Asian Journal of Current Research in Clinical Cancer
ISSN: 3062-4444
2023, Volume 3, Issue 2, Page No: 25-31

Available online at: www.galaxypub.co/page/journals

Galaxy Publication

Lack of Combined Effect of Toluidine Blue and Cytomorphometry in Differentiating Dysplasia in Oral Exfoliative Cytology

Yashi Shrivastava[1], Monal Yuwanati[2]*, Narayanan Ganesh[3]

[1]Department of Oral Pathology and Microbiology, People's College of Dental Science and Research Centre, People's University, Bhopal.
[2]Department of Oral and Maxillofacial Pathology, Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai, 600077, India.
[3]Department of Research, Jawaharlal Nehru Cancer Hospital & Research Centre, Idgah Hills, Bhopal, MP, India.

***E-mail** ✉ monal9817@gmail.com

Received: 19 September 2023; **Revised:** 20 November 2023; **Accepted:** 26 November 2023

ABSTRACT

Several diagnostic methods, including vital staining, autofluorescence, exfoliative cytology, and histomorphometry, have been investigated for the detection of dysplasia, although each shows different levels of sensitivity and specificity. This study aimed to investigate the role of toluidine blue in increasing the precision of histomorphometry for the detection of dysplasia in oral potentially malignant disorders (OPMDs) using exfoliative cytology. A cross-sectional, observational study was conducted on patients visiting dental clinics and oral health camps who were suspected of having OPMDs. Cytology smears were obtained before and after the application of toluidine blue to the lesion areas, following standard protocols. Two pathologists independently performed dysplasia grading and histomorphometry assessments. Statistical analysis, including chi-square and t-tests, was performed ($P < 0.05$). This study showed a positive correlation between toluidine blue positivity and dysplasia in OPMDs. The toluidine blue stain showed a sensitivity of 39.53%, specificity of 97.56%, positive predictive value of 97.14%, and negative predictive value of 43.58%. However, the use of toluidine blue before cytology smears did not significantly increase the diagnostic accuracy of histomorphometry. Furthermore, there was no significant significant in cellular or nuclear morphometry when compared to normal tissue. The study concluded that toluidine blue did not increase the effectiveness of cytomorphometry in detecting dysplasia on cytosmears. However, toluidine blue may still aid clinicians in identifying areas suspicious of dysplasia, which could warrant further biopsy examination.

Keywords: Dysplasia, Cytomorphometry, Oral premalignant lesion, Toluidine blue, Leukoplakia, Erythroplakia

How to Cite This Article: Shrivastava Y, Yuwanati M, Ganesh N. Lack of Combined Effect of Toluidine Blue and Cytomorphometry in Differentiating Dysplasia in Oral Exfoliative Cytology. Asian J Curr Res Clin Cancer. 2023;3(2):25-31. https://doi.org/10.51847/sEjz14Y7qU

Introduction

Oral cancer continues to be a major health concern in India, affecting millions of individuals. It is a preventable disease, with early detection of premalignant changes in the oral cavity playing a crucial role in prevention [1]. Premalignant oral lesions carry a higher risk of progressing to cancer [2]. However, identifying these lesions as dysplastic is challenging, as not all such lesions undergo malignant transformation. To detect oral cancer at an early stage, various diagnostic methods and tools have been explored [3]. Some commonly used tools for early oral cancer detection include toluidine blue or tolonium chloride dye, oral CDx brush biopsy kits, salivary diagnostics, and optical imaging systems. Toluidine blue, with a reported sensitivity of 97.8% and specificity of 100%, has historically been used to predict dysplastic changes in the oral mucosa [4]. Despite its non-invasive nature, simplicity, and cost-effectiveness, toluidine blue can sometimes produce false positives, particularly with inflammatory lesions [5]. Histologically, premalignant lesions display abnormal nuclear and cellular changes that

can be detected through exfoliative cytology [6]. Advances in information technology have led to the development of histomorphometry as a method for analyzing oral premalignant lesions [7]. Due to its ability to minimize pathologist bias, histomorphometry is not only effective for early detection of dysplastic changes but can also serve as a follow-up or screening tool alongside exfoliative cytology. However, comparative evaluations of histomorphometric studies have been lacking, and morphology values can vary [8]. Clinicians also face difficulties in detecting these lesions early, which complicates timely intervention [9]. Additionally, the absence of definitive detection tools hampers effective follow-up [3]. Among the tools mentioned, toluidine blue staining and cytology show promising sensitivity and specificity, but challenges with false negatives and false positives remain.

