A Brief Review on Haematoxylin: An Irreplaceable Tissue Stain

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Abstract

Haematoxylin is the most popular nuclear counterstain used in routine histopathogical techniques for staining tissues. It is derived from the tree Haematoxylum Campechianum (H.campechianum). The history of haematoxylin reveals centuries of war, strife and discovery dating from the Mayans and Aztecs who used it as an ink and fabric dye to the war resulting in the invasion of Mexico by European countries. H.campechianum was later named as logwood and block wood by the invaders that used it to generate haematoxylin crystals to be utilized in textile industry. The recognition to introduce haematoxylin as biological stain goes to Waldeyer. But later Bohmer introduced the technique of coupling a mordant to the dye making it colorfast and thus, haematoxylin remains a very unique and vital stain in histology till date.

Key Words: *Haematoxylin, Histology, Stain, Counterstain*

Introduction

Haematoxylin is the most common nuclear counterstain used in histology. It shall not be an over exaggeration to say that it is the cornerstone of staining tissue sections and the foundation upon which modern histopathological diagnosis is built. Haematoxylin is a natural dye derived from the tree Haematoxylum Campechianum (H.campechianum),that belongs to the order Legumiosae (Genus-Eucaesalpiniea) and is so named because of the reddish nature of its heartwood and young leaves. In Greek haemato means blood and xylo means wood.[1]There are several varieties of Haematoxylin, but H.campechianum gives the best coloured wood. H.campechianum tree grow in swamp lands distributed throughout Guetamala, Belize, and the Yucatan and Campeche area of Mexico (Central America).[2]

Initially, extracts of H.campechianum were used for dyeing in textile industry. For the transportation of H.campechianum from Mexico to Europe by sea the tree was cut into three-foot logs or blocks and hence was also called Logwood or Blockwood. However, the artisans in the textile industry did not know how to use the logwood to make the dye colorfast. Therefore, the fabrics dyed with logwood extract faded quickly.[3] Later a process was developed in which metal was used with a natural dye to make the dye colorfast. This process was widely applied by the industrialists, the metal used was called 'mordant'.[4] In 1810 a French chemist named Michel Eugene Chevreul isolated Haematoxylin crystals. The crystals were obtained by boiling H.campechianum heartwood chips in water, which produced an orange – red solution that turned yellow, then black upon cooling and yielded crude red haematoxylin crystals after

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evaporation of water. Haematoxylin is a neoflavenoid, a phenolic compound related to flower pigments.[5] Haematoxylin is the result of the decomposition of a glucoside that exists in fresh heartwood. Regarding the glucoside itself little is known, except that under the influence of a species of fermentation it is decomposed with formation of sugar and haematoxylin.[6] Waldeyer is frequently credited to have introduced haematoxylin as a biological stain, using it unsuccessfully to stain neuronal axons in 1862. But truly indeed, the potential for haematoxylin in histology should be credited to Bohmer (1865), who inspired by the textile industries, combined a metallic mordant with haematoxylin to stain tissues adequately.

Although the formulation of Bohmer is no longer in use, coupling of haematoxylin with a mordant is still the major form in which the dye is used.[7] The chemical basis of colour is due to certain atomic groupings known as chromophores(Greek=colour bearers) e.g-NO2,N=O,N=N,C=C,C=O,C=N,C=S and the quinoid group .Such groupings introduce 'resonance systems' in the molecule. Resonance systems result when molecular formula can be written in several valid ways. The rapid change resulting from the presentation of possible alternate states involves the absorption of electromagnetic waves and the production of colour. Benzene which does not show colour in white light, shows colour in UV range. The introduction of chromophoric group into an uncoloured molecule will cause it to be coloured, then it is called a chromogen. For a chromogen to be called a dye, it must be composed of an acid and a base and therefore should have salt forming properties. This function is performed by a group called auxochromes (Greek-increasers) which when attached to the dye molecule act as an electron donor to the chromophore. The OH and NH_2 are the main auxochromes; others are COOH(Carboxyl), SO_3H (sulphonic) groups. These can also serve to bind the dye to the tissue besides dissociating and forming compounds.[8] In pure form haematoxylin is a colourless or slightly beige powder which gives a colourless solution with water. Rudolph Nietzki was the first to discuss the chemical nature of haematoxylin in his 1892 publication in the 'Chemistry of Dye stuff'.[9] Pure aqueous haematoxylin cannot stain. The active staining agent is not the principal substance but rather an oxidation product of haematoxylin called haematein.[9,10] Haematoxylin and haematein differ by only hydrogen atom. Removal of this hydrogen from haematoxylin can be accomplished either by atmospheric oxygen or by using mild oxidizing agents and results in a compound with a hydroxyl group adjacent to a carboxyl group. This configuration facilitates formation of co-ordinate complexes with metals.[11]

