INTRODUCTION

Oral cancer is a significant threat to public health all over the world, especially in South and Southeast Asia including the Indian subcontinent, where use of tobacco and betel quid are very popular [1,2]. Annually ~ 300,000 individuals are diagnosed with oral cancer in the world, of which 80% are squamous cell carcinomas (SCC) of the lining mucosa [3]. Advances in the treatment of oral cancer has evolved overtime, but the 5 year survival rate from the time of diagnosis has remained surprisingly static over the past 5 decades despite of advanced therapeutic interventions. Have we analysed the reason for this fact? With early detection of oral cancer, the quality of life of such patients can be improved before its progress to a clinically visible advanced lesion? The late stage diagnosis [in more than 50% cases] of oral cancer is one of the important aspects that top the priority to be rectified. This situation suggests that many providers or their patients or both are failing to recognize early oral mucosal changes that indicate cancer development [2].

The habits of smoking, drinking and areca nut chewing were reported to have higher risk of oral premalignant as well as carcinomatous changes than of people without such habits [1,4]. Cases of oral cancers are also seen in patients who do not use tobacco which comprise of a very small percentage of all oral cancers [5]. Most early stage oral carcinomas appear to be seemingly innocent alterations, in the form of focal color change (red, white or mixed), surface textural change (erosion, keratosis, granularity or fissuring) or both; which represent cellular alterations/ dysplasias [6]. Such oral dysplastic changes bearing the potential to...
transform into invasive squamous cell cancer have been described both clinically and pathologically. Oral lichen planus (OLP) and oral leukoplakia (OLK) are two such commonly encountered premalignant disorders which develops into OSCC \cite{3,4}. Hence periodic clinical examination of the oral cavity and even screening high risk populations for OSCC and precursor lesions is an attractive strategy to reduce the suffering of OSCC. This can reduce mortality from oral cancer by 32% in high-risk individuals and hence enhance the quality of life \cite{1,3}. Thus many researchers engage in finding an efficient chairside non-invasive, objective, predictive, screening (NOPS) method of diagnosis, such as vital tissue staining.

Vital tissue staining involves the topical application of stains to improve localization, characterization, or diagnosis. The contrast between normally stained and abnormally stained epithelium enables to formulate a diagnosis and/or to direct biopsies based on a specific reaction or enhancement of surface morphology \cite{1}. In recent years, there has been a resurgence in interest in this technique such as the use toluidine blue in the form of in-vivo staining which aims to aid Conventional Oral Examination (COE) because it is a simple, safe, quick, widely available, and inexpensive diagnostic tool \cite{7}. The efficacy of this technique has been evaluated in many reports with diverse results, in detecting suspicious malignancies \cite{1}.

Methylene blue is another recently proposed dye used for in-vivo staining in endoscopic examination, having similar chemical structure and physicochemical properties to toluidine blue. The precise mechanism for the uptake of this dye in epithelial cells is still not very clear. But it resembles toluidine blue dye in its acidophilic characteristics, penetrating cells having abnormal increase in nucleic acid, thus resulting in differential uptake between normal and highly dysplastic/malignant cells \cite{1}. These features may help to direct biopsies in patients without a visible lesion in COE \cite{8}. Now what makes it different from toluidine blue? The Material Data Safety Sheet indicates that toluidine blue is probably toxic and is hazardous if swallowed, and was shown to have toxicity to fibroblasts \cite{9}. Methylene blue is less toxic to the human body and has lately been proposed for screening some gastrointestinal or prostate tumors. Its application has also been reported recently in detecting some gastrointestinal abnormalities such as Barrett’s esophagus, gastric cancer, prostate cancers, and also bladder cancer. Only one study has been published in literature so far, using methylene blue in-vivo staining in detecting oral dysplasia \cite{1}. Hence this study was carried out to evaluate the sensitivity and diagnostic reliability of topical methylene blue in-vivo staining in predicting oral dysplastic changes.

**MATERIALS AND METHODS**

The present randomised in-vivo study was conducted in the Department of Oral Medicine and Radiology, Bapuji Dental College and Hospital, Davangere, Karnataka, India with the approval of ethical committee of the institution and Helsinki Declaration have been followed in this investigation on patients reporting to the department. The study consisted of two groups: Group A (study group) and Group B (control group). Each group consisted of 60 patients each. Group A patients were selected randomly comprising of males and females clinically diagnosed with the suspected lesions based on the following criteria:

**Inclusion criteria**

1. Patients of the age group 25-90 years.
2. Patients clinically diagnosed with oral cancer and precancerous lesions according to the guidelines given by Axell et al. and an International Collaborative Group on Oral White Lesions; and International Seminar on oral leukoplakia and associated lesions related to tobacco habits \cite{10,11}.
3. Diagnosis of all OLP cases will be done on the basis of proposed modified W.H.O criterion for Oral Lichen Planus and Oral Lichenoid Lesions \cite{12}.
4. Ulceration: localized and superficial lesions that does not heal after local treatment \cite{13}.

