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Rapid Tissue Clearing in Pre-warmed Xylene Without Compromising Staining Adequacy and Histoarchitecture

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Authors' contributions

This work was carried out in collaboration among all authors. Author KCO conceptualized and designed the study, analyzed and interpreted the data and wrote the article. Authors ONA, STA and OM carried out the experimental work under the supervision of author KCO. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To determine the adequacy of nucleo-cytoplasmic staining characteristics and preservation of nucleo-cytoplasmic morphology of mouse liver tissue cleared in pre-warmed xylene at predetermined temperatures and durations using haematoxylin and eosin staining procedure.
Study Design: Tissues for clearing were first divided into 3 broad experimental groups (A, B and C) based on pre-determined tissue clearing temperature. Each broad group were further divided into 4 sub-groups (A1 to A4, B1 to B4 and C1 to C4) based on duration of tissue clearing.
Place and Duration of Study: Department of Medical Laboratory Science, Babcock University,

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Methodology: Tissues assigned to sub-groups A1 to A4 were cleared in pre-warmed xylene at 25°C for 30, 45, 60 and 90 minutes respectively, while tissues assigned to sub-groups B1 to B4 were cleared in pre-warmed xylene at 30°C for 30, 45, 60 and 90 minutes respectively and tissues assigned to sub-groups C1 to C4 were cleared in pre-warmed xylene at 35°C for 30, 45, 60 and 90 minutes respectively. As a consequence of this procedure, adequacy of nucleo-cytoplasmic staining characteristics and preservation of nucleo-cytoplasmic morphology were subsequently determined.

Results: Adequacy of nuclear and cytoplasmic staining were both assessed using a 2-point grading scale of 0 being inadequate and 1 being adequate, while preservation of both nuclear and cytoplasmic morphology were also assessed using a 2-point grading scale of 0 being poorly preserved and 1 being well preserved. Adequate nuclear and cytoplasmic staining were observed in tissues cleared in pre-warmed xylene at 25°C for 45 and 60 minutes, at 30°C for 30 and 45 minutes, at 35°C for 30, 45, 60 and 90 minutes. Well preserved nuclear morphology were observed in tissues cleared in pre-warmed xylene at 25°C for 30, 45 and 60 minutes, at 30°C for 30 and 45 minutes and at 35°C for 30, 45, 60 and 90 minutes. However, only tissues cleared at 35°C for 30, 45 and 90 minutes exhibited well preserved cytoplasmic morphology.

Conclusion: Adequate nucleo-cytoplasmic staining and well preserved nucleo-cytoplasmic morphology of tissues for histopathologic demonstration may be achieved rapidly by clearing tissues in 35°C pre-warmed xylene for as short as 30 minutes.

Keywords: Pre-warmed; duration; clearing; staining; histoarchitecture.

1. INTRODUCTION

Clearing is an important step in the production of histological sections with the aim of removing alcohol and other dehydrating fluids from tissue prior to infiltration of the embedding material, which is usually paraffin wax. Over the years, xylene which is naturally available in coal tar and petroleum has been widely used as a clearing agent chiefly because of its excellent compatibility with alcohol and paraffin wax, where it causes maximum displacement of alcohol and makes the tissue transparent thus enhancing paraffin infiltration coupled with its use as a deparaffinizing agent in staining and application of coverslip to stained slides [1,2,3,4].

Although, the hazardous effect of xylene is well documented in literature, it is still commonly used in pathology laboratories worldwide [5,6]. In a bid to identify non-toxic, cheaper and commonly available alternatives to xylene as a clearing agent, we embarked on a series of research on locally available plant oils in Nigeria. Interestingly however, in the course of the research, we observed a pattern in staining adequacy and preservation of histomorphology using xylene as a clearing agent which forms the basis of this preliminary report.

Routinely, tissues less than 3mm in thickness are cleared in a single bath of xylene for 1½ hours at room temperature, while tissues between 3 and 5mm in thickness are cleared in two changes of xylene for 2 to 3 hours each at room temperature. Furthermore, tissues between 5 and 8mm in thickness are cleared in two changes of xylene for 3 to 5 hours each at room temperature, thus prolonging the turnaround time [7].

In this study, we report a protocol which drastically reduced the time tissues are immersed in xylene during the clearing step without compromising the staining adequacy and preservation of histomorphology.

