www.jmscr.igmpublication.org
Impact Factor 5.84
Index Copernicus Value: 83.27
ISSN (e)-2347-176x ISSN (p) 2455-0450
crossref DOI: https://dx.doi.org/10.18535/jmscr/v5i8.73

IGM Publication
Journal Of Medical Science And Clinical Research
An Official Publication Of IGM Publication

A Nontoxic, Environment Friendly Method of Deparaffinisation in Hematoxylinand Eosin Staining

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Abstract
Background: *In histopathology labs for Haematoxylin and Eosin staining, xylene is used as the deparaffinising agent. It is costly, hazardous and the working environment will be polluted and there is also the problem of proper disposal. So, it is very useful if we can use an eco-friendly and cheap substitute for xylene in deparaffinisation.*
Objective: *To compare the efficacy of xylene-free deparaffinised sections with the conventional H and E sections in producing adequate H and E staining with simplicity, cost-effectiveness and less turn-around time .*
Materials and Method: *The study was carried out in the Dept. of Pathology and Dept. of MLT in a tertiary level teaching centre in the government sector at Thiruvananthapuram.,for a period of 6 months . Forty paraffin blocks were selected. One section was stained with conventional H and E staining method and the other with xylene -free H and E staining method. Slides were scored for parameters; nuclear staining, cytoplasmic staining, uniformity, clarity and crispness.*
Results & Conclusion: *The xylene-free H and E staining procedure carried out using a simple diluted dish washing soap solution gave excellent positive results and is at par with the conventional H and E staining procedure. It produces a quality staining with sufficient clarity and a crisp nuclear and cytoplasmic staining. It also has added advantages of being nontoxic, economical, nonflammable, nonhazardous, no problem of disposal, reduces staining time and is easy to handle.*
Keywords: *H and E (Haematoxylin and Eosin) staining, xylene.*

INTRODUCTION

The predominant method for producing tissue sections in pathology is by cutting paraffin wax embedded tissue on a microtome and staining it with Hand Ewhich is the most frequently used staining method in anatomical pathology world wide[1]. It is a universal stain used to discriminate between a broad range of cytoplasmic, nuclear and extracellular matrix features. This staining procedure has remained unchanged for over 150 years [2]

Apart from haematoxylin and eosin , the components in the H and E staining procedure are the xylene and graded alcohols. These chemicals are used to carry out the intermediate steps of rehydration and dehydration of tissue sections during the staining. The disadvantage that continue to persist in this age-old procedure are the cost containment, toxicity, problem of disposal of the hazardous chemicals; xylene and methanol, and a polluted working environment.

Exposure to xylene in a laboratory occurs during tissue processing, deparaffinisation of tissue sections, mounting, cleaning tissue processors and recycling[3].. Toxic effects of xyleneinclude acute neurotoxicity, cardiac and renal effects, fatal blood dyscrasias, skin erythema, drying, scaling, and secondary infection[3,4,5]. Exposure to methanol occurs during tissue processing and dewaxing the sections before staining.

Xylene forms an inseparable part of a pathology laboratory. The historical use of xylene in the histology laboratory is an example of a failed substitution. Most of the substitutes available for xylene are less effective more expensive and not that much less hazardous than xylene[10] .Therefore, any technique that minimizes the use of xylene by using non-biohazardous substitutes, reduces staining time, and does not compromise the staining quality will be very valuable for diagnostic reasons as well as for maintaining a healthy laboratory environment, thereby minimizing the risk to the laboratory personnel. In the quest to eliminate the use of xylene from the laboratory, numerous substitute chemicals like limonene reagents, aliphatic hydrocarbons, aromatic hydrocarbons, vegetable oils, olive oil and mineral oil substitutes have been utilized. However, these chemicals were used to substitute xylene as a clearing agent during routine processing, while the exposure and handling of xylene is maximum during dewaxing of the tissue sections.

Thus, in an effort to improve the working conditions in a histopathology laboratory, we investigated the less toxic, cheap, easily available diluted liquid dish wash solution as a deparaffinising agent for H & E staining method.