**Exclusion criteria**

1. Patients having white plaques or patches for which a local cause other than the use of tobacco can be identified.
2. Patients with history of Oral cancer or previous oral surgery.

The control group [group B] consisted of age and sex matched individuals, with no habits of betel quid chewing, smoking or alcohol drinking. Only those subjects who satisfied these inclusion and exclusion criteria and gave a signed informed consent were included in the study. All the patients underwent a conventional oral examination in the Department of Oral Medicine with universal precautions. The first appointment included the collection of patient information, history, clinical examination and diagnosis. Once the clinical diagnosis was established based on the inclusion criteria, the patients underwent routine blood investigations including tests for HIV and HBsAg. After ruling out systemic diseases, the patients were scheduled for biopsy of the oral mucosa, which was carried out in the Department of Oral Medicine.

**Steps in methylene blue staining**
A standard staining procedure was adopted:

**Gargling solution**

A set of methylene blue dye system includes 2 bottles of solution. The dye rinse solution (Bottle A) contains active ingredient Methylene Blue 1%, with the addition of 1% Malachite, 0.5% Eosin, Glycerol and Dimethylsulfoxide. Pre and post rinse solution contains (Bottle B) 1% Lactic acid, vanilla flavor and purified water.

**Staining procedure**

The application of topical methylene blue was as follows. All patients were made to rinse their mouth with Bottle B solution for 20 seconds to remove food debris and excess saliva and to provide a consistent oral environment. The mucosa over the target area is gently dried with a gauze and power air sprayed to ensure that the lesion is not contaminated with saliva. Patient is then made to gargle and rinse with Bottle A solution for 20 seconds and then expectorate. Patient is again made to rinse with Bottle B solution for 20 seconds to wash out the excess dye.

The pattern of dye retention was assessed by the intensity of stain retention on the lesion. Local, stippled, patchy and deep blue stains were marked as positive reaction. This pattern is correlated with the degree of dysplasia evaluated by histopathological evaluation after biopsy. Wide, shallow, or faint blue stains were marked as negative reaction. If the blue stain washed out, negative reaction was recorded. The result of methylene blue dye staining was recorded with photographs and biopsy was performed simultaneously in the stained suspected lesions to compare the accuracy in the diagnostic capability of the stain.

**Biopsy**

The patient was made to sit comfortably on the dental chair, the procedure was explained to the patient, staining was done and biopsy taken from most suitable site judged by staining. If there is no dye uptake in the lesion, the biopsy specimen will be taken from the area judged by a specialist’s experience.

Topical local anaesthetic was sprayed onto the site of injection. 2% lignocaine hydrochloride with 1:80,000 adrenaline was used for local anaesthesia. The solution was administered to the patient using a sterile 26 gauge needle of 1½” length and a 5 ml disposable syringe, approximately 1 cm distal from the site planned for biopsy. After incisional biopsy, the tissue specimen was fixed in 10% neutral buffered formalin. Linterrupted sutures were placed. Histopathological evaluation and grading of dysplasia was done using WHO criteria with basis of Smith-Pindborg method of standardization in the Department of Oral Pathology and Microbiology, Bapuji Dental College and Hospital, Davangere.

**Statistical analysis**

The results of positive and negative uptake were correlated with the histopathological diagnosis. Statistical analysis was performed including sensitivity, specificity, positive and negative predictive values. The association of methylene blue stain uptake and histopathology diagnosis among the oral premalignant/oral cancer group, benign group and control group was analysed using Fisher’s exact parametric test of significance.

**RESULTS**

In group A comprising of 60 subjects, 3 (5%) cases was in benign group which included reticular lichen planus, 41 (68%) cases with potentially malignant disorders and 16 (27%) cases with malignant lesion. In the potentially malignant group (PMD), cases were distributed as follows: Homogenous leukoplakia 35 (86%), Speckled leukoplakia 2 (5%), Nodular leukoplakia 1(2%), Erosive lichen planus 2 (5%), Chronic non healing ulcer 1 (2%). Among group B of 60 patients, with no habit or medical history and with no morphological alteration in mucosa, methylene blue dye was not retained on normal mucosa.