2. MATERIALS AND METHODS

2.1 Fixation of Experimental Animals

Twenty albino mice of mixed sexes were purchased from the animal house of the Department of Zoology, University of Ibadan, Nigeria. They were immediately euthanized by cervical dislocation and subsequently dissected to obtain 3 to 5mm thick liver tissues which were immediately fixed in 10% formol saline for 24 hours.

The fixed tissues were thereafter dehydrated through 70%, 80%, 90% and absolute ethanol for 1 hour each and eventually in a second bath of absolute ethanol overnight.

2.2 Experimental Design

For the clearing process, the dehydrated tissues were then divided into 3 broad experimental groups (A, B and C). Each of these groups were further divided into sub-groups (A1 to A4, B1 to B4 and C1 to C4). Tissues assigned to group A were cleared in pre-warmed xylene at 25^{o}C, while tissues assigned to group B were cleared in pre-warmed xylene at 30^{o}C and tissues assigned to group C were cleared in pre-warmed xylene at 35^{o}C. Thus, tissues in groups A1, A2, A3 and A4 were cleared in pre-warmed xylene at 25^{o}C for 30, 45, 60 and 90 minutes respectively, while tissues in groups B1, B2, B3 and B4 were cleared in pre-warmed xylene at 30^{o}C for 30, 45, 60 and 90 minutes respectively and tissues in groups C1, C2, C3 and C4 were cleared in pre-warmed xylene at 35^{o}C for 30, 45, 60 and 90 minutes respectively.

Each group were thereafter infiltrated in 2 changes of paraffin wax for 1½ hours each and subsequently blocked out. 5µm sections were cut from each block and stained by the routine hematoxylin and eosin method. Adequacy of staining and preservation of histomorphology were then assessed microscopically as indices of the effect of tissue clearing in pre-warmed xylene.

The ethical committee of Babcock University, Ilishan-remo, Ogun state, Nigeria approved the study in compliance with standard laboratory animal care procedures.

2.3 Criteria for Analysis

Multiple slides were made for each parameter under investigation. After previewing all the slides, the best were chosen and used for analysis. Adequacy of both nuclear and cytoplasmic staining were assessed independently using a 2-point grading scale of 0 being inadequate and 1 being adequate while preservation of both nuclear and cytoplasmic morphology were also assessed independently using a 2-point grading scale of 0 being poorly preserved and 1 being well preserved.

3. RESULTS

3.1 Staining Adequacy and Preservation of Tissue Morphology

Table 1 shows the staining adequacy and preservation of morphology of tissues cleared at 25^{o}C for 30, 45, 60 and 90 minutes, while Table 2 shows the staining adequacy and preservation of morphology of tissues cleared at 30^{o}C for 30, 45, 60 and 90 minutes and Table 3 shows the staining adequacy and preservation of morphology of tissues cleared at 35^{o}C for 30, 45, 60 and 90 minutes.

Table 1. Staining adequacy and preservation of morphology of tissues cleared at 25^{o}C

Duration of clearing (minutes)	Staining adequacy		Preservation of morphology	
	Nucleus	Cytoplasm	Nucleus	Cytoplasm
30	0	0	1	0
45	1	1	1	0
60	1	1	1	0
90	0	0	0	0

Grading of staining adequacy was on a scale of 0 (inadequate) to 1 (adequate) while grading of preservation of morphology was on a scale of 0 (poorly preserved) to 1 (well preserved)

Table 2. Staining adequacy and preservation of morphology of tissues cleared at 30^{o}C

Duration of clearing (minutes)	Staining adequacy		Preservation of morphology	
	Nucleus	Cytoplasm	Nucleus	Cytoplasm
30	1	1	1	0
45	1	1	1	0
60	0	0	0	0
90	0	0	0	0

Grading of staining adequacy was on a scale of 0 (inadequate) to 1 (adequate) while grading of preservation of morphology was on a scale of 0 (poorly preserved) to 1 (well preserved)

Adequate nuclear and cytoplasmic staining characteristics were observed in tissues cleared in pre-warmed xylene at 25°C for 45 and 60 minutes (Fig. 1), at 30°C for 30 and 45 minutes (Fig. 2) and at 35°C for 30, 45, 60 and 90 minutes (Fig. 3).

Well preserved nuclear morphology were observed in tissues cleared in pre-warmed xylene at 25°C for 30, 45 and 60 minutes (Fig. 1). Tissues cleared in pre-warmed xylene at 30°C for 30 and 45 minutes (Fig. 2) also exhibited well preserved nuclear morphology.

