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ORIGINAL RESEARCH

Mineral Oil—A Biofriendly Substitute for Xylene in Deparaffinization: A Novel Method

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ABSTRACT

Background: In routine histopathology, xylene has been used in tissue processing and staining. Presently health hazards of xylene are well documented and a safer substitute is an absolute necessity.

Aim: The present study was conducted to evaluate the efficacy of mineral oil as a deparaffinizing agent when compared to that of xylene by using hematoxylin and eosin (H&E) staining.

Materials and methods: Thirty paraffin-embedded tissue blocks were randomly selected and two sections were taken from each block. Thirty sections were stained with conventional H&E method (group A) using xylene as deparaffinizing agent and 30 were stained with xylene free method using refined mineral oil (group B). Sections were blinded and analyzed by two pathologists using the parameters of uniformity, clarity and intensity of nuclear and cytoplasmic staining respectively (satisfactory = score 1, unsatisfactory = score 0). Score ≥ 4 was considered to be adequate for diagnosis.

Results: 100% of sections in group A and 93.3% of sections in group B were adequate for diagnosis (p-value 0.150).

Conclusion: The study recommends refined mineral oil as a biofriendly and effective xylene substitute in deparaffinization of tissue sections.

Keywords: Deparaffinization, Hematoxylin and eosin, Refined mineral oil, Xylene substitutes, Xylene free deparaffinization.

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INTRODUCTION

Pathology laboratory being the hub of various activities which involves translational aspects of histopathology and cytopathology into confirmatory diagnosis exposes the technicians and research workers to an array of hazardous chemicals that pose great threat to health. One such commonly encountered chemical is xylene. It is an aromatic hydrocarbon widely used in tissue processing as a clearing agent and as a deparaffinizing agent in staining and mounting. Maximum handling and exposure occurs during deparaffinizing of tissue sections.[1] OSHA (Occupational Safety and Health Administration) regulation identifies xylene as a hazardous substance[2] and the quest for nontoxic substitutes thus began.

Despite knowing the demerits of xylene, the preparatory steps in routine hematoxylin and eosin (H&E) staining procedure mandates the use of xylene. Thus, xylene being used since ages in laboratories till today reflects our failed attempt in finding a safer substitute. Green chemistry is a challenging arena which explores safer and environmentally friendly alternatives to conventionally used toxic chemicals. Thereby, we explored the feasibility of commonly available refined mineral oil (RMO) as a substitute for xylene in deparaffinization.

Aim of our Study

Comparison of efficacy of refined mineral oil and xylene as deparaffinizing agents using H&E stain.

MATERIALS AND METHODS

Thirty paraffin-embedded tissue blocks were randomly retrieved from the archival collection of the Department of Oral Pathology, MS Ramaiah Dental College and Hospital, Bengaluru. Two sections were cut from each block and one was subjected to routine H&E staining using xylene as deparaffinizing agent (group A). The other section was subjected to H&E staining using RMO as deparaffinizing agent (group B). The conventional staining protocol is elaborated in Table 1, the alternate staining protocol using RMO as deparaffinizing agent is given in Table 2. The slides were blinded and the quality of stained sections was

Table 1: Conventional staining protocol with xylene as deparaffinizing agent

Procedure	*Materials used*	*Time*
Deparaffinization and rehydration	Xylene-I	10 mins
	Xylene-II	10 mins
	70% alcohol	5 mins
	60% alcohol	5 mins
	Water wash	10 mins
Nuclear staining	Harris hematoxylin	7 mins
	One dip in tap water and one dip in 1% acid alcohol	
	Running tap water	10 mins
Cytoplasmic staining	Eosin	
	One dip in tap water	2 mins
Dehydration	60% alcohol	1 min
	70% alcohol	1 min
	Xylene	1 min
Mounting	3 mins	
	Approximate time needed	65 mins

interpreted by two pathologists using the criteria given in Table 3. Based on the parameters, the maximum score a slide could obtain was 6. A score of ≥ 4 was considered as adequate and <4 was considered as inadequate for diagnosis. Chi-square test was used to analyze the data. Interobserver variability was calculated using kappa statistics.

