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Research Article

A BRIEF REVIEW OF THE BASIC CHEMISTRY OF TOLUIDINE BLUE AND ITS APPLICATION IN VITAL STAINING

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ABSTRACT

Toluidine blue is a basic thiazine metachromatic dye with high affinity for acidic tissue components, thereby staining tissues rich in DNA and RNA. It has found wide applications both as vital staining in living tissues and as a special stain owing to its metachromatic nature. Toluidine blue has been used in vivo to identify dysplasia and carcinoma of the oral cavity. This article deals with the basic chemical properties of toluidine blue with an emphasis on its role in vital staining procedure.

INTRODUCTION

Toluidine blue (also known as tolonium chloride) is an acidophilic metachromatic dye that selectively stains acidic tissue components (sulfates, carboxylates, and phosphate radicals).[1] Toluidine blue (TB) has an affinity for nucleic acids, and therefore binds to nuclear material of tissues with a high DNA and RNA content.[2] It is a member of the thiazine group of dyes and is partially soluble in both water and alcohol.[3] The dye has been known for various medical applications since its discovery by William Henry Perkin in 1856, after which it was primarily used by the dye industry. Also known as methylanaline or aminotoluene, it basically has 3 isoforms, namely, ortho-toluidine, para-toluidine, and meta-toluidine. TB has been extensively used as a *vital stain for mucosal lesions.*

Vital staining is a procedure wherein living cells take up certain dyes, which selectively stain certain elements in the cells like mitochondria, lipid vesicles, lysosome, etc. [4] The earliest technique developed by Paul Ehrlich in 1885 involved the immersion of freshly removed tissue in methylated blue. 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. [4] TB was first applied for in vivo staining by Reichart in 1963 for uterine cervical carcinoma in situ. [5]

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Few of the vital stains which can be used as clinical tool are: Toluidine blue, Lugol's iodine, Methylene blue, Rose bengal dye, Acetic acid.

Malignancies of the upper aero digestive tract are one of the common malignancies in the world. Tumors arising from the oral and oropharyngeal malignancies are usually well advanced at the time of diagnosis. The disease is life threatening, with high morbidity resulting from late treatment. However, if it is diagnosed at an early stage, oral cancer is often curable and inexpensive to treat.[6] Precisely, in these cases the role of a simple adjunctive diagnostic tool comes into play for the purpose of early detection of suspicious malignant and premalignant disorder. Among all diagnostic aids, vital staining is simple, inexpensive and sensitive tool for identifying epithelial dysplasia and early squamous cell carcinoma. During 1960s suggestion was made that TB may stain malignant epithelia of the mucous membrane in vivo, whereas normal tissue failed to retain the dye. TB detects relative rather than absolute differences between normal and malignant cells and tissue. [7]

Basic chemical properties of TB

Toluidine blue is also known chemically as tolonium chloride, methylanaline or aminotoluene. Its principal use was in dye industry after its discovery by William Henry Perkin in 1856. TB 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. [8] Its molecular weight is 270.374 g/mol. It is soluble in water (up to 3.5%) and in alcohol (up to 0.5%).[3] It is an

acidophilic dye of the thiazine group that selectively stains acidic tissue components (carboxylates, sulfates, and phosphate radicals)such as deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). [9] It has the staining property of metachromasia, which is due to the presence of repetitive phosphate groups in the nucleic acids and is dependent on temperature and the pH. The recommended pH is 6.0-7.0. The temperature should not exceed 30°C above which the metachromatic property diminishes in strength.

Principle

As the TB 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.[8] TB stains tissues based on the principle of metachromasia. The dye reacts with the tissues to produce a color different from that of the original dye and from the rest of the tissue. Metachromasia was 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. [10]

