ORIGINAL ARTICLE

Comparing the efficacy of coconut oil and xylene as a clearing agent in the histopathology laboratory

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ABSTRACT

Background: The commonly used clearing agent, xylene is supposed to be highly toxic and carcinogenic. As previous research studies have shown the effectiveness of different vegetable oils as clearants, this study was designed to evaluate the efficacy of coconut oil. **Materials and Methods:** Two equal halves of 60 soft tissue specimens were processed simultaneously in xylene and coconut oil as clearing agents. The Xylene-treated specimens (XY-S) and Coconut oil–treated specimens (CO-S) were checked for gross and histological features and comparison was done between the two groups. **Results:** Significant shrinkage was noted in XY-S compared to that in CO-S. No difference was found in either of the sections when checked for cellular details and staining quality. Morphometrically, there was significant reduction in the mean cell area in XY-S compared to that in CO-S. **Conclusion:** Coconut oil may be substituted for the highly hazardous xylene as a clearing agent without compromising the quality of histological details.

Key words: Clearing agent, coconut oil, histopathology laboratory, vegetable oils

INTRODUCTION

Xylene (aromatic hydrocarbon) has been widely used as a de-alcoholization agent of choice, in spite of its toxicity to laboratory personnel and the danger it poses to the environment. The toxic effects of xylene include acute neurotoxicity, cardiac and kidney injury, cancer, blood dyscrasias, skin diseases, gastrointestinal disturbances, musculoskeletal system disorders, fetotoxicity and so on.[1-18] On account of the Occupational Safety and Health Administration (OSHA) regulations, various xylene substitutes, such as, limonene reagents, aliphatic hydrocarbons, vegetable oils and mineral oils were tried in the past to avoid xylene in the laboratory.[11,16,19-23] However, these substitutes were found to be less effective and more expensive. Coconut oil is a commonly used vegetable oil, available throughout the tropical world. It is non-toxic, heat stable, slow to oxidize and has highest resistance to rancidity.[24] In the present study, we have tried to compare the efficacy of coconut oil with that of xylene, as a clearant, as it is readily available, less expensive and a safer alternative to xylene.

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MATERIALS AND METHODS

Totally 60 tissue specimens were considered for this study. Each of the specimens was cut into two equal halves. The first half of the tissue bit was processed in xylene and the other half simultaneously in coconut oil [Figure 1]. The duration of clearing was constant for both the solutions (one hour each: Two changes). The tissue bits were measured before and after clearing, to check for shrinkage. After de-alcoholization, the specimens were also tested for gross changes after clearing. All the sections were stained with hematoxylin and eosin (H and E) to permit evaluation of the histological details. Few of the sections (salivary gland specimens) were subjected to periodic acid Schiff (PAS) also, in order to see whether coconut oil was interfering in this routinely used special staining procedure. The sophisticated technique of computer-assisted morphometry was performed, to observe the morphological features like the mean cell area, to see if any consistent change existed between the study groups.

Sample selection

Specimens for this study were selected from the anatomical structures in the head and neck region, such as, skin, buccal

mucosa, salivary gland, tendon, muscle and lymph node. The inclusion criteria were as follows: Only soft tissue was considered for this study. The specimen size was 0.5 × 1 cm or greater and a thickness of 3-5 mm was taken for processing (for better penetration of the processing fluids). The tissue was then divided into equal halves: During clearing, one was processed in coconut oil and the other in xylene.

Evaluation

Gross tissue specimen: After clearing in two different solvents, the gross tissue features, such as, translucency (surface translucency when viewed for reflected light), rigidity (palpation with two fingers), change after impregnation (change in the rigidity because of infiltration of wax) and ease in section cutting, were noted down for each specimen separately, for CO-S and XY-S. Scoring was done while comparing the parameters for both the agents: The finding of CO-S that was inferior to XY-S was considered as score 0, similar to XY-S as score 1 and superior to XY-S as score 2 [Table 1]. The tissue bits were measured just after clearing, to compare the gross-shrinkage for the two solvents [Figure 2].