RESULTS

In the present study the efficacy of RMO as deparaffinizing agent was evaluated. The scores for uniformity and clarity of cytoplasmic staining ('p' value 0.448 and 0.688 respectively) and clarity of nuclear staining ('p' value 0.688) among conventional method and the novel method were equivalent. But, intensity of nuclear and cytoplasmic staining ('p' value 0.02 and 0.006 respectively) and uniformity of nuclear staining ('p' value 0.038) were decreased with RMO deparaffinization method. Overall the

Table 3: Criteria for evaluation

Parameters	*Scores*	
Cytoplasmic staining	1. Uniformity 2. Clarity 3. Intensity	For each parameter if the results are satisfactory, the score is 1 If unsatisfactory, the score is 0
Nuclear staining	1. Uniformity 2. Clarity 3. Intensity	Total score of ≥4 considered as adequate for diagnosis Total score of <4 considered as inadequate for diagnosis

H&E staining quality of sections using RMO as deparaffinizing agent was at par with that of xylene ('p' value 0.150). Kappa value of 0.792 indicated substantial interobserver agreement. The results are summarized in Graphs 1 and 2. Figures 1 and 2 show the tissues stained with H&E using xylene and RMO as deparaffinizing agents respectively.

DISCUSSION

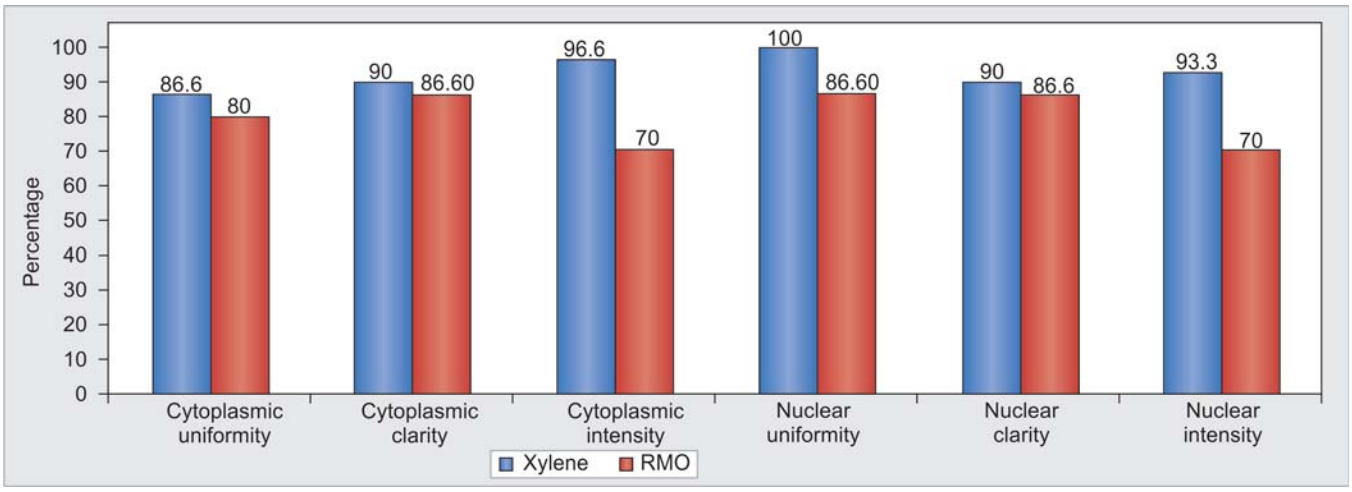
Xylene is an aromatic hydrocarbon with chemical formula of $C_6H_4(CH_3)_2$ i.e. 'dimethylbenzene'. It is a colorless, sweet-smelling liquid or gas occurring naturally in petroleum, coal and wood tar. Xylene is so named because it is found in crude wood spirit (in Greek xylon means 'wood').[3] Laboratory-grade xylene is composed of m-xylene (40-65%), p-xylene (20%), o-xylene (20%) and ethyl benzene (6-20%) and traces of toluene, trimethyl benzene, phenol, thiophene, pyridine and hydrogen sulphide.[3] Xylene has been declared as hazardous by OSHA and the permissible exposure limit is 100 ppm as an 8-hour time-weighted average (TWA) concentration.[2] The various toxic effects of xylene in humans are collated in Table 4.

