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Research Article

Morphological Assessment of Oral Cytological Smears Before and After Application of Toluidine Blue in Smokers and Nonsmokers

Komali Yerlagudda, Venkatesh Vishwanath Kamath, Krishnanand Satelur

Abstract

Background: Oral habits like smoking, chewing tobacco etc. are documented as initiators of dysplastic changes in the oral mucosa. Toluidine blue has been propagated as an intra-vital stain for the demonstration of the dysplasia in vivo. **Aims**: To assess the cytological changes in smokers using Papanicoloau stain against control of non-smokers before and after application of toluidine blue in vivo. **Materials and Methods**: Twenty-five individuals in each group were included in the study. Two smears were taken from each individual and stained using Papanicoloau stain kit. The cytological features were assessed and computed statistically by Mann Whitney test. **Results:** The use of 1% acetic acid as part of the toluidine blue staining protocol increases the clarity of the cytological smears. Toluidine blue enhanced the staining characteristics of the components of Papanicoloau stain in terms of specificity (clinical site delineation) and sensitivity (improved stain quality and enhanced cytological features). Conflicting results were however detected in observation of keratinized cells and cellular clumping. Comparison of cytomorphological changes in the smokers and the nonsmokers groups revealed cellular pleomorphism, increased agglomerations, micronuclei, binucleation of cells, and keratinization in the smears from smokers. **Conclusion**: We conclude that use of toluidine blue is synergistic in assessment of cytological smears with Papanicoloau stain. Tobacco smoking produces cellular alterations in clinically normal buccal mucosa shown in exfoliative cytology and the results of cellular changes in these smears from smokers can be used as an educational tool in smoking cessation counseling.

Key Words: Oral;Cytology;Micronuclei;Dysplastic;Counseling;Tobacco Cessation.

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Introduction

Oral cancer affects as many as 274,000 people worldwide annually, and the frequency of oral cancer around the world is often indicative of the patterns of use of tobacco products. While there are many different forms of tobacco (cigarettes, cigars, cigarellos, beedis, kreteks, pipe tobaccos and smokeless products), the most common is the manufactured cigarette. Cigarette pyrolysis produces smoke containing combustion byproducts and free radicals which have been defined as carcinogenic. These free radicals can and do react with other additives or other pyrolytic products or living cells and cause DNA damage.[1,2] All parts of the oral cavity are susceptible to cancer from tobacco smoking, including the lip, tongue, palate, gum, and cheek. Different in vivo and ex vivo strategies are being pursued for improving oral cancer mortality. In ex vivo strategies, biopsies of normal and malignant tissues, or scrapings containing exfoliated buccal cells, have been explored for many years. In in vivo strategies, dyes like toluidine blue has been used as a mouth stain for identifying high-risk primary oral premalignant lesions.[2]

In smokers with clinically normal oral mucosa it has been determined there exists some changes such as: a higher rate of proliferation of epithelial cells, nuclear and cytoplasmic alterations, as well as an increase in the number of keratinized cells.[3] Characteristic squamous cells are shed from the mucosal surfaces in the incipient stages of tobacco induced changes prior to the conventionally accepted clinical signs. This allows for smears which are obtained by a simple, quick and noninvasive method for tissue analysis.[4] Oral exfoliative cytology was employed in the present study to observe cellular morphological changes if any in smears from buccal mucosa of smokers. Toluidine blue was used as a standard in vivo stain to delineate clinical changes in the buccal mucosa of both the

groups. Highlighted areas, if any, were then sampled and the smears stained with Papanicoloau's stain using standard procedure. Toluidine blue, a popular stain to recognize dysplasia, was used in this study to detect any sensitivity and /or specificity in the cytological changes of oral smears. It is envisioned that cell changes from smears will be used as an adjunct to clinical examination of smokers, which identifies them as being of high risk for oral cancer. Suspect individuals could then be monitored clinically.

Materials & methods

The study was conducted in the Department of Oral Pathology, Dr Syamala Reddy Dental College & Hospital, Bangalore, in accordance with the ethical standards. Two groups, smokers (n = 25) and nonsmokers (n = 25) without clinically apparent lesions were randomly selected. Both, the study and the control group included healthy volunteers; all of them males aged above 18 years. The same examiner collected all smears from the buccal mucosa before and after toluidine blue rinse. The subjects were instructed to rinse the oral cavity with water and then smear was taken using a cytobrush. Subjects then rinsed their mouth with 10ml of 1% acetic acid for 30 seconds, followed by 10ml of toluidine blue rinse and finally repeated the rinse with 1% acetic acid. Another smear was taken using a new cytobrush. Both the smears were spread on to a clean microscopic glass slide, fixed and stained using standard PAP staining protocol.

