ALTERNATIVES TO CLEARING AGENTS - A REVIEW

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Abstract
Chloroform, Xylene, Toluene, Paraffin, Methyl benzoate, methyl salicylate, and citrus fruit oils are examples of clearing agents. Xylene is a natural clearing agent that is miscible with both alcohol and paraffin wax. Clearing is done to extract alcohol from tissues and substitute it with a fluid that is miscible with wax and must be impregnated into the tissue. Because the dehydrating agents are not miscible with paraffin, a clearing agent is required. Both the dehydrating agent and the paraffin wax are miscible with the clearing agent. The main aim of this study is to know the alternatives of clearing agents. The types of clearing agents will be Benzene, Xylene, Toluene, Chloroform, Petroleum ether, Oil of wintergreen (Methyl salicylate), Cedar wood oil, Carbon tetrachloride, Clove oil, Dioxane, Aniline oil. Good clearing agent should remove alcohol quickly, Clear quickly without overhardening, Should not dissolve out aniline dyes, Should not evaporate too quickly in a wax bath.
Keyword: clearing agents, xylene, benzene, cedar wood oil, clove oil, chloroform, toluene.

INTRODUCTION

In order to prepare histopathology slides for light microscopy, clearing is needed. The aim of clearing is to extract dehydrating agents from the tissues and prepare them for embedding agent impregnation. Xylene is a sweet-smelling, colourless, aromatic hydrocarbon found naturally in coal, petroleum, and wood tar in liquid or gas form(1). Much has been written about the use of xylene alternatives that are safer and less costly. Limonene, benzene, toluene, aliphatic and aromatic hydrocarbons, and various oils are examples of alternative clearing agents. Some alternatives, such as oil solvents, take a long time to process. Other chemicals that damage tissue samples include benzene, limonene, and toluene(2). After the tissues have been dehydrated, the alcohol must be removed until they are impregnated and embedded in paraffin(3). Both alcohol and water are immiscible with the paraffin wax used for embedding. As a result, the alcohol must be replaced with a medium that can dissolve both alcohol and paraffin. During wax impregnation, the medium must be removed(4). The use of a clearing agent often improves the resale value. The types of clearing agents will be Benzene, Xylene, Toluene, Chloroform, Petroleum ether, Oil of wintergreen (Methyl salicylate), Cedar wood oil, Carbon tetrachloride, Clove oil, Dioxane, Aniline oil. Good clearing agent should remove alcohol quickly, Clear quickly without overhardening, Should not dissolve out aniline dyes, Should not evaporate too quickly in a wax bath. Chloroform, Xylene, Toluene, Paraffin, Methyl benzoate, methyl salicylate, and citrus fruit oils are examples of clearing agents(5). Xylene is a natural clearing agent that is miscible with both alcohol and paraffin wax. Clearing is done to extract alcohol from tissues and substitute it with a fluid that is miscible with wax and must be impregnated into the tissue(6). Because the dehydrating agents are not miscible with paraffin, a clearing agent is required. Both the dehydrating agent and the paraffin wax are miscible with the clearing agent. Clearing agents are used all over the world(7). Tissue and histology lab in the processes of tissue and histology lab in preparation of the slides (to exclude alcohol and other contaminants) prior to removing other dehydrants from tissues embedding (most commonly in paraffin wax), and prior to mounting, the slides are completed. In addition, they are used to strip paraffin wax after sectioning following microtome cutting(8). Attempts have been made in recent years to replace these hazardous solvents with safer alternatives(9). Although useful, these replacements have presented additional problems. Our team has extensive knowledge and research experience that has translated into high quality publications (10)(11–16)(17–19)(20–29).The aim of the study is to find the alternatives of clearing agents.

XYLENE

Xylene may cause headaches, fatigue, memory loss, irritability, dizziness, giddiness, loss of balance and judgement, respiratory distress or trouble breathing, loss of appetite, nausea, vomiting, shivering, unconsciousness, coma, and death. Ingestion of xylene may cause gastrointestinal irritation including abdominal pain, nausea, and vomiting. Xylene also may affect the liver and kidneys. In histopathology laboratories, it is the most widely used clearing agent. It's a smooth, colourless liquid with a distinct herbal odour.In addition, some xylene substitutes will not absorb water, whereas xylene can absorb small amounts of water, which is useful if a tissue was not fully dehydrated, so this is something else to be aware of, though water tolerance can differ by brand of substitute. In the 70's studies began to confirm that xylene was dangerous. Not only is it flammable, but it is also not great when breathed in for extended periods of time. The vapor is absorbed through the lungs, while the liquid can be absorbed through the skin, then once in the body it is metabolized by the liver. Prolonged exposure in high concentrations became associated with respiratory tract symptoms, anemia, and other symptoms generally associated with central nervous system issues. Xylene is still widely used, but there are now more safety precautions to help prevent these

types of risks. Clinical labs are required to do yearly xylene exposure testing to maintain CAP accreditation under ANP .08216 Formaldehyde/Xylene Safety. If the limits are exceeded, they must develop an action plan for addressing the overexposure and do a repeated test. Xylene substitutes became popular as another way of combating these threats. Some of the substitutes however, come with their own set of risks.Ultimately, neither xylene substitutes nor xylene is perfect. It is up to your lab to decide what makes sense for you and be aware of the risks, and procedural changes needed to use the chemical safely. It is water insoluble but soluble in organic solvents such as ethanol, benzene, and acetone(30). The advantages will be its action is very rapid, Cheap & slightly inflammable, Readily eliminated in the paraffin oven. The disadvantage will be prolonged treatment with this reagent makes the soft tissues like Brain & Spleen quite brittle(31,32).

