Original Article

Evaluation and comparison of the efficacy of coconut oil as a clearing agent with xylene

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Abstract

Background: Xylene is a routinely used clearing agent in histopathology. It is potentially toxic and flammable in nature. Histotechnicians are routinely exposed to this hazardous chemical. Because xylene is used so pervasively in histopathology, it has always been a concern for pathologists and laboratory workers, as its regular and prolonged exposure have serious health effects. Considering its toxicity, different biocompatible xylene substitutes have been evaluated.

Aim and Objective(s): This study was conducted to evaluate the efficacy of coconut oil as a clearing agent and compare with xylene.

Materials and Methods: Two equal halves of 45 soft-tissue specimens were processed simultaneously in xylene and coconut oil as clearing agents. The xylene-treated specimens and coconut oil-treated specimens were checked for gross and histological features, and a comparison was done between the two groups.

Results: Significant shrinkage was noted in xylene-treated specimens compared to that in coconut oil-treated specimens. No difference was found in either of the sections when checked for staining quality, overall morphological features and cellular details.

Conclusion: It may be substituted for xylene without loss of information.

Keywords: Biocompatible, coconut oil, substitutes, xylene

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INTRODUCTION

The term "xylene" is a Greek word meaning "Wood" as it is found in crude wood spirit. It is an aromatic hydrocarbon (dimethylbenzene [$C_6H_4(CH3)_2$]) derived from petroleum, which occurs naturally in petroleum and coal tar and is also formed, to a small extent, during forest fires. It is a colorless, flammable liquid with a sweet odor.[1,2] Apart from its various uses, xylene is commonly used in histopathology laboratories as a de-alcoholization agent during tissue processing and in staining and mounting of tissue sections.[3] It has a high solvency factor that helps in maximum displacement of alcohol rendering the tissue transparent and thus enhances paraffin infiltration. In staining procedures, its excellent dewaxing and clearing capabilities contribute to brilliant staining of the tissue sections.[4] The physical/chemical characteristics of

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coconut oil are listed in Table 1.[5,6] Technical and commercial grades of xylenes often contain substantial amounts of ethylbenzene (10%–50%) and perhaps minor amounts of other solvents as well. Mixtures of xylenes and ethylbenzene are occasionally termed mixed xylenes. Most occupational exposure to xylenes also results in exposure to ethylbenzene.[1,2,7] Xylene was substituted as a safe alternative to other hazardous chemicals such as aniline oil, benzene, chloroform, dioxane and toluene in the histology laboratory in the 1950s. However, this proved to be a failure because of its toxicity which ranges from acute neurotoxicity, cardiac and kidney injury, cancer, blood dyscrasias, skin diseases, gastrointestinal disturbances, musculoskeletal system disorders, fetotoxicity and tissue distortions as a result of long-term immersion of tissue in xylene.[8] By the late 1970s, histopathologists started raising concerns about its safety, with evidence of its acute neurotoxicity being greater than that of benzene or toluene.[9] Since then, the search for a safe xylene substitute has been going on. With that aim, various xylene substitutes such as limonene reagents, aliphatic hydrocarbons, aromatic hydrocarbon mixtures, vegetable oils and mineral oils were tried in the past, but they did not receive much popularity as they were found to be either equally toxic or less effective or expensive.[3,4]

Coconut is available throughout the tropical world. Coconut oil is a commonly used vegetable oil, which is nontoxic and heat stable. It oxidizes slowly and has the highest resistance to rancidity.[10] This study was conducted to evaluate the efficacy of coconut oil as a clearing agent and compare with xylene.