It was seen that there was significant male predilection for the lesions from benign, dysplasia and then to carcinoma with a male to female ratio of 7.4:1 (P=0.007). The age of the study and control groups ranged from 25 to 94 years. PMD was noticed between 25-64 years of age with carcinoma occurring as early as 25 years with a peak between 85-94 years of age. Increase in dysplasia and carcinoma was noted with both smoking and chewing habits. Mild to moderate dysplasia was noted in buccal mucosa and gingiva with highest occurrence in the retrocommissure. In group A, 29 out of 60 in the study group stained positive and exhibited a true positive correlation and 16 exhibited true negative whereas there were 3 with false positive and 13 with false negative correlation. The histopathological results were in concurrence with the clinical finding (Tables 1 and 2).

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The sensitivity of the study was 90.6% and Specificity was 57.1%. The Positive Predictive Value, Negative Predictive Value, Efficiency of staining, False positive rate, False negative rate were 70.7%, 84.2%, 75%, 43% and 9.4% respectively.

**Table 1:** Diagnostic reliability of Methylene Blue staining of benign lesions, potentially malignant disorders and oral carcinoma.
Local staining was shown in 3 subjects among the benign group and 13 of PMD group (Figures 1 and 2). 1 in the PMD group and 2 in the carcinoma group showed patchy staining. Deep blue staining was seen in the 13 among the oral carcinoma group. As the distribution and depth of the stain uptake increased, the diagnostic validity of methylene blue to detect the dysplasia and carcinoma increased, which was highly significant (P<0.001). The patchy stain was taken up in moderately dysplastic lesions as well as carcinomas and deep blue stain entirely by carcinoma whereas the local stain uptake was seen in most of the mild to moderate dysplasia cases (Table 3).

### Table 2: Stain uptake and its correlation with histopathology report.

<table>
<thead>
<tr>
<th>Inference of staining</th>
<th>Benign n (%)</th>
<th>PMD n (%)</th>
<th>Oral carcinoma n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>True Positive</td>
<td>0</td>
<td>14 (34)</td>
<td>15 (100)</td>
</tr>
<tr>
<td>True Negative</td>
<td>3 (100)</td>
<td>18 (44)</td>
<td>0</td>
</tr>
<tr>
<td>False Positive</td>
<td>0</td>
<td>4 (10)</td>
<td>0</td>
</tr>
<tr>
<td>False Negative</td>
<td>0</td>
<td>5 (12)</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>3 (100)</td>
<td>41 (100)</td>
<td>16 (100)</td>
</tr>
</tbody>
</table>

Figure 1. Speckled leukoplakia of left buccal mucosa prior to staining.

Figure 2. Speckled leukoplakia of left buccal mucosa post staining.
DISCUSSION

29 (71%) out of 60 in the study group stained positive and the histopathological results were in concurrence to the clinical findings and the stain uptake, putting forth the sensitivity of the study to 90.6% (94% for oral carcinoma and 78% for PMDs) with a false negative rate of 9.4%. This is comparable with the previous and first study of methylene blue in oral PMDs and oral carcinoma which reported 90% sensitivity with a false negative rate of 10% [1]. Specificity was 57.1% in the study and false positive rate was 43%. In case of specificity, the previous study obtained a value of 69% with a resulting false-positive rate of 31% [1].

Table 3: Extent of staining and its correlation with histopathological dysplastic features.

<table>
<thead>
<tr>
<th>Extent of staining</th>
<th>Benign</th>
<th>PMD</th>
<th>Oral carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Local</td>
<td>3 (19%)</td>
<td>13 (81%)</td>
<td>0</td>
</tr>
<tr>
<td>Patchy</td>
<td>0</td>
<td>1 (33%)</td>
<td>2 (67%)</td>
</tr>
<tr>
<td>Deep Blue</td>
<td>0</td>
<td>0</td>
<td>13 (100%)</td>
</tr>
</tbody>
</table>

All false negatives in the current study were cases of homogenous leukoplakia with mild dysplasia. In the previous study on methylene blue on oral PMDs and oral carcinoma, this was attributed to the ambiguous light blue stains misinterpreted as negative [14]. Vital stain appears to stain only three to four cells deep and thus reflects changes in the epithelial layer alone in such cases. The dysplastic changes in the epithelial strata helps in diffusion of the stain and thereby its retention to the acidogenic nucleic acid components. The underlying tissue is not penetrated by the dye. Hence the presence of dysplastic features confined to the basal area of the epithelium might be the possible reason of the absence of stain uptake. In most of the previously conducted studies using vital stains, the false negative was mainly attributed by low grade dysplasia that can also be seen in our study. As pointed out by Epstein et al. [14] the detection of low-grade (mild/moderate) oral dysplasia has been less consistent, with a significant portion of lesions in PMD not taking up the stain. Zhang et al. [15] reported that vital stains failed to detect 77% of low-grade dysplasia, and according to Juhi Upadhyay et al. [9], 64% of the PMDs exhibited false negative results. This could be attributed to the false negatives in the present study also. Hence blind reliability on staining results in underdiagnosis, which is not justified since malignant transformation of 3-5% has been reported in mild and 3-15% for moderate dysplasia [16].

