Original Article

Evaluation of Efficacy of Toluidine Blue in the Detection of Potentially Malignant Disorders

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Received: 06-08-2013
Revised: 11-09-2013
Accepted: 22-10-2013

Abstract:

Background: Early detection of oral cancer at potentially malignant disorders (PMDs) stage reduces the morbidity and improves the survival rates and quality of life of patient. Toluidine blue has been developed as an adjunct to conventional examination for early detection of dysplastic lesions. This study was conducted to assess the efficacy of toluidine blue in the detection of PMDs and to compare the results with histopathological examination.

Materials and Methods: 50 patients with PMDs were taken from the outpatients attending the Department of Oral Medicine and Radiology and subjected to conventional oral examination followed by toluidine blue test and biopsy for histopathological confirmation.

Results: The sensitivity, specificity, positive predictive value(PPV), negative predictive value(NPV) and accuracy of toluidine blue was 43.75%, 88.89%, 87.50%, 47.06 % and 60% respectively. A statistically significant association was observed between histopathology results and toluidine blue results.

Conclusion: Toluidine blue staining is a easy, safe, minimal time consuming and noninvasive technique that can be a useful adjunct to conventional examination for detection of PMDs.

Key words: Dysplasia, Leukoplakia, Oral cancer, Toluidine blue.

This article may be cited as: Chaudhary A, M Manjunatha, Gupta I. Evaluation of Efficacy of Toluidine Blue in the Detection of Potentially Malignant Disorders. J Adv Med Dent Scie 2013;1(2):19-24.

Introduction:

Cancer is among the most dreadful diseases human race is suffering since age. Oral Cancer ranks as the sixth most common cancer gobally.[1] The annual incidence of oral cancer is around 275,000 with India having the highest incidence rate of oral cancer around the world.[2] The 5-year survival rate for oral cancer patients is still 50% despite advances in cancer therapies. This can be attributed to their late detection at advanced stages. However, if diagnosed at an early asymptomatic stage, oral cancer is often curable and inexpensive to treat. The focus therefore, has shifted towards early diagnosis and prompt treatment of these lesions for improving the prognosis. The natural history of oral cancer is such that it is usually preceded by a precancerous stage in the form of potentially malignant disorders (PMDs).[3] These PMDs cannot be differentiated from early oral cancers by visual examination alone regardless of the

expertise of the clinician.[4-6] Also, the clinical examination alone cannot distinguish between dysplastic and non-dysplastic lesions.

The gold standard for diagnosis of dysplasia is histopathological examination. But scalpel biopsy is an invasive procedure with disadvantage of tumor seeding. It is usually done when the lesion displays either symptoms or clinical features of malignancy while many innocuous appearing early stage oral cancerous lesions are merely observed clinically and left undiagnosed. Thus various adjunctive and noninvasive tools have been developed at both the clinical and molecular level to assess the oral lesions of uncertain biologic significance. One such technique is vital staining including toluidine blue.

Vital staining is the process of dyeing living cells or tissues. The staining reveals unapparent cytological details. Toluidine blue was developed as tolonium chloride by Abbott laboratories and has been used as a dye for wool and silk, in medicine, as an antiheparin compound and as a histologic stain.[7] It has been used as a vital stain to disclose dysplasia and carcinoma in situ of uterine cervix.[8] Its application for the detection of oral premalignant and malignant lesions was first reported by Neibel and Chomet in 1964.[9]

Toluidine blue is a basic metachromatic dye that stains the acidic cellular components. Since, cancer cells contain quantitatively more DNA and RNA than normal epithelial cells, toluidine blue delineates areas of malignancy. It is a simple, fast, and inexpensive technique. Toluidine blue has been shown to have a high false positive rate due to its retention in inflammatory regions, ulcerations, fissures[9-11] but this can be reduced by restaining after 2 weeks.[12,13] The aim of the study was to assess the efficacy of toluidine blue in the detection of PMDs and to compare the results with histopathological examination.

Materials and Methods:

50 patients with clinically suspected PMDs were selected from the outpatients attending the Department of Oral Medicine and Radiology. Adult Individuals belonging to any gender or race presenting with PMDs like leukoplakia(white patch), erythroplakia (red patch), erythroleukoplakia/speckled leukoplakia (mixed area of white and red) were enrolled in the study. The study was approved by the institutional review board and ethical clearance was obtained.