Ripening

The process of generation of haematein from haematoxylin is called ripening. Haematein can be produced naturally through exposure to air and sunlight or UV light. This natural process of ripening can take upto 3 months or longer. Examples of use of naturally ripened dye are the methods of Delafield and Ehrlich's haematoxylin.[12] Chemical oxidants can also produce haematein from haematoxylin by the process of ripening in a shorter period, but does carry the risk of developing effective reaction products, a solution with a shorter shelf life than the naturally ripened dye.[13] Chemicals like potassium permanganate, iodine, sodium iodate, sodium periodate, hydrogen peroxide and mercuric oxide are example of oxidants used to ripen haematoxylin. Common stains like Mayer's and Ehrlich's formulation use sodium iodate while Harris' preparation relies on vigorous boiling and addition of mercuric oxide to produce haematein. Although chemical oxidation produces a working dye in very short period, it has to be performed very carefully to avoid over-oxidation of haematoxylin. Several substances may be produced as a consequence of over oxidation, but the most significant is a quinine-carboxylic acid, which is called oxy-haematein. Another important aspect is that haematoxylin powder may oxidize during prolong storage.If such a dye is used with usual amount of oxidant, it would unavoidably give rise to over oxidation products. One of the means of avoiding this problem has been to use half the normally required amount of chemical oxidant. The half oxidized solution has a greater shelf life sine unoxidised haematoxylin forms a reservoir which through natural ripening gradually replaces haematein that is over oxidized or exhausted by staining.[14] Haematein forms rapidly in alkaline solutions but slowly in acidic solvent. Addition of alcohol or glycerol further slows down oxidation, alcohol can also act as preservative.[15]

Mordants

Mordants are chemical substances that by their physico chemical structure aid in attaching a stain or dye to the tissues. They are essential to haematoxylin staining; the mordants used are always divalent or trivalent salts or hydroxides of metals. They probably combine as hydroxides with the dye by displacing hydrogen atom from it, and their remaining valences serve to attach or bind the dye-mordant complex to tissue components specially phosphate groups of nucleic acids.[14] The chelate complex of dye and mordant is called a 'lake'. The term 'lake' is derived from 'lac', a mordant dye obtained from an insect in India and from which shellac is obtained. Over the time the term 'lac' has changed to 'lake' and has come to be the generic term for all dye-mordant complexes.Two types of bonds are involved in the fundamental reaction between the dye and mordant. One is a covalent bond with hydroxyl oxygen, the other is a co-ordinate bond with another oxygen (electron donor).The alum salts are most commonly used because they were more readily available. Mordants and dyes can be applied in three ways:[15]

· Onchrome: Mordant is applied first followed by the dye. Heidenhain's iron haematoxylin is a classic example.

· Metachrome: Mordant and dye are mixedtogether and then applied.It is the most common type.Example-alum haematoxylins.

· Afterchrome: The dye is applied first followed by mordant.

Progressive and Regressive staining

In progressive staining, the staining is continued until the desired intensity of colouring of the different tissue elements is attained. e.g.-Mayer's, Delafied's, Harris' and Gill's haematoxylin formulae. In regressive staining the tissues are overstained and the excess dye is then removed selectively until the desired intensity of colour is attained. e.g. Ehrlich's, Harris' and Delafield's formulae. The designation 'selectively ' is due to the fact that when excess dye is removed, it is cleared from certain cell constituents before others, while other cell constituents are still strongly stained. This process of selective removal of excess dye is called 'differentiation'. Generally if the dye used is a basic one, differentiations are carried out by an acid solution and vice versa. For both basic and acidic dyes, alcohol acted as a fairly efficient differentiator and probably acts by simply dissolving out the excess dye.[16]

Differentiators are of three types:[16]

· Acid differentiator —These act by combining with metal, thus breaking the latters association with the tissue or cell components. e.g.-acetic acid and hydrochloric acid.

· Oxidising differentiator — These act by oxidizing the dye to a colourless form (leuco form). Cell components holding the least dye get bleached first. e.g-Potassium ferricyanide, Chromic acid, Potassium permanganate, Potassium dichromate and picric acid.

· Mordant differentiator — The process happens due to mass action. When a section that has been stained by a mordant dye in a solution of mordant, the latter is present in great excess and the dye gradually leave the tissue to combine with free mordant in the solution. Also iron alum mordants can oxidize haematoxylin to a colourless compound, tissue component, which hold the least dye gets decolourised first.e.g-aniline used with gentian violet, Phenol used with carbolthionone.