Furthermore, tissues cleared in pre-warmed xylene at 35°C for 30, 45, 60 and 90 minutes (Fig. 3) also exhibited well preserved nuclear morphology. However, only tissues cleared in pre-warmed xylene at 35°C for 30, 45 and 90 minutes (Fig. 3) exhibited well preserved cytoplasmic morphology.

4. DISCUSSION

Tissue processing in histology and cytology is a physical process that involves chemical solutions reacting with biological specimen with profound effects if not properly handled. Biopsy and autopsy specimen require processing prior to histopathological diagnosis. One of the processes is clearing of the tissue in a chemical agent. The term clearing, derives from the fact that the clearing agent often have the same refractive index as proteins. As a result when the tissue is completely infiltrated in the clearing agent, it becomes translucent. This change in appearance is often used as an indication of the effectiveness and completeness of the clearing process [8].

Table 3. Staining adequacy and preservation of morphology of tissues cleared at 35°C

Duration of clearing (minutes)	Staining adequacy		Preservation of morphology	
	Nucleus	Cytoplasm	Nucleus	Cytoplasm
30	1	1	1	1
45	1	1	1	1
60	1	1	1	0
90	1	1	1	1

Grading of staining adequacy was on a scale of 0 (inadequate) to 1 (adequate) while grading of preservation of morphology was on a scale of 0 (poorly preserved) to 1 (well preserved)