To address these concerns, Caruntu *et al*. [10] introduced a quantitative method for constructing a classifier to detect dysplastic or cancerous cells in cytology smears. While exfoliative cytology has proven valuable in cervical cancer detection, its use in oral cancer and precancer detection has been limited, mainly serving as an adjunct due to its unreliability. The addition of toluidine blue staining enhances the sensitivity of cytology but does not fully overcome its limitations. A synergistic effect can be achieved by combining exfoliative cytology with toluidine blue staining, which allows for a more comprehensive evaluation of cellular morphology. Integrating quantitative histomorphometry provides additional insights into this traditional yet reliable technique, especially given its low cost and non-invasive nature. With these considerations in mind, the current study was designed to evaluate whether the application of toluidine blue staining could enhance the effectiveness of cytomorphometry in distinguishing dysplasia.

Materials and Methods

Study design

This cross-sectional, observational, and analytical study employed a single-blind approach, targeting individuals aged 15 to 35 years. Ethical approval was granted by the Institutional Ethics Committee before proceeding with the study. The data were collected over one month from participants residing in a metro city and its nearby areas. Oral health camps were organized in different locations for the initial screening of oral potentially malignant disorders (OPMDs), following the WHO criteria. The principal investigator conducted clinical assessments, with guidance provided by a supervising expert. Patients suspected of having oral premalignant lesions were selected for the study, and necessary details were recorded on a data sheet. The recruitment process continued until the required sample size was reached. Participants were thoroughly informed about the study, and written consent was obtained. Those allergic to toluidine blue or unwilling to participate were excluded. Dysplasia severity, cytomorphometric parameters, and the expertise of the pathologists were considered dependent and independent variables. Factors such as tobacco use, areca nut chewing, and candidiasis were treated as potential confounders.

Investigation process

Upon identifying the suspected oral premalignant lesions, participants were instructed to rinse their mouths, and the first cytology sample **(Figure 1)** was collected and systematically labeled. Subsequently, toluidine blue staining was applied with proper precautions, and results were recorded in the data sheet **(Figures 2a and 2b)**. Another cytology sample was obtained from the toluidine blue-positive area and labeled accordingly. Patients suspected of having malignant lesions based on clinical examination were referred for a biopsy after obtaining their consent. Two independent pathologists performed the grading of dysplasia in the cytology samples. Following this, photographs of the cytology smears were taken, and the sample was given a new code for blinding purposes before histomorphometric analysis. The analysis was conducted using morphometry software (Magnus Pro), with the patient's normal mucosa serving as a control for comparison. Data from the study were organized and analyzed to evaluate the following outcomes: 1) correlation between cytology findings and dysplasia, 2) comparison of cytomorphometric findings for dysplasia against normal mucosa, and 3) correlation between cytomorphometry and toluidine blue staining results.

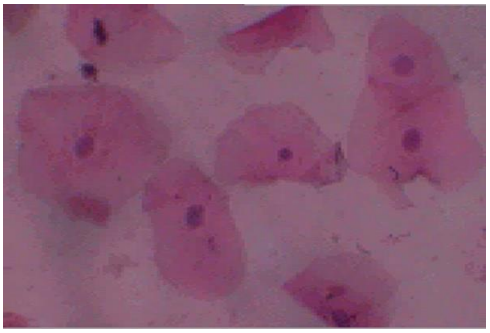
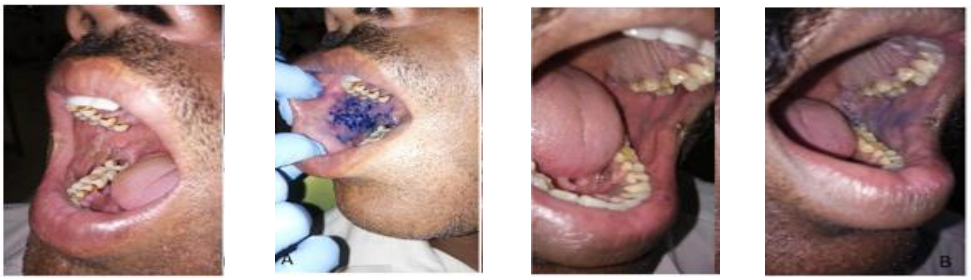


