Haematoxylin and eosin staining technique using liquid dish washing soap as a dewaxing agent replacing xylene -A biocompatible technique to demonstrate histoarchitecture of tissue in teaching laboratories.

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Abstract

Background: Histology slides are used in teaching to help students learn about microstructure of human and animal biological tissues.Interpretation of histological sections is greatly aided by the use of different stains. Haematoxylin and Eosin (H &E)Staining being the most popularly used staining technique utilizes xylene as an efficient dewaxing agent during routine procedural steps.Xylene although contribute to well stained slides it is known to be atoxic reagent posing serious health risk problems.This article attempts to discuss the utilization of liquid dishwashing soap as an alternative to xylene in conventional H &E Staining technique.

Aim: To produce histological slides of academic value by substituting xylene by liquid dishwashing soap as a dewaxing agent and thereby observe the advantages of xylene free sections over conventional H &E sections.

Methods: Sample of tissues from full term dead foetuswere collected from department of Anatomy.Fifty paraffin embedded tissue blocks were prepared.Two sections of five microns each were cut.A total of 100 unstained slides were prepared.Fifty slides were subjected to conventional H &E Staining technique(Group A) and remaining fifty slides were subjected to xylene free technique(Group B) and slides were scored based on their staining pattern.

Results: Adequate nuclear staining was observed in 88% of group A sections when compared with 74% of group B sections.(P>0.05; Z=1.78).Adequate cytoplasmic staining was observed in 62% of group A sections when compared with 68% of group B sections(P>0.05,Z=0.63).Clarity of staining was present in 68% of group A sections and 72% in group B sections(P>0.05;Z=0.44).Uniform staining was present in 64% of group A sections and 54% in group B sections(P>0.05;Z=1.02).Crisp staining was observed in 68% of group A sections and 60% in group B sections(P>0.05;Z=0.83).The staining was found to be acceptable for histological studies in 76% of group A sections when compared with 72% of group B sections(P>0.05;Z=0.46).

Conclusion: Liquid dish washing detergent can be used as an alternative to xylene in routine H &E Staining procedure to produce slides that can be used routinely for practical demonstration of slides of academic value and create a safe working environment.

Key words: Xylene, Liquid dishwashing detergent, haematoxylin, eosin,slides.

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Introduction

Histology is an essential tool of medicine**[1]**.The perspective of histology can illuminate many other aspects of medicine,enriching and strengthening understanding in other areas**[**2].

The histological architecture of the tissues can be revealed by various techniques utilizing the methods of cytology, histochemistry,electron microscopy and tissue culture.However the general features can be studied microscopically by processing the tissue and subjecting the tissue sections to various methods of stainingto allow distinctions to be made between various tissue components.

Histology slides are often used in teaching to help students learn about themicrostructure of human and animal biological tissues**[1]** .The most prevalent staining procedure for the preparation of histology slides is H &E Staining procedure which has remained unchanged for many years**[3]**.The conventional method utilizes xylene during routine procedural steps.Recent studies have found xylene to be toxic,though laboratories use xylene routinely**[4]**.Literature search revealed that xylene poses serious health risk problems such as fatal blood dyscrasias,acute neurotoxity,cardiac and kidney injuries[5]. The exposure to xylene is maximum during dewaxing of tissue sections[5].However while staining the histological slides, the excellent dewaxing property of xylene contribute to well stained slides.

The innovative concept of using "liquid dish-washing detergent" to dewax the tissue sections by eliminating both xylene as well as alchohol(ethanol/methanol) from the staining task was experimented by Falkeholm et.al in an attempt to address to problems common to all laboratories such as cost containment,turn around time and safe working environment**[5]**.Various research works published by Buesa et al. and Ankle and Joshi have also used liquid dish washing detergent instead of xylene and alchohol(ethanol/methanol) and concluded that staining the tissues with xylene free technique was at par with conventional staining procedure to stain tissue sections**[6].**

This experimental study focuses on observing and evaluating the distilled dish washing detergent as a potential alternative to xylene and there by rejuvenating the technique that eliminates xylene and alchohol in routine H &E staining procedure.

