Received: 27th July 2016 *Accepted: 15th October 2016* *Conflicts of Interest: None*

Source of Support: Nil

Original Research

Doi: 10.2047/jioh-08-12-10

Quest for Biofriendly Xylene Substitutes in Histopathology: A Comparative Study

Snehashish Ghosh[1], Roopa S Rao[2], Shwetha Nambiar[3], Vanishri C Haragannavar[4], Dominic Augustine[4], S V Sowmya[5], Shankargouda Patil[6]

Contributors:

[1]Post-graduate Student, Department of Oral Pathology & Microbiology, Faculty of Dental Sciences, M.S. Ramaiah University of Applied Sciences, Bengaluru, Karnataka, India; [2]Professor and Head, Department of Oral Pathology & Microbiology, Faculty of Dental Sciences, M.S. Ramaiah University of Applied Sciences, Bengaluru, Karnataka, India; [3]Tutor, Department of Oral Pathology & Microbiology, Faculty of Dental Sciences, M.S. Ramaiah University of Applied Sciences, Bengaluru, Karnataka, India; [4]Assistant Professor, Department of Oral Pathology & Microbiology, Faculty of Dental Sciences, M.S. Ramaiah University of Applied Sciences, Bengaluru, Karnataka, India; [5]Associate Professor, Department of Oral Pathology & Microbiology, Faculty of Dental Sciences, M.S. Ramaiah University of Applied Sciences, Bengaluru, Karnataka, India; [6]Associate Professor, Department of Maxillofacial Surgery and Diagnostic Sciences, Division of Oral Pathology, College of Dentistry, Jazan University, Jazan, Saudi Arabia.

Correspondence:

Dr. Ghosh S. Department of Oral Pathology & Microbiology, Faculty of Dental Sciences, M.S. Ramaiah University of Applied Sciences, MSRIT Post, MSR Nagar, Bengaluru - 560 054, Karnataka, India. Email: ghoshrocks2004@gmail.com

How to cite the article:

Ghosh S, Rao RS, Nambiar S, Haragannavar VC, Augustine D, Sowmya SV, Patil S. Quest for biofriendly xylene substitutes in histopathology: A comparative study. J Int Oral Health 2016;8(12):1101-1104.

Abstract:

Background: Xylene is an aromatic hydrocarbon widely used in histopathology laboratory. However, exposure to xylene is a well-documented occupational hazard. This study intended to find a natural, non-toxic, economical substitute for xylene to minimize its hazardous effects and make the histopathological laboratory an biofriendly environment.

Objectives: (1) To evaluate the efficacy of extra virgin olive oil and refined sunflower oil (RSO) as a clearing and deparaffinizing agent in histopathological staining, (2) To compare the efficacy of olive oil and RSO as a clearing and deparaffinizing agent with regard to xylene.

Materials and Methods: Commercially available fresh goat tissue was procured and fixed in 10% neutral buffered formalin for 48 h. The tissues were divided into three experimental groups (Groups I, II, and III). Hematoxylin and eosin staining was performed for all the three groups. Group I was processed and stained using xylene as clearing and deparaffinizing agent. Groups II and III were processed and stained using olive oil and RSO, respectively. Evaluation was done by three pathologists and the entire procedure was blinded. Statistical analysis was accomplished by Kruskal–Wallis analysis of variance, Mann–Whitney U-test, Chi-square test, and Kappa statistics.

Results: Results showed both the oils had the ability to clear and deparaffinize tissues. Extra virgin olive oil was comparatively better, it maintained the tissue integrity, and the overall staining quality was also better when compared to RSO.

Conclusion: To conclude, extra virgin olive oil and RSO can be used as a biofriendly substitute to xylene in histopathological laboratory.

Key Words: Clearing, deparaffinization, olive oil, xylene

Introduction

The aim of histotechnology is to reproduce the microscopic composition of the tissues to their original configuration. In histopathological laboratories, xylene is routinely used for tissue processing, hematoxylin and eosin (H and E) staining and coverslipping.[1,2]

Xylene is an aromatic hydrocarbon which is colorless, sweet-smelling liquid or gas occurring naturally in coal, petroleum, and wood tar. Therefore, it is used as the safest alternative to hazardous chemicals such as aniline, chloroform, and toluene in histopathological slide preparation since 1950's. However, over the last few decades, its routine use in histopathological laboratory is questionable, on account of compelling evidence of its toxicity on skin, eyes, nose, nervous system, and musculoskeletal system.[3,4] Although there are inadequate evidence for the carcinogenicity of xylene in humans, few studies mentioned the role of xylene in the pathogenesis of blood dyscrasias.[5]

Therefore, considering its hazardous potential, the discovery of biofriendly alternatives to xylene is the need of the hour. In pursuit of such an alternative substitute, this study was designed to replace xylene from tissue processing as well as staining procedures.