Figure 1. Cytology smear of patients.



a) b)

Figure 2. Toluidine blue staining: a) positive staining, and b) negative staining

Results and Discussion

A total of 126 participants with oral potentially malignant disorders (OPMDs) were included in the study, comprising 99 males and 27 females, with an average age of 30 years. The most prevalent habit among the participants was tobacco use, followed by the consumption of betel nuts and cigarettes. Clinical lesions observed in the participants included leukoplakia (67 cases), oral submucous fibrosis (53 cases), erythroplakia (29 cases), and lichen planus (11 cases). Cytological analysis of the smears from all 126 participants revealed dysplastic changes in 86 individuals. A comparison of these cytological results with toluidine blue staining revealed that only 34 out of the 86 dysplastic cases were positive for the stain. A detailed breakdown of these findings is provided in **Table 1**.

Table 1. Characteristics of the study group

Gender	Male		99	
	Female		27	
Habit	Tobacco		119	
	Betel nut		71	
	Cigarette		63	
Duration of habit	yrs.	Tobacco	Cigarette	Betel nut
	0-1	07	64	55
	1-5	17	11	08
	6-10	23	10	13
	11-15	30	17	18
	15-20	30	14	20
	20-25	11	11	12
Type of lesion	Leukoplakia		67	
	Erythroplakia		29	
	Oral lichen planus		11	
	Oral submucous fibrosis		53	
Toluidine blue test	Positive		35	

	Negative	91
Dysplasia	Present	86
	Absent	40

Link between cytology diagnosis of dysplasia and toluidine blue staining

A chi-square analysis was used to assess the association between dysplasia presence and toluidine blue positivity **(Table 2)**. The results showed a significant correlation (X2 (1, n = 126) = 18.6656, P = .000016). Dysplastic lesions exhibited higher positivity compared to non-dysplastic (normal/benign) lesions. The toluidine blue stain demonstrated a sensitivity of 39.53%, specificity of 97.56%, positive predictive value of 97.14%, and negative predictive value of 43.58%. Toluidine blue, a vital stain, is absorbed by cell nuclei because of its affinity for DNA and RNA. To evaluate whether the application of toluidine blue improves the accuracy of detecting dysplasia through exfoliative cytology, another chi-square test was performed. This analysis showed a significant relationship (X2 (n = 126) = 81.457, P < .00001) **(Table 3)**. Additionally, the Kappa statistic of 0.785 suggested moderate agreement between observers in identifying dysplasia.

Table 2. Correlation of the presence of dysplasia with toluidine blue positivity

		Dysplasia			
		Present	**Absent**	**Total**	**P value**
Toluidine blue staining	Positive	34	1	35	0.000016
	Negative	52	39	91	
	Total	86	40	126	

Table 3. Inter-observer agreement on dysplasia (After toluidine blue application)

		First Cytology smear		**Total**	**P value**
		Dysplasia	**Normal**		
Second cytosmear	Dysplasia	35	12	47	0.00001
	Normal	0	79	79	
Total		35	91	126	

Comparison of cytomorphometric analysis for dysplasia detection in normal and dysplastic mucosa

To assess changes in morphometric characteristics, a comparison was made between cells from dysplastic and normal mucosa. Dysplastic cells exhibited larger nuclear parameters, while cellular parameters were reduced, leading to an increase in the ratio of nuclear-to-cellular area and diameter. A statistical analysis using the t-test showed no significant difference in the morphometric measurements between the normal and dysplastic mucosa **(Table 4)**. Further analysis compared key cytomorphometric values, including cell area (CA), nuclear area (NA), the ratio of nuclear-to-cell area (NA: CA), cell diameter (CD), nuclear diameter (ND), and the ratio of nuclear-to-cellular diameter (ND: CD), to determine whether toluidine blue staining affected these measurements. No significant changes were observed in the mean values of these parameters after toluidine blue application **(Table 4)**. However, a comparison of dysplastic cells before and after the application of toluidine blue showed significant alterations in CA, NA, CD, and ND. Specifically, CA, NA, and CD decreased, while ND, NCA, and NCD increased post-staining. This suggests that while toluidine blue staining impacts some morphometric values, it does not improve the ability of cytomorphometry to discriminate dysplastic changes.

