Evaluation of biosafe alternatives as xylene substitutes in hematoxylin and eosin staining procedure: A comparative pilot study

Taneeru Sravya, Guttikonda Venkateswara Rao, Masabattula Geetha Kumari, Yerraguntla Vidya Sagar, Yeluri Sivaranjani, Kondamarri Sudheerkanth

Departments of Oral Pathology and Microbiology and ‡Public Health Dentistry, Mamata Dental College, Khammam, Telangana, India

Abstract

Background: Xylene is synthetic hydrocarbon produced from coal tar known for its wide usage as universal solvent which has many hazardous effects. The aim of this study is to compare the efficacy of xylene-free hematoxylin and eosin (H and E) sections with conventional H and E sections.

Materials and Methods: The study included ninety paraffin-embedded tissue blocks. Of these, sixty blocks were processed with sesame oil (xylene alternative) and thirty blocks with xylene. The study sample was divided into three groups. Sixty sections which are taken from sesame oil-processed blocks were stained with xylene-free H and E staining method. In xylene-free staining method, 95% diluted lemon water (Group A) and 1.7% dish washing solution (DWS, Group B) were used as deparaffinizing agents whereas the remaining 30 sections were processed with xylene and stained with conventional H and E staining method (Group C). Slides were scored for the following parameters: (i) nuclear staining (adequate = score 1, inadequate = score 0), (ii) cytoplasmic staining (adequate = score 1, inadequate = score 0), (iii) uniformity (present = score 1, absent = score 0), (iv) clarity (present = score 1, absent = score 0) and (v) intensity (present = score 1, absent = score 0). Score ≤2 was considered inadequate for diagnosis while scores 3–5 were considered adequate for diagnosis.

Results: Adequate nuclear staining was noted in 90% of sections of Group A and 100% each in Group B and Group C (P < 0.05); adequate cytoplasmic staining in 96.7% in Group A and 100% each in Group B and Group C (P > 0.05); adequate uniformity of staining in 53.3% of sections of Group A, 70% in Group B and 83.3% in Group C (P < 0.05); adequate clarity of staining in 73.3% sections of Group A, 80% in Group B and 83.3% in Group C (P > 0.05) and adequate intensity of staining in 76.7% sections of Group A, 93.3% in Group B and 100% in Group C (P < 0.05). Group C sections stained adequate for diagnosis (93.3%) followed by Group B (88.7%) and Group A (78%; P < 0.05).

Conclusion: Tissues processed with sesame oil and stained using 1.7% DWS were found to be effective alternative to xylene.

Keywords: Deparaffinization, diluted lemon water, dish washing solution, xylene
INTRODUCTION

Histopathology laboratories perform various investigative analyses on patient’s specimen. In the process of such investigative procedures, a wide range of chemicals that are potentially harmful are employed in laboratories, which includes toxic chemicals, radiochemicals and pathogenic microorganisms. According to the Occupational Safety and Health Administration regulations, the two fundamental chemicals that are frequently used in histopathology procedure and are known for their long-term effects include formalin and xylene.[1,2] Among these two chemicals, xylene is considered to be more hazardous compared to formalin [Table 1].

Xylene, a synthetic hydrocarbon which is produced from coal tar, is widely used as universal solvent. In histopathology, xylene is used as a clearing agent, which gives translucency to tissues enhancing paraffin infiltration. Exposure to xylene can occur via inhalation, ingestion and eye or skin contact.[3] In our histopathology laboratory, we personally experienced a problem with xylene while doing hand-immersion technique. Because of frequent exposure to xylene in the laboratory, the fingers that are in contact revealed vasodilation along with dryness, itching and scaling of that particular area [Figure 1]. A closed patch test was done and then it was diagnosed as allergic dermatitis secondary to xylene. Similar findings were also noticed by Engström et al.[4] and Riihimäki and Pfäffli.[5] Various other hazardous effects due to xylene include acute neurotoxicity, cardiac and kidney injuries, hepatotoxicity and fatal blood dyscrasias and also have carcinogenic effect.[6]

Various substitutes have been employed as xylene alternatives, which include limonene reagents, aliphatic and aromatic hydrocarbons, vegetable oils, olive oil and mineral oil substitutes.[7] To eliminate the use of xylene, the present study was designed with eco-friendly materials which are nontoxic, less hazardous and economical. As xylene is used in both tissue processing and staining procedure, a study was designed to replace xylene in tissue processing using limonene oil and sesame oil. The results revealed that sesame oil has shown 96.7% while limonene oil revealed 60% of adequacy for the diagnosis.[8] This study was continued further and is now aimed to replace xylene in the staining procedure. The study was conducted using alternatives to xylene, i.e., sesame oil, in tissue processing and 95% diluted lemon water (DLW) and 1.7% dish washing solution (DWS) as deparaffinizing agents in hematoxylin and eosin (H and E) staining procedure.