The procedure was explained to the patient and written consent was obtained. Each patient underwent a conventional soft tissue examination under incandescent light followed by toluidine blue test. All the lesions that stained positive by toluidine blue were restained after 2 weeks to reduce the false positive rate. Following that the lesions were biopsied. For toluidine blue examination, the lesional area was dried. The toluidine blue solution (containing 0.5% Tolonium chloride, purified water, acetic acid, sodium acetate, hydrogen peroxide, dehydrated alcohol) was applied over the lesion and pressed firmly in a painting motion for about a minute. About 2 cm area around the visible lesion was covered. Following this, cotton pellets soaked in 1% acetic acid (decolorizing agent) were pressed firmly in a painting motion using a reasonable mechanical effort to remove the blue stain. Dysplastic cells containing more DNA will retain the toluidine blue stain while cells with normal nuclear cytoplasmic ratio will get decolorized by acetic acid. The retention of stain regardless of intensity was defined as “positive” test (Figure 1) and absence of any stain was defined as “negative” test. Incisional or excisional biopsy of the lesion was carried out and histopathological examination was done. The tissue specimen was obtained from areas of toluidine blue stain retention. The grading of dysplasia was

Table1: Formulae used:

S.No.	Definition	Formula
1.	**Sensitivity** – it is defined as the proportion of truly diseased persons in the screened population who are identified as diseased by the screening test.[19] It is also called as the true positive rate.	TP/TP+FN x 100*
2.	**Specificity** – it is defined as the proportion of truly non-diseased persons who are so identified by the screening test. It is also called as the true negative rate.[19]	TN/TN+FPx 100*
3.	**Positive predictive value** – it is defined as the probability that a person with a positive test is a true positive (i.e. does have the disease).[19]	TP/TP+FP x 100*
4.	**Negative predictive value** – it is defined as the probability that a person with a negative test does not have the disease.[19]	TN/TN+FNx 100*
5.	**Accuracy** – it is the measure of the overall agreement between the diagnostic test and the gold standard. The more accurate the test, the fewer the false negative and false positive results.[19]	TP+TN/total no. of cases*
6.	Chi square test**	$\chi^2 = \sum \frac{(O-E)^2}{E}$

*TP= true positive, TN= true negative, FP= false positive, FN= false negative,
**O is the observed frequency and E stands for expected frequency.

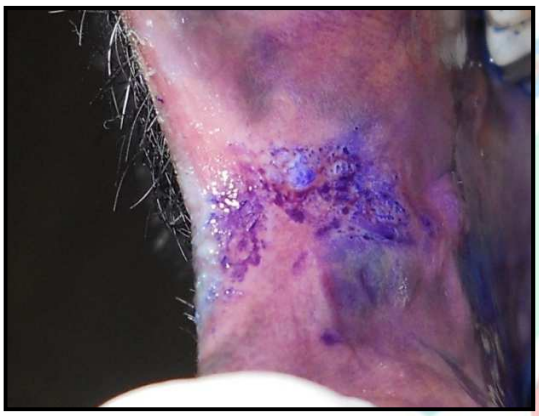

Figure 1: Speckled leukoplakia on right retrocommissure showing toluidine blue stain retention.

done according to the thickness of epithelium showing dysplastic changes. The presence of dysplasia was considered as "positive test" and absence of it was considered as "negative test". The collected data was entered through the SPSS version 18.0 software. Sensitivity, Specificity, Positive Predictive Value (PPV), Negative Predictive Value (NPV) and Accuracy were calculated for toluidine blue (Table 1). The results were compared with histopathological results using Chi-Square test with p value set as < 0.05.

Table 2: Demographic data

	n=50(%)
Gender	
Male	37(74%)
Female	13(26%)
Age group	
20-40	24(48%)
40-60	20(40%)
60-80	4(8%)
80-100	2(4%)
Site	
Buccal mucosa	41(82%)
Commisure	5(10%)
Labial mucosa	1(2%)
Tongue	1(2%)
Palate	1(2%)
Habit	
Smokeless tobacco	30(60%)
Smoking	16(32%)
Both	4(8%)

Results:

The demographic data of 50 patients enrolled in the study is given in table 2. Out of 50 lesions examined 37 lesions (74%) were categorized as homogenous leukoplakia, 13 lesions (26%) were categorized as non-homogenous leukoplakia (speckled leukoplakia)

