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Review Article

Vital Staining: Diagnostic Adjunct for Early Diagnosis of Oral Epithelial Dysplasia and Carcinoma

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Abstract

Early diagnosis is the single most important factors that improve the prognosis and survival rate of cancer patients. Numerous adjuncts are available to help in its diagnosis. Vital tissue staining is one such adjunct used in the diagnosis of cancer. Though not a new technique, its application to cancer diagnosis especially in its premalignant stage is still uncertain. While the efficiency of toluidine blue (TB) is established to certain extent, the role of other vital stains needs to be researched. This article reviews the various vital tissue staining techniques available in the diagnosis of oral precancer and cancer.

Introduction

Oral cancer occurs as a multistep process, progressing from a precancerous stage to the stage of cancer. Vital staining gives the advantage of diagnosing it in an early stage before it progresses into cancer. In spite of occurring in stages, most often cancer is diagnosed in its advanced stages. The prognosis still remains poor with the 5-year survival rate approximately 50% for the last 50 years.[1] There are numerous diagnostic adjuncts available for its early diagnosis. Cytological methods, tissue staining techniques, and molecular methods have been used and tried.

Vital staining is a procedure where living cells take up certain dyes, which selectively stains some elements in the cells like mitochondria, lipid vesicles, lysosome, etc. Among all diagnostic aids, vital staining is simple, in expensive and sensitive tool for identifying epithelial dysplasia and early squamous cell carcinoma.

It is of two types:

1. Intravital staining.
2. Supravital staining.

- When the technique is applied in vivo, it is referred as intravital staining.
- If the technique is applied in vitro (living cells outside the body) then it is called supra vital staining.
- To reduce the toxicity of these stains they are used in dilute solutions ranging from 1:5000 to 1:500000. Sensitivity and specificity was found 100%.[2]

History :

- Discovery of toulidine blue ---William Henry perkin in 1856
- In vivo staining (TB) ----- first applied in 1963 by Reichart--- for uterine cervical carcinoma in situ.
- Application of vital stains to detect oral premalignant and malignant lesions ----- first reported by Neibel HH and Chomet B in 1964.[3]

Few of the vital stains which can be used as clinical tool are:

1) Toluidine blue
2) Lugol's iodine
3) Acetic acid
4) Rose bengal dye
5) Methylene blue

Supravital staining has long been used as an adjunct in the early diagnosis of malignant lesions. In 1928, Schiller reported the use of Lugol's iodine solution in carcinoma of the uterine cervix. *In vivo* staining has been extensively used in gynecological practice for the detection of malignant change of the cervix during colposcopy. [2],[3] The technique has been applied in the oral setting for over 30 years by means of the dye toluidine blue (TB). [2],[4] Apart from TB, other stains such as methylene blue (MB), Lugol's iodine, and acetic acid have also been tried in the diagnosis of cancerous lesions.

1) **TOLUIDINE BLUE (TB) STAINING :[2,3]**

- It is also known as tolonium chloride, methylanaline, aminotoluene.
- Its principal use was in dye industry after its discovery by ---William Henry Perkin in 1856.
- it is an acidophilic metachromatic dye which has the ability to bind to acidic tissue components, thereby it binds to the nuclear material of the tissues having high DNA and RNA content.

METACHROMATASIA:

- Discovered in 1875--- by Cornil,Jurgens, and Ranvier.
- Metachromasia is important as it is highly selective and only certain tissue structures can stain

metachromatically.

- It is a phenomenon whereby "a dye may absorb light at different wavelengths depending on its concentration and surroundings and it has the ability to change its color without changing its chemical structure."
- For metachromasia to occur there must be free electronegative groups on the surface of tissues.
- Metachromasia is attributed to stacking of dye cations at the sites of high density of anionic groups in the tissue.
- Stacking shortens the wavelength of maximum absorption, a hypsochromic shift, so that the maximum wavelength in the spectrum of the transmitted light is longer making the observed color red instead of blue.
- Substances that can be stained in this way are called chromotropes and they include mucins, mast cells.
- The chromotropes carry acidic groups with a minimum density of not more than 0.5 nm between adjacent negatively charged groups.
- These alter the color of metachromatic dyes.
- Principally, van der Waals forces hold the dye together to form dimers, trimers, or polymers
- Other forms that play a lesser role are hydrogen and hydrophobic bonding.
- Dye exists in a normal monomeric (orthochromatic) form to a potential polymeric (metachromatic) form.
- Negative charges on the chromotropes attract the positively charged polar groups on the dye leading to dye-to-dye aggregation in a specialized orderly form forming a polymeric form.
- Three forms of metachromasia alpha (a), beta (b), and gamma(g) giving a range of colors [Table 1].

