Diagnostic efficiency of toluidine blue with Lugol's iodine in oral premalignant and malignant lesions

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ABSTRACT

Background and Objectives: *In vivo* stains are prompt resources, which have emerged, in the recent years, to aid as clinical diagnostic tools in detecting early premalignant and malignant lesions. The aim of the study was to determine the diagnostic efficiency of toluidine blue with Lugol's iodine in oral premalignancies and malignancies and to evaluate the reliability of *in vivo* staining with toluidine blue and Lugol's iodine in the lesions at risk of malignancy.

Materials and Methods: The study group comprised 30 subjects with clinically suspicious premalignant lesions and 30 subjects with clinically suspicious malignant lesions. All the lesions were stained consecutively with toluidine blue and Lugol's iodine and the dye retention were recorded with photographs. Depending on the retention of the dyes, the biopsy site was determined. The biopsy specimens were sent for histological confirmation and results were statistically analyzed.

Results: The overall diagnostic accuracy of Lugol's iodine when used consecutively with toluidine blue stain in distinguishing premalignant lesions and malignant lesions was 90%. As the degree of differentiation of malignant lesions progressed toward more severity, they failed to show the retention of Lugol's iodine and the result was highly significant statistically, with a P value <0.001.

Interpretation and Conclusion: Lugol's iodine when used with toluidine blue helped in delineating the inflammatory lesions and was the mean source in determining clinically the degrees of differentiation of malignant lesions as the poorly differentiated malignant lesions without glycogen content failed to show Lugol's iodine retention. Toluidine blue with Lugol's iodine can be used as a pretherapeutic assessment of the biologic aggressiveness of the disease.

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Oral cancer when caught at an early stage is often curable, inexpensive to treat and affords a better quality of life.[1] With this aim, various techniques have been developed to supplement clinical examination and improve the diagnosis of premalignant and early malignant lesions.[2]

In vivo staining has been used extensively in gynecologic practice for the detection of malignant changes of the cervix during colposcopy, and this technique has been applied in the oral setting for over 30 years by means of dyes like toluidine blue and Lugol's iodine.[2,3]

Toluidine blue selectively stains acidic tissue components, DNA and RNA, and as the dysplastic and anaplastic cells contain quantitatively more nucleic acids than normal tissues, can show a varied clinical picture when stained with toluidine blue.[4] However, staining of the Lugol's iodine depends on the glycogen content present in the normal epithelium and this selective character of staining helps in delineating the inflammatory or carcinomatous epithelium from the normal epithelium where the glycogen content is low.[4-6]

This foregoing makes it imperative to study the diagnostic efficiency of toluidine blue with Lugol's iodine in detecting the biopsy sites and thus establishes an accurate diagnosis in oral premalignant and malignant lesions.

MATERIALS AND METHODS

The present study was conducted in the Department of Oral Medicine and Radiology, Bapuji Dental College and Hospital, Davangere, Karnataka.

The study subjects were taken from the outpatients attending the Department of Oral Medicine and Radiology, Bapuji Dental College and Hospital, Davangere, and the

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outpatient Department of ENT, J.J.M. Medical College and Hospital, Davangere.

The study group consisted of 60 subjects of both the sexes, 30 subjects with clinically suspicious premalignant lesions and 30 subjects with clinically suspicious malignant lesions.

The above subjects who fulfilled the following criteria were selected for the study:
Leukoplakia: Nonscrappable elevated white patch or plaque with a history of tobacco chewing or smoking and smooth or wrinkled surface and sometimes traversed by small cracks or fissures.[7]
Speckled Leukoplakia: Mixed red and white lesion with keratotic white nodule or specks or patches distributed over an atrophic erythematous background.[8,9]
Erosive lichen planus: Red irregular erosion or ulceration associated with a reticular form, especially in the peripheral region of the lesion and with a pseudomembrane covering the ulcerated areas.[10]
Oral malignancy: White patch-like lesion with ulcerated area within (or) adjacent to it. An ulcerated area with rolled borders and hard indurated edges with a velvety-red irregular base (infiltrative variety). A proliferative growth with single or multiple ulcers around it with indurations (exophytic–verrucous variety), white patch-like lesion with interspersed reddish areas, which ulcerates.[11] Staging of the malignant lesion was carried out according to the TNM classification of the American Joint Committee for cancer staging.[12]

A provisional diagnosis of leukoplakia, speckled leukoplakia, erosive lichen planus (premalignant lesions) and oral malignancies were made on basis of clinical examination.

