ORIGINAL ARTICLE

Bleached vegetable oil as a suitable bio-safe alternative to xylene: An exploratory study

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Abstract

Background: Xylene is used as a clearing and dewaxing agent during routine tissue processing in histopathology laboratories. Despite its excellent clearing property, xylene is associated with adequate amount of toxicity. Therefore, various substitutes for xylene such as mineral oil and vegetable oil mixtures have been explored and are well documented in English literature.

Aims and Objectives: This study had aimed at assessing the efficacy of bleached vegetable oil as a clearing and a dewaxing agent. The objectives were to compare the clearing ability of bleached vegetable oil with that of xylene.

Materials and Methods: A total of 12 normal oral mucosal samples (n = 12) were cut into pairs forming two groups - Group A tissues (n = 12) were processed in xylene and Group B tissues (n = 12) were processed in bleached vegetable (palm) oil. The parameters such as transparency of the tissues, nuclear and cytoplasmic staining were assessed between the two groups.

Results: Tissues from both the study groups were transparent and had yielded good serial sections; adequate nuclear and cytoplasmic staining was observed in 91.7% of sections treated with bleached palm oil.

Conclusion: Bleached vegetable oil may be used as a safer substitute for xylene in tissue processing.

Introduction

Xylene is a routinely used chemical in histopathology laboratories. Xylene finds application as a dealcoholization agent during tissue processing and as a dewaxing agent during staining. Other uses of xylene are before cover slipping, as a solvent to free the microscope objective from synthetic immersion oil, in cleaning tissue processors, and in recycling of used glass slides.[1]

Xylene is naturally found in petroleum, coal, and wood tar. It is used as a liquid which has a sweet smell. The sweet smell of xylene is deceiving, considering its level of toxicity to the handling personnel as well as to the environment.

The ill effects of xylene have been reported on various systems of the body such as central nervous system, respiratory system, reproductive system, and so on.[2]

Despite the toxic effects of xylene, it has been used as a popular clearing and dewaxing agent for the past several years. There are striving attempts reported in English literature, searching for bio-safe alternatives for this sweet-smelling toxic substance; Mineral oil mixture, vegetable oil, cedar wood oil, and liquid dish washing soap are a few to name.[3-9]

Vegetable oils like palm oil are saturated fat that exist as semisolids at room temperature.[10] Conversion of a vegetable or plant-based food into oil involves complicated chemical process. The process of bleaching is the physical and chemical interaction of a fat or oil with various materials, to improve its quality. Bleaching of a vegetable oil involves various mechanisms and adsorption is one, concerned with the removal of colored particles. The temperature also has an effect on bleaching. The temperature affects viscosity of the oil and adsorption kinetics.[11-13]

Only one study has been reported in English literature which had adopted bleached palm oil as an alternative to xylene in processing of bird tissues.[14]

Any experiment reducing health hazards in histology laboratories definitely deserves to be tried. Therefore, this study had aimed to compare the efficacy of a bleached vegetable oil with that of xylene in human oral mucosal tissue processing.

Materials and Methods

The study was conducted on 12 normal oral mucosal tissue samples (n = 12) which were obtained from the patients undergoing minor oral surgical procedures (from the Departments of Oral and Maxillofacial Surgery, and Periodontics).

The oral mucosal sample with inflammation; patients with systemic diseases and allergic conditions and tissue specimens measuring <0.75 cm were excluded from this study.

Written informed consent was taken from the patients and clearance was obtained from the institutional ethical board. The confidentiality of case details procured for the study purpose was maintained.

The biopsied normal oral tissues were cut in pairs, forming two groups - Group A and Group B. Group A tissues (n = 12) were processed with xylene as a clearing agent and Group B tissues (n = 12) were processed with bleached vegetable (palm) oil as a clearing agent.

The armamentarium for the study included: 10% neutral buffered formalin, NO. 3 sterile BP handle and No. 15 BP blade, ethanol, xylene, bleached palm oil, paraffin wax, plastic cassettes, plastic embedding rings, metal molds, semiautomatic soft tissue microtome (MICROM MODEL HM340E), tissue embedding center (MICROM MODEL EC350), frosted clean glass slides and suitable adhesive, hematoxylin and eosin (H & E) stains, and Research microscope (Olympus BX51).