The smears were then observed under 40X & 100X magnifications. An eyepiece grid was used and 100 cells per slide were counted to note the changes. The cytological features in the smears were grouped as follows: a) Architectural changes; agglomerations or clumping of squamous cells and altered nuclear cytoplasmic ratio, b) Cellular changes; cellular pleomorphism and presence of micronuclei, c) Nuclear changes; nuclear pleomorphism and binucleation and d) Other changes; includes presence of bacterial colony units and keratin flakes.

Statistical analysis: *Null Hypothesis:* There is no significant difference between smokers and non-smokers i.e. $\eta_1 = \eta_2$. *Alternate Hypothesis:* There is a significant difference between smokers and non-smokers i.e. $\eta_1 \neq \eta_2$. *Level of Significance:* α=0.05. *Statistical Technique Used:* Mann Whitney test. *Decision Criterion:* The decision criterion is to reject the null hypothesis if the p-value is less than 0.05. Otherwise we accept the null hypothesis.

Results

The computation of the various parameters was done separately for each group; before the toluidine blue rinse and after. Table 1 and Table 2 depict the results with statistical observations (Mann-Whitney test) in these groups.

We observe that higher mean numbers of clumps are present in smokers compared to non-smokers and the difference between them is found to be statistically significant ($P < 0.05$). Highly statistically significant difference was noticed between smokers and non-smokers with respect to cellular pleomorphism ($P < 0.001$). Higher mean cellular pleomorphism was found in smokers compared to non-smokers. Mean micronuclei were found to be higher in smokers compared to non-smokers and the difference between them was found to be statistically significant ($P < 0.05$). Smokers recorded a higher mean binucleation compared to non-smokers and the difference between them is found to be statistically significant ($P < 0.05$). No statistically significant difference was observed between smokers and nonsmokers with respect to the number of bacterial colony units ($P > 0.05$). The difference in number of keratin flakes was found to be statistically significant ($P < 0.05$). Higher mean numbers of keratin flakes were found in smokers compared to non-smokers.

Statistically significant difference was observed between smokers and nonsmokers with respect to cellular pleomorphism ($P < 0.05$). Higher mean cellular pleomorphism was found in smokers compared to non-smokers. Higher mean number of clumps, micronuclei, binucleation, number of bacterial colony units and number of keratin flakes was found in smokers compared to non-smokers but the difference between them is not statistically significant ($P > 0.05$). Mean of clumping of cells was more in smokers and was statistically significant (Fig 1a). Clumping of cells was found to be less after toluidine blue rinse. The cells showing altered nuclear cytoplasmic ratio were very few and were not statistically evaluated. Cells showing pleomorphism were found to be higher in

smokers both before and after toluidine blue rinse and was statistically significant (Fig 1b).

Presence of micronuclei in cells was found to be higher in smokers before toluidine blue rinse and was statistically significant (Fig 1c). After toluidine blue rinse there was no significant difference statistically between smears of smokers and nonsmokers in cells showing micronuclei. Before toluidine blue rinse, cells showing binucleation were found to be significantly higher in smokers (Fig 1d). After the rinse, statistically significant difference was not observed between both the study groups. Cells showing nuclear pleomorphism were insufficient for statistical evaluation. There was no statistically significant difference in the cells showing presence of bacterial colonies (Fig 1e) between two groups studied both before and after toluidine blue rinse. However bacterial colonies appeared significantly less both in smokers and in nonsmokers after the rinse. Keratinized cells (Fig 1f) were found to be higher in smokers before toluidine blue rinse and were statistically significant. Statistically no significant difference in the number of keratinized cells was found after the rinse. Cellular features generally were more enhanced and clear in smears after toluidine blue than before (Fig 1g & 1h).

