Toluidine Blue with a Synergistic Effect in Morphological Assessment of Oral Cytosmears

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Abstract

Objectives: One percent toluidine blue being the most effective adjunct is often used to detect dysplasia. Not much attention has been given to the effect of toluidine blue in enhancement of cytological smears. The present study assessed the smears before and after application of toluidine blue in smokers against non-smokers using three different stains [Papanicolaou (PAP), Hematoxylin and Eosin (H and E), and Giemsa]. Study Design: Twenty-five individuals from each group participated in the study. The oral cytосmears were obtained before and after application of toluidine blue and assessed for clumping of squamous cells, cellular and nuclear pleomorphism, micronuclei, binucleation, bacterial colony units, and keratin flakes. Results: In smokers, the maximum enhancement in cytological smears post-toluidine blue application was shown by Giemsa stain than PAP and H and E stains. Among the individual parameters, nuclear pleomorphism exhibited greatest significant difference between smokers and non-smokers. Conclusion: Toluidine blue enhanced the staining characteristics both in terms of sensitivity and specificity and thereby was found to be synergistic in assessment of cytосmears. The cellular alterations noticed in the smears of smokers with clinically normal buccal mucosa can be used as a means of education tool in counselling for smoking cessation.

Keywords: Smoking, stains, toluidine blue

INTRODUCTION

Oral health is essential in improving one’s quality of life. Any abnormality ranging from dental decay to fatal oral cancer affects individual’s well-being. Oral cancer is considered to be a significant threat to the public health as it is often not diagnosed until it is advanced.[1] Various methods have been developed to supplement clinical examination and to improve diagnosis of oral cancer in its early stage. The most evaluated adjunct for lesion detection is toluidine blue, a metachromatic acidophilic dye. Dysplasia in premalignant lesions contains much more deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) than the normal epithelium due to active cellular proliferation. So use of in vivo staining by means of toluidine blue is based on the fact that it selectively stains acidic tissue components such as DNA and RNA.[2]

It has been determined that smokers with clinically normal mucosa exhibit certain cytological alterations such as higher rate of proliferation of epithelial cells, nuclear and cytoplasmic abnormalities, and increase in keratinized cells.[3] So before the clinical signs appear, characteristic squamous cells are shed from oral mucosa in the incipient stages which can be observed using exfoliative cytology.[4]

Further, a study carried out recently concluded that toluidine blue enhanced the staining characters of Papanicolaou (PAP) stain by improving cytological features and stain quality.[5] This present study is an attempt to validate this effect of toluidine blue using three different stains.

MATERIALS AND METHODS

The study sample included randomly selected 25 healthy volunteers with clinically normal oral mucosa and 25 smokers without clinically apparent lesions.

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The written consent was obtained from all the subjects. Ethical approval certificate was obtained from the Institutional Review Board. All of them were male and aged 18 years or more. Patients with provisional/confirmed diagnosis of any cancer, with any clinical apparent lesions, diabetes, heart, and neurological diseases were excluded.

The cytomsears were obtained from one side of buccal mucosa before and after application of toluidine blue. The subjects were instructed to rinse the oral cavity with water for 20 s and three smears were obtained using cytobrush. Later, a rinse with 10 mL of 1% acetic acid for 30 s followed by 1% toluidine blue (Mashberg preparation) application for 20 s was carried out. The final rinse with 1% acetic acid was needed to remove excess toluidine blue. Three more smears were taken post-toluidine blue application in each subject. All the smears were transferred to dry glass slides, fixed with microanatomy fixative, and stained separately using the PAP [Figure 1a and b], H and E [Figure 1c and d], and Giemsa stains [Figure 1e and f].

The smears were observed under 40× and 100× magnifications. A cell counter was used and 100 cells per slide were counted and following cytological parameters were assessed: clumping [Figure 2a], bacterial colony units of squamous cells [Figure 2b], micronuclei [Figure 2c], keratin flakes [Figure 2d], cellular pleomorphism [Figure 2e], and binucleation [Figure 2f].