Methodology

The subjects comfortably seated in the dental chair were examined following the methods described by Kerr *et al.*[13] and the relevant data were entered and an informed consent was obtained from each subject for carrying out the diagnostic procedure.

Formulation of 1% toluidine blue solution

- Toluidine blue 1 g
- Acetic acid 10 cc
- Absolute alcohol 4.19 cc
- Distilled water 86 cc
- pH adjusted to 4.5

Formulation of Lugol's iodine solution

- Iodine 2 g
- Potassium iodide 4 g
- Distilled water 100 cc

Staining procedure

After recording the clinical features and photographs of the clinically suspicious lesions, the lesion areas were applied prior with 1% acetic acid with cotton bud for 20 s and further rinsed with water.[4]

1% toluidine blue was applied with a cotton bud for 10–20 s and was decolorized with 2% acetic acid using a cotton bud for 20–30 s, and a photograph was taken [Figures 1 and 2].

Lugol's iodine was applied with a cotton bud for 10–20 s and another photograph was taken [Figures 3 and 4].

Interpretation of the toluidine blue stain

Dark blue stain was considered as positive for lesions suspicious of malignancy, light blue retention was considered as positive for premalignant lesions unless proved otherwise by biopsy and the lesions without any retention of stain were considered as negative.[14]

Interpretation of the Lugol's iodine stain

Brown stain was considered as positive for lesions while lesions without any retention of stain were considered as negative.[15]

Biopsy site was selected on the basis of clinical appearance and dye retention and in the sites where no retention of the stain occurred, clinical judgment directed the biopsy.

Interpretation of the microscopic slides

The stained sections were seen under a Leica Binocular Light Microscope (Leica Microsystems GmbH, Ernst-Leitz-Straße 17-37, Wetzlar 35578, Germany). Histopathological grading for premalignant lesions was performed as per the pathologic features suggested by Axell *et al.*,[7] which were grouped on the basis of degree of dysplasia into those with no dysplasia, mild dysplasia, moderate dysplasia and severe dysplasia. Oral malignancies were graded into well-differentiated (Grade I), moderately differentiated (Grade II) and poorly differentiated (Grade III) squamous cell carcinoma (SCC) according to Broder's system of grading.[11]

Statistical analysis

Data are presented by number and percentage. Chi-square test was used to compare the groups and assess the association. Diagnostic validity tests, viz., sensitivity, specificity, positive predictive values (PPV), negative predictive values (NPV) and diagnostic validity/diagnostic accuracy (DA) were performed to determine the utility of the test results for predicting the various condition of the disease.

RESULTS AND OBSERVATIONS

Effects of stains

The safety of toluidine blue and Lugol's iodine as a vital stain has been assessed and confirmed over numerous studies. In the present study too, we observed no harmful effects or persistent staining in any of the lesions or in the adjacent mucosa.

Degree of dysplasia in premalignant lesions

Of the 30 premalignant lesions, 15 (50%), 12 (40%) and three (10%) lesions comprised of homogenous leukoplakia, speckled leukoplakia and erosive lichen planus, respectively.

Out of 30 cases of premalignant lesions, 25 cases were proved histologically as having dysplastic changes. Thirteen cases of leukoplakia and 10 cases of speckled leukoplakia were of mild dysplasia while two cases of speckled leukoplakia were of moderate dysplasia.

Degree of differentiation in malignant lesions

Of the 30 cases of malignant lesions, 11 cases were well-differentiated SCC, 16 cases were moderately differentiated SCC while three cases were poorly differentiated SCC.