Extra virgin olive oil and refined sunflower oil (RSO) were selected as the alternative agents. These potential substitutes were selected since they are nontoxic, noninflammable, easy to handle, economical, easily available, and disposable. It was also found that these products reduce the working time for H and E staining, as compared to xylene.[6] Therefore, this study aimed to evaluate the efficacy of olive oil and RSO as clearing and deparaffinizing agents in comparison to that of xylene.

Materials and Methods

Sample size and procedure

A total of 120 specimens of commercially available fresh goat tissue (buccal mucosa, lymph node, liver, and salivary gland

tissue 30 each) were procured. Each tissue was cut into three equal bits (0.5 cm × 0.5 cm × 0.5 cm) and was included in three experimental groups (Groups I, II and III). The tissues were fixed in 10% neutral buffered formalin for 48 h. Tissues in Group I were processed using xylene as a clearing agent. Tissues in Groups II and III were processed using extra virgin olive oil and RSO as clearing agent, respectively. Following processing, H and E staining was done for all the three groups of tissues. The staining was performed by conventional method for Group I and xylene was replaced by extra virgin olive oil and RSO in Groups II and III, respectively.[1,6] The stained slides were blinded, and the quality of the slides were evaluated by three pathologists using the criteria given in Table 1.[6]

Statistical analysis

The results were tabulated and subjected to statistical analysis using Kruskal–Wallis test, Mann–Whitney U-test, Chi-square test, and Kappa statistics via Statistical Package for the Social Sciences 10.5 software. A $P < 0.001$ was considered significant.

Results

This study was conducted to compare the xylene free method of tissue processing and H and E staining with the conventional method. The tissue sections were evaluated and compared for cellular outline, cytoplasmic and nuclear details, staining quality, clarity, and tissue integrity. Four different types of tissues were used in this study, namely buccal mucosa, lymph node, liver, and salivary gland. Extra virgin olive oil and RSO were used as clearing and deparaffinizing agent with xylene as positive control.

The H and E staining quality of sections of four different types of tissues cleared and deparaffinized using extra virgin olive oil were at par with xylene cleared and deparaffinized tissues. Although the H and E staining quality of tissue sections cleared and deparaffinized using RSO were inferior to xylene and extra virgin olive oil, they were adequate for histopathological interpretation. The results obtained were statistically significant ($P < 0.001$) in all the three groups.

The Kappa value of 0.825 showed substantial inter-observer agreement. The results of the study are summarized in Graph 1. The photomicrographic demonstration of the H and E stained tissues are represented in Figure 1.

Discussion

Tissue processing and H and E staining are the prime step in histopathology. Clearing is an important step in tissue processing, in which, the dehydrating solutions are being removed, making the tissue receptive to the infiltrating medium. Deparaffinization is the process which facilitates the removal of paraffin wax from the tissue sections, making them accessible for staining. Both clearing and deparaffinization necessitate the use of a colorless, volatile, aromatic compound, xylene.[1,4] The high solvency factor of xylene allows maximal displacement of alcohol, enhancing paraffin infiltration into the tissue. Apart from being a good clearing agent, it has an excellent dewaxing capability.[7]

Lacunae continue to persist in this age-old procedure, such as toxicity and pollution of the working environment. Neurotoxic effects of xylene were first discovered, and subsequently, it was also found to be toxic to multi-organ systems such as gastrointestinal system, skin, liver, lung, kidney, musculoskeletal system, and reproductive system. Moreover, its low flash point of 28.9°C makes it a flammable solvent.[4]

Table 1: Criteria for evaluation.

