Available online at http://www.journalijdr.com

IJDR

International Journal of DEVELOPMENT RESEARCH

ISSN: 2230-9926

International Journal of Development Research
Vol. 06, Issue, 10, pp.9770-9773, October, 2016

Full Length Research Article

ANTIOXIDANT ENRICHED MOUNTING MEDIA – A REMEDY FOR FADING PROBLEM OF HAEMATOXYLIN AND EOSIN STAINED HISTOLOGY SLIDES'

***Yogesh Ashok Sontakke, Sonali Adole and Parkash Chand**

Department of Anatomy, Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), An Institute of National Importance Under Ministry of Health and Family welfare, Government of India, Pondicherry 605006

ARTICLE INFO

Article History:
Received 17th July, 2016
Received in revised form
11th August, 2016
Accepted 26th September, 2016
Published online 31st October, 2016

Key Words:

Staining,
Histology,
Antioxidants,
DPX.

ABSTRACT

Histology teaching and research depend on the quality of haematoxylin and eosin (H&E) stained sections. Over a period, slides fade and become uninterpretable even with good attempts for preservation. One of the principal causes of fading of H&E stained slides is the quality of the mounting media (DPX). Earlier studies have suggested the use of mounting media with added antioxidants to prevent fading in fluorescent staining. For the preservation of H&E stained sections, we utilised antioxidant (1% butylhydroxytoluene) enriched mounting media. The paraffin embedded tissue was sectioned at 5 microns to prepare slides. Each section was stained with H&E. One group of slides mounted with normal DPX while another with antioxidant enriched DPX. The group of slides mounted with antioxidant enriched mounting media showed very less fading when observed after one year on comparison with that of the slides mounted with usual mountant. This finding was consistent even on continuous exposure of slides to natural light for one year. Thus, use of antioxidant enriched mounting media can be a useful technique for preservation of histology slides.

INTRODUCTION

Medical students encounter microscopy in histology, pathology and the laboratory medicine. Maintaining and preserving good teaching histology material is complicated and cumbersome. The main constraint in laboratories is prevention of fading of stained microscopic slides as they tend to fade and become uninterpretable over a period of the time.Commonly hematoxylin and eosin (H & E) are used as the nuclear and cytoplasmic stains respectively. Fading of H&E stained slides depends on the type of hematoxylin, type of staining procedure, clearing agents, and mounting media. Storage conditions are also responsible for fading of stained sections. Stained slides exposed to air and sunlight for prolonged periods result in fading (Takizawa *et al*., 2000). Reducing exposure to the direct sunlight and air resulted in reduced the fading (Ono *et al*., 2001). Contaminated clearing agents also causes fading of the sections, but this also can be avoided by using a better quality clearing agent.

****Corresponding author: Yogesh Ashok Sontakke***
Department of Anatomy, Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), An Institute of National Importance Under Ministry of Health and Family welfare, Government of India, Pondicherry 605006

DPX is a mixture of distyrene (a polystyrene), a plasticiser (tricresyl phosphate), and xylene. It is suitable mounting media for all staining techniques which are compatible with the use of alcohol and aromatic (xylene & toluene) clearing agents. DPX is a clear, colourless and will not discolour with age. For mounting, DPX is dispensed onto a stained specimen slide in liquid form, and the evaporation of carrier solvent results in its hardening to form a solid colourless film.As DPX has a refractive index similar to glass, it will not interfere with observation of the specimen under the microscope. After mounting, even with permanent mounting media, haematoxylin tend to fade probably due to its continued oxidation (Avwior G, 2011). Most commonly the acidic pH of the mountant causes fading of the hematoxylin.The pH of the mountant should frequently be checked to keep the mountant at neutral pH (Johnson *et al*., 1982). Several commercial ready-to-use and homemade antifade mounting media are available for fluorescence and confocal microscopy (Ono *et al*., 2001). For routine H&E staining, mounting media with an antifade agent is not yet available commercially. Butylhydroxytoluene (BHT) is phenolic antioxidant with broad biological activities. Due to its anti-oxidizing property, it is also used as the preservative in lipsticks and other

cosmetics, moisturisers and canned food items (Elgazar AF, 2013). It is entirely soluble in xylene and toluene and does not alter the colour of the solvent. Hence, in the present report we are focusing on the use of the BHT enriched DPX as the mounting media for preventing fading of H & E stained slides at the different conditions and in the different environment.