Diagnostic validity

Of the 60 cases (30 cases of premalignant and 30 malignant lesions), either of the stains was retained in 59 (98.33%) cases whereas one (1.67%) case failed to retain both the stains. Among the 59 cases that retained either of the stain, 55 cases were confirmed histologically as having dysplastic/carcinomatous changes while the remaining four cases and one unstained lesion were confirmed as benign lesions. The sensitivity was found to be 100% while the specificity was 20%. The PPV and NPV were 93% and 100%, respectively. The DA when either of the stain was retained was 93%.

Of the 60 cases (30 cases of premalignant and 30 malignant lesions), both the stains were retained in 52 (86.66%) cases whereas four cases of malignant lesions (three cases of poorly differentiated SCC and one case of moderately differentiated SCC) and two cases of premalignant lesions (erosive lichen planus) failed to retain Lugol's iodine, and in the remaining two cases, one case failed to retain both the stains while one case failed to retain toluidine blue stain.

Among the 52 cases that retained both the stains, 51 cases were confirmed histologically as having dysplastic/carcinomatous changes while one case was confirmed as a benign lesion. When both the stains were negative, the lesions were proved with histological features as benign/inflammatory lesions. The sensitivity was found to be 93% while the specificity was 80% and the PPV and the NPV were 98% and 50%, respectively. The DA when both the stains were retained was 92%.

Diagnostic validity of Lugol's iodine with histologically graded malignant lesions

Lugol's iodine retained in 11 cases of well-differentiated SCC and 15 cases of moderately differentiated SCC, while one case of moderately differentiated and three cases of poorly differentiated SCC failed to retain the stain, and the difference was statistically significant, with a *P* value of <0.001.

DISCUSSION

The concept of a two-step process of cancer development in the oral mucosa, i.e., the initial presence of a precursor (premalignant or precancerous) lesion subsequently developing into cancer is well established[16] and an early detection of oral mucosal epithelial dysplasias could potentially halt the progression of these lesions into malignant transformation.[2] Thus, the establishment of useful and objective techniques adjunctive to clinical judgements and microscopic diagnosis have contributed to the control of oral cancers.[17]

In vivo staining reveals cytological details that might otherwise not be apparent. However, staining can also reveal where certain chemicals or specific chemical reactions are taking place within cells or tissues[18] and thus aid in accelerating biopsies, diagnosis and treatment.

Toluidine blue, an acidophilic metachromatic dye of the thiazine group, selectively stains acidic tissue components (sulfates, carboxylates and phosphate radicals), thus staining DNA and RNA. It is used as an *in vivo* stain based on the fact that dysplastic and anaplastic cells may contain quantitatively more nucleic acids than normal tissues. Also, the malignant epithelium may contain intracellular canals that are wider than the normal epithelium, which may facilitate penetration of the dye.[4]

The selective character of staining the intact mucosa with Lugol's iodine is dependent on the glycogen content present in the normal epithelium and this selective character of staining helps in delineating the inflammatory or carcinomatous epithelium from the normal epithelium where the glycogen content is low.[4-6]

Both the stains have been used separately in various studies, with Lugol's iodine application pertaining to the cervix and esophageal areas while toluidine blue has been established as a diagnostic adjunct in detecting oral lesions related to invasive carcinomas, carcinoma *in situ* or early asymptomatic oral carcinomas. This study was conducted with the aim to assess the diagnostic accuracy of toluidine blue with Lugol's iodine in oral malignant lesions and lesions at risk of malignancy.

Diagnostic accuracy of Lugol's iodine with toluidine blue in premalignant and malignant lesions

When Lugol's iodine was used consecutively with toluidine blue (30 cases), the sensitivity of the stains in detecting premalignant lesions (dysplastic changes) was 100% (25 cases), while the specificity was 60% (three cases out of five) [Figure 5] and the PPV, NPV and the DA were found to be 93%, 100% and 93%, respectively, whereas the sensitivity and DA of the stains in detecting malignant lesions was 87% [Figure 6].