MATERIALS AND METHODS

The study included ninety paraffin-embedded tissue blocks. Of these, sixty blocks were processed with sesame oil as xylene alternative and thirty blocks with routine xylene [Table 2]. One paraffin section of 3 μm thickness each was taken from all the blocks. The study sample was divided into three groups. Sixty sections which are taken from sesame oil-processed blocks were stained with

| Table 1: Differences between formalin and xylene |
|-----------------------------------------------|
| **Features** | **Formalin** | **Xylene** |
| Time weighted averages (ppm) | 0.75 | 100 |
| Usage form | Diluted form (10% formalin) | Pure form |
| Amount of usage | Less | More |
| Exposure time | Less | More |
| Uses in histopathology laboratory | Fixative agent | Clearing agent |
| In histopathology procedure | During tissue processing | Both tissue processing and staining procedure |

Table 2: Xylene and xylene-free methods for tissue preparation

| Methods | Time |
|---------|------|
| Conventional xylene method | 24 h |
| Formalin | 24 h |
| Water | 15 min |
| 50% alcohol | 60 min |
| 70% alcohol | 60 min |
| 90% alcohol | 60 min |
| 100% alcohol | 60 min |
| Xylene I | 60 min |
| Xylene II | 60 min |
| Paraffin | 720 min |
| Xylene-free method | 24 h |
| Formalin | 24 h |
| Water | 15 min |
| 50% alcohol | 60 min |
| 70% alcohol | 60 min |
| 90% alcohol | 60 min |
| 100% alcohol | 60 min |
| Sesame oil I | 60 min |
| Sesame oil II | 60 min |
| Paraffin | 60 min |

Figure 1: Fingers revealing vasodilation along with dryness and scaling because of exposure to xylene
xylene-free H and E staining method [Tables 3 and 4]. In xylene-free staining method, 1.7% DWS (1.7 ml DWS in 98.3 ml distilled water) and 95% DLW (95 ml lemon water in 5 ml of distilled water) were used as deparaffinizing agents. The remaining thirty sections were processed with xylene and stained with conventional H and E staining method [Table 5].

Group A (n = 30): Tissue sections which were processed with sesame oil and stained with xylene-free method where 95% DLW used as a deparaffinizing agent.

Group B (n = 30): Tissue sections which were processed with sesame oil and stained with xylene-free method where 1.7% DWS used as a deparaffinizing agent.

Group C (n = 30): Tissue sections which were processed with xylene and stained with conventional H and E method.

Each section was scored based on the following five parameters:
- Nuclear staining (adequate = score 1, inadequate = score 0)
- Cytoplasmic staining (adequate = score 1, inadequate = score 0)
- Uniformity (adequate = score 1, inadequate = score 0)
- Clarity (adequate = score 1, inadequate = score 0)
- Intensity (adequate = score 1, inadequate = score 0)

All sections were analyzed by two pathologists who were blinded. The average score was taken into consideration. The scores of each slide were totaled, and if the total score is ≤2, it was graded as inadequate, and if the score ranged from 3 to 5, it was graded as adequate for diagnosis. The scores were recorded and subjected for statistical analysis.

RESULTS

Adequate nuclear staining was noted in 90% of sections of Group A and 100% each in Group B and Group C (P < 0.05); adequate cytoplasmic staining in 96.7% in Group A and 100% each in Group B and Group C (P > 0.05); adequate uniformity of staining in 53.3% of sections of Group A, 70% in Group B and 83.3% in Group C (P < 0.05); adequate clarity of staining in 73.3% sections of Group A, 80% in Group B and 83.3% in Group C (P > 0.05) and adequate intensity of staining in 76.7% sections of Group A, 93.3% in Group B and 100% in Group C (P < 0.05) [Table 6 and Figures 2-5].

On comparing the adequacy of diagnosis of stained sections obtained by different staining methods on the basis of scores obtained, it was apparent that more number of diagnostically adequate and less number of inadequate sections were obtained by Group B immediately next to the Group C sections. Group C sections stained adequate for diagnosis (93.3%) followed by Group B (88.7%) and Group A (78%) [Figure 6].

