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Assessment of Staining Quality of Curcumin as a Substitute for Eosin in Hematoxylin and Eosin Staining in Histopathology

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ABSTRACT

Background and objectives: Hematoxylin and Eosin staining is the globally accepted staining technique for histology and histopathology sections. Though hematoxylin is a natural dye, eosin is synthetic dye manufactured from chemicals. Eosin acts as a counterstain to hematoxylin giving sharp contrast to its blue colour. Eosin being a chemical can cause health hazards and environmental pollution. Using ecofriendly materials and going organic is the demand of this era. The present study was conducted to evaluate the staining qualities of Curcumin longa(turmeric) a natural substitute of eosin and the results of Hematoxylin and curcumin (H & C)was compared with Hematoxylin and Eosin(H&E).

Methods: The study was conducted in the Histopathology lab of a tertiary health care centre in South India after getting Institutional Ethical Committee Clearance. For each staining method 100 sections were prepared from 20 collagen tissues, 20 epithelial tissues, 20 smooth muscle tissues, 20 bony tissues and 20 adipose tissues. The staining qualities were assessed by an experienced pathologist and the results were scored as excellent, good and poor. After entering the scores in Microsoft excel, the results were statistically analyzed.

Results: H and C staining gave comparable results with H and E staining in all the five types of tissue sections studied (p value<0.05) with intense affinity on collagen and muscle fibres.

Interpretation and conclusions: Curcumin is a safer and cheaper alternative to Eosin stain in histology and histopathology sections.

Key words: Curcumin, Ecofriendly stains, Eosin substitutes, Natural dyes, Nontoxic stains, Turmeric

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INTRODUCTION

Hematoxylin and Eosin staining is the globally practiced staining technique for histology and histopathology studies. Hematoxylin and eosin (H and E) stains have been used for at least a century and are still essential for recognizing various tissue types and the morphologic changes that form the basis of contemporary cancer diagnosis. In a typical tissue, nuclei are stained blue by the basic dye hematoxylin, whereas the cytoplasm and extracellular matrix have varying degrees of pink staining with acidic eosin.

Hematoxylin is a natural dye, obtained from the Mexican tree Hematoxylin campechianum while the Eosin is a synthetic dye. Synthetic dyes are often efficient but may display hazards to human and animal health. This has resulted in the withdrawal of several dyes [1]. With the worldwide concern over the use of eco-friendly and biodegradable materials, the use of natural dyes obtained from plants has again gained interest. Moreover, as many developing countries can no longer afford the ever-increasing cost of synthetic dyes, the use of cheaper, naturally occurring dyes from plants is being viewed as an alternative to synthetic dyes. Based on this facts

Curcuma longa was investigated as a natural dye with potential histopathological application [2].

Eosin is a name of several fluorescent acidic compounds which bind to and form salts with basic or eosinophilic proteins such as arginine and lysine. It stains them dark red or pink because of the actions of bromine on fluorescein. In addition to staining the proteins in cytoplasm, it can be used to stain collagen and muscle fibres for microscopic examination. Structures that stain readily with eosin are termed eosinophilic.

Curcumin, or diferuloylmethane, is a major chemical component of turmeric (*Curcuma longa Linn*) that has been consumed as a dietary spice. Chemically, curcumin is a diarylheptanoid, belonging to the group of curcuminoids, which are natural phenols responsible for turmeric's yellow colour. It contains flavonoids, which are typically polyphenolic compounds. Phenols are acidic, due to their ability to release the hydrogen from their hydroxyl group, hence the ability of *C. longa* to stain the basic parts of the cell [3]. *C. longa* was used as a counter stain for haematoxylin with the reaction like that of eosin in the hematoxylin and eosin technique except for its yellow coloration. The present study was conducted to assess the staining quality of curcumin as an eosin substitute in hematoxylin and eosin staining.

METHODOLOGY

The study design was a comparative study conducted in the Histopathology laboratory, of a tertiary health care centre in South India. The sample size was calculated as 100 for each staining technique. The period of study was six months after Institutional ethical committee approval (IEC.NO.08/11/2017/MCT dated 28/7/2017).

Methods of preparing turmeric stain

Slender dried pieces of turmeric are first powdered, and 15 gm of this powder is mixed with 100ml of 70% alcohol. The contents are mixed well and kept for an hour for sedimentation. After centrifugation, the supernatant fluid is taken for staining.

Methods of preparing eosin hematoxylin stain

1g% Alcoholic eosin is prepared by mixing 1 gram of commercially available eosin powder with 100 ml of alcohol.

Methods of preparing hematoxylin stain

Harris's hematoxylin is prepared by dissolving 2.5 grams of Hematoxylin in 50 ml absolute alcohol. Alum solution is prepared by adding 50 mg of ammonium alum in hot water. The two solutions are heated up to boiling, 1.5 grams mercuric oxide is added, and mixture is cooled rapidly. Addition of 20 ml Glacial acetic acid improves nuclear staining of hematoxylin stain.