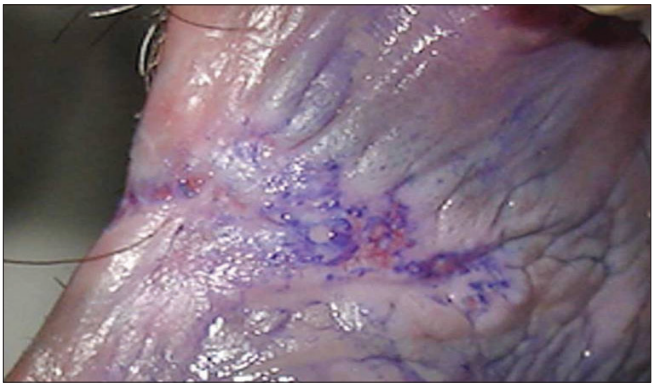

Figure 1: Speckled leukoplakia after toluidine blue staining

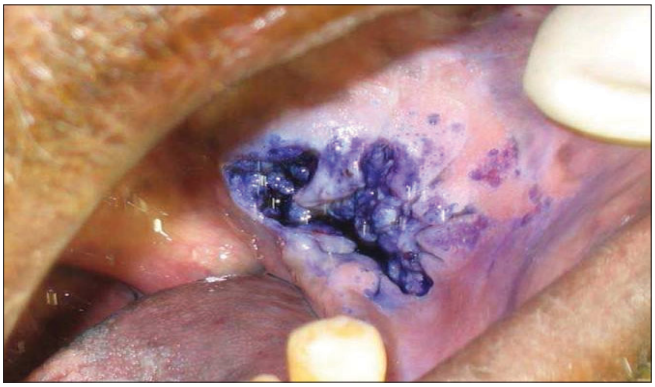

Figure 2: Squamous cell carcinoma after toluidine blue staining

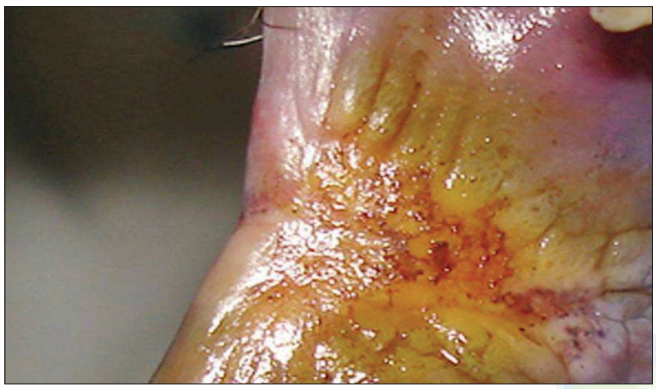

Figure 3: Speckled leukoplakia after Lugol's iodine staining

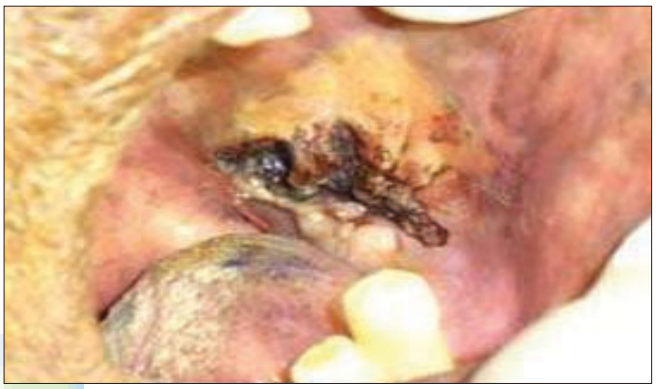

Figure 4: Squamous cell carcinoma after Lugol's iodine staining

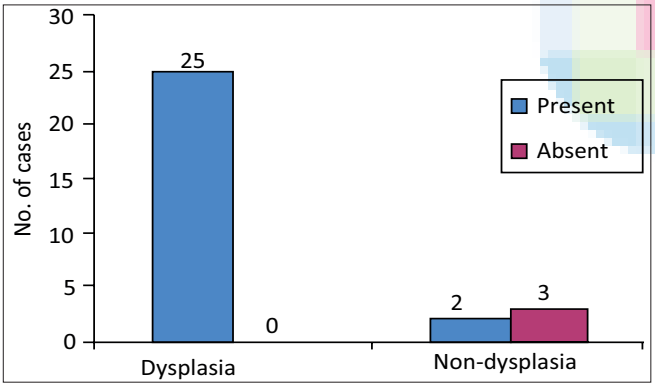

Figure 5: Diagnostic accuracy of Lugol's iodine with toluidine blue in premalignant lesions