Aims and Objectives:

a) To produce histological slides using dilutedliquid dish washing detergent.

b) To observe the advantages of xylene free sections over conventional H& E sections.

c) To check the feasibility of xylene free technique from the view points of histological clarity,staining properties, affordability in its utilization for academic puposes.

Materials and Methods:

The study was carried out in the department of anatomy.Sample of tissues that included liver,cardiac muscle, glands, thymus,spleen,cartilage,pancreas, trachea,lung,intestine,kidney and ureter were collected from full term dead foetus.The tissues were trimmed, fixed in 10% formalin and were subjected to the routine tissue processing procedures. The process was manually conducted(Table 1).

50 paraffin embedded tissue blocks were prepared.Two sections of five microns each were cut from 50 embedded tissue blocks.Thus a total of 100 unstained slides

were prepared. 50 sections were stained with conventional H& E staining method(group A; Table2) and 50 sections were stained with xylene free method(group B; Table3).

Each section was scored by a single anatomist who was blinded.Slides were scored based on scoring system used by Ankle for parameters that included clarity of nuclear, cytoplasmic staining,crispness (clear well defined image) and uniformity(adequate=score 1, Inadequate=score 0)**[5].**On totallingthe scores, slides that scored≤2 was considered unacceptable for histological studies and slides scoring 3-5 was considered acceptable for histological studies. Z testwas used for the statistical analysis of the data obtained(Table 4).

Table 1: Histology Process steps.

Gross Examination

↓

Tissuesretrieved from full term deadfoetus

↓

10%Formalin fixation→Tissue Processing

Paraffinembedding

Microtomy

Slide preparation

Staining (conventional H&E) Xylene free technique

Mounting(DPX)

Analyzed by Anatomist Table 2:Conventional H&E Staining Procedure

Deparaffinisation and rehydration	Xylene I	5min
	Xylene II	5min
	90% alcohol	5min
	70% alcohol	5min
	Water wash	10 min
Nuclear Staining	Harri'shaematoxylin	8min
	Water wash	2min
Differentiation	1% Acid alchohol	1 dip
	Water wash	10 min
Bluing	1% lithium carbonate	1 min
	Water wash	10 min
Cytoplasmic Staining	1% Eosin	1 min
Dehydration	70% alchohol	30 sec
	90% alcohol	30

		sec
	Absolute alcohol	30sec
	Xylene I	5 min
	Xylene II	5 min

Approximate time required- 70-75min

Table 3:Xylene free H&E Staining procedure

Deparaffinisation	2% diluted liquid dishwashing detergent-I	At 90^0 C	1 min
	2% diluted liquid dishwashing detergent-II	At 90^0 C	1 min
	Distilled water-I	At 90^0 C	30sec
	Distilled water-II	At 90^0 C	30sec
	Wash slides in distilled water	At 45^0 C	30sec
	Wash slides in distilled water	At room temperature	30sec
Nuclear Staining	Harri'sHaematoxylin	At room temperature	7 min
	Tepid water wash		4 min
Differentiation	1% acid alcohol Water wash		1min 30sec
Bluing	Water wash	At room temperature	5 min
Cytoplasmic staining	Eosin 1%	At room temperature	1 min
	Wash slides in distilled water		1 min
Dehydration	Over drying the sections	At 60^0C	10 min

Approximate time required-30-36 min
(2% diluted dish washing detergent -30ml of liquid dishwashing detergent added to 1500 ml of distilled water.

Table 4: Scoring Pattern and statistical data, Total number of sections = 100,Group A=50,Group B=50

		Group A	Group B	Z	P	Significance
Nuclear Staining	Adequate	44	37	1.78	>0.05	NS
	Inadequate	06	13			
Cytoplasmic Staining	Adequate	31	34	0.63	>0.05	NS
	Inadequate	19	16			
Clarityof staining	Adequate	34	36	0.44	>0.05	NS
	Inadequate	16	14			
Uniformity of staining	Present	32	27	1.02	>0.05	NS
	Absent	18	23			
Crispness of Staining	Present	34	30	0.83	>0.05	NS
	Absent	16	20			
Acceptablity for histological studies	Acceptable	38	36	0.46	>0.05	NS
	Unacceptable	12	14			