MATERIALS AND METHODS

During the routine histology lab work in the Department of Anatomy, Elrich'shematoxylin and 1% eosin was prepared by the method as described earlier (Bancroft *et al*., 2013). The paraffin embedded tissue was sectioned at 5 microns using the rotary microtome (Thermo Fischer). Each of the 12 slides section was deparaffinised using xylene and rehydrated using descending grades of alcohol. Then each section was stained for 10 minutes in Erlich'shematoxylin and differentiated in 1%acid-alcohol. After blueing in the water bath for 10 minutes, all sections were stained with 1% eosin for 30 seconds and dehydrated rapidly using ascending grades of alcohol and xylene (Fischer *et al*., 2008). Afterwards, these H&E stained slides were grouped into two groups (group A and B), each containing six slides. Two types of mounting media were used, one as usual DPX without added BHT and another DPX enriched by adding one gram of BHT in 100ml of DPX.

A slides were grouped into set A1 with the three slides and set A2 with the three slides. Similarly, the group B slides were also divided into set B1 with the three slides and set B2 with the three slides. Set A1 and B1 slides were stored in the slide rack and kept on the laboratory platform to expose to the daylight. Set A2 and B2 slides were kept in the slide cabinet and stored in the dark place. All the slides were observed on the first day of preparation and then after one year. All slide observations were documented and microphotographed using Olympus BX-53 microscope and cellSens Olympus software.

RESULTS

There was no difference in staining quality between the observations at the first day of preparation of slides. After one year, all the slides of set A1 showed fading. It was difficult to recognise the nuclei and to differentiate nuclei from cytoplasm (Figure 1a and 1b). Similarly, there was the fading in all slides of the set A2 though it was slightly lesser as compared to the set A1 (Figure 1c and 1d). Slight fading was also observed in set B1, but in set B2 there was no fading (Figure 2a-d). Thus, we found that the fading of slides mounted using DPX without added BHT irrespective of storage conditions. In slides mounted using DPX enriched with 1% BHT, there was minimal to no fading regardless of the storage conditions.