Table 4. Comparisons of cytomorphometric parameters for normal mucosa and dysplastic mucosa before and after TB application; CA, NA, CD, and NCD showed significant changes in values.

Normal Mucosa				**Dysplasia**					
Before TB application		**After TB Application**		**Before TB application**		**After TB Application**		**Normal vs Dysplasia (After TB Application)**	**Pre and Post TB application (dysplastic mucosa)**
Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**t-test (p-value)†**	**p-value**

CA	687.8532	108.3428	620.2732	81.16636	748.1616	60.16166	610.5762	87.02875	0.1541	0.009*
NA	16.0594	1.173549	14.2784	1.012995	15.5528	1.244192	14.826	1.641975	0.2632	0.032*
NA: CA	0.02392	0.004922	0.023456	0.004298	0.020913	0.002626	0.024679	0.004246	0.1313	0.334
CD	33.794	1.783708	32.3504	1.970659	34.4218	3.305812	29.644	4.045592	0.3592	0.011*
ND	4.446	0.386546	4.5468	0.300343	4.2592	0.730135	4.241	0.713402	0.3133	0.749
ND: CD	0.131974	0.014864	0.140967	0.012500	0.123114	0.012186	0.143854	0.023911	0.1664	0.018*

† Comparison of after TB application values of normal mucosa and dysplastic mucosa, * statistically significant; CA: cellular area, NA: nuclear area, CD: cellular diameter, ND: nuclear diameter, TB: toluidine blue

Oral premalignant lesions can progress into malignant tumors. Early detection, along with interventions such as the cessation of harmful habits, plays a crucial role in preventing these lesions from transforming. Exfoliative cytology, a non-invasive and simple technique, has been historically used for the early detection of oral and cervical cancers. Despite its ease of use, the method is susceptible to variations, influenced by factors such as the level of expertise and experience of the cytologists. Toluidine blue vital staining has been utilized as an adjunct to help clinicians pinpoint areas with dysplastic changes. However, this staining technique is also subject to inconsistencies due to factors like tissue changes, inflammation, or benign growth. In light of these challenges, cytomorphometric analysis, a computerized approach for cell examination, has been introduced and proven effective in providing an objective assessment of dysplasia. The goal of this study was to evaluate the effectiveness of combining cytomorphometry with toluidine blue vital staining for the early detection of dysplasia in oral premalignant lesions.

Cytological identification of dysplasia is based on changes in cellular and nuclear characteristics, such as nuclear hyperchromasia, increased nuclear size, decreased cell size, and altered nuclear-cytoplasmic ratio. A pathologist observes these features under a microscope to diagnose dysplastic lesions. In the study, 35 cases exhibited dysplasia via exfoliative cytology. This technique primarily collects superficial cells, which limits its sensitivity. However, depending on the brush used, such as the OralCDx, deeper cells can sometimes be retrieved. Despite the effectiveness of the OralCDx brush, it is expensive and may not be practical or affordable in rural areas. To evaluate the performance of cytology in detecting dysplasia, the researchers used a toothbrush as a cytobrush. Their findings indicated that brush biopsy showed a strong correlation with histopathological diagnoses of dysplasia and malignancy. Studies that modified the brush technique have indicated that cytology is a sensitive and reliable tool for detecting dysplasia in low-resource settings. However, cytological interpretation can vary, particularly in cases of false negative results, which can arise from factors like sampling errors, laboratory preparation issues, staining problems, and pathologist experience. Exfoliative cytology is known to have a high false negative rate [7].

A key factor contributing to false negative results in exfoliative cytology may be improper site selection [11]. The use of toluidine blue vital stain has shown promise in identifying the right areas for biopsy, which helps reduce the likelihood of false negatives [12]. A study by Parakh *et al*. [13] explored the utility of toluidine blue staining for guiding biopsy site selection in potentially malignant conditions. Their findings revealed that toluidine blue positivity strongly correlates with the detection of dysplasia, proving to be a reliable tool for ruling out potentially malignant disorders. In the current research, 27% of the cases were positive for toluidine blue. The study reported a sensitivity of 39.53%, a specificity of 97.56%, a positive predictive value of 97.14%, and a negative predictive value of 43.58%. Earlier studies have similarly noted low sensitivity [13-15], potentially due to factors like surface keratosis or prolonged exposure to toluidine blue staining [13-15]. The reduced sensitivity observed here may stem from the higher prevalence of leukoplakia and oral submucous fibrosis cases, both of which exhibit surface keratosis.