Results

- Adequate nuclear staining was observed in 88% of group A sections when compared with 74% of group B sections (P>0.05; Z=1.78).
- Adequate cytoplasmic staining was observed in 62% of group A sections when compared with 68% of group B sections(P>0.05,Z=0.63).
- Clarity of staining was present in 68% of group A sections and 72% in group B sections(P>0.05;Z=0.44).
- Uniform staining was present in 64% of group A sections and 54% in group B sections(P>0.05;Z=1.02).
- Crisp staining was observed in 68% of group A sections and 60% in group B sections(P>0.05;Z=0.83).
- The staining was found to be acceptable for histological studies in 76% of group A sections when compared with 72% of group B sections (P>0.05;Z=0.46).

Discussion

Exposure to xylene in a laboratory occurs during tissue processing, deparaffinization of tissue sections, staining,cover slipping,cleaning tissue processors and recycling**[5].** Xylene is an aromatic hydrocarbon widely used in industry and medical technology as a solvent.It is a colorless, sweet smelling liquid or gas existing in three isomeric forms:ortho-, met- and para-xylene**.[7]**Xylene became the clearing agent of choice in histo labs when chloroform was declared a carcinogen and a safer substitute was needed. But when xylene was also identified as a health hazard, replacing it with safer chemicals became the major objective of researchers and manufacturers alike. However, all focused on its use as aclearing agent even when the exposure is greater during dewaxing and staining**[8].**

Exposure to xylene can occur via inhalation, ingestion,eyes and skin**[6].** Toxic effects of xylene include heart and kidney injuries,some fatal blood dyscrasias and other less dangerous problems such as skin erythema,drying and scaling, secondary infections associated with its use.Other documented effects of xylene include bilateral auditory neuropathy(retrocochlear hearing loss) and possibly epithelial and stromal keratopathy,either as splash contamination or through its fumes**[8]**. It has also been recognized as a reproductive toxin as a reproductive study of workers exposed to workplace solvents such as xylene

concluded that it was prudent to minimize the women's exposure to organic solvents during pregnancy because xylene readily passes through the placental barrier and is even present in maternal milk**[9].** The majority of population who are routinely exposed to xylene are the histotechnicians.It is therefore worthwhile to substitute costly and hazardous compound xylene with a less toxic compound without impairing the morphology and staining characteristics of the sections**[4].**

The xylene free technique utilizes liquid dish washing detergent as a substitute to xylene as it performs the same fuction (deparaffinization)as that of xylene and thus lessen the hazardous effects of xylene.Liquid dish washing detergent is usually a high foaming mixture of surfactants primarily usedfor handwashing of glasses,plates,cutlery and cooking utensils, easily available and cheap**[10].**The detergents are divided into two categories:automatic dish washing detergents and hand dishwashing detergents.Automatic dishwashing detergents used in automatic dish washers are known to produce skin irritations and are poisonous if swallowed.Hand dishwashing detergents routinely used by householders are more mild to the skin and if swallowed, it may cause irritation to the mouth,throat and nausea but not death**[11].** In this technique since liquid dishwashing detergent is diluted and used in meagre amount (2%) it has less chances of being toxic when compared to the harmful effects of xylene, to the histotechnicians and students in researching laboratories.

In the present study out of the 100 sections studied ,50 sections were subjected to routine H&E staining procedure(Group A) and the rest of the 50 sections (Group B)were subjected to xylene free technique.

Since the paraffin sections were invariably attachedto the glass slides and the presence of paraffin were likely to interfere with the staining technique, these sections were deparaffinized.In the current study 2% liquiddish washing detergent (at 90^0C) was used for deparaffinisation of tissue sections.Detergents are primarily surfactants. Surfactants lower the surface tension of water,essentially making it wetter so that it is less likely to stick to itself and more likely to interact with oil and grease.Detergents have hydrophobic or water hating molecular chains and hydrophilic or water loving components.The hydrophobic hydrocarbons are repelled by water, but are attracted to oil, grease,etc.The hydrophilic end of the same molecule means one end of the molecule will be attracted to water,while the other side is binding to oil.Warm or hot water melts fats and oils so that it is easier for the detergent to dissolve them and pull it away into the rinse water**[12].**Probably the same mechanism of detergent action works while dewaxing the tissue sections with 2% liquid dish washing detergent (at 90^0C) wherein the hydrophobic end of detergent binds to the paraffin and aid in dissolving it and pull it away into the rinse water.