MATERIALS AND METHODS

A total of 45 tissue specimens preserved in formalin were retrieved from the archives of the Department of Oral and Maxillofacial Pathology and selected for this study. Only soft tissue was considered for this study. The tissue specimens included in this study comprised of epithelial hyperplasia, hyperkeratosis, fibrous hyperplasia, dentigerous cyst, ameloblastoma, odontogenic fibroma and peripheral giant cell granuloma. The average specimen size was 0.5 cm × 0.5 cm × 0.3 cm. A thickness of 3–5 mm was taken for processing for better penetration of the processing fluids. A tissue specimen was divided into two equal halves [Figure 1]: during clearing, one was processed in (edible coconut oil of Patanjali brand available in market) and the other in xylene. Thus, Group A comprised of tissues cleared in coconut oil and Group B of tissues cleared in xylene. The duration of clearing was constant for both the solutions 2 hours in the same solution of coconut oil and 1 hour each in two changes of solution of xylene. The tissue specimens were measured before and after processing to check for shrinkage. Tissue sections of 4 μ were obtained and stained with hematoxylin and eosin (H&E) to permit evaluation of the histological details. The sections were evaluated under light microscopy.

After processing, the tissue specimens were subjected to gross evaluation and measured for any change in dimension. Sectioning was done, and ease of sectioning and presence of tissue folding in the slides were noted for each specimen. For ease of sectioning, a score was assigned on a scale of 1–5 (1 – poor, 2 – average, 3 – good, 4 – very good and 5 – excellent). Sections were stained, and each stained section was assigned a code. The sections were evaluated by two histopathologists who were blind to the procedures. The sections were evaluated under the light microscope for the following criteria: cellular outline, nuclear detail, staining quality and overall morphology. Each histomorphologic criterion was rated on a scale of 1–5 (1 – poor, 2 – average, 3 – good, 4 – very good and 5 – excellent). The data were subjected to statistical analyses; Mann–Whitney U-test and Spearman's correlation coefficient test were applied. The study was approved by the Institutional Ethics Committee.

RESULTS

There was no significant shrinkage in the tissue specimens after clearing in coconut oil. However, in comparison to xylene, there was a significant difference ($P < 0.0001$) with regard to the shrinkage in tissue [Table 2], which means that the xylene-treated tissue specimens shrank significantly.

The "ease of sectioning" was not found to be significantly different between the two groups [Table 3].

The presence of tissue folds in the slides was found to be significantly more in the xylene group as compared to coconut oil [Figure 2].

There was no difference in staining quality and tissue architecture in both kinds of specimens [Figures 3 and 4]. The comparison of the results of Observer 1 and Observer 2 on staining quality and cellular details that include cellular outline, nuclear detail and overall morphology showed that there is a significant correlation [Table 4]. This evaluation suggests that all preparations are equivalent to controls.

DISCUSSION

The specimen preparation influences the microscopic appearance of the biological material. The water present in tissue resists the entry of embedding media. Therefore,

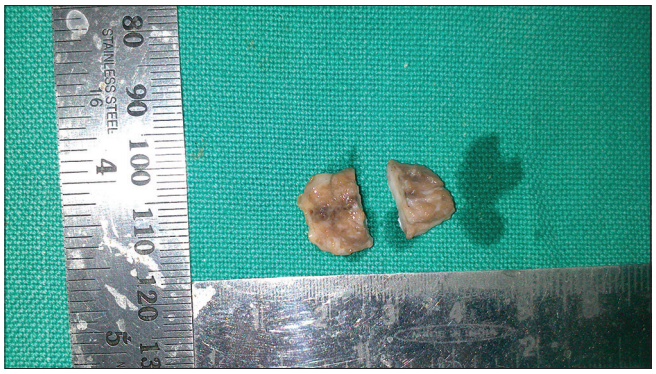

Figure 1: Each equal half of every tissue cleared in parallel solutions of either xylene or coconut oil