The diluted liquid dish wash solution is an extremely cheap, non toxic substitute for xylene. It is readily available in any stationary stores. The liquid dish wash solution is composed of sodium laurethsulphate, sodium dodecyl benzene sulphonate, cocamidopropylbetaine and non ionic surfactants. These components are anionic surfactants commonly used in detergent soaps and shampoos[7]. These chemicals form a part of the products which are used daily. Their concentration in these products is already well monitored by the manufacturing companies. Moreover, we are diluting only 25 ml of the liquid dish wash solution in 1500ml of distilled water. Thus , there are very rare chances of this product being toxic to the laboratory personnel.

MATERIALS AND METHODS

The objective of this study was to evaluate the efficacy of xylene-free deparaffinisation in haematoxylin and eosin staining procedure as compared to the conventional haematoxylin and eosin staining. This was a cross sectional study conducted in a tertiary level teaching centre in the government sector at Thiruvananthapuram for a period of six months from March, 2015 to August, 2015 after the ethics committee clearance.. 40 blocks of biopsy and autopsy specimens received in the Histopathology divisionwere taken . Two paraffin sections of 5 microns each cut from each of the 40 paraffin blocks.

One was deparaffinised with xylene as in conventional H&E staining method and the other section wasdeparaffinised using diluted dish wash solution which contain sodium laurethsulphate, sodium dodecyl benzene sulphonate, cocamidopropyl betaine etc as surfactants.Tissue sections with well preserved morphology was selected for the study. Tissue sections with necrotic material, under fixed and showing artifacts were excluded. Haematoxylin& Eosin staining using Harris's haematoxylin regressively and alcoholic eosin was done.

STAINING PROCEDURE– MODIFIED METHOD[6]

1. Deparaffinisation

Diluted dish wash solution 1.7% 1st 75^0C - 1min
Diluted dish wash solution 1.7% 2nd 75^0 C - 1min
Distilled water 1st 75^0C - 30 sec
Distilled water 2nd 75^0C - 30 sec
Wash slides in distilled water 45^0C - 30 sec
Wash slides in distilled water R.T - 30 sec

2. Nuclear staining

Harris' haematoxylin 05 min
Wash with water

3. Differentiation

1% Acid alcohol 03 dips

4. Blueing

Running tap water 15 min

5. Cytoplasmic staining

1% Alcoholic eosin 01 min

6. Dehydration

95% alcohol 02 min
Absolute alcohol 03 min
Absolute alcohol 05 min

7. Air dry

8. Treatment with xylene

Xylene 1st 05 min
Xylene 2nd 05 min

9. Mount in DPX

Criteria of assessment parameters
Slides will be scored for the parameters
Nuclear staining & cytoplasmic staining
(adequate- score 1, inadequate- score 0)
Uniformity, clarity, crispness
(present- score 1, absent- score 0)
(score 2→ inadequate for diagnosis
Score 3-5→ adequate for diagnosis)

RESULTS

Five parameters of the staining characters are evaluated by modified method and conventional method and was compared by McNemar test and comparisons of overall staining score between conventional and modified method was analysed by paired t test. A P value of 0.05 was taken as the level of significance. The five parameters are nuclear staining, cytoplasmic staining, uniformity, clarity & crispness.

a) Nuclear staining

The observed P value is 0.375 and there is no significant difference between modified method and conventional method in the aspect of nuclear staining. (Table1)

b) Cytoplasmic staining

The observed P value is 0.508 and there is no significant difference between modified method and conventional method in the aspect of cytoplasmic staining.(Table 2)

c) uniformity

The observed P value is 0.687 and there is no significant difference between modified method and conventional method in the aspect of uniformity in staining. (Table 3)

d) Clarity

The observed P value is 1.000 and there is no significant difference between modified method and conventional method in the aspect of clarity in staining. (Table 4)

e) Crispness

The observed P value is 0.219 and there is no significant difference between modified method and conventional method in the aspect of crispness in staining. (Table 5)

The overall staining character of the five parameters (Table 6)