Harris haematoxylin was used for nuclear staining.Harris stain can be used progressively,but is usually used regressively and most widely used in Indian laboratories**[6].**However incomplete differentiation following regressive staining can result in inadequate cytoplasmic staining due to binding of aluminiumhematin in the cytoplasm**[5].**Hence the slides were differentiated in 1%acid alchohol. Since the tap water was enough alkaline (pH=7.1)Bluing of sections was achieved by

thorough washing of sections in running tap wateronly.Eosin Y was used to counter stain the slides.

In our study,adequate nuclear staining was observed in 88% of group A sections when compared with 74% of group B sections (P>0.05; Z=1.78).Thirteen slides out of the fifty belonging to group B showed a decline in the nuclear clarity.This inaccuracy could be due to incomplete deparaffinization, inadequate staining time, contaminants in rinsing solution,insufficient pre rinsing with water prior to staining with haematoxylin.These errors were rectified and adequate nuclear staining was obtained(Fig:a).

Adequate cytoplasmic staining was accounted in 68% of Group B sections as compared to 64% in Group A sections and statistically there was no significant difference in the two staining methods(Z=0.63,P>0.05)(Fig:b,c).72% of group B sections showed sufficient clarity as compared to 68% of group A sections however therewas a significant decline in the uniformity of Group B sections, 54% in group B (Fig:f,g) as compared to 64% of group A sections(Fig:d,e).A similar result was obtained by Ankle et al where in the uniformity of the stained section was downgraded using xylene free technique.Statistical analysis revealed no significant difference in the clarity and uniformity in both the staining methods [uniformity(Z=1.02,P>0.05), Clarity(Z=0.44,P>0.05)]

The compromise in the uniformity could be suggestive of fold or tear in sections, moisture on cover slip or improper removal of wax**.[5,6].**68% of Group A sections showed crisp staining as compared to 60% of Group B sections with no significant difference statistically(Z=0.83,P>0.05). A similar result was obtained by Ramulu et al but in contrary to the result obtained by Ankle et al where there was a significant upgradation in the crispiness of xylene free sections.

Staining of conventional sections was preceded by rehydration and followed by dehydration before mounting.While the xylene free sections were stained immediately after deparaffinisation.Drying the xylene free sections before mounting was sufficient**[5].** 30-36min was the total time consumed in staining the slides using xylene free H &E method (Table3) when compared to70-75min time taken to stain the slides with conventional H &E method (Table2)**[6].**

On totalling the scores, 72% of xylene free sections was found to be acceptable for histological study as compared to 76% of routine H&E sections with statistically no significant difference.Thusconcluding that the xylene free technique can be used as an alternative to conventional H&E staining technique for histological studies.

Conclusion

Present study observed that xylene free staining technique using dish washing detergentis at par with the routine H&E staining procedure that produced slides with sufficient nuclear,cytoplasmic staining,clarity and crispiness.Although this technique produces slides of diagnostic value as suggested by various research workers, it can be used routinely for preparation and practical demonstration of slides for academic purposes due to its added advantages of being less hazardous, non-expensive,less time consuming and easily disposable. This technique does not totally avoid xylene as it is used during tissue processing and mounting procedures but

itattempts to minimize the utilization of xylene while staining of slides (deparaffinization) and thereby preventing the exposure of xylene to histotechs in the histology teaching laboratories.Further the scope of the study would be to device a technique so as to eliminate xylene during tissue processing and mounting procedures and also evaluate the xylene free H&E slides for its stability and longevity.

Students of first level courses in anatomy may not be required to demonstrate practical skills in histology techniques but it is useful to have general awareness of steps involved in preparing histology slides**[8].** Not only does such appreciation contribute to a general insight into laboratory techniques used in medical research but also know the merits and demerits of the constituents used during routine staining procedures and the knowledge of which can aid researchers in histology laboratories.

It is the responsibility of those authorities concerned in histology laboratories to implement such newer techniques and thereby promote the elimination of such hazardous elements in the routine preparation of slides and thus provide a safe working environment.

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