Xylene is considered as a hazardous waste too (under RCRA: Resource Conservation and Recovery Act).[2] Besides

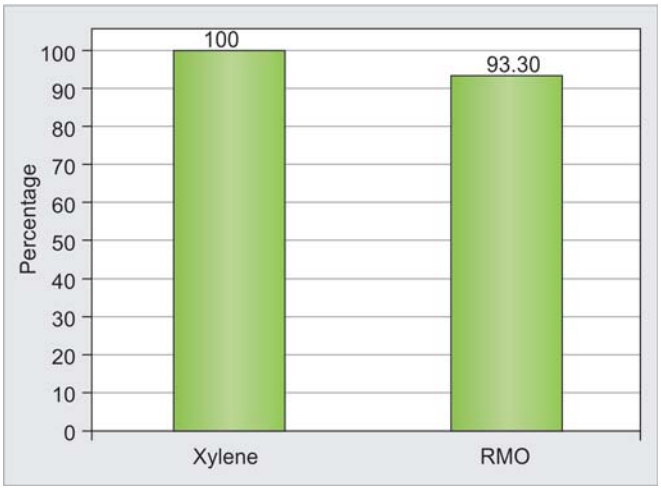
Table 2: Standardized alternate procedure using RMO as deparaffinizing agent

Procedure	*Material used*	*Temperature***	*Time*
Deparaffinization and rehydration	RMO-I	90°C	2 mins
	RMO-II	90°C	2 mins
	Distilled water-I	90°C	2 mins
	Distilled water-II	90°C	2 mins
	Wash slides in distilled water	45°C	1 min
	Wash slides in distilled water	RT*	1 min
Nuclear staining	Harris hematoxylin	RT	15 mins
	One dip in tap water and one dip in 1% acid alcohol		
	Running tap water		5 mins
Cytoplasmic staining	Eosin	RT	2 mins
	One dip in tap water		
Dehydration	Dry the sections in air		10 mins
Mounting			3 mins
Approximate time required			45 mins

*RT: Room temperature; **The fluids were heated in stainless steel containers using an electric stove and the temperature was measured using a standard mercury thermometer.



Graph 1: Comparison of quality of H&E staining between group A (xylene as deparaffinizing agent) and group B (RMO as deparaffinizing agent)



Graph 2: Overall H&E staining quality using xylene and RMO as deparaffinizing agents

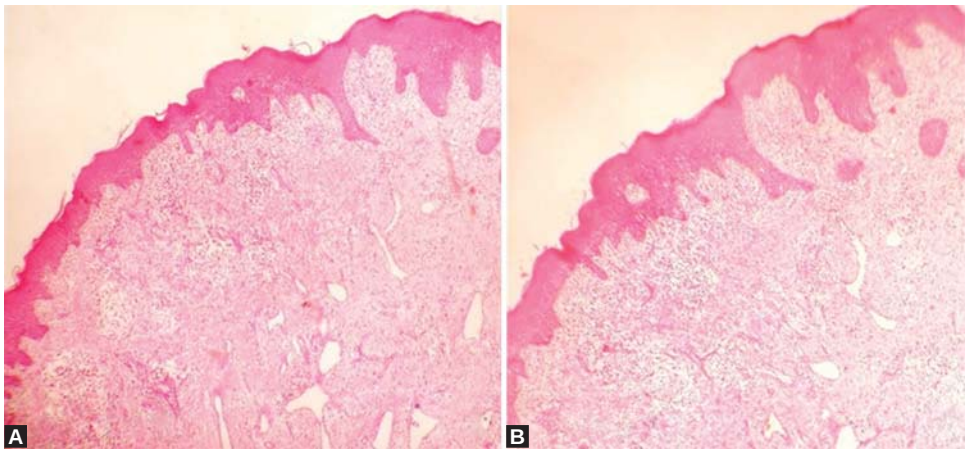
occupational exposure, the principal pathway of human contact is via soil contamination. Xylene can leak into the soil, surface water or ground water and it may remain there for months. However as xylene evaporates easily, most of it goes into the air and gets broken down by sunlight into other less harmful chemicals.[3] It is approved for land disposal as long as the concentration of xylene in the waste or treatment residual does not exceed 28 mg/kg. Also, xylene may be disposed off in an organometallic or organic lab pack.[2]