DISCUSSION

Clearing agents are among the most noxious and hazardous chemicals found in histopathology laboratories. Xylene is a colorless, sweet-smelling liquid or gas which is commonly used in histopathology laboratory as a clearing agent. Technically, xylene is a combination of three isomers:
orth, para and meta and this mixture is known as “xylol.”[3] The National Institute for Occupational Safety and Health recommended exposure limits for xylene at 100 ppm as a Time Weighted Average (TWA) for up to a 10-h work shift and a 40-h work week and 200 ppm for 10 min as a short-term limit.[11]

Due to new regulations, several xylene substitutes have been developed in recent years. Currently, many studies are being carried out as substitutes which are biohazardous, improve healthy laboratory environment, do not compromise the staining quality and are adequate for diagnosis. Hence, the present study was aimed to replace xylene during the tissue processing with sesame oil and followed by 95% DLW and 1.7% DWS as deparaffinizing agents.

Xylene-free method for paraffin sections was developed since 1995 in Vrinnevi hospital.[12] Buesa RJ used mineral oil (pure and mixed with ethanol and isopropyl) as a xylene substitute and found equivalent results to xylene. [13] Falkeholm et al. conducted a study using randomized mix of 180 xylene-free sections and ranked, of which 74% of sections were good and 26% were poor when compared to conventional xylene sections.[14]

Ankle and Joshi[7] and Ramulu et al.[11] conducted a study using dish washing soap as a substitute for alcohol and xylene in conventional H and E procedure. They concluded that liquid dish washing soap can be used as an effective substitute to xylene and ethanol in routine H and E staining. Premalatha et al. evaluated the efficacy of mineral oil over xylene in routine H and E staining and found that 93.3% sections were adequate for diagnosis.[15]

Taneeru et al. used sesame oil and limonene oil as xylene alternatives in tissue processing and found 96.7% adequacy with sesame oil and 60% with limonene oil.[8] Negi et al.[10] and Metgud et al.[9] used DWS as a deparaffinizing agent in

| Table 5: Routine (conventional) hematoxylin and eosin staining procedure |
|---|---|---|
| Steps | Procedure | Temperature | Time |
| Deparaffinization | Xylene I | 5 min | 5 min |
| | Xylene II | 5 min | 5 min |
| | 90% alcohol | 5 min | 5 min |
| | 90% alcohol | 5 min | 5 min |
| | Water wash | 10 min | |
| Nuclear staining | Harris hematoxylin | At room | 8 min |
| Tap water wash | Temperature | 2 min |
| Differentiation | 1% acid alcohol | At room | 1 dip |
| Water wash | Temperature | |
| Blueing | 1% lithium carbonate | 1 min | |
| Tap water wash | 10 min | |
| Cytoplasmic staining | 1% eosin | At room | 1 min |
| Dehydration | 90% alcohol | 30 s | |
| | 70% alcohol | 30 s | |
| | Xylene I | 5 min | |
| | Xylene II | 5 min | |
| Approximate time required | 70‑75 min | |

| Table 6: Staining pattern in Group A, Group B and Group C |
|---|---|---|---|---|---|---|
| Parameter | Group A | Group B | Group C | Total | Pearson \( \chi^2 \) | \( P \) | Significance |
| Nuclear staining | Adequate | 27 | 30 | 30 | 87 | 6.207 | 0.045 | S |
| | Inadequate | 3 | 0 | 0 | 3 | 20.23 | 0.036 | S |
| Cytoplasmic staining | Adequate | 29 | 30 | 30 | 89 | 2.022 | 0.364 | NS |
| | Inadequate | 1 | 0 | 0 | 1 | 0.12 | 0.72 | NS |
| Uniformity of staining | Adequate | 16 | 21 | 25 | 62 | 6.325 | 0.042 | S |
| | Inadequate | 14 | 9 | 5 | 28 | 0.934 | 0.627 | NS |
| Clarity of staining | Adequate | 22 | 24 | 25 | 71 | 0.934 | 0.627 | NS |
| | Inadequate | 8 | 6 | 5 | 19 | 1.90 | 0.39 | NS |
| Intensity of staining | Adequate | 23 | 28 | 30 | 81 | 9.630 | 0.008 | S |
| | Inadequate | 7 | 2 | 0 | 9 | 1.69 | 0.19 | NS |

S: Significant, NS: Not significant
Sravya, et al.: Xylene substitutes in hematoxylin and eosin staining procedure

H and E staining and found 1.7% DWS to be an effective alternate over xylene. Indu et al. used 8% cedarwood oil to produce quality staining with sufficient clarity and uniformity.[16] Pandey et al. used DWS as an alternative to xylene and alcohol in H and E staining and found that 84% sections which were routinely stained with xylene and 84% sections with liquid DWS were adequate for diagnosis.[6]

Ananthaneni et al. used 1.5% DWS and 95% DLW as deparaffinizing agents in routine H and E staining and found that 1.5% DWS revealed comparatively superior uniform staining and less retention of wax compared to 95% DLW.[17] Sermadi et al. used coconut oil as an alternative in tissue processing and found it as an efficient substitute for xylene causing less shrinkage of the tissue.[18]