Table 1: Various forms of metachromasia

Type	Color	Structure	Results
α—Orthochromatic	Blue	Monomeric	Negative
β—Weakly metachromatic	Purple	Dimer and trimer	Weak positive
γ—Strongly metachromatic	Red	Polymeric	Strong positive

- color shift is always from a blue or violet dye to yellow or red stain meaning that the color absorption shifts to shorter wavelengths, leaving only the longest wavelengths to be seen.
- This is believed to represent polymerization of the dye.
- Greater the degree of polymerization, the stronger is the metachromasia.
- Metachromasia requires water between dye molecules to form the polymer and does not survive dehydration and clearing.

Principle:

- As the toluidine blue has the ability to bind to acidic components of the tissue, it is based on the fact that dysplastic and neoplastic cells contain more nucleic acid quantitatively than normal cells.
- Also the intercellular canals are wider in malignant epithelium than the normal epithelium, thereby enhancing the penetration of dye.
- For intraoral use, 1% of toluidine blue is used.

Composition:

100 ml of 1% TB contains 1 gm of toluidine blue powder, 10 ml of 1% acetic acid, 4.19 ml of absolute alcohol and 86 ml of distilled water, pH maintained at 4.5.

Procedure :

- Rinse the mouth twice with water for 20s to remove debris.
- Apply 1% of acetic acid for 20seconds to remove ropey saliva and then apply 1% toluidine blue either with cotton swab or can be given as rinse.

- Then, 2 rinses with 1% acetic acid are done to reduce the mechanically retained stain.
- Finally rinse the mouth with water.
- Then the color change is assessed.
- Interpretation is based on the color change of mucosa. Dark royal blue is considered as positive, light blue is doubtful while no color is negative.

False positive results are seen with following lesions:

- Epithelial hyperplasia, hyperkeratotic lesions, inflammatory and traumatic lesions, hyperplastic candidiasis can retain 60% of stain.
- The decision making can also be attributed to the experience of the clinician.
- Repeat the test after 10-14 days to allow the inflammatory lesions to resolve.
- This reduces the false positive by 8.5%.

False negative results are recognized in:

- Low grade dysplasia, lichenoid dysplasia

Advantages :

1. Inexpensive and simple procedure
2. Can be used for screening high- risk patients who may have asymptomatic malignant lesions of oral cavity.
3. Helpful for surgeon in operating room to evaluate free surgical margins.
4. Toluidine blue staining of oral epithelium will not interfere with histologic staining or interpretation.

Disadvantages:

1. Both false positive and false negative results are more.
2. Filiform papillae retain the dye due to high protein synthesis rate.
3. TB appears to stain only three to four cells deep and thus reflects changes in the epithelial layer alone.
4. Invaded underlying tissue is not penetrated by the dye. So the extent of submucosal spread is difficult to appreciate.

Application of Toluidine Blue:

1. Connective tissue mucins, especially acid mucins. Tissue stains purple to red,while the background is stained blue.
2. Mast cell granules stain purple in color due to the presence of heparin and histamine.
3. Amyloid stain blue but under polarized light they give a bright red birefringence.
4. Endocrine cell granules are stained purple to red. The concentration of stain used here is 0.01%.
5. Sulfatides stain red brown or yellow. Only lipids that are sufficiently acidic to induce a metachromatic shift are stained.
6. Corneybacterium diphtheria contains granules with polymerized inorganic polyphosphate, which stains red violet color.
7. Helicobacter stains dark blue against a variably blue background. The concentration of TB used is 1%.
8. TB can also be used to stain frozen section because of the rapidity of the staining procedure (10–20 s) and better clarity of the cells.