With this background, a substitute for xylene has become a dire necessity. Thus, we explored the feasibility of commonly available RMO (commercial nonstick hair oil with 80%-RMO, 20%-coconut oil) as a xylene substitute. RMO is generally used in baby lotions, cold creams, ointments, hair conditioners, nonstick hair oil, laxatives, in livestock vaccines as an adjuvant, as overlay covering of culture medium in petri dishes. It is easily available, non hazardous hydrocarbon. This colorless, odorless aliphatic hydrocarbon is produced from distillate of petroleum. The comparison of properties of xylene and RMO are collated in Table 5. To standardize the alternate procedure, efficacy of RMO for deparaffinization was checked at 60°C, 70°C, 80°C and 90°C. It was observed that rate of deparaffinization increased with increase in temperature and optimal results were obtained at 90°C. The standardization protocols are summarized in Table 6.

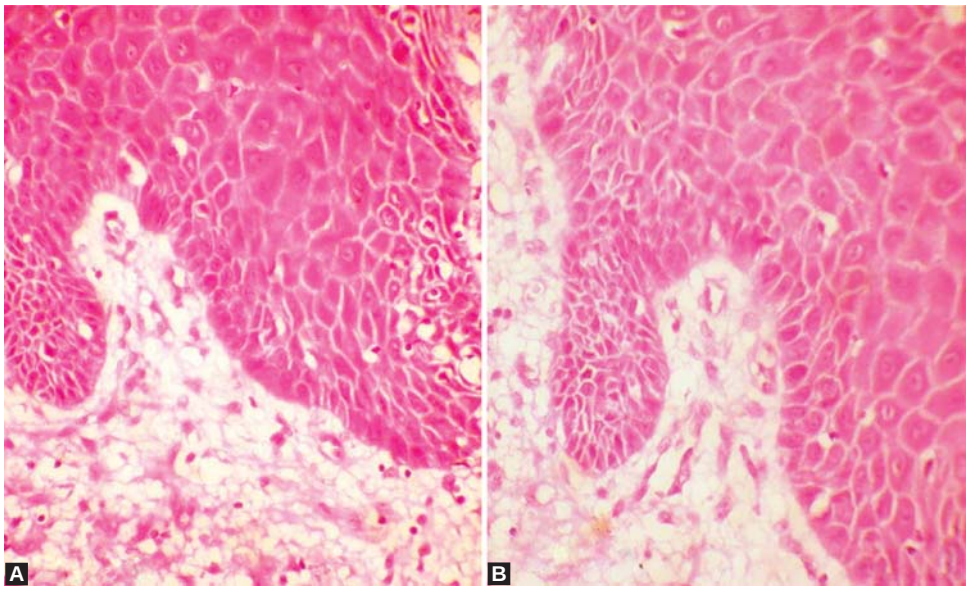
We also checked for stability of H&E stain with the alternate method over a period of 6 months and found it to

Table 4: Toxic effects of xylene on various organ systems

Organ system	*Toxic effects*
CNS	CNS depression, headaches, irritability, depression, insomnia, agitation, extreme tiredness, tremors, impaired concentration and short-term memory[3]
Respiratory system	Chest pain, shortness of breath (exposure ≥ 200 ppm), pulmonary edema (over exposure at a confined space)[4]
Liver and kidney	Liver and kidney injury[3] (>300 ppm causes fat deposition in liver and kidney in rats)[5]
GIT	Nausea, vomiting and gastric discomfort[3]
Musculoskeletal system	Reduced grasping power and muscle coordination in extremities[3]
Skin	Contact dermatitis, flaking and cracking of skin[3]
Eye	Damages surface layer of eye[3]
Reproductive system	Reaches developing fetus and contaminates breast milk (fetotoxic effects like delayed ossification and behavioral effects in animals noted)[3]
Cancer	Inadequate evidence[3,4]



Figs 1A and B: Tissue stained with H&E (4×) using xylene (A) and RMO (B) as deparaffinizing agents



Figs 2A and B: Tissue stained with H&E (40×) using xylene (A) and RMO (B) as deparaffinizing agents

Table 5: Comparison of properties of xylene and RMO[6]