Cellular architecture: (a) For cellular details, distinct architecture and good nuclear-cytoplasmic contrast is considered as score 1 and indistinct/blurred nuclear-cytoplasmic contrast as score 0. (b) For nuclear details, distinct chromatin condensation, prominent nuclear membrane and crisp staining of the nucleus is considered as score 1 and indistinct smudging and pyknosis of the nuclei as score 0.

Quality of staining: The staining of tissues was evaluated as poor, satisfactory and good. Poor indicated that the tissue failed to take up the stain adequately, stained unevenly (score = 0). 'Satisfactory' pointed toward details like not visualized up to the mark (score = 1). 'Good' designated good contrast between the nucleus and cytoplasm and visibility of details, along with brilliance of staining (score = 2).

Morphometric analysis: After reviewing, the sections were further subjected to morphometric analysis. The images were captured using a three-chip CCD camera attached to a trinocular research microscope with a 100X objective. The final image captured on the monitor had a magnification of 1000X. For each specimen, five most representative fields were selected.

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

The selected fields included representative cells where distinct cellular and nuclear outlines were seen, avoiding overlapping. A total of 100 cells (20 cells in five different high-power fields) were randomly selected and measured for any difference in the XY-S specimens and CO-S specimens. Histologically identifiable acini, adipocytes and epithelial cells in the para basal layer were subjected for measurement. The images were classified, transferred and stored in the computer. The actual measurements of the morphometric parameters were done using the image analyzer software Image-Proexpress (Media Cybernetics, Silver Spring, MD, USA). The cell area (CA) was measured in square microns when the perimeter was traced; the software automatically calculated the CA (number of pixels detected, converted to micrometers) [Figure 3].

As most of the evaluative criteria were subjective, the scoring and assessment was carried out by three different observers and the mean scoring was considered, which would prevent interobserver and intraobserver bias. The obtained data was subjected to statistical analysis using the Wilcoxon matched pair test and the Mann-Whitney U Test.

RESULTS

Most of the specimens (73%) were more rigid in XY-S when compared with CO-S. Although in 16 specimens, the rigidity was same in both the groups [Table 1]. Translucency was visibly better in all CO-S than XY-S [Table 1]. However, there was no difference observed in the tissue bits as far as rigidity after impregnation and ease of sectioning was concerned, in both the groups [Table 1]. There was no significant shrinkage in the tissue bits after clearing in coconut oil (P = 1.000).

Table 1: Comparison of gross features of CO-S with respect to XY-S

Sample	Score 0	Score 1	Score 2
Rigidity	73% (less rigid)	27% (rigidity is same)	0
Translucency	0	0	100% (more translucent)
Impregnation	0	100% (no change in impregnation)	0
Sectioning	0	100% (no change in sectioning)	0

Score 0: Inferior to xylene, Score 1: Equivalent to xylene, Score 2: Superior to xylene

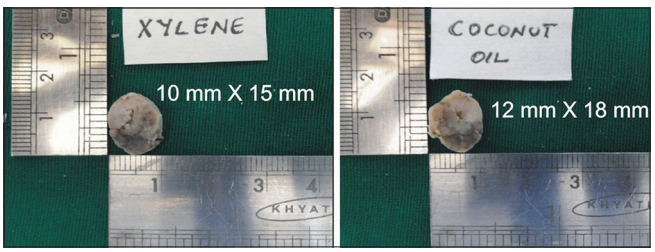

Figure 2: Measurements for gross tissue shrinkage

However, with respect to XY-S, the specimen shrank significantly, when compared with the measurements taken before clearing (P = 0.0117) [Table 2 and Figure 2]. There was no difference in staining quality and tissue architecture in both kinds of specimens [Table 3 and Figure 4 and 5A]. CO-S, when stained with Periodic acid-Schiff (PAS), showed similar details as seen in XY-S [Figure 5B]. Morphometrically, there was a significant decrease in the mean area of the individual cells in XY-S, compared to CO-S (P = 0.0006), [Table 4 and Figure 3].