BENZENE

Benzene is preferred by some as a clearing agent in the embedding process of tissues because it penetrates and clears tissues rapidly. The advantages of benzene are it is rapid acting, hence is recommended for urgent biopsies (15 – 60 minutes) and routine purposes, It volatizes rapidly in paraffin oven and is therefore easily eliminated from the tissue, It is miscible with absolute alcohol, It does not make tissues hard and brittle, It causes minimum shrinkage and it makes tissues transparent. Since it is a carcinogen and may cause cancer, benzene is rarely used as a clearing agent. It's a colourless, flammable liquid with a distinctive sweet, gasoline-like odour. It's water-insoluble but fully soluble in organic solvents including acetone, ethanol, and chloroform. The advantages of benzene will be it penetrates the tissues rapidly, it causes minimum shrinkage, it is a Cheap clearing agent(33). Benzene is preferred by some as a clearing agent in the embedding process of tissues because it penetrates and clears tissues rapidly. The disadvantage will be it is a carcinogen and potentially causes cancer, it is a flammable liquid(34).

CLOVE OIL AND CEDAR WOOD OIL

Chloroform is used for routine clearing of tissues during the embedding process. The advantages are it is recommended for routine work (6 – 24 hours), It is miscible with absolute alcohol, It is recommended for tough tissues (e.g., skin, fibroid and decalcified tissues) for nervous tissues, lymph nodes and embryos because it causes minimum shrinkage and hardening of tissues, It is suitable for large tissue specimens and It is not inflammable. The disadvantages are it is relatively toxic to the liver after prolonged inhalation; adequate room ventilation and proper caution may prevent this when handling the specimen, Wax impregnation after Chloroform clearing is relatively slow, it does not make tissue transparent, Its vapor may attack the rubber seal used in vacuum impregnation bath, Complete clearing is difficult to evaluate, Tissues tend to float in chloroform; this may be avoided by wrapping the tissues with absorbent cotton gauze to facilitate sinking of the section in solution and it evaporates quickly from a waterbath. Clove oil is an excellent clearing medium, but it has the power of extracting certain stains, especially safranin stain, and so it cannot be used in all cases. Coconut oil is a commonly used vegetable oil, available throughout the tropical world. It is an excellent clearing agent for tissues but less commonly used due to its slow penetration rate(35). It is light yellow to pale brown colored viscous fluid with a characteristic woody odor. It is insoluble in water but soluble in organic solvents like ethanol, acetone, etc(35).

TOLUENE

Toluene may be used as a substitute for Xylene or Benzene for clearing both during the embedding and mounting process. Time recommended for clearing is 1 – 2 hours. The advantages are it is miscible with both absolute alcohol and paraffin, It acts fairly rapidly and is recommended for routine purposes, Tissues do not become excessively hard and brittle even if left in toluene for 24 hours and it is not carcinogenic. The disadvantages of toluene are it is relatively slower than Xylene and Benzene, It tends to acidify in a partially filled vessel, Highly concentrated solutions will emit fumes that are toxic upon prolonged exposure and It is more expensive. It is also a good clearing agent but less commonly used in histopathology laboratories. It is a colorless, flammable and clear liquid with a characteristic aromatic odor. It is insoluble in water but soluble in Organic solvents like Acetone, Chloroform, Ethanol, Benzene etc(6,36). The advantages of toluene as clearing agents will be that tissues can be kept in this for a longer period, its action is similar to benzene but it is less toxic. The disadvantages will be it causes irritation if accidentally inhaled or come into contact with skin or eyes(37).

CHLOROFORM

Besides its various uses in Pharmaceuticals, Dyes & Pesticides industries and in Refrigerant industries, it can also be used as a clearing agent in histopathology laboratory. It is a Colorless, volatile liquid with a characteristic ether-like odor. It is slightly soluble in water but completely soluble in organic solvents like ethanol, benzene, acetone etc(38). The advantages will be it is widely used for its hardening effect, ideal for hard & delicate tissues like Bone and the Brain. The disadvantages will be Its action is slower than other clearing agents, it can cause faintness if inhaled(39).

CONCLUSION

From the above studied agents the alternatives had been studied well and the furthermore original researches and studies will lead to better understanding and can find the best alternative of clearing agents.