Parameter	Group	n	Mean	Std dev	Median	Mean difference	Z - Value	P - Value
No. of Clumps of cells	Smokers	25	14.28	10.65	13.00	6.840	-2.380	0.017*
	Non-Smokers	25	7.44	5.01	7.00			
Cellular Pleomorphism	Smokers	25	21.96	8.84	22.00	11.360	-4.157	<0.001 *
	Non-Smokers	25	10.60	8.02	8.00			
Micro Nuclei	Smokers	21	7.86	6.78	6.00	4.594	-2.201	0.028*
	Non-Smokers	19	3.26	1.91	3.00			
Bi Nucleation	Smokers	12	2.92	3.37	2.00	1.792	-2.101	0.036*
	Non-Smokers	8	1.13	0.35	1.00			
Bacterial Colony units	Smokers	15	7.33	8.36	4.00	2.733	-0.985	0.325
	Non-Smokers	10	4.60	5.17	2.50			
No. of Keratin Flakes	Smokers	19	4.16	3.39	3.00	0.558	-2.212	0.027*
	Non-Smokers	10	3.60	5.27	2.00			

∗ denotes significant difference

Table 1: Architectural, cytological, nuclear and other changes in PAP smears before toluidine blue rinse in the study group.

Parameter	Group	n	Mean	Std Dev	Median	Mean difference	Z - Value	P - Value
No. of Clumps of Cells	Smokers	24	4.46	2.57	4.00	-0.492	-0.071	0.943
	Non-Smokers	20	4.95	4.03	4.00			
Cellular Pleomorphism	Smokers	24	11.42	8.28	8.00	5.286	-2.106	0.035*
	Non-Smokers	23	6.13	3.98	5.00			
Micro Nuclei	Smokers	20	4.00	3.57	3.00	0.294	-0.311	0.756
	Non-Smokers	17	3.71	3.02	3.00			
Bi Nucleation	Smokers	6	2.00	1.67	1.00	1.000	-1.061	0.289
	Non-Smokers	3	1.00	0.00	1.00			
Bacterial Colony units	Smokers	11	2.91	2.77	2.00	0.509	-1.137	0.256
	Non-Smokers	10	2.40	3.75	1.00			
No. of Keratin Flakes	Smokers	14	4.29	3.69	2.00	0.286	-0.679	0.497
	Non-Smokers	15	4.00	5.59	2.00			

∗ denotes significant difference

Table 2: Architectural, cytological, nuclear and other changes in PAP smears after toluidine blue rinse in the study group.

Discussion

The most common type of oral cancer is squamous cell carcinoma, which develops from the stratified squamous epithelium that lines the oral cavity and pharynx. Tobacco use affects mainly the surface epithelium, resulting in changes in the appearance of tissues. Behavioral intervention to quit smoking may be efficient if smokers are assigned a perceptible and visual individual risk of dysplastic changes. Buccal cells are shed spontaneously (e.g., exfoliative cells)

and daily from healthy buccal mucosa. The exfoliative buccal cells are end-stage cells of differentiation and seldom display mitotic figures. Tobacco-associated buccal cell changes have been reported to be the biomarkers of disease progression.[5] Presence of two or more of the following features were consistent with atypia: nuclear enlargement, associated with the increased nuclear/cytoplasmic ratio, nuclear hyperchromatism, chromatin clumping with prominent nucleation, irregularity of nuclear membranes, bi or multi-nucleation, increased keratinization.[6] Assessment of these changes was incorporated in our study.

Agglomerations or clumping of cells without layering appears to occur more in inflammations and malignancy. Agglomerations indicate high turnover rate. We observed cellular features of clumping more in smokers compared to nonsmokers. Surprisingly post toluidine blue there was lesser number of areas with cellular clumping. This is very significant in view of the fact that clumping of cells is an accepted sign of dysplasia.[4] Whether use of toluidine blue masked this feature or enhanced the cytological smear quality in rendering smears free of the physical action of clumping due to procedural and sampling errors is debatable. We opine that the latter view would seem plausible especially since adhesion of dysplastic cells (if the case may be) is an ionic and biochemical phenomenon unlikely to be affected by use of the dye and its procedural components. The obvious inference would then be that use of toluidine blue has helped in removing the false positive status of the smear.

The size and shape of cells and nuclei in dysplasia usually differs (pleomorphism) from normal cells of the same origin.[7] In our study, cellular pleomorphism was seen more in smokers and the features do not seem to be affected after toluidine blue.

The frequency of occurrence of micronuclei is a measure of chromosome breakage in early cell divisions. The number of micronuclei is known to increase with carcinogenic stimuli.[5] Buccal cell micronuclei were more frequent among cigarette smokers as shown by previous workers.[8,9] Higher frequency of micronuclei in cells collected from smokers was also observed in our study. This is consistent with previous studies. Interestingly in the same group these features were less discernible after the toluidine blue rinse. These results were not statistically viable and it is again surmised that enhancement of the cytological stain by toluidine blue may have removed a few of the false positives leading to a more factual representation of the cellular detail and count.