The results were expressed as Mean ± SD (standard deviation), median, and range values. Since the measurements were in counts, non-parametric methods were used for the analysis. Mann-Whitney test was used for the two group comparisons (smokers vs. non-smokers).

*P value* of 0.05 or less was considered for statistical significance. Within each stain changes occurring before and after application of toluidine blue were assessed using Wilcoxon signed-rank test.

**Results**

Most of the smokers and non-smokers of this study belonged to the age group of 18 to 25 years. Overall the parameters in cytomsears exhibited statistically significant difference between smokers and non-smokers [Table 1].

The ratio of means of each parameter of smokers and non-smokers were taken and sum of the ratios of all parameters for each stain was calculated. This ratio denoted the amount of difference between the smokers and non-smokers in the cytomsears. The difference between smokers and non-smokers increased after toluidine blue application using Giemsa (27.31 to 30.26) and PAP stain (23.40 to 25.18), while it decreased on using H and E stain (29.93 to 21.82) [Table 2].

The changes occurring within each stain before and after application of toluidine blue was assessed using Wilcoxon Matched Pairs Test in smokers. These changes were not of...
much concern in non-smokers as toluidine blue was ineffective in them. Within each stain, the ratio of mean values of each parameter after and before application of toluidine blue were taken in smokers and sum of the ratios was obtained. The sum denoted the amount of difference present between before and after application of toluidine blue in smokers. The sum of ratios in Giemsa, PAP, and H and E stains were 10.20, 8.55, and 8.37 respectively. Therefore, among smokers, Giemsa stain showed maximum variation between before and after application of toluidine blue followed by PAP and H and E [Table 3].

There was an overall increase in mean frequency of cellular pleomorphism, nuclear pleomorphism, and keratin squames, and decrease in mean frequency of bacterial units post-toluidine blue application using all stains. The cytological features were more enhanced and clear post-toluidine blue application in the smears.

**Discussion**

The efficiency of *in vivo* staining with toluidine blue have been evaluated by various studies in detecting dysplasia and malignant lesions. Toluidine blue being a metachromatic acidophilic dye of thiazine group selectively stains acidic tissue components of dystrophic and cancer cells. This dye when applied to smokers acts as a visual aid to monitor high risk individuals without any visible clinical lesions. Exfoliative cytology when applied, further benefits in detecting cytological alterations, if any, in the smears of smokers. This being a non-invasive technique is a better option for early diagnosis of potentially malignant lesions in smokers. In smokers exhibiting clinically normal mucosa existence of certain alterations such as a higher rate of proliferation of epithelial cells, nuclear and cytoplasmatic alterations, and increase in the number of keratinized cells has been determined. In the study by Ahmed *et al.*, increase in nuclear size, nuclear cytoplasmic (N/C) ratio, and multi-lobed nuclei, while a decrease in size of cytoplasm in smears of smokers as compared to non-smokers was reported. These results were consistent with the present study.

PAP and H and E stains are most easily available and routinely used stains in oral exfoliative cytology. One of the studies compared diagnostic accuracy of PAP stain with Giemsa and concluded Giemsa stain to be more superior in oral cytology diagnosis. Therefore, present study included all the three stains—PAP, H and E, and Giemsa stains. Though many studies with toluidine blue used various stains for brush cytology and histopathological evaluation of biopsy respectively, none of them emphasized the effect of toluidine blue in enhancement of staining. One of the recent studies showed that toluidine blue enhanced the staining characters of PAP by improving cytological features and stain quality. Thus, in the present study an attempt had been made to evaluate the role of toluidine blue in enhancing the staining characteristics of PAP, H and E, and Giemsa stains in oral smears of smokers against non-smokers.

The results of the present study indicate occurrence of certain alterations in the cytosmears post-toluidine blue application.