Metachromasia may be defined as the staining of tissue or tissue components such that the color of the tissue-bound dye complex differs significantly from the color of the original dye complex to give a marked contrast in color (Pearse 1960). [11] Typically, there is a shift in the absorption of light by the tissue dye complex toward the shorter wavelengths with an inverse shift in color transmission or emission towards the longer wavelengths. This is believed to represent polymerization of the dye. The 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. [12] Azure A and toluidine blue are small planar cationic dyes that typically stain tissues blue. Under conditions of metachromasia, these dyes stain tissue components purple-red. The use of such dyes to identify charged mucins and proteoglycans is one of the oldest of the histochemical techniques for carbohydrates. Metachromasia is believed to result from a specific form of dye aggregation that is characterized by the formation of new intermolecular bonds between adjacent dye molecules (Pearse 1960). [11] The bonds between the dye molecules only occur in situations in which the molecules are brought into close proximity to one another (Sylven 1954; Bergeron & Singer 1958). 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. [13] Substances that can be stained in this way are called chromotropes and they include mucins, mast cells, and so on. [12] The absorption spectrum of TB with an orthochromatic tissue is maximum at about 630 nm and staining result is blue. With a metachromatic substance the absorption spectrum is maximum at 480–540 nm and the staining is red in color. [4] Van der Waals attraction between TB and polyanions contribute to affinity when binding to DNA or RNA as does hydrophobic bonding. [13]

Vital Staining

The use of TB as a vital stain was first proposed by Reichart to disclose dysplasia and carcinoma in situ of the uterine cervix. Neibel and Chomet and Shedd and co-workers were the first to report vital application of TB for the detection of premalignant and malignant lesions of the oral cavity.[14] TB is used based on the fact that dysplastic and neoplastic cells may contain quantitatively more nucleic acids than normal tissues. Also, malignant epithelium may contain intracellular canals that are wider than normal epithelium, which may facilitate penetration of the dye.[1] The other proposals about the uptake of TB in dysplastic and carcinomas include the high density of nuclear material, loss of cell cohesion, and increased mitosis. [3]

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.[15]

Technique of staining -TB can be used in two ways. It is either applied to the site of the lesion with a cotton applicator or it is used as mouth rinse. The procedure of staining is as follows:

- Oral examination
- Rinsing the mouth twice with water for 20 secs to remove the debris
- Application of 1% acetic acid for 20 secs to remove any ropey saliva
- Application of 1% TB solution for 20 secs either with cotton swab when a mucosal lesion is seen or given as a rinse
- Application of 1% acetic acid to reduce the extent of mechanically retained stain
- Rinsing oral cavity with water
- Oral examination and recording of the stained areas. [15]

Interpretation

A dark blue (royal or navy) stain of either the entire lesion or a portion of it is considered as positive stain, lack of color absorption by the lesion as negative stain, and light or pale blue staining as doubtful. These cases are usually due to mechanical surface retention or inadequate removal of the stain. [16] Mashberg suggests some areas not to be considered positive if it retains stain. These areas include the nucleated scales covering the papillae on the dorsum of the tongue, pores of seromucinous glands in the hard palate, dental plaques, gingival margins around each tooth, diffuse stain of soft palate transferred from the retained stain on dorsum of tongue, and ulceration lesions.[5],[16]

Several studies have been performed over the years to determine the sensitivity and specificity of in vivo TB staining. Earlier studies have found sensitivity in the range of 86-100% and specificity in the range of 44–100%. [17], [18] Lingen *et al*, [19] in their review, mentioned the sensitivity and specificity of TB in the detection of oral cancer to be in the range of 78–100% and 31–100%, respectively. Epstein *et al*.[20] while screening for recurrence in patients who had previously been

treated for upper aero- digestive tract malignancies, found that the use of tolonium chloride rinse is more sensitive than clinical examination alone in detecting lesions that might be found on biopsy to be carcinoma or carcinoma in situ.

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. Test is to be repeated 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. [21]

Onofre *et al* [22] evaluated the TB staining in premalignancies, and superficial oral ulceration suggesting malignancy. The study showed 100% sensitivity in the detection of in situ and invasive carcinoma and no false-negative results occurred. TB application is an important adjunct to the clinical examination because it may not only increase the clinical suspicion of the examination but also assist in identifying sites in need of biopsy and delineating margins of the lesions, which may lead to a more timely diagnosis allowing benefits of earlier treatment and in directing surgical management. [23]

DISCUSSION

Early detection and timely intervention is the essence of any premalignant and malignant treatment protocol. Toluidine blue staining is very useful in these regards; specially in the developing countries like India because it's very simple, economical, widely available, noninvasive, and easy to use. TB is useful in raising clinical suspicion of malignancy or pre-malignancy and performs as an adjunctive method to gold standard histopathology. Further future research and studies in the days to come will perform to strengthen the role of TB as an emphatic screening procedure and will help in diagnostic and prognostic aspects as well as during performing biopsy as a useful adjunct.

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