In the present study, among all the parameters, the uniformity of staining was found to be reduced in Group A (53.3%) and Group B (70%) compared to Group C (83.3%). This is mainly because at the time of tissue processing, the tissue became hard which resulted in thick and uneven sections. When all the scores were totaled, it was found that sesame oil can be used as an alternative to xylene in tissue processing and 95% DLW and 1.7% DWS as deparaffinizing agents in conventional H and E staining and found that the sections stained with 1.7% DWS revealed superior staining quality with adequacy of 88.7% over the sections stained with 95% DLW which revealed 78% of adequacy.

CONCLUSION

The results of the present study infer that 1.7% DWS is an efficient substitute for xylene, as it is nonhazardous,
Sravya, et al.: Xylene substitutes in hematoxylin and eosin staining procedure

inexpensive compared to 95% DLW. All the substitutes should be analyzed properly, before concluding the better alternative. Further studies with larger samples need to be carried out to conclude 1.7% DWS as a better and safer substitute for xylene.

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

REFERENCES

1. US Department of Labor. Occupational Safety and Health Administration Regulations (Standards-29 CFR). Table Z-1 Limits for air Contaminants; 2006.
2. Buesa RJ. Histology safety: Now and then. Ann Diagn Pathol 2007;11:334-9.
3. Rajan ST, Malathi N. Health hazards of xylene: A literature review. J Clin Diagn Res 2014;8:271-4.
4. Engström K, Husman K, Riihimäki V. Percutaneous absorption of m-xylene in man. Int Arch Occup Environ Health 1977;39:181-9.
5. Riihimäki V, Pfäffli P. Percutaneous absorption of solvent vapors in man. Scand J Work Environ Health 1978;4:73-85.
6. Pandey P, Dixit A, Tanwar A, Sharma A, Mittal S. A comparative study to evaluate liquid dish washing soap as an alternative to xylene and alcohol in deparaffinization and hematoxylin and eosin staining. J Lab Physicians 2014;6:84-90.
7. Ankle MR, Joshi PS. A study to evaluate the efficacy of xylene-free hematoxylin and eosin staining procedure as compared to the conventional hematoxylin and eosin staining: An experimental study. J Oral Maxillofac Pathol 2011;15:161-7.
8. Taneeru S, Guttikonda VR, Vanajakshi CN, Korlepara R. Xylene free method for tissue processing: A pilot study. Health Sci 2013;2:J8004.
9. Metgud R, Astekar MS, Soni A, Naik S, Vanishree M. Conventional xylene and xylene-free methods for routine histopathological preparation of tissue sections. Biotech Histochem 2013;88:235-41.
10. Negi A, Puri A, Gupta R, Chauhan I, Nangia R, Sachdeva A, et al. Biosafe alternative to xylene: A comparative study. J Oral Maxillofac Pathol 2013;17:563-6.
11. Ramulu S, Konuru A, Ravikumar S, Sharma P, Ramesh D, Patil R. Liquid dish washing soap: An excellent substitute for xylene and alcohol in hematoxylin and eosin procedure. J Orofac Sci 2012;4:37-42.
12. Falkeholm L. Going green: Using water, not xylene. Lab Med 1996;27:638.
13. Buesa RJ. Mineral oil: The best xylene substitute for tissue processing yet? Journal of Histotechnology 2000;23:143-9.
14. Falkeholm L, Grant CA, Magnusson A, Möller E. Xylene-free method for histological preparation: A multicentre evaluation. Lab Invest 2001;81:1213-21.
15. Premalatha BR, Patil S, Rao RS, Indu M. Mineral oil – a biofriendly substitute for xylene in deparaffinization: A novel method. J Contemp Dent Pract 2013;14:281-6.
16. Indu S, Ramesh V, Indu PC, Prashad KD, Premalatha B, Ramadoss K, et al. Comparative efficacy of cedarwood oil and xylene in hematoxylin and eosin staining procedures: An experimental study. J Nat Sci Biol Med 2014;5:284-7.
17. Ananthaneni A, Namala S, Gaduru VS, Ramprasad VV, Ramisetty SD, Udayashankar U, et al. Efficacy of 1.5% dish washing solution and 95% lemon water in substituting perilous xylene as a deparaffinizing agent for routine h and e staining procedure: A short study. Scientifica (Cairo) 2014;2014:707310.
18. Sermadi W, Prabhu S, Acharya S, Javali S. Comparing the efficacy of coconut oil and xylene as a clearing agent in the histopathology laboratory. J Oral Maxillofac Pathol 2014;18:S49-53.