2) LUGOL'S IODINE STAINING:[5]

- Lugol's iodine was first made in 1829, which is named after the French physician Lugol .
- Upto the end of 19th century, it was used as an antiseptic and disinfectant.
- Other names for Lugol's solution are I2KI (iodine–potassium iodide), markodine, strong solution (systemic), aqueous iodine solution.
- In 1929, Schiller W described Iodine test to delineate areas of cervical precancers.

Composition:

Iodine 2 g , potassium Iodide 4 gm in 100cc of distilled water.

Principle :

- Principle is based on glycogen content of the cytoplasm and the reaction is known as the iodine–starch reaction, visualized by a colour change.
- As there is enhanced glycolysis in cancer cells, do not promote the iodine–starch reaction.
- Hence there is no color change in dysplastic epithelium, whereas due to high glycogen content

of normal epithelial cells, brown color can be noticed.

- Vital dye with Lugol's solution is also called Schiller's test.

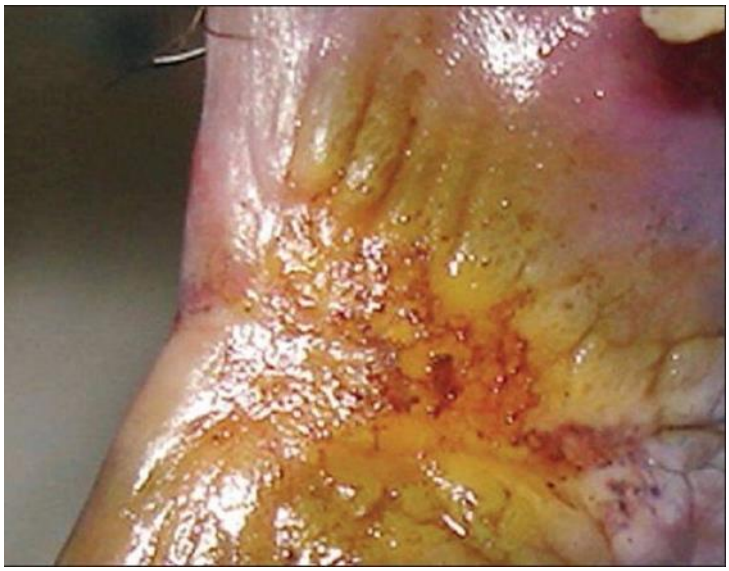

Figure 3: Speckled leukoplakia after Lugol's iodine staining

Procedure:

- Rinse with water/ carbocisteine syrup 250 mg/5 ml and dry with a gauze to clear the mucin.
- Then apply Lugol's iodine until parakeratinized epithelium is stained a brown or black.
- After one to two minutes, interpret the stain reaction.
- Most effective method is to stain the lesion with 3% Lugol's solution followed by 5% Lugol's solution.
- Lugol iodine is cheap, widely available, easy to use and only about 5 min are necessary to perform the staining procedure.

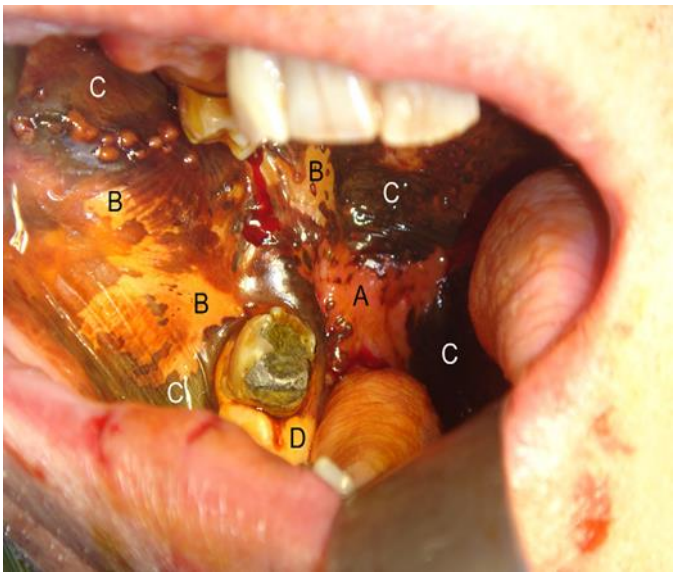

Figure 4. (A) Invasive squamous cell carcinoma not stained by iodine; (B) dysplastic areas not stained by iodine; (C) normal parakeratinised mucosa stained brown or black by iodine; and (D) normal orthokeratinised mucosa not stained by iodine.