Staining method

For H and E staining deparaffinized sections are first treated with 2 changes of xylene followed by hydration of the tissue sections with descending grades of alcohol and water. After that, the sections are treated with Harris hematoxylin solution for 5 minutes. This is followed by differentiation in 1% acid alcohol and Bluing in running tap water. The sections are dehydrated in 95% alcohol, counterstained with eosin for 45 seconds and again dehydrated in ascending grades of alcohol. Finally, the sections are again treated with xylene and mounted with DPX. H and C staining also follows same methods except 5 minutes staining in curcumin instead of 45 seconds eosin staining in H and E method.

The study was carried on the tissue specimen received at the histopathology lab. Five types of tissue sections were selected for this study include:

Epithelium: Skin specimens for keratinized stratified squamous epithelium (10 slides) and Intestinal specimens for glandular epithelium (10 slides).

Collagen: Keloid specimens for collagen.

Muscle: Leiomyoma specimens for Muscle.

Bone: Tissue from bony lesions for Bone.

Adipose tissue: Fat rich specimens like lipoma for Adipose tissue.

For each tissue type, 20sections stained with H and E and 20 sections stained with H and C were studied. The staining qualities were studied by a pathologist who had 8 years of experience in Histopathology. The staining quality of slides was graded into following three scores.

Score 1- P (Poor): Refers for difficulty in appreciation of a tissue Structure.

Score 2 G (Good): Refers for sufficient appreciation of a tissue structure.

Table 1: Scoring of observer.

	Method	Type of tissue					
		Collegen	Epithelium	Muscle	Bone	Adipose tissue	Total sections
Observer score	H&E	20E	9E	16E	0E	0E	100
		0G	10G	4G	5G	20G	
		0P	1P	0P	15P	0P	
	H&C	0E	0E	0E	0E	0E	100
		19G	1G	15G	20G	5G	
		1P	3P	5P	0P	15P	

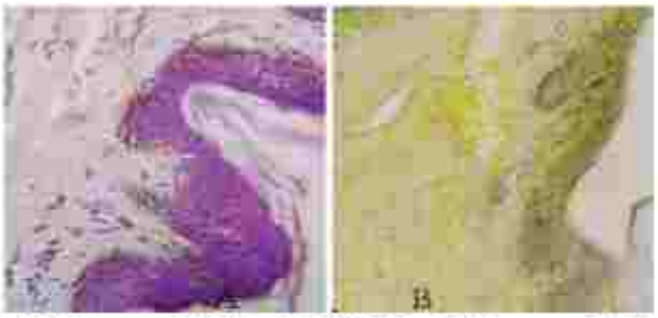

Figure 1: A. Stratified Squamous Epithelium H&E 40X. B. Stratified Squamous Epithelium H& C 40X.

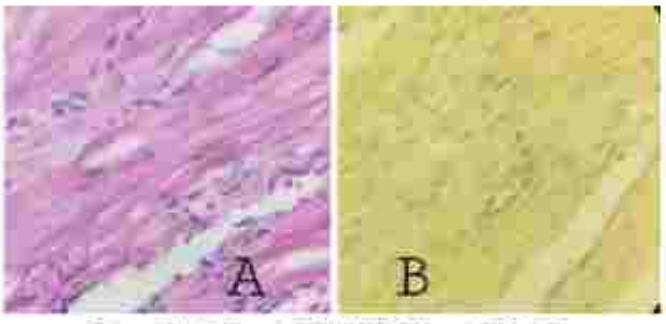

Figure 2: A. Collagen H&E 40X. B. Collagen H&C 40X.

Figure 3: A. Bone H&E 40X. B. C-Bone H&C 40X.

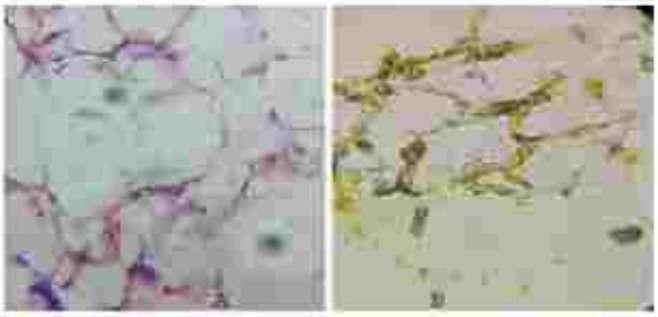

Figure 4: A: Adipose tissue H&E 40X. B: Adipose tissue H&C 40X.

Score 3 E (Excellent): Refers for fine appreciation of a tissue structure.

The scores of the pathologist were entered in Microsoft excel and the scores were summed up for each five parameters and statistical significance was studied.

RESULTS

The present study evaluated the comparison between Hematoxylin and Curcumin staining and conventional Hematoxylin and Eosin staining. Scoring done by observer is depicted in Table 1 and staining characters all the studied five tissue elements are depicted in Figures 1-4.