Histomorphologic criteria	Grading scale for each criteria
Cellular outline	0 - Non diagnostic
Cytoplasmic details	1 - Poor
Nuclear details	2 - Average
Staining quality	3 - Good
Clarity	4 - Very good
Tissue integrity	5 - Excellent

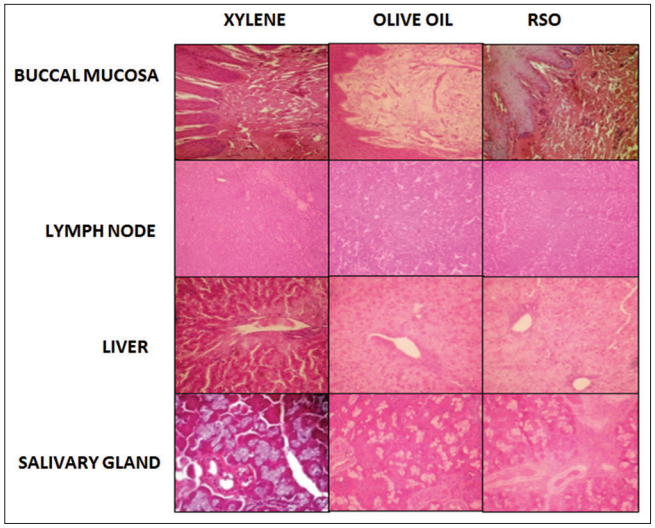
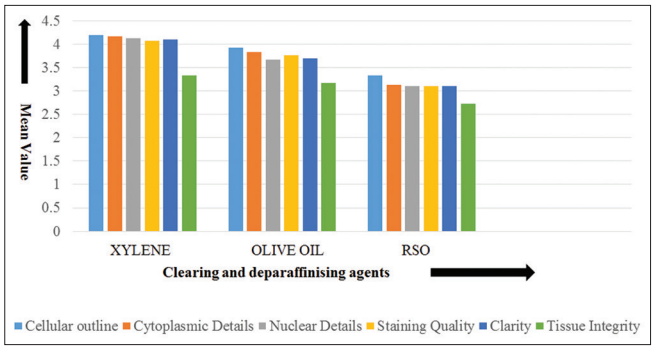


Figure 1: Photomicrograph depicting the comparison of hematoxylin and eosin stained buccal mucosa, lymph node, liver, and salivary gland tissues among the three clearing and deparaffinizing agents (×400 magnification).



Graph 1: Comparison of mean value of the hematoxylin and eosin staining among the three clearing and deparaffinizing agents.

With this background, a substitute for xylene, without compromising the quality of clearing and deparaffinization of tissues has become the need of the hour. Many potential substitutes were tried by different researchers to replace xylene from the histopathological laboratory. Those included isopropanol, vegetable oils namely olive oil, palm oil, coconut oil, hexanes, dishwashing solution, isopropanol, and propylene glycol methyl ether. Isopropanol showed promising results. Dishwashing solution proved to be an effective deparaffinizing agent but its role in clearing was questionable. However, hexanes proved to be hazardous to health and 1.41 times expensive than xylene.[8,9] Clearing of tissue was tried by directly transferring it into paraffin wax, following dehydration. In this procedure, paraffin wax itself acted as a clearing agent. This procedure had different levels of success but could only eliminate xylene from tissue processing, not from staining.[6]

In this study, xylene was replaced by extra virgin olive oil and RSO during tissue processing and H and E staining. Apart from being non-toxic, other advantages of using extra virgin olive oil and RSO as clearing and deparaffinizing agent are noninflammable, noncarcinogenic, easy handling properties, and easily disposable.

The results obtained after analysis showed that 100% of the tissues processed and stained using all the 3 clearing and deparaffinizing agents were suitable for histopathological interpretation, indicating that extra virgin olive oil and RSO had clearing and deparaffinizing ability. This can be attributed to a number of reasons. The refractive index of extra virgin olive oil (1.467) and RSO (1.474) are closer to the tissue proteins (ranging between 1.33 and 1.4), which allowed easy infiltration into the intercellular spaces of the tissues.[4,6] Similar refractive indices also causes reduction in scattering of light and enhances the optical clearance of the tissues, making them more transparent.[8-10] The properties of xylene, olive oil, and RSO are summarized in Table 2.[6,8,9]

Extra virgin olive oil and RSO do not have the ability to dissolve fat at room temperature, which xylene does; therefore, they were used at 30°C, 60°C, and 90°C as an increase in temperature causes the tissue fats to dissolve readily, ensuring proper clearing of tissues. Optimum results were obtained at 60°C. In addition to that increase in the temperature, increases the kinetic energy and the rate of diffusion of oil molecules, thereby leading to decrease in viscosity. This ensured greater penetration of the extra virgin olive oil, RSO and faster removal of the dehydrating medium. Similarly, deparaffinization procedure was also tried at 30°C, 60°C, and 90°C, and the optimum results were obtained at 60°C. There were certain advantages noted while using extra virgin olive oil and RSO as clearing and deparaffinizing agents (Table 3). The density of extra virgin olive oil and RSO is closer to that of the density of animal fat, which allowed them to cause displacement of fat, rather than dissolution, as xylene does. Time taken for staining was comparatively less than the conventional procedure which is in accordance with the study by Premalatha *et al.*, in 2013.[6]