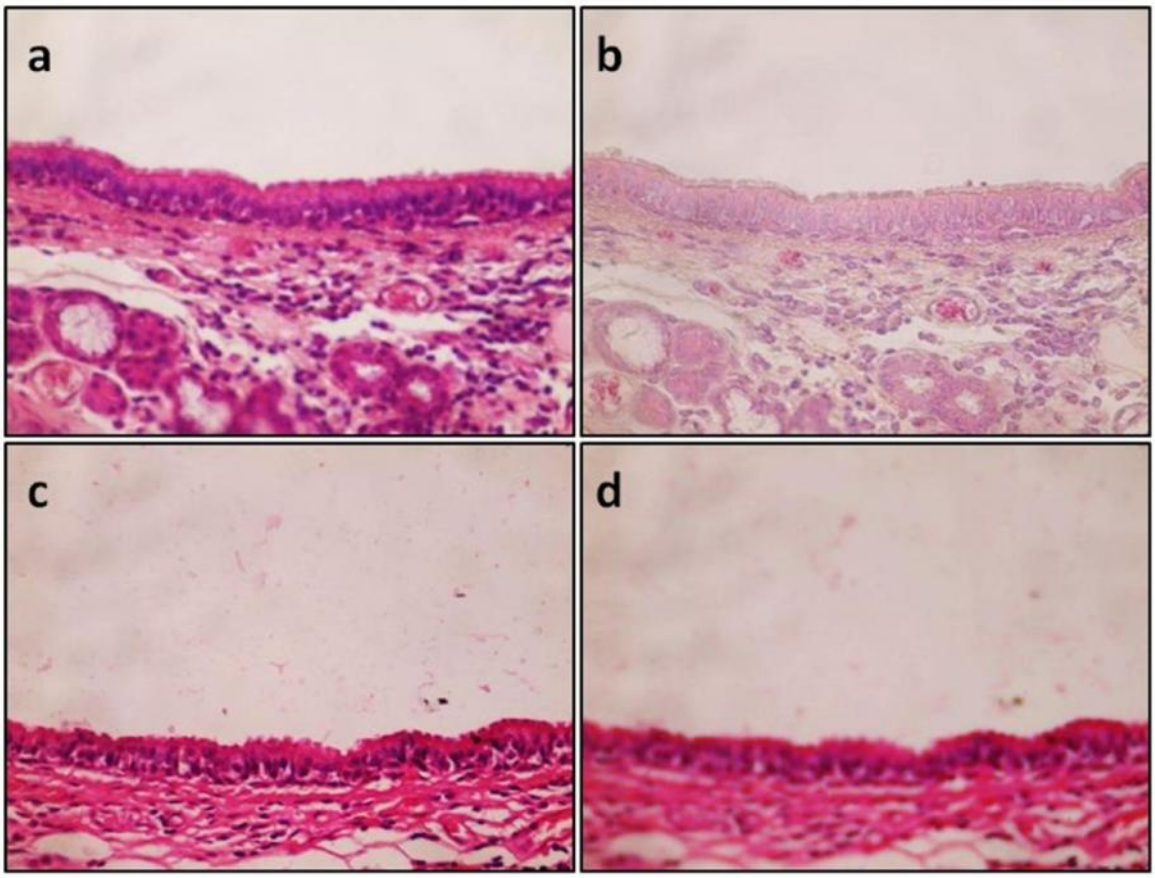

Figure 1. Photomicrograph of group A slides mounted with DPX without BHT enrichment
a: Set A1 slide on the first day of staining
b: Set A1 slide after one year
c: Set A2 slide on the first day of staining
d: Set A2 slide after one year

Out of the twelve stained slides, six slides (group A) were mounted in DPX without added BHT, and remaining six slides (group B) mounted in 1% BHT enriched DPX. Then the group

The slides stored in the dark, fade considerably less and take the longer period to fade than the slides exposed to light.