Binucleation is the presence of two nuclei within a cell. It is a nuclear abnormality seen in dysplastic cells and is found to be increased in smokers.[10] Binucleus formation is considered as indicator of cytotoxicity.[2] In the present study significant increase in binucleus frequency was observed in smokers group. This feature was less evident after toluidine blue rinse.

Cigarette smoke extract enhances binding of bacteria to the epithelial cells. Gordon (2002) reported that buccal cells from smokers bound significantly more bacteria than those from nonsmokers.[5] In our study there was no significant variation in the number of cells showing bacterial colonies between two groups. The results are inconsistent with the previous studies. However, there was significantly less number of bacterial colonies in smears taken after toluidine blue rinse. This was in our opinion due to the effect of 1% acetic acid rinse, which is a part of the toluidine blue stain protocol. The mild acid probably cleaves the weak attachments of superficial bacterial colonies present in the smears and removes them from the surface.

Smoking influences cell keratinization indices in the buccal mucosa of healthy smokers. It is postulated that increase in the production of keratinized cells would be caused by the direct stimulation of the heat of the cigarette and the chemical action of volatile products of the tobacco, with the purpose of protecting itself from these injurious agents.[3] Bustoc et al (2004) showed smokers with clinically normal mucosa exhibiting a greater percentage of keratinized cells.[3] There was significant increase in number of keratin flakes in smears of smokers in our study, which is in concordance with previous studies.

Toluidine blue is a basic chromatic dye used in screening for the demonstration of dysplasia in vivo.[11] Oral mucosa of smokers absorb more toluidine blue than that of nonsmokers.[12] It was under this assumption that the present study was designed and

executed. The observations from our study on the enhancement of cellular and nuclear detail brought about by toluidine blue support our assumptions that this in vivo staining dye not only helps delineate suspect areas clinically but also aids in filtering of cells with false positive features. This was especially so in regard to features like keratinized cells, clumping of cells and binucleation We also observed that the use of 1% acetic acid as part of the toluidine blue staining protocol increased the clarity of the cytological smears by removing background clutter and increasing the quality of the smears.

Recent advances in the clinical visualization and detection of the oral mucosa have made the viability of cytological procedures more specific and sensitive. Contact endoscopy and use of autofluorescence devices are the forerunners in this group. In a study Ramos et al[13] compared detection sensitivity and specificity of toluidine blue stained areas of lower lip with contact endoscopy (stomatoscopy). The chair side procedure which uses a contact laryngoscope attached to a digital camera, a light source and digital recording device was found to be highly specific and sensitive with a sensitivity of 100%, specificity-98%, accuracy-90.3%. According to Lingen et al [14] a screening test must have the five characteristics of being: simple, safe and accepted by the population; able to detect the disease early in its natural evolution; able to preferentially detect those lesions that are prone to progress; able to detect those lesions that are treatable or have prevented their progression with an intervention, and; have high sensitivity (few false negatives) and high positive predictive value. The authors opine that the procedure of contact endoscopy satisfies almost all the requirements of good screening procedure.