To improve oral cytology, researchers have recently introduced modified brush and oral rinse techniques [16, 17]. Despite this, one of the major challenges in cytology remains the subjective bias from pathologists, especially when they lack sufficient training and experience in interpreting cytology. This introduces intra-observer variability [18], with studies generally reporting poor to moderate agreement, a result similar to the findings of this study [19]. Premalignant lesions typically exhibit poor inter-observer consistency. To mitigate this issue, computerized cytological analysis has been introduced, eliminating the subjective bias and improving observer agreement [7, 20, 21]. Histomorphometric evaluations of exfoliative cells from normal mucosa and oral

premalignant lesions have been effective in distinguishing between dysplastic and non-dysplastic cells, thus aiding in dysplasia diagnosis. These studies reported lower cellular area (CA) and cellular density (CD) along with higher nuclear area (NA), nuclear density (ND), nuclear-cytoplasmic area (NACA), and nuclear-cytoplasmic density (NDCD) ratios in dysplastic cells compared to normal ones. As the grade of dysplasia increases, nuclear parameters rise, while cellular parameters decrease, aligning with the criteria in established grading systems, including the WHO and Binary systems. Although previous studies reported variations in cytomorphometric values between normal and dysplastic cells, the current study did not find such discrepancies, which could be attributed to the early-stage nature of most cases and the limitations of cytology in sampling only superficial cells rather than deeper ones.

Toluidine blue vital staining has been explored for its role in enhancing the diagnostic accuracy of cytology for dysplastic lesions [22]. Ratna Kumari and Ahmed Mujib [22] assessed cytological smears obtained before and after the application of toluidine blue, noting that staining significantly improved the morphological features. The most notable improvement was observed in nuclear pleomorphism. In this study, a positive correlation between toluidine blue application and cytological diagnosis was evident. Other studies have also reported similar enhancements in staining properties due to toluidine blue [23]. However, further large-scale studies, including randomized case-control trials with histopathology as the gold standard, are necessary to confirm these findings.

Conclusion

The findings of this study show a noticeable association between toluidine blue positivity and the occurrence of dysplasia. Dysplastic lesions were observed to be more frequently positive for toluidine blue than their non-dysplastic counterparts. Despite this, the sensitivity of toluidine blue staining (39.53%) and its negative predictive value (43.58%) were relatively low. These results indicate that toluidine blue does not significantly improve the pathologist's ability to identify dysplasia in cytology smears. Additionally, it did not provide meaningful support in differentiating dysplastic cells during histomorphometric analysis.

Acknowledgments: None

Conflict of Interest: None

Financial Support: The research was partially funded by the STS Program under the Indian Council of Medical Research, New Delhi, India [Reg. No: 2019-04212].

Ethics Statement: Ethical approval for this study was granted by the Institutional Ethics Committee (Approval letter: PCDS/ACAD/8/2019/03, dated 23 January 2019).

References

1. Steele TO, Meyers A. Early detection of premalignant lesions and oral cancer. Otolaryngol Clin North Am. 2011;44(1):221-9.
2. Yardimci G, Kutlubay Z, Engin B, Tuzun Y. Precancerous lesions of oral mucosa. World J Clin Cases. 2014;2(12):866-72.
3. Lingen MW, Kalmar JR, Karrison T, Speight PM. Critical evaluation of diagnostic aids for the detection of oral cancer. Oral Oncol. 2008;44(1):10-22.
4. Singh D, Shukla RK. Utility of toluidine blue test in accessing and detecting intra-oral malignancies. Indian J Otolaryngol Head Neck Surg. 2015;67(Suppl 1):47-50.
5. Warnakulasuriya KA, Johnson NW. Sensitivity and specificity of OraScan (R) toluidine blue mouthrinse in the detection of oral cancer and precancer. J Oral Pathol Med. 1996;25(3):97-103.
6. Navone R, Marsico A, Reale I, Pich A, Broccoletti R, Pentenero M, et al. Usefulness of oral exfoliative cytology for the diagnosis of oral squamous dysplasia and carcinoma. Minerva Stomatol. 2004;53(3):77-86.
7. Verma R, Singh A, Badni M, Chandra A, Gupta S, Verma R. Evaluation of exfoliative cytology in the diagnosis of oral premalignant and malignant lesions: a cytomorphometric analysis. Dent Res J (Isfahan). 2015;12(1):83-8.