Total score for each slide

Based on the total scores obtained for each slides the observed P value is 0.041 and there is significant difference between modified method and conventional method in the aspect of total scores for each slides. (Table 7)

Table 1 Nuclear staining

NUCLEAR STAINING		CONVENTIONAL		Total
		Inadequate	Adequate	
MODIFIED	Inadequate	0	1	1
	Adequate	4	35	39
	Total	4	36	40

McNemar test P=0.375

Table 2 Cytoplasmic staining

CYTOPLASMIC STAINING		CONVENTIONAL		Total
		Inadequate	Adequate	
MODIFIED	Inadequate	1	3	4
	Adequate	6	30	36
	Total	7	33	40

McNemar test P=0.508

Table 3 uniformity

UNIFORMITY		CONVENTIONAL		Total
		Inadequate	Adequate	
MODIFIED	Inadequate	3	2	5
	Adequate	4	31	35
	Total	7	33	40

McNemar test P=0.687

Table 4 Clarity

CLARITY		CONVENTIONAL		Total
		Inadequate	Adequate	
MODIFIED	Inadequate	0	5	5
	Adequate	6	29	35
	Total	6	34	40

McNemar test P=1.000

Table 5 Crispness

CRISPNESS		CONVENTIONAL		Total
		Inadequate	Adequate	
MODIFIED	Inadequate	1	1	2
	Adequate	5	33	38
	Total	6	34	40

McNemar test P=0.219

Table 6 The overall staining character of the five parameteres

Distribution of adequate staining character	MODIFIED		CONVENTIONAL		McNemar test
	N	%	N	%	p
NUCLEAR STAINING	39	97.5	36	90.0	0.375
CYTOPLASMIC STAINING	36	90.0	33	82.5	0.508
UNIFORMITY	35	87.5	33	82.5	0.687
CLARITY	35	87.5	34	85.0	1.000
CRISPNESS	38	95.0	34	85.0	0.219

Table 7 Total scores for each slides

Total score		CONVENTIONAL			Total
		3	4	5	
MODIFIED	3	1	1	0	2
	4	1	10	2	13
	5	2	11	12	25
Total		4	22	14	40

McNemar test P=0.041

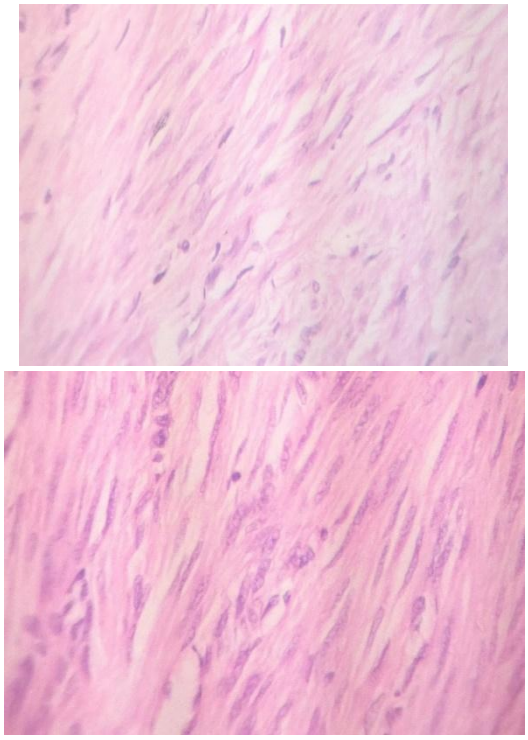

Fig 8 H and E sections conventional method and modified method 10x

DISCUSSION

An amazing variety of histopathological conditions can be studied and diagnosed just on the basis of the H&E staining method alone. The method is cheap, quick to perform and amenable to automation.Xylene traditionally has been employed as a clearing, dewaxing, and mounting agent for histology. It is well-documented as an environmental hazard and highly toxic to humans.In addition, xylene has many shortcomings, such as being highly flammable and volatile, much lower boiling point (137-143°C), a flash point (25°C) and an ignition point (25°C).