Giemsa stain exhibited maximum variation in cytosmears after application of toluidine blue than PAP and H and E stains as revealed by the sum of ratios of means of parameters obtained before and after application of toluidine blue [Table 3].

The variations in smears can be attributed to the effect of toluidine blue in enhancing the staining in terms of sensitivity by improving cytological features and specificity by means of clinical site delineation. The pre-rinse acetic acid along with toluidine blue would have removed the false positives. The acidic effect of the acid must have removed the false positives. The acidic effect of the acid must have removed the false negatives caused by bacterial cells. The variations in smears can be attributed to the effect of toluidine blue in enhancing the staining in terms of sensitivity by improving cytological features and specificity by means of clinical site delineation. The pre-rinse acetic acid along with toluidine blue would have removed the false positives. The acidic effect of the acid must have removed the background clutter and improved the stain. Increase in the number of parameters exhibiting significant difference between both groups post-toluidine blue application using...
Giemsa stain and decrease in the same using H and E stain was observed.

Giemsa stain being superior in determining nuclear chromatin, cellularity, cytoplasmic detail, background material, and ability in making a definite diagnosis, exhibited least number of false positives.\[9\]

False positives due to misinterpretation of nuclear anomalies like karyorrhexis, karyolysis, condensed chromatin, and binucleates as micronuclei were common with DNA non-specific stains like PAP and H and E along with the evidence of keratin granules, contamination of bacteria, and small dye granules.\[10\]