	Xylene	*RMO*
Synonym	Dimethylbenzene	Liquid paraffin
Chemical structure	Aromatic hydrocarbon	Aliphatic hydrocarbon
NFPA rating (0-4)*		
Health	2	0
Fire	3	1
Reactivity (Chemical)	0	0
Exposure limit (TWA ppm)	100 ppm	No limit
Flammability	Flammable	Slightly flammable
Ignition	Readily	Not readily
Solubility in water	Insoluble	Insoluble
Solubility in alcohol	In absolute alcohol	Insoluble
Density (gm/ml)	0.86	0.82-0.88
Refractive index	1.50 (20°C)	1.468 (20°C)
Melting point	–25°C	–15°C
Boiling point	135-145°C	260-316°C

*NFPA: National fire protection association; 0: Minimal hazard; 1: Slight hazard; 2: Moderate hazard; 3: Serious hazard; 4: Severe hazard

be unaltered and stable. There are certain noted advantages of using RMO. Firstly paraffin wax can be dissolved in mineral oil completely.[7] Solubility of solid paraffin in hydrocarbons like mineral oil increases rapidly with increase in temperature.[8] Secondly; it can be used as clearing agent, as the density of mineral oil is closer to that of average

Table 6: Standardization protocols of H&E staining using RMO as deparaffinizing agent

Procedure	*Materials used*	*Protocol 1 temperature*	*Protocol 2 temperature*	*Protocol 3 temperature*	*Protocol 4 temperature*	*Time*
Deparaffinization	RMO-I	60°C	70°C	80°C	90°C	2 mins
	RMO-II	60°C	70°C	80°C	90°C	2 mins
	Distilled water-I	60°C	70°C	80°C	90°C	2 mins
	Distilled water-II	60°C	70°C	80°C	90°C	2 mins
	Wash in distilled water	45°C	45°C	45°C	45°C	1 min
	Wash in distilled water	RT*	RT	RT	RT	1 min
Nuclear staining	Harris hematoxylin	RT (7 mins)	RT (10 mins)	RT	RT	15 mins
	One dip in tap water and one dip in 1% acid alcohol					
	Running tap water					5 mins
Cytoplasmic staining	Eosin	RT	RT	RT	RT	2 mins
	One dip in tap water					
Dehydration	Air drying of the sections					10 mins

*RT: Room temperature

Table 7: Advantages of RMO over xylene

	Xylene	*RMO*
Health risk	Hazardous	Nonhazardous
Personal protective equipment	Required	Not required[6]
Cost	Costly (₹ 456/1,000 ml)	Cost effective (₹ 340/1,000 ml)
Disposal	Difficult	Easy; mix it with used paraffin and incinerate[6]
Need of alcohol in staining procedure	Needed	Not needed
Quality of staining	Good	Good
Compatibility with advanced techniques like PCR	Good	Good[7]
Time needed for staining	65 mins	45 mins

density of human fat when compared to that of xylene. This allows mineral oil to remove tissue fat by displacement rather than by dissolution as xylene does.[6] Also alcohol mediated rehydration and dehydration was not needed in this novel method; thus bringing down the cost drastically. Time taken for total staining procedure was less with mineral oil (45 minutes) when compared to the conventional method (65 minutes). According to Jianghai et al mineral oil can even be used for deparaffinization in high quality genomic DNA extraction from formalin-fixed and paraffin-embedded samples using PCR.[7] The advantages of RMO over xylene are summarized in Table 7.

Other deparaffinizing agents in the literature are hexane,[9] dish wash solution[10] and propylene glycol methyl ether (PGME).[11] Commercially available xylene substitutes are histoclear and trilogy. However, hexane is hazardous to health and 1.41 times costlier than xylene.[12] Dish wash solution was effective as deparaffinizing agent.[1] But its role as a clearing agent is questionable.

In a study conducted by RJ Buesa, mineral oil was employed as a clearing agent in tissue processing (pure and mixed with ethanol and isopropyl alcohol) with excellent results equivalent to processing tissue with xylene and also much safer to personnel and environment.[12] Scope for further studies involves evaluation of long-term stability of stained sections cleared and deparaffinized with RMO.

CONCLUSION

As pathologists the onus lies on our side to reduce the use of hazardous chemicals in histopathology laboratories without compromising the quality of diagnostic procedures. The staining quality provided by xylene free method, using RMO is equally effective as the conventional method. In addition to that, this method is safer, faster and cost-effective. The idea of using mineral oil as a xylene substitute is a small step toward a giant leap into the future of ecofriendly pathology laboratories!

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