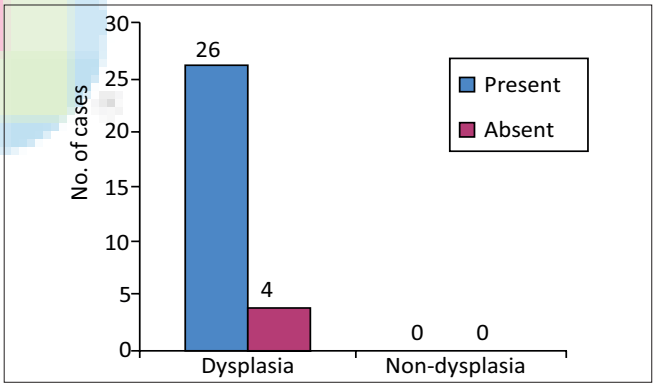

Figure 6: Diagnostic accuracy of Lugol's iodine with toluidine blue in malignant lesions

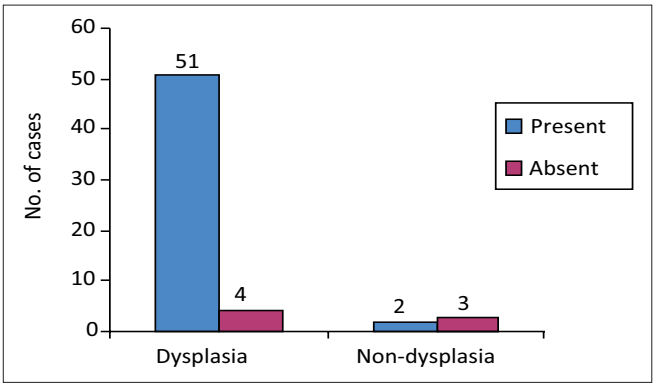

Figure 7: Overall diagnostic accuracy of Lugol's iodine with toluidine blue in premalignant and malignant lesions

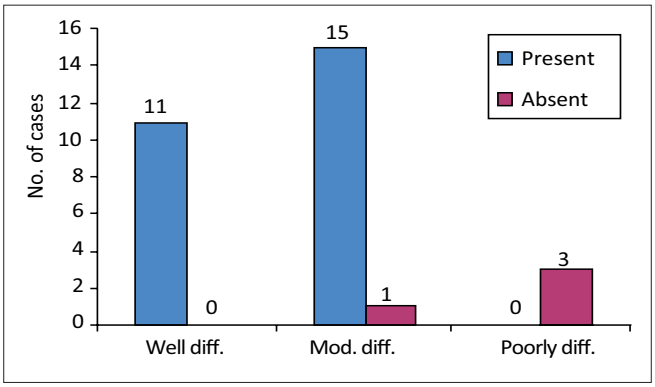

Figure 8: Dye retention of Lugol's iodine in comparison with histological grading of squamous cell carcinoma

To the best of our knowledge, only one study has been documented in the literature, which assessed the DA of Lugol's iodine with toluidine blue in detecting premalignant lesions and early malignant lesions, but their results comprised of overall DA of Lugol's iodine with toluidine blue in detecting both premalignant and malignant lesions. Thus, an individual comparison of our results with this study was not possible.

Overall diagnostic accuracy of Lugol's iodine with toluidine blue

In this study, the overall sensitivity of Lugol's iodine when used consecutively with toluidine blue in detecting dysplasia or malignant lesions was 92.7% while the specificity was 60% and the PPV, NPV and DA were 96%, 43% and 90%, respectively [Figure 7].