DISCUSSION

Considering the toxicity of xylene and its hazards, various substitutes, including vegetable oils and mineral oils, have been tried in the past.[11,16,19-23] However, most of them showed an inconsistent outcome, which motivated us to take up this study. Coconut oil was selected, as it is, profusely available in the tropical world, especially in South Asia, it is less expensive and non-hazardous. When compared with xylene, it is not harmful to the environment.[24]

The results of the present study showed that CO-S, after clearing, was apparently more translucent compared to XY-S. Although less rigid in contrast to XY-S, it did not adversely affect impregnation and section cutting.

Morphometrically, the shrinkage was relatively less in CO-S when compared with XY-S, which was noted as a statistically significant difference in the mean cell area of individual cells between sections (P = 0.0006). However, there was no change in cellular, nuclear and cytoplasmic staining, when both groups were compared.

Buesa used a mixture of ethanol, isopropyl alcohol and mineral oil as an alternative for xylene and found the mixture to be as efficient as xylene in de-alcoholization.[19] Instead, we considered the environment-friendly, readily-available alternative, coconut oil, as we wanted to avoid chemicals such as ethanol and isopropyl alcohol, which were also

Table 2: Comparison of gross shrinkage after clearing

Groups	Area of the specimen	Mean	Std Dv	*P* value
Xylene-treated specimen	Before clearing	1.6361	0.4266	0.0117*S
	After clearing	1.4870	0.4396	
Coconut oil-treated specimen	Before clearing	1.6680	0.4303	1.0000
	After clearing	1.6690	0.4368	

*S: Statistically significant as P <0.05. Std Dv: Standard deviation

Table 3: Comparison of staining quality

Cellular architecture and staining	Score 0 (in percentage)	Score 1 (in percentage)
Cytoplasm	0	100
Nucleus	0	100
Quality	0	100

Score 0: Inferior to xylene, Score 1: Equivalent to xylene

Table 4: Morphometric analysis of mean area of individual cell

Groups	Mean area of individual cells	St Dev	*P* value
Xylene-treated specimen	5.2820	0.7014	0.0006*S
Coconut oil-treated specimen	7.0440	0.8983	

*S: Statistically significant as P <0.05. Std Dv: Standard deviation

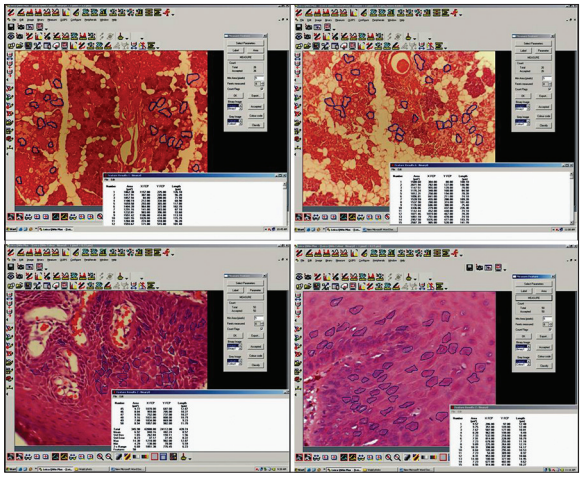

Figure 3: Morphometrical analysis of the mean area of an individual cell to assess shrinkage at the cellular level