3) **ACETIC ACID STAINING:**[4]

- In the past, 3–5% acetic acid was used as a vital stain for detection of cancer in developing countries.
- Sankaranarayanan et al. used 5% acetic acid for the detection of cervical cancer.
- Further, Bhalang et al. used 5% acetic acid as a clinical marker for the detection of oral cancer .

Composition :

100 ml of 1% acetic acid rinse contains 1 ml of glacial acetic acid with 99 ml distilled water.

Principle :

- Application of acetic acid causes reversible coagulation / precipitation of cellular proteins and causes swelling of the epithelial tissue, particularly abnormal squamous epithelial areas, dehydration of the cells and it helps in coagulating and clearing the mucous secretions.
- Normal squamous epithelium appears pink and the columnar epithelium red, due to the reflection of light from the underlying stroma, which is rich in blood vessels.

- If the epithelium contains a lot of cellular proteins, acetic acid coagulates these proteins, which may obliterate the colour of the stroma.
- Resulting aceto whitening is seen distinctly as compared with the normal pinkish colour of the surrounding normal squamous epithelium.

Procedure:

- A piece of gauze soaked with 5% acetic acid is applied to a cleaned and dried lesion for 60 seconds.
- After that gauze is removed and characteristic color change can be noted.
- Color change to opaque white is considered positive and transparent white as negative.
- The Sensitivity, specificity and accuracy is about 83.33%, 84.21% and 83.64% respectively.

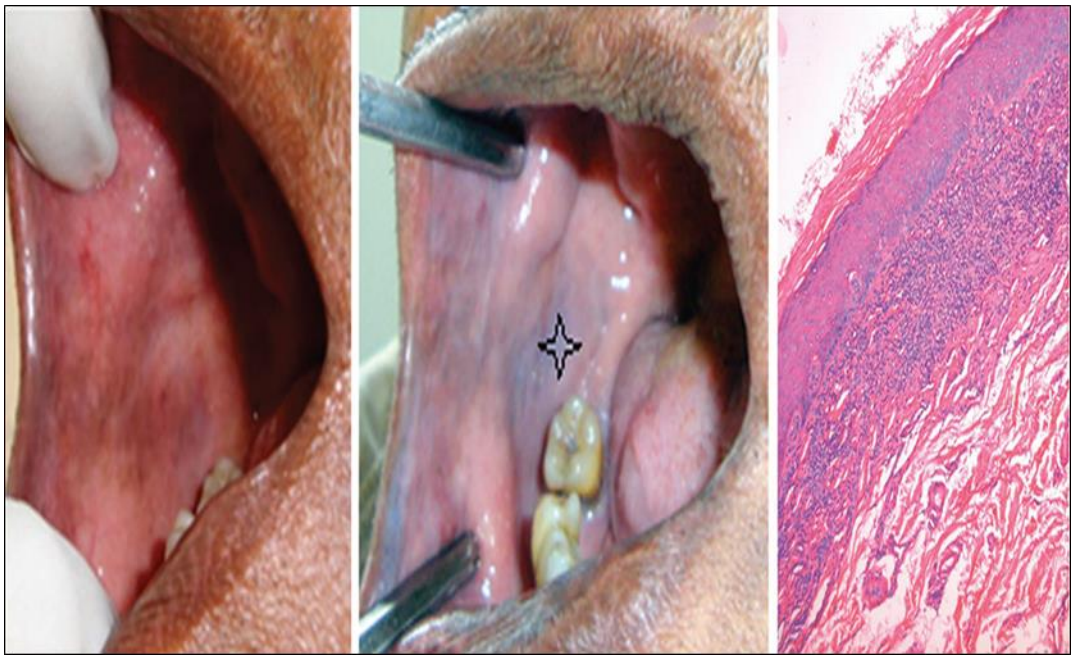

Figure5 :(a) Group I : Before application of the vital stain. (b) After application of vital stain, a showing biopsy site. (c) Histopathology showing hyperkeratosis (H&E stain, ×100)

It is associated by false positive reaction with small aphthous-like ulcerated lesion that might not routinely be biopsied turned opaque white after the application of acetic acid and the histopathologic result was moderate epithelial dysplasia .