The pre-rinse acetic acid along with toluidine blue would have

| Parameters          | Stain         | Smoker (S)/ non-smoker (NS) | Before | After | Ratio of means | P       | Ratio of means | P       |
|---------------------|---------------|-----------------------------|--------|-------|----------------|---------|----------------|---------|
| No of clumps        | Giemsa stain  | S                           | 14.56  | 0.00001* | 12.52          | 0.0215* |
|                     |               | NS                          | 9.16   |        |                |         | 10.2           |         |
| Cellular pleomorphism | S             | 0.52                        | 0.0991 | 1.48   | 0.0023*        |         | 0.48           |         |
|                     |               | NS                          | 0.2    |        |                |         | 0.48           |         |
| Micronuclei         | S             | 13.64                       | 0.0006* | 15     | 0.00001*       |         | 4.68           |         |
|                     |               | NS                          | 6.28   |        |                |         | 4.68           |         |
| Bi-nucleation       | S             | 0.4                         | 0.086  | 0.52   | 0.086          |         | 0.52           | 0.086  |
|                     |               | NS                          | 0.04   |        |                |         | 0.04           |         |
| Bacterial colony units | S            | 15.2                        | 0.0043* | 14.8   | 0.0026*        |         | 6.48           |         |
|                     |               | NS                          | 6.16   |        |                |         | 6.48           |         |
| No. of keratin squames | S           | 0.6                         | 0.0991 | 1.08   | 0.0134*        |         | 0.4            |         |
|                     |               | NS                          | 0.2    |        |                |         | 0.4            |         |
| Nuclear pleomorphism | S             | 5.48                        | 0.00001* | 7.24  | 0.00001*       |         | 7.24           | 0.00001* |
|                     |               | NS                          | 1      |        |                |         | 1.52           |         |
| Sum of the ratios   |               |                             | 27.31  |        |                |         | 30.26          |         |
| No. of clumps       | H and E stain | S                           | 11.48  | 0.0032* | 12.16          | 0.0008* |
|                     |               | NS                          | 8.36   |        |                |         | 8.44           |         |
| Cellular pleomorphism | S            | 1.08                        | 0.0130* | 1.6    | 0.187          |         | 0.84           |         |
|                     |               | NS                          | 0.28   |        |                |         | 0.84           |         |
| Micronuclei         | S             | 23.12                       | 0.0024* | 21.36  | 0.0032*        |         | 13.4           |         |
|                     |               | NS                          | 14.44  |        |                |         | 13.4           |         |
| Bi-nucleation       | S             | 0.4                         | 0.4551 | 0.2    | 1              |         | 0.2            |         |
|                     |               | NS                          | 0.24   |        |                |         | 0.2            |         |
| Bacterial colony units | S           | 11.16                       | 0.3084 | 8.48   | 0.2523         |         | 8.48           |         |
|                     |               | NS                          | 7.44   |        |                |         | 6.84           |         |
| No. of keratin squames | S           | 1.08                        | 0.0057* | 2.32   | 0.00001*       |         | 0.24           |         |
|                     |               | NS                          | 0.08   |        |                |         | 0.24           |         |
| Nuclear pleomorphism | S             | 3.6                         | 0.00001* | 6.76  | 0.00001*       |         | 6.76           | 0.00001* |
|                     |               | NS                          | 0.56   |        |                |         | 1.36           |         |
| Sum of the Ratios   |               |                             | 29.93  |        |                |         | 21.82          |         |
| No. of clumps       | PAP stain     | S                           | 11.12  | 0.0107* | 12.88          | 0.0001* |
|                     |               | NS                          | 8.88   |        |                |         | 7.48           |         |
| Cellular pleomorphism | S            | 1                           | 0.0305* | 1.2    | 0.0064*        |         | 0.32           |         |
|                     |               | NS                          | 0.24   |        |                |         | 0.32           |         |
| Micronuclei         | S             | 14.32                       | 0.00001* | 15.16  | 0.0001*        |         | 7.16           |         |
|                     |               | NS                          | 6.16   |        |                |         | 7.16           |         |
| Bi-nucleation       | S             | 0.24                        | 0.7785 | 0.28   | 0.4492         |         | 0.28           |         |
|                     |               | NS                          | 0.16   |        |                |         | 0.12           |         |
| Bacterial colony units | S           | 15.56                       | 0.0059* | 12.56  | 0.0019*        |         | 5.56           |         |
|                     |               | NS                          | 6.64   |        |                |         | 5.56           |         |
| No. of keratin squames | S           | 0.92                        | 0.0134* | 1.64   | 0.0002*        |         | 0.2            |         |
|                     |               | NS                          | 0.12   |        |                |         | 0.2            |         |
| Nuclear pleomorphism | S             | 3.48                        | 0.00001* | 4.8    | 0.00001*       |         | 4.8            | 0.00001* |
|                     |               | NS                          | 0.84   |        |                |         | 1              |         |
| Sum of the ratios   |               |                             | 23.4   |        |                |         | 25.18          |         |

PAP: Papanicolaou, H and E: Hematoxylin and Eosin
removed the false positives reducing the significant difference in H and E stain. PAP stain, on the other hand was not much influenced by the toluidine blue application.

The identification of two or more of the following features would be consistent with atypia: nuclear enlargement associated with increase in nuclear cytoplasmic ratio, hyperchromatism, chromatin clumping with prominent nucleoli, irregular nuclear membrane, bi/multinucleation, scant cytoplasm, variation in size and/or shape of cell and nucleus, and increased keratinization.\[11\] Assessment of these features was incorporated in the present study.

### Table 3: Comparison of means of all parameters before and after application of toluidine blue in each of the stains in smokers and non-smokers