8. In 6 years, oral cancer cases rise by 114% in India: Study; 2018. Available from: https://timesofindia.indiatimes.com/india/in-6-years-oral-cancer-cases-rise-by-114-in-india-study/articleshow/66644066.cms
9. Messadi DV. Diagnostic aids for detection of oral precancerous conditions. Int J Oral Sci. 2013;5(2):59-65.
10. Caruntu ID, Scutariu MM, Dobrescu G. Computerized morphometric discrimination between normal and tumoral cells in oral smears. J Cell Mol Med. 2005;9(1):160-8.
11. Chitturi R, Rathinam E, Santo R, Yoithapprabhunath T. The role of exfoliative cytology and molecular biology in oral potentially malignant disorders. J Health Res Rev. 2017;4(2):43. doi:10.4103/jhrr.jhrr_22_1
12. Bánóczy J. Exfoliative cytologic examinations in the early diagnosis of oral cancer. Int Dent J. 1976;26(4):398-404.
13. Parakh MK, Jagat Reddy RC, Subramani P. Toluidine blue staining in identification of a biopsy site in potentially malignant lesions: a case-control study. Asia Pac J Oncol Nurs. 2017;4(4):356-60.
14. Awan Kh, Yang Y, Morgan P, Warnakulasuriya S. Utility of toluidine blue as a diagnostic adjunct in the detection of potentially malignant disorders of the oral cavity--a clinical and histological assessment. Oral Dis. 2012;18(8):728-33.
15. Ramanathan A, Deepak TA, Manjunath M, Krishna S, Annaji AG. Assessment of oral mucosa in high-risk and precancers using chemiluminescent illumination and toluidine blue supravital staining. Int J Oral Health Med Res. 2016;2(5):28-32.
16. Cancela-Rodríguez P, Cerero-Lapiedra R, Esparza-Gómez G, Llamas-Martínez S, Warnakulasuriya S. The use of toluidine blue in the detection of pre-malignant and malignant oral lesions. J Oral Pathol Med. 2011;40(4):300-4.
17. Pereira T, Kesarkar K, Tamgadge A, Bhalerao S, Shetty S. Comparative analysis of oral rinse-based cytology and conventional exfoliative cytology: a pilot study. J Cancer Res Ther. 2018;14(5):921-5.
18. Olms C, Hix N, Neumann H, Yahiaoui-Doktor M, Remmerbach TW. Clinical comparison of liquid-based and conventional cytology of oral brush biopsies: a randomized controlled trial. Head Face Med. 2018;14(1):9.
19. Shubhasini AR, Praveen BN, Hegde U, Uma K, Shubha G, Keerthi G, et al. Inter- and intra-observer variability in diagnosis of oral dysplasia. Asian Pac J Cancer Prev. 2017;18(12):3251-4.
20. Kujan O, Khattab A, Oliver RJ, Roberts SA, Thakker N, Sloan P. Why oral histopathology suffers inter-observer variability on grading oral epithelial dysplasia: an attempt to understand the sources of variation. Oral Oncol. 2007;43(3):224-31.
21. Pandey P, Agarwal S, Ralli M, Dixit A, Singh D. Oral brush liquid-based cytology: a study of concordance between a cytotechnologist and a cytopathologist. Acta Cytol. 2018;62(2):121-9.
22. Ratna Kumari TVN, Ahmed Mujib BR. Toluidine blue with a synergistic effect in morphological assessment of oral cytosmears. J Cytol. 2018;35(1):8-14.
23. Yerlagudda K, Kamath VV, Satelur K. Morphological assessment of oral cytological smears before and after application of toluidine blue in smokers and non-smokers. Int J Oral Maxillofac Pathol. 2012;3(1):8-14.