Methodology (for Group A tissue processing and staining)[15,16]

After formalin fixation, Group A tissues were dehydrated through ascending grades of ethyl alcohol (70%, 90%, and 100%) for 1 h in every solution. The dehydrated tissues were dealcoholized (cleared) using two changes of xylene for 1 h each. For Group B, the tissues were dehydrated through ascending grades of ethanol (70%, 90%, and 100%) for 1 h in every solution. The dehydrated tissues were dealcoholized (cleared) using two changes of preheated bleached vegetable oil (in hot air oven) for 1 h each.

The bleaching of palm oil was carried out by heating the oil between 55°C and 60°C for 20-30 min.

For both the study groups, the tissues were checked for transparency - the end point of clearing by macroscopic observation.

The cleared tissues (of Group A and Group B) were infiltrated in molten paraffin wax. Then the tissues were embedded in molten paraffin wax using plastic embedding rings and metal molds and they were allowed to solidify before microtomy. The tissue blocks were all sectioned at 4-5 μm with a semiautomatic soft tissue rotary microtome; the degree of ease of sectioning was made a note for both the study groups. The tissue sections were then floated in a warm water bath and each section was picked up in pairs on albuminized glass slides.

For Group A, the slides were dewaxed in xylene for 30 min.

For Group B, the tissue sections were dewaxed in preheated bleached palm oil at 60°C for 15 min. Excess oil was drained off from the slides by making them stand upright for 1 min and the slides were rinsed in 2 changes of 2% dish washing soap solution preheated at 60°C for 2 min each, to degrease the sections before rinsing in water.

The slides (for Group A and Group B tissue sections) were then passed through descending grades of ethanol (100%, 90%, and 70%) and were rinsed in water. The hydrated sections were stained in Harris hematoxylin solution for 10 min and were rinsed in water. The tissue sections were differentiated in acid alcohol for 4-5 s followed by water wash for 15-20 min under running tap water (Bluing).

For Group A tissues, the sections were counterstained with eosin for 5 min, dehydrated through graded concentrations of alcohol, cleared in xylene, and mounted with DPX.

For Group B tissues, the sections were counterstained in 1% eosin solution for 1 min, rinsed in water, air dried and mounted in DPX mounting medium.

Wherever required the procedures were done with strict aseptic precautions.

The histological slides prepared by using xylene and by bleached palm oil as clearing agents were coded and then were observed under the research microscope. Three oral pathologists had participated in the study as observers. The slides were viewed under low power (×100) and high power (×400) magnifications and photomicrographs were then captured. The findings were tabulated for further analysis and interpretation.

Results

In this study, 12 human oral mucosal biopsy tissues had been considered (n = 12), which were grossed into pairs and divided as Group A (12 tissues cleared by xylene) and Group B (12 tissues cleared by bleached palm oil). The macroscopic observation showed that after clearing, all the tissues (100%) in both Group A and Group B were found to be transparent. Sectioning test showed ease of sectioning with ribbon formation in all the tissues (100%) from both the study groups. Overall clarity of the sections was compared between two groups; it was 100% for xylene treated sections and 88.80% for bleached palm oil treated sections [Table 1].

The slides were reviewed by three observers. Nuclear staining was adequate (meaning distinctness of the features with interpretation as satisfactory and good) in 100% sections in Group A [Figure 1] and in 91.7% sections in Group B [Table 2 and Figure 2]. Adequacy (meaning distinctness of the features with interpretation as satisfactory and good) of cytoplasmic staining was noticed in 100% sections in Group A [Figure 1] and in 91.6% sections in Group B [Table 3 and Figure 2].