4) ROSE BENGAL STAINING :[6]

- It is 4, 5, 6, 7 tetrachloro-2, 4, 5, 7 tetraiododerivate of fluorescein, can stain the desquamated ocular epithelial cells.
- RB staining is used to delineate the extent of the corneal and conjunctival neoplasms and even oral epithelial dysplasia and OSCC.

Principle :

- With an exposure for 1 second, RB predominantly stains the cell membranes.
- An increasing the concentration or time of exposure, it produces predominant nuclear staining.
- A primary epithelial abnormality such as dysplasia, metaplasia, virus infected cells or other forms of epithelial keratitis can render the inability of epithelium to interact with the mucous layer, thus allowing the RB staining.

Procedure :

- Distilled water is used to rinse the mouth for 1 minute in order to clean the lesions.
- Apply RB solution with cotton with 2 minutes and again rinse the mouth with distilled water for 1 min to remove excess stain.
- RB staining is more favorable in revealing the dysplasia than toluidine blue because even the mild dysplasia can be detected by this dye.

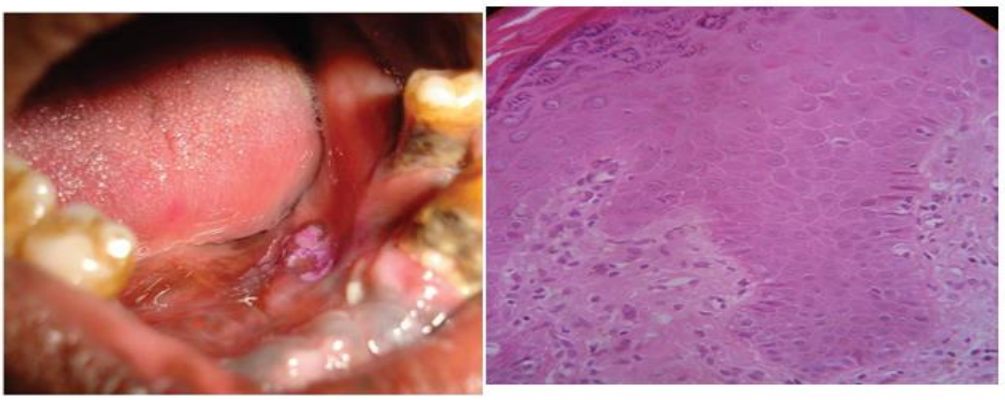

Figure 1:a) Mild staining with Rose Bengal of homogeneous leukoplakia b) Mild dysplasia

Applications of vital stains :

1) To highlight the potentially malignant oral lesions
2) To identify early lesions which could be missed out on clinical examination .
3) To outline the full extent of dysplastic epithelium or carcinoma prior to excision
4) To detect multicentric or second primary tumors
5) Selecting the biopsy sample site in premalignant lesions
6) Help in follow up the patients with oral cancer.
7) Useful in obtaining the marginal control of carcinoma.
8) Can be used as effective screening modality to assess the intra operative margins alterative to the frozen sections.
9) Recognition of post-treatment recurrence.
10) Also in educating the patient.

Conclusion:

Being simple, economical, widely available, noninvasive, and easy to use; vital staining can be used in all the clinical settings especially in developing countries where the advanced diagnostic modalities are unavailable. Though numerous studies have been carried out using TB, the same are lacking for other vital stains. Studies with well-designed methodologies with due consideration to the nature of samples are essential to evaluate the efficacy of MB, Lugol's iodine, and acetic acid; so that their role in the diagnosis of precancer and cancer can be accurately determined.

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