Blueing

Acid solutions of alum haematoxylin are reddish in colour while the aluminium lake of haematein is blue in colour. This is because Alum (Potassium aluminium sulphate) is watery solution and tends to dissociate: the aluminium combines with the OH group of water to form insoluble aluminium hydroxide; the free hydrogen from water tends to form suphuric acid by uniting with suphate from alum. However, if excess of acid (Sulphuric or any other)is present aluminium hydroxide does not form. Under these conditions, in an alum haematoxylin dye the insoluble dye lake cannot form because of lack of hydroxyl ions. Hence alkaline treatment is required to neutralize the acid to restore blue colour to the tissues.[17]

Various types of Haematoxylin stains

There are six common types of haematoxylin stains in use:[18]

1. Alum haematoxylin-Alum haematoxylin

solutions contain potassium alum or ammonium alum, but the active principle being aluminium(mordant).They includeDelafield's,Ehrlich's,Harris',Mayer's,Carazzi's, Cole's, Gill's and Iyiola and Avwioro'shaematoxylin formulations. Alum haematoxylins are used when the counterstain doesnot contain an acid.

2. Iron haematoxylin-In iron haematoxylin stains, iron salts are used both as the oxidizing agent and as mordant. The most commonly used iron salts are Ferric chloride and Ferric ammonium sulphate. Generally, iron haematoxylin stain requires differentiation. E.g - Heidenhain's haematoxylin, Anderson's Iron haematoxylin, Weigert'shaematoxylin, Verhoeff's haematoxylin.

3. Tungsten acid haematoxylin-There is only one widely used tungsten haematoxylin, although many variants on the main technique of Mallory's phosphotungstic acid haematoxylin (PTAH)has been described.

4. Molybdenum haematoxylin-Haematoxylin solutions that use molybdic acid as the mordant are rare, the method of Thomas (1941)is the most accepted method and described by McManus and Mowry. It is used to stain collagen, reticulin, argentaffin cell granules.

5. Lead haematoxylin-Lead haematoxylin stain is relatively recent in development, mostly used for demonstration of granules in the endocrine cells of alimentary tract and other regions.

6. Haematoxylin with chromium-As in Weigert – Pal technique (Weigert1885,Pal 1886).This is a hybrid technique as it employs Weigert's mordant, Pal's differentiator and usually Kultschitzky's (1889) haematoxylin. Mordating in chrome salts forms a chromium dioxide complex so that normal myelin forms a lake with haematoxylin. Only long standing myelin degeneration will show lack of staining.

7. Haematoxylin without mordant-Under appropriate conditions, free haematein is weakly anionic and will stain cationic tissue components-particularly collagen, but also elastin, erythrocytes and contractile elements in smooth muscle-yellow to orange-brown. Non-mordanted, dilute solutions of haematoxylin (haematein) also colour nuclei through binding to basic nuclear proteins.In the Clara haematoxylin procedure, non-mordanted haematoxylin reacts with arginine residues and stains keratin, keratohyalin, and basic nucleoproteins.

Uses of Haematoxylin[16,18]

· Carbohydrates: Connective tissue mucin can be stained with Mayer's haematoxylin. Haematoxylin has also been used as a nuclear stain in some techniques when demonstrating carbohydrates. These techniques include Best's carminefor glycogen, Periodic Acid Schiff for neutral mucopolysaccharides and congo red for amyloid.

· Lipids: Baker's acid haematein method is used for demonstration of phospholipids, while the alum haematoxylins are used as nuclear counterstains when oil red O is used for staining the lipids.

· Connective tissue fibers: Verhoeff's haematoxylin has been used for staining elastic fibers black. Weigert's haematoxylin and Celestine blue haemalum sequence are used for nulclear stains.

· Nervous tissue: Weigert-Pal's, Loyez and Weil's methods are used for staining myelin. The haematoxylin used in these methods is the active ingredient to stain astrocytes, myoglia.

· Intracellular substances: Heidenhain's haematoxylinis an excellent stain which stains chromosomes nucleoli, mitochondria, and cross striations of muscle fibres.

· Cytology: Papanicolaou is very popular cytological technique for epithelial cells to distinguish between malignant and non-malignant cells, here haematoxylin plays major role as a stain.

· Microfilaria and amoeba: The differential diagnosis of microfilaria based on nuclear arrangement is enhanced when their nuclei are stained by hot haematoxylin. Amoeba also stains well with alum haematoxylin.

Conclusion

Undoubtedly haematoxylin is one of the most important biological stains. It is valuable not only because it is a powerful nuclear stain and a chromatin

stain par excellence, but also because it has polychrome properties. With proper differentiation it is possible to get several shades between blue to red to show in the same preparation.

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