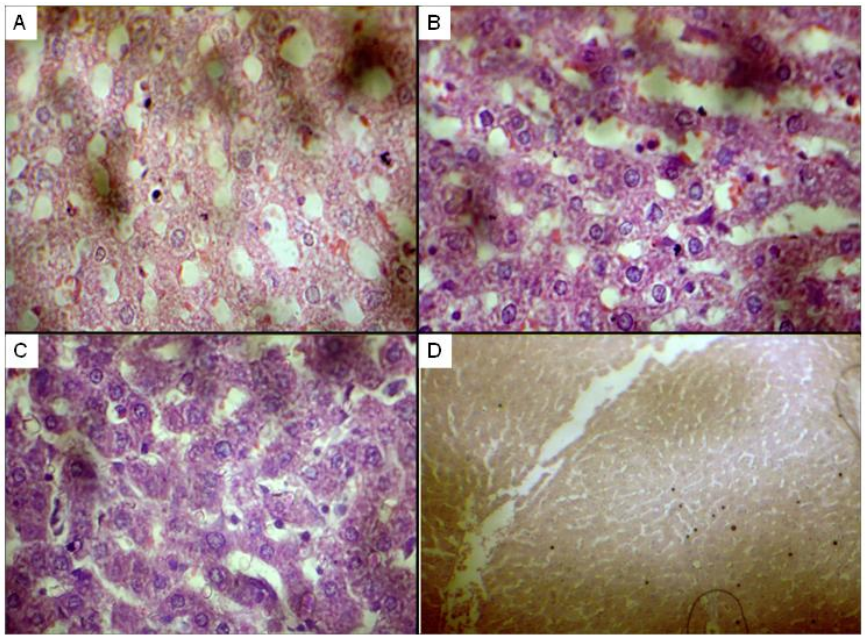

Fig. 1. Pre-warmed 25°C. A-30 min; B-45 min; C-60 min; D-90 min

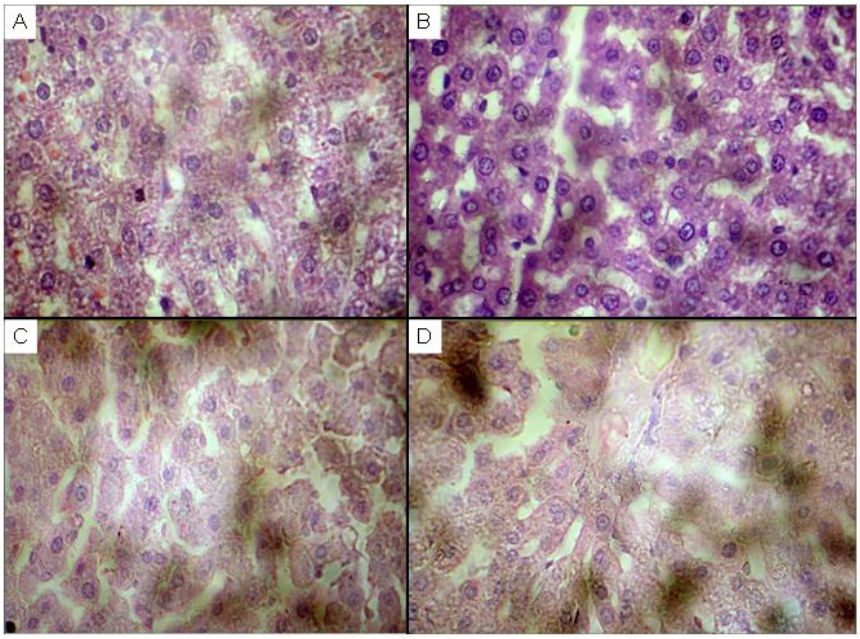

Fig. 2. Pre-warmed 30°C. A-30 min; B-45 min; C-60 min; D-90 min

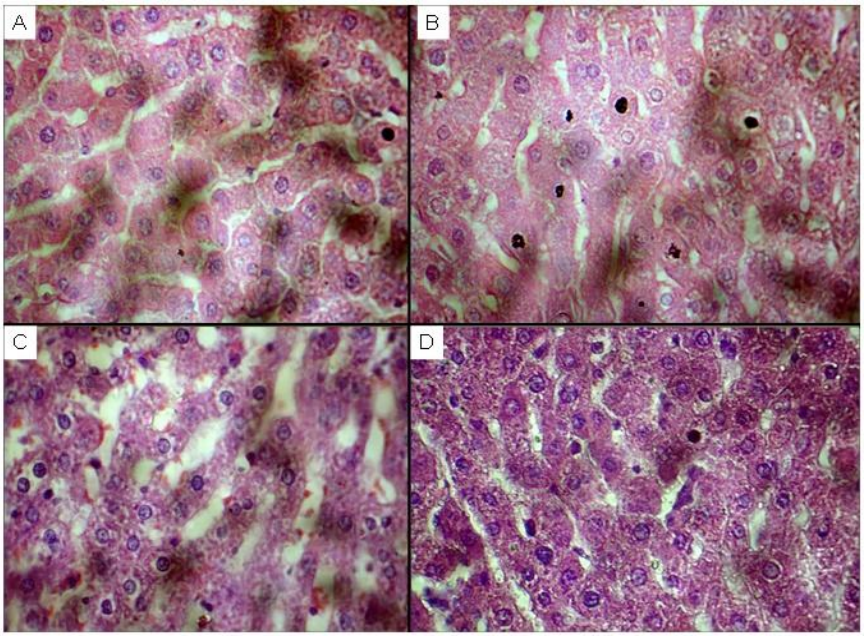

Fig. 3. Pre-warmed 35°C. A-30 min; B-45 min; C-60 min; D-90 min

The chemical agent most commonly used is xylene. It works reasonably well for short-term clearing of tissue blocks, whereas, long-term immersion of tissues in xylene results in tissue distortion [8]. Although the hazards and toxicities caused by xylene to humans are well documented in literature [9,10,11,12,13,14], and although many substitutes have been commercially developed [15], they fail to completely replace xylene partially due to their variable effectiveness and cost.

In histology laboratories, standard operating procedures requires that tissues be cleared in

two changes of xylene or other clearing agents at room temperature (25°C) for at least 1½ hours and up to 10 hours each depending on the size of the tissue sample. In our laboratory, samples are routinely cleared in two changes of xylene for 1½ hours each irrespective of the size. However, in the course of this study, we were able to achieve adequate nucleo-cytoplasmic staining characteristics and well preserved nucleo-cytoplasmic morphology in pre-warmed xylene at shorter clearing times. We therefore report that although adequate nucleo-cytoplasmic staining characteristics were observed in tissues cleared in pre-warmed xylene across the three experimental temperature ranges, however, our results show that adequate nucleo-cytoplasmic staining characteristics suitable for pathological diagnosis were observed in tissue samples cleared in pre-warmed xylene at 30°C and 35°C for 30 minutes (Figs. 2 and 3) as against 45 minutes for tissue samples cleared in pre-warmed xylene at 25°C (Fig. 1). Furthermore, our results also show that clearing of tissues in pre-warmed xylene at 35°C for 30 minutes (Fig. 3) produces well preserved nucleo-cytoplasmic morphology suitable for pathological diagnosis. Efforts to locate similar work done previously in our literature search for comparison proved abortive.