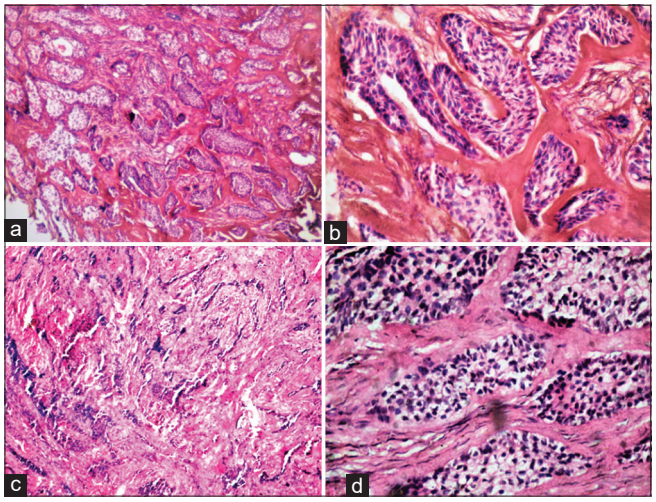

Figure 3: Histopathological image of odontogenic fibroma cleared in coconut oil (a [×4], b [×40]) and xylene (c [×4], d [×40])

Table 1: Physical and chemical properties of coconut oil[5,6]

Parameter	Coconut oil
Boiling point (°C)	Above 300
Melting point (°C)	23-26°C
Specific gravity	0.9
Self-ignition point (°C)	Above 250°C
Iodine number	7.5-10.5
Free acids (%)	<0.05
Water content (ppm)	100-200
Saponification value	250-254
Refractive index	Between 1.448 and 1.454

Table 2: Measurement of tissue specimens (length × width × thickness)

	Group	*n*	Mean	SD	*P*
Before tissue processing	Xylene	45	115.689	37.499	0.789 (NS)
	Coconut oil	45	112.667	65.508	
After tissue processing	Xylene	45	46.933	14.824	<0.0001 (S)
	Coconut oil	45	73.111	27.262	

P<0.05. S: Statistically significant, NS: Nonsignificant, SD: Standard deviation

this water needs to be removed from the tissue, which is usually achieved by the use of alcohol. Clearing, on the other hand, replaces this alcohol with a reagent that is

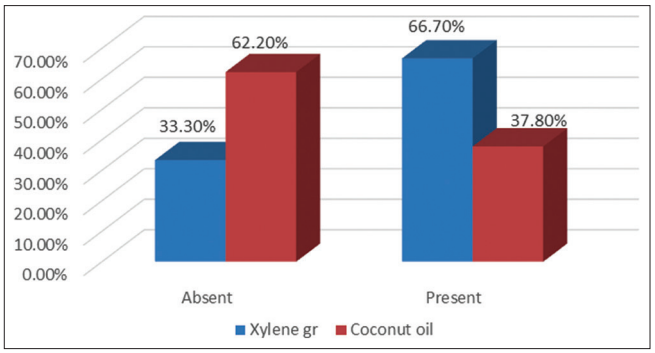

Figure 2: Presence of tissue folds in the slide

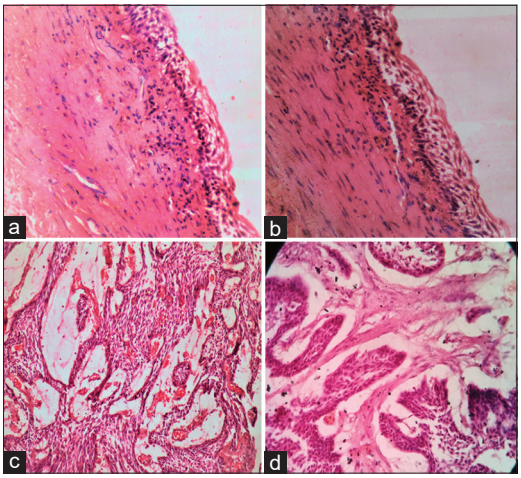

Figure 4: Histopathological image of dentigerous cyst (a [×20] cleared in coconut oil, b [×20] cleared in xylene) and ameloblastoma (c [×10] cleared in coconut oil, d [×20] cleared in xylene)

miscible with paraffin or other embedding media. Xylene continues to be used routinely as a clearing agent despite its known hazards.[11] Xylene substituted chloroform when its carcinogenic effects came to light, but when health hazards of xylene were identified, replacing it with safer substitutes has always been a concern.[9] From an environmental point of view as well, it is highly desirable to replace xylene with safer substitutes.[6]