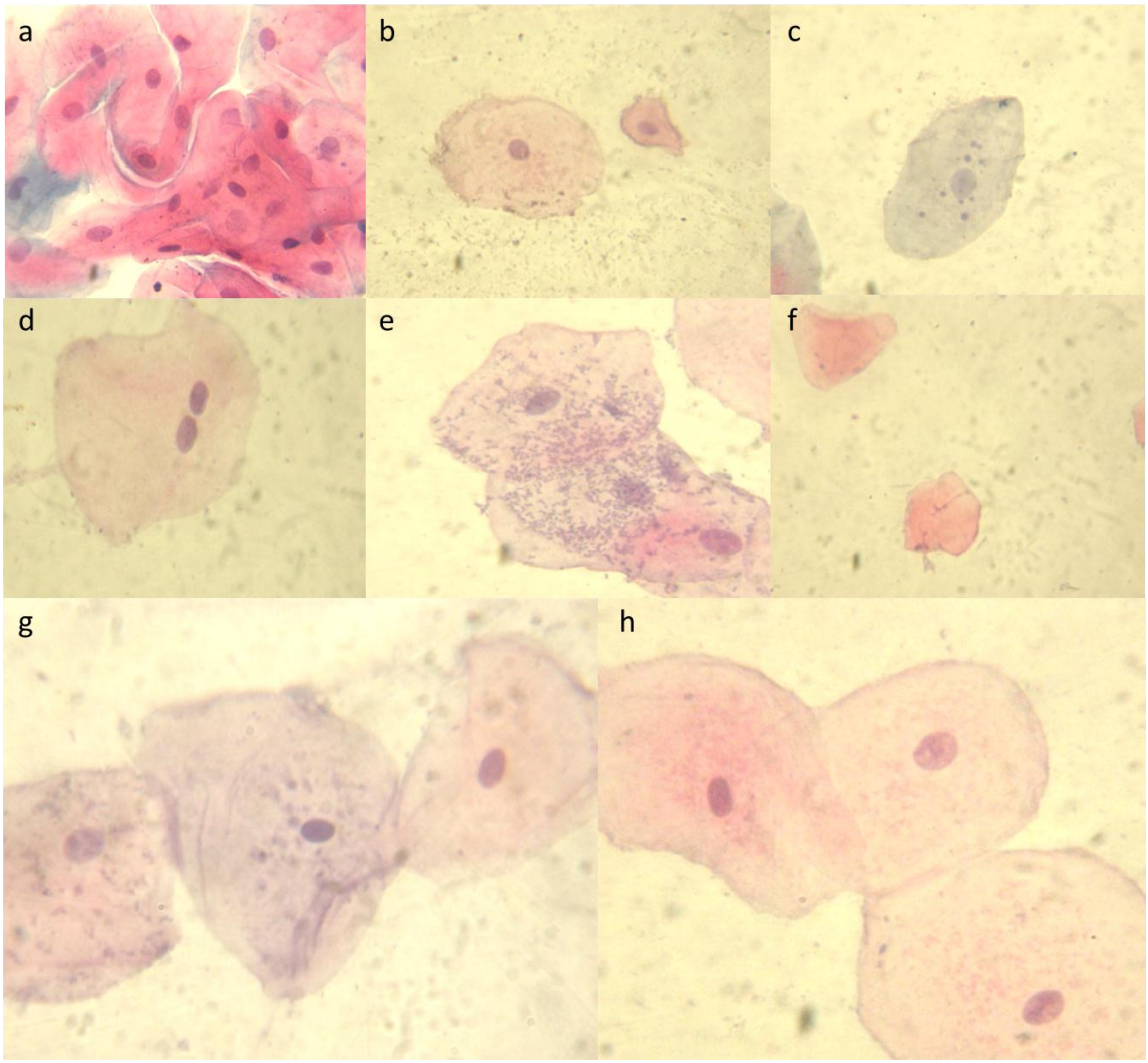

Figure 1: The photomicrograph at low power shows Clumping of cells (a), and under high power demonstrates cellular pleomorphism (b), micronuclei (c), binucleation (d), bacterial aggregates (e) and Keratin flakes (f). The cells observed before (g) and after the toluidine blue rinse (h).

The cells of the oral mucosa have inherent autofluorescent properties. These get altered when dysplastic changes occur in the tissues. The alterations can be captured and visualized using hand-held devices which emit light in the desired wavelength. Significant differences in the fluorescent spectra of normal buccal mucosa and dysplastic oral tissues in the excitation bandwidth of 410nm were observed by Ingrams et al[15] and form the basis of modern day autofluorescent devices. Commercial devices approved by the FDA (VizLite, VelScope etc) are available for the clinician to peruse in diagnosis of potential malignant lesions.

While the technical and financial feasibilities of such devices in a standard clinical setting will probably take some time, the benchmark of diagnosis will be microscopic tissue examination. Cytological smears will hold their stead as highly specific, sensitive, easy to use and reproducible procedures in routine or mass screening of population for potentially and frankly malignant conditions of the oral cavity.

Summary & Conclusion

The results are consistent with previous studies in that tobacco smoking produces cellular alterations in clinically normal buccal mucosa shown in exfoliative cytology. Oral smears stained with PAP are non-invasive techniques and easy procedures for screening in high risk groups. The results of cellular changes in these smears from smokers can be used as an educational tool in smoking cessation counseling. The present study demonstrated that use of toluidine blue prior to staining cytological smears with PAP caused a difference in certain cellular features. We feel that this difference is due to the synergistic effect of toluidine blue on PAP and probably increases the sensitivity of the cytological stain by reducing false positives. However more studies using larger samples may be needed to bear out this fact. The technique of slide preparation of smears can be enhanced by the use of 1% acetic acid rinse as a routine additive in the procedures.

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