Temperature is known to increase the kinetic energy of molecules, which in turn increases the rate at which molecules diffuse across tissue membranes [16,17]. It may therefore be inferred that xylene molecules at higher temperatures of 30°C and 35°C were able to penetrate the tissues faster than at 25°C thereby displacing ethanol from the tissues for better clearing efficiency that resulted in adequate nucleo-cytoplasmic staining characteristics and well preserved nucleo-cytoplasmic morphology.

5. CONCLUSION

We therefore conclude that immersion of tissues for 30 minutes in pre-warmed xylene at 35°C produces adequate nucleo-cytoplasmic staining characteristics, as well as, well preserved nucleo-cytoplasmic morphology suitable for histopathological diagnosis. We recommend that further studies should be conducted with actual pathological tissue samples and possibly extended to antibody detection studies.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that the study was approved by the ethical committee of Babcock University in compliance with standard animal care procedures. The approval number is NHREC/17/12/2013 BUHREC 125/15.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Matthews JB. Influence of clearing agent on immunohistochemical staining of paraffin embedded tissue. J. Clin. Pathol. 1981;34:103-105.
2. Pollard K, Lunny D, Holgate CS, Jackson P, Bird CC. Fixation, processing and immuno-histochemical reagent effects on preservation of T-lymphocyte surface membrane antigens in paraffin-embedded tissue. J. Histochem. Cytochem. 1987;35: 1329-1338.
3. Kunhua W, Chuming F, Tao L, Yanmei Y, Xin Y, Xiaoming Z et al. A novel non-toxic xylene substitute (SBO) for histology. Afr. J. Trad. Alte. Med. 2012;9(1):43-49.
4. Ananthaneni A, Namala S, Guduru V, Ramprasad V, Ramisetty S, Udayashankar U et al. Efficacy of 1.5% dishwashing solution and 95% lemon water in substituting perilous xylene as a deparaffinizing agent for routine H and E staining procedure: A short study. Scientifica; 2014.
DOI:doi.org/10.1155/2014/707310.
5. Bush C.L, Nelson G.E. Xylene (a warning on its use in the histology and cytology laboratory. Histologic. 1977;7(1):93.
6. Revilla AS, Pestana CR, Pardo-Andreu. Potential toxicity of toluene and xylene evoked by mitochondrial uncoupling. Toxicology in vitro. 2007;21(5):782-788.

7. Baker FJ, Silverton RE, Pallister CJ. Baker and Silverton's Introduction to Medical Laboratory Technology. Bounty Press limited under licence from Edward Arnold. 1995;202.
8. Ofusori DA, Ayoka AO, Adeeyo OA, Adewole SO. Mixture of kerosene and xylene: A contribution to clearing agents. Int. J. Morphol. 2009;27(1):211-218.
9. Gamberale F, Annwall G, Hultengren M. Exposure to xylene and ethylbenzene: Effects on central nervous functions. Scan. J. Work Environ. Health. 1978;4:204-211.
10. Hass U, Lund SP, Simonsen K, Fries AS. Effects of prenatal exposure to xylene on postnatal development and behavior in rats. Neurotoxicol. Tertol. 1995;17:341-349.
11. Kum C, Kiral F, Sekkin S, Seyrek K, Boyacioglu M. Effects of xylene and formaldehyde inhalations on oxidative stress in adult and developing rat livers. Exp. Anim. 2007a;56:35-42.
12. Chattergee A, Babu RJ, Ahaghotu E, Singh M. The effects of occlusive and unocclussive exposure to xylene and benzene on skin irritation and molecular responses in hairless rats. Archiv. Toxicol. 2005;79:294-301.
13. Sandikci M, Seyrek K, Aksit H, Kose H. Inhalation of formaldehyde and xylene induces apoptotic cell death in the lung tissue. Toxicol. Ind. Health. 2009;24:455-461.
14. Kum C, Sekkin S, Kiral F, Akar F. Effects of xylene and formaldehyde inhalations on renal oxidative stress and some serum biochemical parameters in rats. Toxicol. Ind. Health. 2007b;23:115-120.
15. Buesa RJ. Histology safety: Now and then. Ann. Diag. Pathol. 2007;11(5):334-339.
16. Winsor L. Tissue processing. 2000; In Woods and Ellis edition. New York, Springer-Verlag.
17. Udonkang M, Eluwa M, Ekanem T, Asuquo O, Akpantah A. Bleached palm oil as a sub-stitute for xylene in histology. J. Pharm. Clin. Scien. 2014;8:8-17.

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