The results of this study regarding the sensitivity, PPV and DA were in accordance with the findings of Epstein *et al.*,[4] who reported a sensitivity of 87.5% and PPV and DA of 92.1% and 86% respectively and an increase in specificity values when both the stains were used. The results regarding the NPV were not in accordance to each other, but the variations in the NPV can be attributed to our exclusive clinical judgement with regard to the selection criteria of the cases, which minimized the total number of benign lesions in the study sample.

Comparison of Lugol's iodine staining in histological grading of malignant lesions

In the present study, Lugol's iodine retention interspersed in between toluidine blue stain was documented in 11 cases of well-differentiated SCC and 15 cases of moderately differentiated SCC, while one case of moderately differentiated SCC and all the three cases of poorly differentiated SCC failed to document the interspersed Lugol's iodine stain [Figure 8]. This difference can be attributed to the decreased content of glycogen in poorly differentiated SCC, and this difference was statistically significant with a P value <0.001.

Because no studies were available regarding the comparison of Lugol's iodine in histologically graded malignant lesion, a comparison was not carried out and the results of this study can throw a light for the future studies in analyzing the carbohydrate content in malignant lesions as the disease progresses.

Comparison of diagnostic accuracy of toluidine blue with both the stains in premalignant and malignant lesions

When toluidine blue stain and Lugol's iodine were used consecutively, the diagnostic retention of the dye enhanced the visual inspection of the suspicious lesions while the decreased retention of Lugol's iodine in the inflammatory lesions helped to increase the specificity of these stains to 60%. Although the difference in the values of sensitivity and NPV in either occasion was similar, there was betterment in the PPV and DA when both the stains were used consecutively.

In the present study, the sensitivity, specificity, PPV and DA of toluidine blue in detecting malignant lesions was found to be 100%. When toluidine blue stain and Lugol's iodine were used consecutively, sensitivity and DA of Lugol's iodine with toluidine blue was reduced to 87% as four false-negative results were noted. This difference can be attributed to the decreased content of glycogen in poorly differentiated SCC.

The ideal way of analyzing both the stains as a diagnostic adjunct is to undertake a longitudinal study of untreated cases from the earlier stages of manifestation of a disease to their most severe manifestation. However, this clearly would be impossible on ethical and moral grounds. Future studies are suggested with larger samples, performed longitudinally to evaluate the progress of the lesions based on the intensities and patterns of retention of the stains and to draw substantial conclusions.

CONCLUSION

In vivo stains are the prompt resources in diagnosing the molecular changes or some specific chemical reactions taking place within cells or tissues during the process of carcinogenesis.

The diagnostic efficiency of toluidine blue and Lugol's iodine in detecting the premalignant and malignant lesions was assessed.

We concluded as follows:

- A significant difference was observed in the staining pattern of toluidine blue in homogenous leukoplakias and speckled leukoplakias owing to the differences in the epithelial alterations in both the stages of the disease.
- A significant difference was observed in the staining pattern and intensities of toluidine blue in premalignant lesions and malignant lesions owing to the difference in the activity of cells in both the stages of the disease.
- Consecutive use of toluidine blue and Lugol's iodine identified the inflammatory lesions echoing premalignant lesions, as Lugol's iodine failed to retain in inflammatory lesions.
- A significant difference was observed in the dye retention of Lugol's iodine in premalignant lesions and malignant lesions owing to the differences in the glycogen content of cells, which gets minimized as the mitotic activity increases.
- No significant difference in the staining pattern of toluidine blue was observed in the stage II to stage IV-malignant diseases, but was highly efficient in detecting

and delineating the margins of stage II to stage IV-malignant lesions.

- A statistically significant difference existed between the Lugol's iodine retention in well-differentiated, moderately differentiated and poorly differentiated SCC, with a *P* value <0.001.

Staining should be routinely used as a method to assist in the choice of biopsy site and in the follow-up of premalignant lesions. Further studies with more number of cases are suggested to establish these stains as sound diagnostic adjuncts.

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