95% of slides stained with H and C staining were good and 100% slides are excellent with H and E staining for collagen (x^2=80p<0.001). For epithelium H and E staining, 45% of slides were graded as excellent and 52.5% as good and 2.5% as poor. In epithelium H and C staining 87.5% slides were graded as good and 12.5% as poor (x2=24.167 p<0.001). For muscle tissue H and E staining gave excellent score in 75% of slides, good score in 25% of slides. For H and C staining 72.5% slides were good and remaining were graded poor in muscle tissue (x2=50.256p<0.001). Most of the slides stained with H and E and H and C staining for bone tissue were graded as poor, only 20% slides with H and E staining showed good quality(x2=10.141p=0.001). In adipose tiisue100% slides with H and E staining showed excellent staining, only 30% slides in H and C showed good score(x2=43.077p<0.001).

When used as a counter stain, *C. longa* stain showed a similar reaction to that of eosin in the Hematoxylin and Eosin technique except for its yellow coloration. Although statistically Eosin have proved to be better over turmeric, turmeric has shown good and comparable staining to eosin. Turmeric dye stains collagen and muscle fibres with deep yellowish orange color.

DISCUSSION

Hematoxylin and Eosin stain (H and E stain or HE stain) is the most popular histology stain all over the world. Hematoxylin is a naturally occurring chemical and the cytoplasmic stain eosin is a manufactured from coal tar. Synthetic dyes became popular because of longer shelf life than many natural dyes. However, synthetic dyes may cause harmful effects on the environment and human beings. Eosin is an irritant for skin, eyes, and mucous membranes. Acute exposure to this synthetic dye can cause chelitis, stomaitis, dermatitis etc. There have been indefinite reports that this substance is an animal carcinogen [4]. Environmental pollution with laboratory chemicals and dyes are also reported from places where safe disposal of lab wastes is not attended well.

Replacement of chemical dyes with natural ones is truly relevant in this era of global warming and environmental pollution. Organic methods and materials gain so much global attention. The role of eosin in H and E staining is to act as a counter stain giving a sharp contrast color in cytoplasm with the hematoxylin's blue nuclear color. Carmine, carcade, henna, wing fruit, china rose are few natural dyes [5]. Those natural dyes which have a contrast colour with hematoxylin can be used as a substitute for eosin. Various types of natural substitutes have been an interesting topic for many other researchers before [6].

In a similar study conducted by Sachin, et al. they observed that *C.longa* deeply stains collagen and muscle fibres with deep yellowish orange color [2]. "*Curcuma longa* extract as a histological dye for collagen fibres and red blood cells", a study conducted by Avwioro et al. concluded that the turmeric dye stains collagen and muscle fibres with deep yellowish orange colour suggesting its stronger affinity to these structures [7]. Staining qualities of Zingiber roscoe (ginger) and Curcuma longa studied in an Oral Pathology centre in 2018 concluded that ginger has better staining qualities than turmeric and both can be used as substitutes for eosin [8]. Lawsonia inermis (henna) extract used as eosin substitute by Lizbeth, et al. observed both henna and eosin gave comparable staining reactions [9].

The basic principle in the theory of attaining is the ionic bond between the tissue components and the dye, which is associated with electrostatic attraction between the dissimilar ions. Due to strong affinity of *C. longa* for the cytoplasm, it can be deduced that the *C. longa* extract dye is acidic in nature. The main components of turmeric are flavonoids and tannins, which are responsible for its staining properties. Flavonoids are typically polyphenolic which show acidic nature by their

ability to release hydrogen from their hydroxyl group. This gives ability of *C. longa* to stain the basic parts of the cell. The ability of turmeric dye to stain specific tissue structures is determined by the acidity of the stain.

Traditional extraction techniques from natural sources, known as maceration and soxhlet methods were also studied and compared by few researchers. Avwioro et al., Baseey et al. conducted the study on soxhlet extracts of turmeric [7,10], while Kumar et al in 2014 did the study on tissue sections using with maceration extracts of turmeric [2]. Although turmeric stain is a natural one, it tends to achromatize when stored over a long period of time. The shelf life of turmeric was found to be inferior to eosin and Bassey et al. Avwioro et al. Kumar, et al. stated that addition of a mordant to Curcuma longa can increase the shelf life of this stain [2,7,10].

CONCLUSION

To go organic and ecofriendly is the necessity of modern era. Implementing ecofriendly substitutes in laboratories which are handling harmful chemical substances contributes so much to this noble concept of environment protection. The tissue sections, in the absence of stains appear colorless and make morphological features indiscernible. In our study we compared the staining qualities of eosin with its natural substitute *C. longa*. When *C. longa* was used as a counter stain for Haematoxylin; collagen, muscle fibers and epithelium stained better than adipose and bone tissue. The nuclei took the blue colouration which enabled a clear contrast to be made between the different structures of the cells. Turmeric showed a comparable staining quality to that of eosin. The strong affinity of turmeric to collagen and muscle fibres needs to be studied further. With further modifications and standardization, natural dye Curcumin can be used as a histological stain in place of synthetic and toxic eosin dye. Curcumin is also cheap and readily available.

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