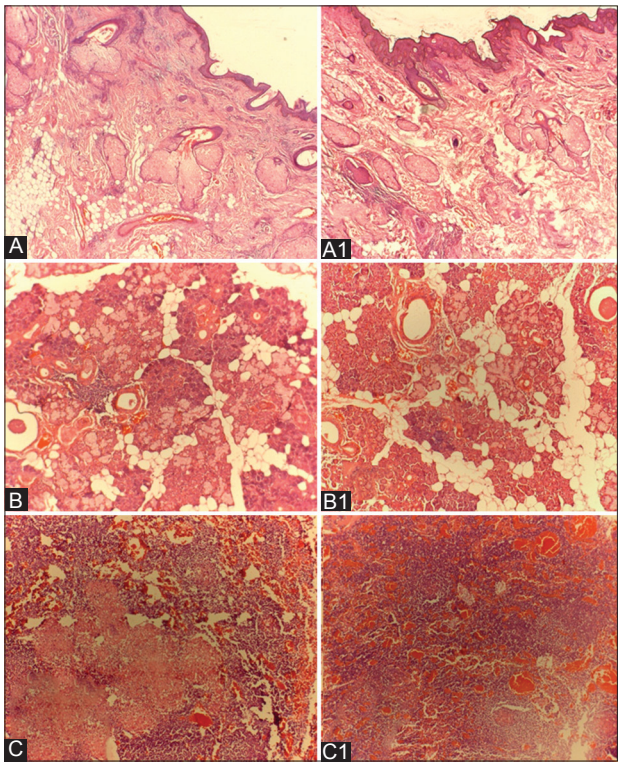

Figure 4: A,B,C — Hematoxylin and Eosin stained tissue sections of Xylene-treated specimen. A1, B1,C1 — Hematoxylin and Eosin stained tissue sections of Coconut oil–treated specimen. [A and A1 — skin tissue, B and B1 — salivary gland tissue, C and C1 — lymph node tissue]

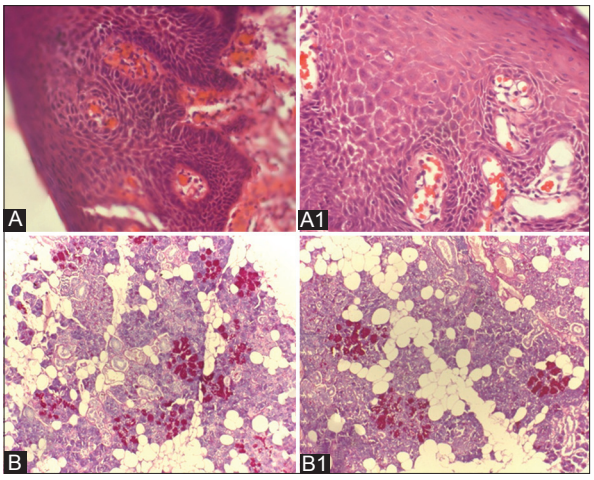

Figure 5: A, B — Xylene treated specimen. A1, B1 — Coconut oil–treated specimen. [A and A1 — oral epithelium; B and B1 — salivary gland stained with PAS]

hazardous. A mixture of coconut oil and olive oil was tried by Rasmussen *et al.* and they noted incomplete impregnation, leading to problems in the cutting sections and therefore, they concluded that this mixture was ineffective as a clearing agent.[20] In contrast to their observation, we found that CO-S, when used alone, was as effective as xylene, without interfering with further impregnation and cutting. This difference could be because of the olive oil in the mixture, which would have adversely affected the procedure, counteracting with the favorable properties of coconut oil. Instead, there was increased translucency and less rigidity.

A study by Andre *et al.*[23] substituted xylene with a mixture of peanut oil, soyabean oil, coconut oil and cotton oil and concluded that it was a poor alternative, as the quality of sections with respect to XY-S were better. The present study showed sections with similar cellular architecture and better staining quality. Even the special staining procedure showed good results, proving no interference by coconut oil with the tissue composition and it just acted as a transient media. As the result of our study showed less shrinkage in CO-S, compared to XY-S, we would suggest that this would be a preferred procedure, where morphometric studies have to be carried out. The only drawback associated with coconut oil, is its tendency to get solidified at a lower temperature. However, this can be overcome by performing the clearing procedure in an incubator, maintaining the required temperature. This research study is unique, as we have tried to assess the efficacy of two clearing agents at different stages of the histopathological procedure, such as, processing, impregnation, sectioning, staining and microscopic evaluation, including morphometry.

CONCLUSION

The results of the present study infer that coconut oil is an efficient substitute for xylene, as it is non-hazardous, less expensive and causes less shrinkage of the tissue. It can be used as a de-alcoholization agent in the histopathological laboratory, without losing the quality of the histological details. All the xylene-substitutes have to be analyzed thoroughly, before concluding which alternative is better. Further research in this area is expected, where the coconut oil–treated specimen can be subjected to all stains and advanced histological procedures (like immunohistochemistry), in order to consider this agent as a better and safer substitute for xylene.

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