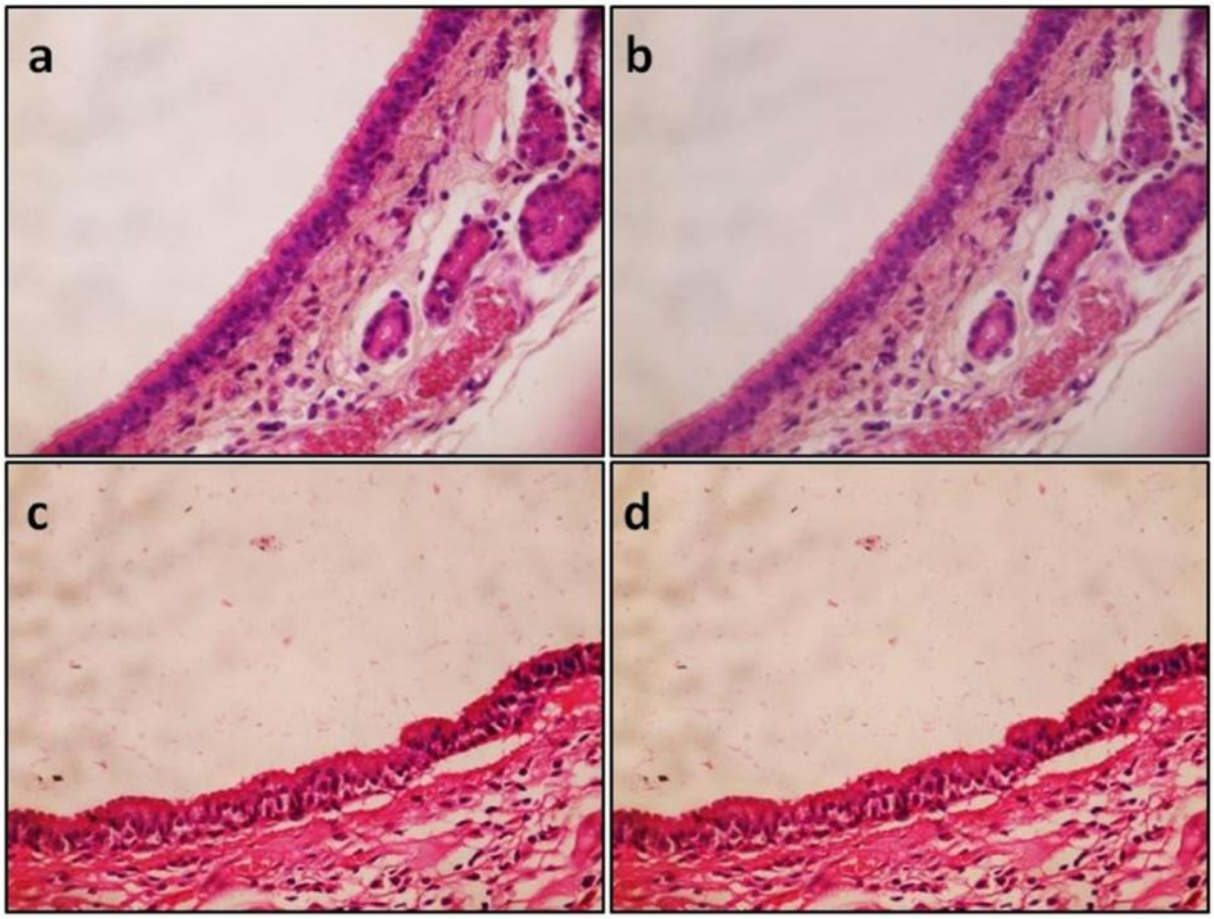

Figure 2. Photomicrograph of group B slides mounted with DPX enriched with 1% BHT
a: Set B1 slide on the first day of staining
b: Set B1 slide after one year
c: Set B2 slide on the first day of staining
d: Set B2 slide after one year

DISCUSSION

The preparation and preservation of good histology slides are needed as a good teaching material for microanatomy. The problem of fading is well known since antiquity and has been thoroughly investigated. Holde Puchtler and Amyl L. McGowan studied extensively standards of fastness properties of dyes to dye concentration, light exposure, different light sources, acids and alkalis (Heinrich Z, 2003).In past, various antifade mounting media were developed for fluorescence and confocal microscopy (Ravikumar *et al*., 2014). In the fluorescent staining method, the utility of antioxidant beta-mercapto phenol (BME) in conjunction with mountant for retarding the fading of fluorescence was proven earlier. The increased stability of Cy3, Cy5, and Alexa488 fluorophores by antioxidant such as ascorbic acid and n-propyl gallate was also shown (Aitken *et al*, 2008). They recommended the storage of the stained slides in the dark for reducing the photo bleaching of fluorescence. Commonly used commercial mounting media vary markedly in their chemical constitution. Some stains retain their colour in the acid media, provided in case of eosin, that the acidity is not greater than pH 4.0 (Johnson *et al*., 1982). Though H&E is most routinely used stain in histotechnology, earlier its fading problem was not studied extensively. Durability of H&E stain is very desirable for long-term research and collection; it seemed timely to review the information on the antifade agent to be added in mounting media to prevent or minimise the fading of H&E stained slides.

Earlier researchers reported light exposure and duration after slide preparation as the two main factors responsible for the fading of H&E stained sections. We applied the principal of utilised antioxidant for retarding the fading of fluorescenceto prevent the fading of H&E stain slides (Aitken *et al*. 2008). In the present study, we used BHT which is the phenolic antioxidant like BME. The utility of BHT as the antifading agent to counteract the effect of light exposure and storage condition wastested. It was seen that the efficacy of BHT as the antifading agent was found when employed as 1% added component for the mounting media in H&E stained slide preservation over a period of one year. The reason behind the utilisation of the antioxidant in the present study can be understood by focusing on their mechanism of action. An atom or molecule with greater oxidation potential than a second atom or molecule is the antioxidant agent. Hence, the antioxidant is preferentially oxidised instead of the second atom or molecule. Thus, the antioxidant can have a greater oxidation potential than haematein which helps to prevent the oxidation of haematein to oxyhaematein. Furthermore, the antioxidant can also function as a reducing agent that converts the oxyhaematein back to the haematein (Koch *et al*., 2000). Phenolic antioxidants have the property of free radical formation and by this means, they can interrupt the propagation of the autoxidation (Lobo *et al*., 2010). Synthetic phenolic antioxidants which include propyl gallate, butylated hydroxyanisole, and butylated hydroxytoluene (BHT) inhibits oxidation. Koch *et al*. (2000) also utilised the enzyme assay with the inhibition of the autooxidation of the haematoxylin to

red coloured hematin by superoxide dismutase (SOD). It indicated that the antioxidant property of the SOD was responsible for the inhibition of autooxidation by spectrophotometry on the addition of taurine, an agent which inhibits the antioxidant property. The researcher formulated a concept of the stable haematoxylin. It includes ripened haematoxylin and an antioxidant. This composition exhibits the sufficient stability to be utilised in an automated staining process without undue degradation before use for the staining biological samples (Kosmeder *et al*., 2008).

Conclusions

Light exposure and time lapse after preparation of histology slide were the factors responsible for the fading of H&E stained microanatomy slides. The slide storage in the slide cabinet, i.e., away from the light can reduce the fading. The mounting media (DPX) enriched with antioxidant (1% BHT) can be utilised as the antifading agent for the preservation of H&E stained histology slides.

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