| Parameters                  | Stain         | After (A)/before (B) | Smokers | Nonsmokers | Ratio of means | P       | Ratio of means | P       |
|-----------------------------|---------------|----------------------|---------|------------|----------------|---------|----------------|---------|
| No of clumps                | Giemsa Stain  | A                    | 12.52   | 0.00001*   | 10.2           | 0.0215* |                |         |
|                             |               | B                    | 14.56   | 9.16       |                |         |                |         |
| Cellular pleomorphism       |               | A                    | 1.48    | 0.0991     | 0.48           | 0.0023* |                |         |
|                             |               | B                    | 0.52    | 0.2        |                |         |                |         |
| Micronuclei                 |               | A                    | 15      | 0.0006*    | 4.68           | 0.00001*|                |         |
|                             |               | B                    | 13.64   | 6.28       |                |         |                |         |
| Bi-nucleation               |               | A                    | 0.52    | 0.086      | 0.04           | 0.086   |                |         |
|                             |               | B                    | 0.4     | 0.04       |                |         |                |         |
| Bacterial colony units      |               | A                    | 14.8    | 0.0043*    | 6.48           | 0.0026* |                |         |
|                             |               | B                    | 15.2    | 6.16       |                |         |                |         |
| No of keratin squames       |               | A                    | 1.08    | 0.0991     | 0.4            | 0.0134* |                |         |
|                             |               | B                    | 0.6     | 0.2        |                |         |                |         |
| Nuclear pleomorphism        |               | A                    | 7.24    | 0.00001*   | 1.52           | 0.00001*|                |         |
|                             |               | B                    | 5.48    | 1          |                |         |                |         |
| Sum of ratios               | H and E stain | A                    | 12.56   | 0.0032*    | 8.44           | 0.0008* |                |         |
|                             |               | B                    | 11.12   | 8.36       |                |         |                |         |
| Cellular pleomorphism       |               | A                    | 1.64    | 0.0130*    | 0.84           | 0.187   |                |         |
|                             |               | B                    | 1.12    | 0.28       |                |         |                |         |
| Micronuclei                 |               | A                    | 20.88   | 0.0024*    | 13.4           | 0.0032* |                |         |
|                             |               | B                    | 22.92   | 14.44      |                |         |                |         |
| Bi-nucleation               |               | A                    | 0.12    | 0.4551     | 0.2            | 1       |                |         |
|                             |               | B                    | 0.48    | 0.24       |                |         |                |         |
| Bacterial colony units      |               | A                    | 7.44    | 0.3084     | 6.84           | 0.2523  |                |         |
|                             |               | B                    | 11.04   | 7.44       |                |         |                |         |
| No of keratin squames       |               | A                    | 2.32    | 0.0057*    | 0.24           | 0.00001*|                |         |
|                             |               | B                    | 1.08    | 0.08       |                |         |                |         |
| Nuclear pleomorphism        |               | A                    | 6.76    | 0.00001*   | 1.36           | 0.00001*|                |         |
|                             |               | B                    | 3.76    | 0.56       |                |         |                |         |
| Sum of ratios               | PAP stain     | A                    | 12.88   | 0.0107*    | 7.48           | 0.0001* |                |         |
|                             |               | B                    | 11.12   | 8.88       |                |         |                |         |
| Cellular pleomorphism       |               | A                    | 1.2     | 0.0305*    | 0.32           | 0.0064* |                |         |
|                             |               | B                    | 1       | 0.24       |                |         |                |         |
| Micronuclei                 |               | A                    | 15.16   | 0.00001*   | 7.16           | 0.0001* |                |         |
|                             |               | B                    | 14.32   | 6.16       |                |         |                |         |
| Bi-nucleation               |               | A                    | 0.28    | 0.7785     | 0.12           | 0.4492  |                |         |
|                             |               | B                    | 0.24    | 0.16       |                |         |                |         |
| Bacterial colony units      |               | A                    | 12.56   | 0.0059*    | 5.56           | 0.0019* |                |         |
|                             |               | B                    | 15.56   | 6.64       |                |         |                |         |
| No. of keratin squames      |               | A                    | 1.64    | 0.0134*    | 0.2            | 0.0002* |                |         |
|                             |               | B                    | 0.92    | 0.12       |                |         |                |         |
| Nuclear pleomorphism        |               | A                    | 4.8     | 0.00001*   | 1              | 0.00001*|                |         |
|                             |               | B                    | 3.48    | 0.84       |                |         |                |         |
| Sum of ratios               |               | A                    | 8.55    | 7.78       |                |         |                |         |

PAP: Papanicolaou, H and E: Hematoxylin and Eosin
Clumping or agglomerations of cells is accepted to be a sign of dysplasia.