Discussion

Xylene is very popular in its use as a clearing and a dewaxing agent during routine tissue processing. Xylene is a synthetic hydrocarbon well known for its toxic effects not only to the personnel but also to the environment. The metabolites of xylene are known to cause toxic effects on various systems of the

Table 1: Comparison of the overall clarity of the tissues between 2 study groups by 3 observers using Chi-square test

Clearing agent	Grading	Observer-1 (%)	Observer-2 (%)	Observer-3 (%)	Total (%)	$\chi 2$ value	*P*-value
Xylene	Good	12 (100)	12 (100)	12 (100)	36 (100)		
Palm oil	Poor	1 (8.30)	1 (8.30)	2 (16.70)	4 (11.10)	3.000	0.56
	Satisfactory	5 (41.70)	4 (33.30)	7 (58.30)	16 (44.40)		
	Good	6 (50)	7 (58.30)	3 (25)	16 (44.40)		

Table 2: Comparison of the nuclear staining property between 2 study groups by 3 observers using Chi-square test

Dewaxing agent	Grading	Observer-1 (%)	Observer-2 (%)	Observer-3 (%)	Total (%)	$\chi 2$ value	*P*-value
Xylene	Good	12 (100)	12 (100)	12 (100)	36 (100)		
Palm oil	Poor	0 (0)	1 (8.30)	2 (16.70)	3 (8.30)	3.533	0.47
	Satisfactory	5 (41.70)	7 (58.30)	6 (50)	18 (50)		
	Good	7 (58.30)	4 (33.30)	4 (33.30)	15 (41.70)		

Table 3: Comparison of the cytoplasmic staining property between 2 study groups by 3 observers using Chi-square test

Dewaxing agent	Grading	Observer-1 (%)	Observer-2 (%)	Observer-3 (%)	Total (%)	$\chi 2$ value	*P*-value
Xylene	Good	12 (100)	12 (100)	12 (100)	36 (100)		
Palm oil	Poor	0 (0)	1 (8.30)	2 (16.70)	3 (8.30)	4.757	0.31
	Satisfactory	5 (41.70)	4 (33.30)	7 (58.30)	16 (44.40)		
	Good	7 (58.30)	7 (58.30)	3 (25)	17 (47.20)		

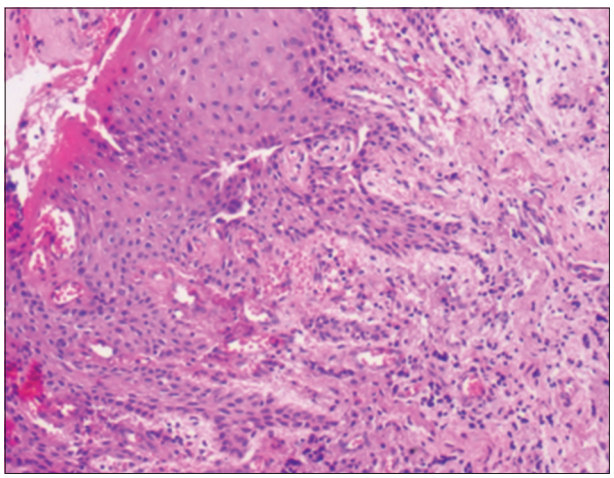

Figure 1: Photomicrograph of a tissue section using xylene as a clearing agent (hematoxylin and eosin, ×40)

body.[1,2] Owing to this, there has been a continuous search for bio-safe substitutes for xylene.[3-9]

Any attempt to reduce the health hazards in histopathology laboratories provides scope for a trial. Hence, this study was attempted to assess the role of bleached vegetable oil (palm oil) as a suitable alternative to xylene in processing of oral mucosal tissues.

A total of 12 human oral biopsy tissues (n = 12) had constituted the study material, which was subdivided into two groups; Group A tissues (12) were processed in xylene and Group B (12) in bleached palm oil.

All the tissues treated by xylene and by bleached palm oil had become transparent and were easy to section during microtomy. Three oral pathologists had participated in the study as observers. After completion of H and E staining protocol for tissue sections of both the study groups, the slides were reviewed under the research microscope. Nuclear staining was adequate in 100% sections of xylene treated tissues [Figure 1] and was 91.70% in bleached palm oil treated tissue sections [Table 2 and Figure 2]. The adequacy of cytoplasmic staining was 100% in xylene treated tissue sections [Figure 1] and was 91.6% in bleached palm oil treated tissue sections [Table 3 and Figure 2].