Coconut oil or copra oil is extracted from the kernel or meat of the mature coconuts obtained from the coconut palm (*Cocos nucifera*). It slowly oxidizes because of its high saturated fat content and is thus resistant to rancidification. Coconut oil is a commonly used vegetable oil, available all over the tropical world. It is nontoxic and heat stable.[12]

In histopathology, it may find its use as a clearing agent replacing xylene. Studies on clearing tissues with coconut oil report promising results. The first study by Caxton-Martins *et al*. in 1987 used an oil obtained from the dried endosperm of coconut, known as adi agbon, and reported that it is a

good substitute for conventional clearing agents as tissues cleared in it appear transparent a property possessed by other clearing agents. Tissues can also be left in it for a long time without becoming brittle, as occurs with xylene or benzene.[5] Another study by Shokunbi *et al.* on adi agbon reported similar staining quality with normal tissue architecture and light microscopic details but found some tissue shrinkage in embryonic brain tissue. They concluded that it may be useful for routine work but not suitable for quantitative histological studies.[13] Sermadi *et al.* reported in their study that tissues cleared with coconut oil are found to be more translucent, less rigid (with no interference in impregnation and cutting) and demonstrate less shrinkage as compared to the tissues cleared with xylene.[3] In our study as well, we have found that there is less shrinkage in coconut oil-cleared tissues and there was no problem in sectioning.

Rasmussen *et al.* used olive oil for the clearing process and coconut oil for deparaffinization before staining. They found that in 91% of the cases, there were no differences in the quality of the two slides. The oil-prepared tissue was evaluated as identical to or better than the xylene-prepared tissue in 94% of the cases. They concluded that vegetable oil may be substituted for xylene without loss of information.[6] Andre *et al.* conducted a study with clearing and infiltration mixture (CIM) containing unsaturated oil, monosaturated oil and saturated oil with paraffin. They found that except breast tissue demonstrating noticeable challenges in microtomy and only occasional minor difficulties in embedding, microtomy and H-&E-staining procedures, paraffin alone or the experimental CIMs can be used as effective xylene substitutes and provide satisfactory processing of liver, brain and breast tissue stained with H&E.[14] In our study, we have found that coconut oil alone is an effective clearing agent.

Sermadi *et al.* reported in their study that there is no difference between the tissue groups as far as ease of sectioning is concerned. In our study, we found similar results but also noted that the paraffin blocks prepared by using coconut oil as a clearing agent had proper hardness and were easy to be cut into 4-μm serial sections.[3]

Table 3: Ease of sectioning

Group	*n*	Mean score	SD	*P*
Xylene	45	4.000	0.000	0.09 (NS)
Coconut oil	45	4.156	0.601	

P>0.05. NS: Nonsignificant, SD: Standard deviation

Table 4: Comparison of staining quality and cellular details

Group	Parameter	Spearman's correlation coefficient between Observer 1 and Observer 2	*P*
Xylene	Staining quality	0.661	<0.0001 (S)
	Cellular outline	0.604	<0.0001 (S)
	Nuclear detail	0.723	<0.0001 (S)
	Overall morphology	0.577	<0.0001 (S)
Coconut oil	Staining quality	0.892	<0.0001 (S)
	Cellular outline	0.882	<0.0001 (S)
	Nuclear detail	0.835	<0.0001 (S)
	Overall morphology	0.932	<0.0001 (S)

