. Prakash N reported that the sensitivity of AO stain in the demonstration of malignant cells was found to be 85% as against 70% sensitivity of Papanicoloau stain in suspicious cases category. Reddy SP reported that the sensitivity of AO confocal microscopy is (93%) i.e., same as that of the Pap stained smears seen under light microscope [13].

LIMITATION

Rapidly proliferating lesions such as traumatic ulcers have rapid protein synthesis and increased nucleic acid content. This increased amount of nucleic acids in these lesions may lead to false positive results with AO staining. AO fluorescent technique does not give satisfactory morphological details. For comparison, we don't have many similar studies to assess the reliability of AO staining for diagnosis of oral lesions.

CONCLUSION

Hence, we conclude that the fluorescent AO method proved more reliable and more sensitive than Papanicolaou stain in demonstrating malignant cells in oral lesions that were clinically suggestive of cancer. This was confirmed using the biopsy findings but histopathology still remains the "gold standard" for the detection of oral cancer. Pap stain was more sensitive than AO stain in ruling out the presence of malignant cells in oral lesions. The AO technique can be used as a marker for screening of oral lesions. It can be used reliably for the screening of carcinomas and it is especially helpful in the follow-up detection of recurrent carcinoma in previously treated cases.

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