The false positive results in the present study might be due to the stain retention in the necrotic slough and inflammation in the erosive lichen planus lesions and entrapment of stain in the irregular fissured surface texture in case of the homogenous leukoplakia. In the previous study also, the high false-positive rate was discussed to be related to the retention of stain in inflamed and trauma areas. Other causative factors included the irregular, papillary or digital surfaces of the lesions, which caused the mechanical retention of dye, contamination of saliva and plaque, retention of dye material in papilla of the tongue or minor salivary gland ducts over the mucosa [1, 17, 19]. False-positives associated with retention of dye in inflammatory and traumatic lesions have been extensively documented [17, 19]. False positive in vital staining was mainly attributed to epithelial hyperplasia and hyperkeratotic lesions [9]. Mashberg [17] reported 9.2%, Onofre et al. [18] 2%, Epstein et al. [14] 64%, Zhang et al. [15] 26% and Siddiqui et al. [17] 13% of false positive results of vital staining in PMDs. A 10-14 day waiting period was suggested to allow inflammatory lesions to resolve thus decreasing the false positives [17].

Local staining was shown in 3 cases (19%) among the benign group and 13 (81%) cases of PMD group. 1 (33%) cases in the PMD group and 2 (67%) cases in the carcinoma group showed patchy staining. Deep blue staining was seen in the 13 (100%) cases among the oral carcinoma group. As the distribution and depth of the stain uptake increased, the diagnostic validity of methylene blue to detect the dysplasia and carcinoma increased, which was highly significant (P<0.001). The patchy stain was taken up in moderately dysplastic as well as carcinomas and deep blue stain entirely by carcinoma whereas the local stain uptake was seen in most of the mild to moderate dysplasia cases. In the previous study, 26 of 29 pathologically proven PMDs and Oral carcinoma showed positive staining with deep and focal methylene blue dye [1]. In another study of 100 patients with PMDs, staining of specific lesions with vital stains (toluidine blue) correlated with clinicopathologic and malignant progression to invasive carcinoma [20]. Staining in the study correlated with degree of dysplasia, with only 5 of 19 (26%) non-dysplastic lesions compared with 16 of 17 (96%) with severe dysplasia which stained blue [20].

The present study found that topical methylene blue stain was taken up by PMDs and oral carcinoma with significant staining in correlation with the degree of dysplasia and carcinoma. There was a significant relationship between increase in the age of the patient and the susceptibility of acquiring dysplasia and carcinoma. It was also seen that such lesions were more prevalent in males than females. The potential of dysplasia and carcinoma had a significant increase with habits. However it was discerned that, as the distribution and depth of the stain uptake increased, the diagnostic validity of methylene blue to detect the dysplasia and carcinoma increased, which was highly significant (P<0.001). Further it was found that the vital stains were diagnostic in detecting increased degrees of dysplasia and carcinoma rather than its efficacy in determining mild dysplasia. Since changing trends of habit and carcinoma prevalence in young adult population have developed due to the early usage of tobacco related habits, it is necessary to diagnose the PMDs and early oral carcinomas at its early dysplastic stages to decrease the rates of disability and increase in quality of life. Topical Methylene blue due to its less toxicity, easy availability and low cost can be used as a valuable chairside diagnostic tool in the early detection of such lesions.
CONCLUSION

Clinical appearance and diagnosis of a lesion in the oral cavity is not adequate to determine its premalignant nature as not all white lesions turn malignant \[13\]. Vital staining have been used to improve recognition of these early neoplastic lesions \[9,20\]. Hence we validated the use of topical methylene blue *in-vivo* staining, which has less toxic effects on the human body, ease of availability and low cost and can be used as a valuable chairside diagnostic tool in the early detection of oral dysplastic lesions thereby enhancing the quality of life by significantly reducing the morbidity and mortality in such cases. We found that topical methylene blue stain was taken up by majority of dysplastic lesions in and the depth of the stain uptake increased with the degree of dysplasia in PMDs and oral carcinoma. Further studies with larger samples should be considered in the future prospective of the study.

Conflict of interest

None declared.

Acknowledgment

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REFERENCES