S: Statistically significant

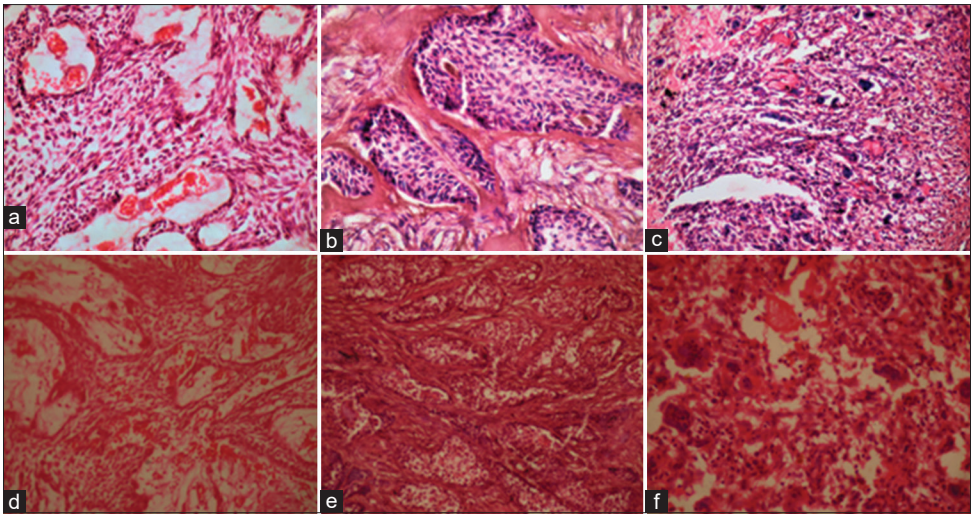

Figure 5: Histopathological image of ameloblastoma (a [×20] immediate, d [×10] after 6 months), odontogenic fibroma (b [×40] immediate, e [×20] after 6 months) and peripheral giant cell granuloma (c [×10] immediate, f [×20] after 6 months)

Assessment of the stained sections revealed a good maintenance of cell morphology and structure. Caxton-Martins *et al*. reported that following clearing in adi agbon, nuclear and cytoplasmic tissue constituents appear sharper, crisper and brighter following H&E staining.[5] We also observed the same findings. The cytoplasm and the nucleus were well stained in H&E, and they were sharper, crisper and brighter. The quality of stain, overall morphology, cellular detail including cellular outline and nuclear detail of the coconut oil-cleared tissues were as good as the xylene-cleared tissues. Sermadi *et al*. also reported that there was no difference in staining quality and tissue architecture in both kinds of specimens. They also performed periodic acid–Schiff staining and obtained similar details as seen in xylene-cleared tissues.[3]

The drawbacks associated with coconut oil are as follows. Coconut oil is 2.21 times more expensive than xylene.[9] However, vegetable oils are recyclable, which is an economical alternative to disposal and an effective cost-saving measure.[15] It has a tendency to get solidified at a lower temperature, which can, however, be overcome by performing the clearing procedure in an incubator, maintaining the required temperature.[3] We conducted the study during the summer days when the temperatures in North India were between 38°C and 45°C, and hence, we did not encounter the problem of solidification. However, we evaluated the slides after 6 months and what we noted was the leaching of the stain when the slides were stored at room temperature during the summer days [Figure 5]. Hence, their proper storage at proper temperature is very important.

The study has the following limitations. Our study had a small sample size, and further research can be performed with a larger sample size including various types of tissues. We did not perform any special stain procedure, and this study is unable to make any remark on that. While preserving the tissue blocks, there is still an unresolved risk that oil-processed tissue may become rancid. We have not evaluated the deterioration in tissue quality by making new sections from the preserved blocks.

CONCLUSION

Although our study suggests that a comparable staining result can be achieved in the coconut oil and xylene-processed specimens, the criteria for assessing it were subjective. We mainly focused in our study on the shrinkage of the tissue specimen, ease of sectioning, quality of the slides, staining quality, preservation of overall morphology, cellular and nuclear outlines and whether the definition of the cell structure was distinct. However, it is difficult to give a clear definition of the ideal appearance of a tissue section by using microscopes. Nevertheless, our study revealed that coconut oil can be used as a clearing agent. It may be substituted for xylene without loss of information, while recognizing that it may be necessary to investigate a broader range of tissues before making any general recommendation.

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