Any technique that minimizes the use of xylene by using non-biohazardous substitutes, reduces staining time with unequivocal cell morphology will be indispensable for diagnostic reasons as

well as for maintaining a healthy laboratory environment, thereby minimizing the risk to the laboratory personnel. Thus, in this study presents the results of a new deparaffinizing and H and E staining method that involves the use of easily available, nontoxic and eco-friendly liquid diluted DWS by completely eliminating expensive and hazardous xylene fromdeparaffinizing prior to staining, so as to device an optimal staining technique which is easily available, less toxic, time-saving, and cost-effective.

The nucleus reflects the reproductive potential of the cell. The size and staining intensity (chromasia) of the nucleus is a critical factor in the evaluation of the cell. Correct hematoxylin staining shows crisp staining of the nuclear chromatin, demonstrated well delineated nuclear membranes and sharply stained condensed chromatin against an unstained nucleoplasm.

The results showed that of the 40 sections studied, 97.5% of the modified method slides (xylene free deparaffinisation) showed adequate nuclear staining as compared with 90% of the conventional H&E staining method. The difference was not statistically significant (P-0.375) suggesting that there was no difference in the two staining methods in producing adequate nuclear staining. The nuclear staining in both the methods were done using Harris haematoxylin. In the study of Madhuri R ankle &Priya S joshi, they found that 98.33% of the xylene free deparaffinised slides showed adequate nuclear staining compared with 96.66% of the conventional H & E staining method and the difference was also not statistically significant.

90% of xylene free deparaffinised sections had adequately stained cytoplasm as compared with the 82.5% of the conventional H & E stained sections (P value 0.508). This is also statistically not significant. Commercially available water-soluble 1% Eosin Y was used in both the methods. Eosin Y brought out the maximum details of the cytoplasm in36 of 40 xylene free deparaffinised sections studied. Only four sections showed a deteriorated cytoplasmic stain. That sections appeared bluish. Eosin is an acidic dye with optimum staining occurring at a pH of 5.2 to 5.4. It will rinse out in any alkaline solution. The pH of eosin was found to be adequate. The other sources of alkalinity was the tap water wash used before and after the eosin staining step. In the study of Ankle et al they found that only 83.33% of the xylene free deparaffinised slides showed adequate cytoplasmic staining compared with 93.33% of the conventional H & E staining method and the difference was statistically significant downgradation of cytoplasmic staining in the xylene-free H & E stained sections.

The uniformity with xylene free deparaffinised sections was 87.5% as compared with 82.5% of uniformity in conventional H & E staining. No statistically significant difference was noted (P value- 0.687) in the two staining methods followed , suggesting that the xylene free H & E staining are at par with conventional H & E in producing uniformity in staining. In the study ofAnkle et al they found that only 50% of the xylene free deparaffinised slides are uniformly stained compared with 70% of the conventional H & E staining method and the difference was statistically significant downgradation of uniformity in staining in the xylene-free H & E stained sections.

The clarity of the xylene free deparaffinised sections was 87.5% as compared with 85% of clarity in conventional H & E staining method. No statistically significant difference was noted (P value- 1.0) in the two staining methods followed, suggesting that the xylene free H & E staining are at par with conventional H & E in producing clarity in staining. Out-of – focus areas can be due to the reasons like tear or rip of section, introduction of extraneous tissue, unclean blade, dirty microscopic lenses, thick section, and moisture on coverslip.Similar findings were found in the experimental study by Ankle et al.as well as Ramulu et al[8] .95% of the xylene free deparaffinised sections revealed a crisp staining as compared with 85% of the conventional H&E stain. Statistically not significant (P value- 0.219)

but we can see that 10% more slides show good crispness compared with the conventional method. The combination of 1.7% liquid dish wash solution along with Harris haematoxylin and Eosin Y brings about an ideal degree of crispness. Deparaffinisation with diluted dish wash solution is achieved only in 4 min unlike 30 minutes required for deparaffinisation with xylene in the routine H & E staining. This saves time and simplifies the staining procedure. In the study of Madhuri R ankle &Priya S joshi, they found that 83.33% of the xylene free deparaffinised slides are having good crispness compared with 76.66% of the conventional H & E staining method and the difference was statistically significant.