The present study showed greater number of clumping of cells in smokers compared to non-smokers. Clumping or agglomeration of cells in the present study did not show any specific pattern post-toluidine blue application in all the stains. The size and shape of cells and nuclei get altered in conditions of dysplasia when compared to normal cells of same origin.

In the present study, both cellular and nuclear pleomorphism parameters were more in smokers than non-smokers and improved post-toluidine blue application. The results were in accordance with the previous studies. Nuclear pleomorphism mean frequency was found to be more using Giemsa stain in both groups.

The micronucleus represents a small, additional nucleus formed due to lag in mitosis caused by exclusion of fragment or whole chromosome. The micronucleus rates reflecting chromosomal breakage and impaired mitotic apparatus activity have been used as a biomarker for genomic instability and cancer risk since the last few decades. The buccal cell micronuclei frequency was more in cigarette smokers according to previous studies similar to that of present study. The micronuclei mean frequency was highest with H and E stain in both groups before and after application of toluidine blue. The possible explanation for increased frequency of micronuclei in H and E stain could be due to misinterpretation of nuclear anomalies like karyorrhexis, karyolysis, and condensed chromatin as micronuclei and similar results were obtained in a study carried over by Grover et al.

Binucleation, the presence of two nuclei within a cell is a nuclear abnormality seen in cases of dysplastic cells, in conditions of smoking and considered as a marker of cytotoxicity. The results of the present study showed significant increase in binucleation frequency in smokers.

Bacterial binding to epithelial cells is enhanced by cigarette smoke extract. According to Gordon et al., buccal cells from smokers bound significantly more bacteria than those from non-smokers. Consistent with the previous studies, there was an overall significant increase in bacterial colony units in smokers compared to non-smokers in the present study. There was a decrease in the frequency of bacterial colony units in smears obtained after toluidine blue application using all the stains. The reason could be the effect of 1% acetic acid pre-rinse which cleaved the weak attachments of superficial bacterial colony units present in the smears. This was in accordance with a study by Yerlagudda et al.

The cigarette smoking had an influential effect on cell keratinization indices in buccal mucosa of healthy smokers. There was a significant increase in number of keratin squames in smears of smokers in present study, which is in concordance with previous studies. Increase in the keratinization of cells was thought to be an act of protective mechanism to escape from the direct stimulation of heat of cigarette and also from chemical action of volatile products. Further, there was significant increase in the number of keratin squames in the smears obtained after toluidine blue application in both smokers and non-smokers using all the stains. The possible reason would be the enhancement of staining by toluidine blue.

Further the present study suggests, incorporation of 1% acetic acid rinse as a routine additive into slide preparation to achieve enhancement of staining. These cellular alterations observed in the smears can be used as a mode of educational tool in counselling for smoking cessation. Moreover in smokers, cellular alterations in smears should be used in addition to clinical examination to identify their risk to develop cancerous lesions.

Though all the three stains were DNA-nonspecific, the reason why Giemsa stain was more enhanced by toluidine blue application warrants further evaluation as scientific literature lacks optimum relevant data in context to use of this stain with toluidine blue.

However, more studies should be carried out on larger populations to validate this synergistic effect of toluidine blue.

**Conclusion**

Toluidine blue being an acidophilic dye is taken up more by dysplastic cells. Oral mucosa of smokers undergoing such changes takes up more of the dye. Further the smears obtained from such visualized areas not only exhibited the specificity in terms of site delineation, but also exhibited enhanced cellular and nuclear detail. This could be attributed to the synergistic effect of toluidine blue along with pre-rinse acetic acid in removing false positives and enhancing the staining.

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Conflicts of interest

There are no conflicts of interest.

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