A limited number of studies have been conducted on olive oil as a clearing and deparaffinizing agent. Bruun Rasmussen *et al.*, in 1992, and Swamy *et al.*, in 2015,[11,12] used olive oil as clearing agent and obtained good results that were suitable for histopathological interpretation, which is in concordance with the results of this study. As per our knowledge, no study has been conducted using RSO as clearing and deparaffinizing agent.

Table 2: Comparison of properties of xylene, extra virgin olive oil, and RSO.[6,8,9]

Properties	Xylene	Extra virgin olive oil	RSO
Synonym	Dimethyl benzene	Vegetable oil	Sunflower oil
Chemical structure	Aromatic hydrocarbon	Aliphatic hydrocarbon	Aliphatic hydrocarbon
Exposure limit (TWA ppm)	100 ppm	No limit	No limit
Flammability	Flammable	Non-flammable	Non-flammable
Ignition	Readily	Not readily	Not readily
Solubility in water	Insoluble	Insoluble	Insoluble
Solubility in alcohol	Soluble	Insoluble	Insoluble
Density (g/ml)	0.86	0.88-0.92	0.92
Refractive index	1.50	1.467	1.474
Melting point	−25°C	−6°C	−17°C
Boiling point	135-145°C	207°C	227°C

RSO: Refined sunflower oil

Table 3: Advantages of extra virgin olive oil and RSO over xylene.[6,8,9]

Criteria for comparison	Xylene	Extra virgin olive oil	RSO
Health risk	Hazardous	Non-hazardous	Non-hazardous
Personal protective equipment	Required	Not required	Not required
Disposal	Difficult	Easy	Easy
Quality of staining	Very good	Very good	Good
Time needed for staining	65 min	50 min	50 min

RSO: Refined sunflower oil

Conclusion

As pathologists, it is our duty to minimize the use of toxic chemicals in the laboratory without compromising on the diagnostic quality of the tissue sections. The overall clearing and deparaffinizing capacity of extra virgin olive oil and RSO were commendable. Scope for further studies involves the assessment of long-term stability of the tissue sections cleared and deparaffinized using extra virgin olive oil and RSO.

References

1. Bancroft JD, Gamble M. Theory and Practice of Histological Techniques, 6th ed. Philadelphia, PA, USA: Churchill Livingstone, Elsevier; 2008.
2. 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(2):161-7.
3. Buesa RJ, Peshkov MV. Histology without xylene. Ann Diagn Pathol 2009;13(4):246-56.
4. Kandyala R, Raghavendra SP, Rajasekharan ST. Xylene: An overview of its health hazards and preventive measures. J Oral Maxillofac Pathol 2010;14(1):1-5.
5. Patil S, Rao RS, Ganavi BS, Majumdar B. Natural sweeteners as fixatives in histopathology: A longitudinal study. J Nat Sci Biol Med 2015;6(1):67-70.
6. 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(2):281-6.
7. Falkeholm L, Grant CA, Magnusson A, Möller E. Xylene-free method for histological preparation: A multicentre evaluation. Lab Invest 2001;81(9):1213-21.
8. Frankel EN. Nutritional and biological properties of extra virgin olive oil. J Agric Food Chem 2011;59(3):785-92.
9. Saurabh T, Patnaik M, Bhagt S, Renge V. Epoxidation of vegetable oils: A review. Int J Adv Eng Technol 2011;2(4):491-501.
10. Majumdar B, Rao RS, Patil S. Tissue preservation with natural fixatives: An immunohistochemical evaluation. World J Dent 2016;7(2):87-91.
11. Bruun Rasmussen B, Norring Hjort K, Mellerup I, Sether G, Christensen N. Vegetable oils instead of xylene in tissue processing. APMIS 1992;100(9):827-31.
12. Swamy SR, Nandan SR, Kulkarni PG, Rao TM, Palakurthy P. Bio-friendly alternatives for xylene - Carrot oil, olive oil, pine oil, rose oil. J Clin Diagn Res 2015;9(11):ZC16-8.