When the scores were totaled, 91.5% of the xylene free deparaffinised sections were found to be adequate for diagnosis as compared with 85% of the slides stained with routine H & E. In the study of Madhuri R ankle &Priya S joshi, they found that when the scores were totaled, 90% of the xylene free deparaffinised slides were adequate for diagnosis compared with 88.33% of the conventional H & E staining method. In another study by Falkeholm L, Grant C A, Magnusson A and Moller found that the xylene free deparaffinised sections were ranked as good as or better than their conventional counterparts in 74% of the comparisons and poorer in 26%. In an attempt to completely eliminate xylene from staining procedure Buesa and Peshkov demonstrated that the use of dishwasher solution to dewax sections and oven drying prior to coverslipping will eliminate xylene from the staining procedure[9]

CONCLUSION

The objective of this study was to compare the efficacy of xylene free deparaffinisation in tissue sections using surfactants such as diluted dish wash solutions with the conventional deparaffinisation using xylene in H&E staining. That is to compare with the conventional method with respect to simplicity, cost effectiveness and decreased turnaround time of results. All the results obtained were evaluated according to nuclear staining, cytoplasmic staining, clarity, uniformity and crispness of staining. The stastitical evaluation was done and the following conclusions were made. The described modified xylene free deparaffinisation method gives excellent nuclear staining character, excellent cytoplasmic staining character,clarity, uniformity in staining character and excellent crispness in staining character.

Thus the present study shows that the xylene free deparaffinisation in H & E staining procedure carried out using a simple diluted liquid dish washing soap solution is at par with the conventional H & E procedure. It also has added advantages of being nontoxic, economical, nonfl-ammable, nonhazardous, no problem of disposal, reduces staining time, and easy to handle.

Since in a time period of 6 months more trials on various tissues were limited, we have to perform more studies on this technique to popularise this excellent staining technique to be used as a routine method of staining. In short, this modification improve utilization of reagents, while reducing and eliminating their safety concerns and economic burden; and improve personnel-friendly workflow and place the laboratory in a better position to meet the demands of decreased turn around time.

Source of funding: none

REFERENCES

1. Cook HC.Origins of tinctorial methods in histology.J Clin Pathol 1997;50:716-20 [PMC free article]
2. Falkeholm L, Grant CA, Magnusson A, Moller E. Xylene-Free Method for Histological preparation: A Multicentre Evaluation. Lab Invest 2001;81:1213-21
3. Buesa RJ, Peshkov MV. Histology without xylene. Ann DiagnPathol 2009; 13:246-56.
4. Reena Kandyala, SumanthPhani C Ragha-vendra & Saraswathi T Rajasekharan :

Xylene An overview of its health hazards and preventive measures; J Oral Maxillo-fac Pathol. 2010 Jan- Jun; 14 (1): 1-5.

5. Kandyala R, Raghavendra SP, Rajasekharan ST.Xylene:An overview of its health hazards and preventive measures .J Oral Maxillofac Pathol.2010;14:1-5
6. Madhuri R ankle, Priya S joshi; A study to evaluate the efficacy of xylene-free Haematoxylin and eosin staining procedure as compared to the conventional H and E staining : An experimental study.J Oral Maxillofac Pathol.2011 May-Aug;15(2):161-167
7. PinkiPandey,Alok Dixit et al:A comparative study to evaluate liquid dish washing soapas an alternative to xylene and alcohol in deparaffinisation and hematoxylin and eosin staining. J Lab Physicians.2014 Jul-Dec;6(2):84-90.
8. RamuluS,KoneruA,RavikumarS.An excellent substitute for xylene and alcohol in Hematoxylin and Eosin staining procedure .J Orofac Sci.2012;4:37-42.
9. BuesaRJ,PeshkovMY.Complete elimination of xylene in practice of a histology laboratory.Arkh Patol.2011;73: 54-60.[PubMed]
10. BuesaR J. Mineraloil: The best xylene substitute for tissue processing yet? Histotechnol. 2000;23:143-9