On first staining 35 lesions (70%) showed positive result. On staining after 2 weeks only 16 lesions (32%) showed stain retention. On histopathological examination, 64% of the lesions were diagnosed with dysplasia. On the basis of the grades of dysplasia 42% were categorized as showing mild dysplasia, 14% moderate dysplasia, 4% severe dysplasia and 4% carcinoma-in situ. Out of 16 toluidine blue positive lesions, 14 lesions (87.5%) were identified as dysplastic following histopathological examination. Out of 34 (68%) lesions that were tested negative by toluidine blue, 18 lesions (62.9%) were positive for dysplasia as tested by histopathology (Table 3). A statistically significant association was observed between the toluidine blue test results and histopathology results (χ^2 = 5.640) at p-value ≤0.018 where p value was set at ≤0.05

Table 3: Association between Toluidine Blue results and Histopathology results

Toluidine Blue	Histopathology				Total	χ^2	p-Value
	Positive		Negative				
	n	%	n	%			
Positive	14	87.5%	2	12.5%	16	5.640	0.018*
Negative	18	52.9%	16	47.1%	34		
Total	32		18		50		

*denotes significant association

Discussion:

This study assessed the adjunctive utility of toluidine blue in detecting dysplastic oral lesions on comparison with histopathology. On initial staining 35 lesions were positive. Out of these only 16 lesions retained stain after 2 weeks rendering 19 lesions as false positives. The number of false positives had reduced considerably after restaining at 2 weeks. This is in accordance with the study by Mashberg et al.[13]

Out of 16 toluidine blue positive tests, 13 lesions (81.25%) were speckled leukoplakia and among these, 11 lesions (84.6%) were positive of dysplasia. The toluidine blue retention was mainly observed in the erythematous/atrophic areas in speckled leukoplakia. This finding is similar to that observed by Sharma et al[14] and Rajmohan et al.[15] Out of 34 lesions tested as negative, 18 lesions (52.9%) were diagnosed as dysplastic on histopathology resulting in high number of false negatives. This is in contrast to the result obtained by Epstein et al[16] where false negatives was 0%.

The exact mechanism of action has been controvertial. Although it has affinity towards nucleic acids, the haphazard arrangement of tumor cells also facilitates penetration and retention of the dye in the intercellular spaces.[17] A thick layer of keratin present in homogenous leukoplakias prevents penetration of the dye resulting in false negative test. The atrophic areas in speckled leukoplakia favour dye penetration and retention as seen in our study. Also false negatives could be due to the fact that surface layer of keratin contains pyknotic or no nuclei. This is in contrast to the result obtained by Epstein et al[16] where intensely keratotic tissues, verrucous hyperplasias were shown to retain stain. That could be attributed to the mechanical retention of the dye in the irregularities of verrucous hyperplasia and fissures in leukoplakia.

The efficacy of any diagnostic test is mainly tested by the number of false negatives as it prevents rendering of any therapeutic services that can have a significant bearing on the survival of patients. The high number of false positives may lead to overtreatment and may have a significant burden on the mental status of the patient fearful of having cancer. Toluidine blue has been shown to have a high number of false positives[16] or false negatives (present study). But the number of false positives can be reduced by restaining at 2 weeks as seen in present study Also a well defined criteria for the staining pattern, intensity of stain has not been established.

The main disadvantage of toluidine blue is that it gives information only about the surface changes. The depth of the lesion which is more important in predicting the malignant behavior cannot be assessed as it has been shown that toluidine blue stains to a depth of only two to ten cell layers.[17] The limitation of the present study was that the efficacy of stain was tested only in clinically apparent lesions which can be adequately diagnosed by visual examination and palpation. This was done mainly to identify the dysplastic or high risk lesions. The efficacy was not evaluated in innocuous lesions as in patients with high risk habits or in previously treated squamous cell carcinomas to detect secondary tumors.

Although toluidine blue has been promoted as valuable adjuncts in the early detection of PMDs but there is no evidence that use of this modality will negate further evaluation of a clinically suspicious lesion. In addition there is controversy regarding the subjective interpretation of mucosal staining and criteria for positive results (dark royal blue vs pale blue staining).[18] So further studies with well defined objectives, larger sample sizes and with histopathological confirmation are required.

Conclusion:

Toluidine blue has only adjunctive utility in diagnosing and delineating dysplastic oral lesions. It is more accurate in detecting non-homogenous leukoplakia as compared to homogenous leukoplakia. The number of false positives is high but it can be reduced by restaining at 2 weeks. The interpretation of stain retention is very subjective, so well defined criteria for staining needs to be established.

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Source of Support: Nil
Conflict of Interest: None declared