The overall clearing of the tissues by xylene as checked by the observers was 100% and was 88.8% for bleached palm oil [Table 1].

The observations of this study indicate that bleached palm oil has clearing property similar to that of xylene. The various factors attributed to this clearing mechanism of bleached palm oil could be: First, the matching of refractive index of bleached palm oil (1.45 at 50-55°C) with that of the tissue proteins (varies from 1.3 to 1.4); this reduces the scattering of light and increases the optical clearance of the tissue. The second factor could be dissolution of tissue fats under higher temperature (55-60°C) followed by displacement of the alcohol by the bleached palm oil thereby ensuring tissue clearing. The third factor could be the raised temperature itself allowing easy diffusion of alcohol out of the tissue and penetration of the oil into the tissue with less difficulty.

Commenting on quality of staining in bleached palm oil treated sections, nuclear staining was adequate in 91.7% sections and cytoplasmic staining in 91.6% sections, respectively. Immersion

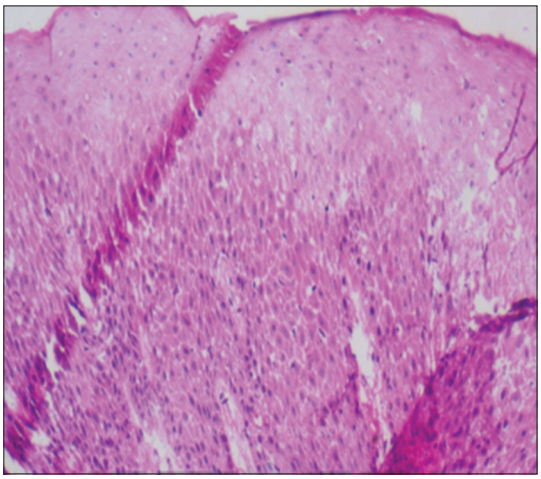

Figure 2: Photomicrograph of a tissue section using bleached palm oil as a clearing agent (hematoxylin and eosin, ×40)

of sections in preheated oil and degreasing in preheated 2% dish washing soap solution could have ensured clearing of the sections, allowing penetration of dyes during staining.

On comparing the findings of this study with that of the reported literature, the following observations were made:

Bleached palm oil had been used to clear bird tissues by Udonkang *et al.*[14] In their study, 93.3% tissues treated with bleached palm oil had shown to be transparent. In contrast to this finding, this study has shown that all the studied tissues (100%) had cleared the transparency test. This could be due to change in the inherent composition of the tissues studied.

Considering microtomy test, this study has shown easy microtomy with serial section for all the tissues, whereas only 73.7% had cleared this test under bleached palm oil category, in the study by Udonkang *et al.*[14] In their (Udonkang *et al.*) study, clarity of staining by xylene treated tissues was observed in 93.3% tissues and it was 100% in this study. Considering quality of staining for bleached palm oil treated tissues, it was 86.7% in Udonkang *et al.*'s study and 91.6% in this study. These differences could be attributed to study of various parts of the bird in Udokang *et al.*'s study in contrast of study of only oral mucosal tissue samples from humans in this study.

In accordance with the studies incorporating vegetable oils/mineral oil mixtures/liquid dish wash soap as substitutes for xylene, along with studies on xylene free staining techniques, it may be inferred that bleached palm oil may be used as a bio-safe substitute for xylene in routine tissue processing.

In addition, use of bleached palm oil may be nonhazardous, nonflammable, nontoxic, economic, biodegradable, readily available, and is easy to handle.

Additional advantages of bleached palm oil usage had reduced the exposure to xylene as the sections were air dried before cover slipping.

Bleached palm oil may be used as a safer and suitable alternative to xylene in tissue processing. It may be further tried with various histochemical and immunohistochemical stains.

Conclusion

This study has shown that bleached vegetable (palm) oil may be used as a suitable bio-safe substitute for xylene for human oral mucosal tissue processing. However, it has to be tried with a larger sample along with its trial for various special stains including immunohistochemical analysis.

Clinical significance

Bleached vegetable oil may be used as a bio-safe substitute for xylene in tissue processing.

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