References

1. *Alwahaibi N, Aljaradi S, Alazri H. Alternative to xylene as a clearing agent in histopathology. J Lab Physicians. 2018 Apr;10(2):189–93.*
2. *Sermadi W, Prabhu S, Acharya S, Javali S. Comparing the efficacy of coconut oil and xylene as a clearing agent in the histopathology laboratory. J Oral Maxillofac Pathol. 2014 Sep;18(Suppl 1):S49–53.*
3. *Prabhu S, Acharya S, Javali SB, Sermadi W. Comparing the efficacy of coconut oil and xylene as a clearing agent in the histopathology laboratory [Internet]. Vol. 18, Journal of Oral and Maxillofacial Pathology. 2014. p. 49. Available from: http://dx.doi.org/10.4103/0973-029x.141348*
4. *Alwahaibi NY, Aldughaishi SH. A substitute to xylene in deparaffinization and clearing prior to coverslipping in histopathology. J Lab Physicians. 2019 Apr;11(2):118–22.*
5. *Temel SG, Noyan S, Cavusoglu I, Kahveci Z. A simple and rapid microwave-assisted hematoxylin and eosin staining method using 1,1,1 trichloroethane as a dewaxing and a clearing agent. Biotech Histochem. 2005 May;80(3-4):123–32.*

6. *Institute NC, National Cancer Institute. Clearing Agent [Internet]. Definitions. 2020. Available from: http://dx.doi.org/10.32388/o4uoum*
7. *Skinner, Skinner. The Value of Methyl Salicylate as a Clearing Agent [Internet]. Journal of Histotechnology. 1986. Available from: http://dx.doi.org/10.1179/014788886794641180*
8. *Onyegbula KC, Afahaene ON, Awolaja ST, Makinde O. Rapid Tissue Clearing in Pre-warmed Xylene Without Compromising Staining Adequacy and Histoarchitecture [Internet]. Current Journal of Applied Science and Technology. 2019. p. 1–7. Available from: http://dx.doi.org/10.9734/cjast/2019/v34i230120*
9. *Ofusori DA, Ayoka AO, Adeeyo OA, Adewole SO. Mixture of Kerosene and Xylene: a Contribution to Clearing Agents [Internet]. Vol. 27, International Journal of Morphology. 2009. Available from: http://dx.doi.org/10.4067/s0717-95022009000100036*
10. *Princeton B, Santhakumar P, Prathap L. Awareness on Preventive Measures taken by Health Care Professionals Attending COVID-19 Patients among Dental Students. Eur J Dent. 2020 Dec;14(S 01):S105–9.*
11. *Mathew MG, Samuel SR, Soni AJ, Roopa KB. Evaluation of adhesion of Streptococcus mutans, plaque accumulation on zirconia and stainless steel crowns, and surrounding gingival inflammation in primary molars: randomized controlled trial. Clin Oral Investig. 2020 Sep;24(9):3275–80.*
12. *Sridharan G, Ramani P, Patankar S, Vijayaraghavan R. Evaluation of salivary metabolomics in oral leukoplakia and oral squamous cell carcinoma. J Oral Pathol Med. 2019 Apr;48(4):299–306.*
13. *R H, Hannah R, Ramani P, Ramanathan A, Jancy MR, Gheena S, et al. CYP2 C9 polymorphism among patients with oral squamous cell carcinoma and its role in altering the metabolism of benzo[a]pyrene [Internet]. Vol. 130, Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology. 2020. p. 306–12. Available from: http://dx.doi.org/10.1016/j.oooo.2020.06.021*
14. *Antony JVM, Ramani P, Ramasubramanian A, Sukumaran G. Particle size penetration rate and effects of smoke and smokeless tobacco products - An invitro analysis. Heliyon. 2021 Mar;7(3):e06455.*
15. *Sarode SC, Gondivkar S, Sarode GS, Gadbail A, Yuwanati M. Hybrid oral potentially malignant disorder: A neglected fact in oral submucous fibrosis. Oral Oncol. 2021 Oct;121:105390.*
16. *R H, Hannah R, Ramani P, Tilakaratne WM, Sukumaran G, Ramasubramanian A, et al. Author response for "Critical appraisal of different triggering pathways for the pathobiology of pemphigus vulgaris—A review" [Internet]. 2021. Available from: http://dx.doi.org/10.1111/odi.13937/v2/response1*
17. *Chandrasekar R, Chandrasekhar S, Sundari KKS, Ravi P. Development and validation of a formula for objective assessment of cervical vertebral bone age. Prog Orthod. 2020 Oct 12;21(1):38.*
18. *Subramanyam D, Gurunathan D, Gaayathri R, Vishnu Priya V. Comparative evaluation of salivary malondialdehyde levels as a marker of lipid peroxidation in early childhood caries. Eur J Dent. 2018 Jan;12(1):67–70.*
19. *Jeevanandan G, Thomas E. Volumetric analysis of hand, reciprocating and rotary instrumentation techniques in primary molars using spiral computed tomography: An in vitro comparative study [Internet]. Vol. 12, European Journal of Dentistry. 2018. p. 021–6. Available from: http://dx.doi.org/10.4103/ejd.ejd_247_17*
20. *Ponnulakshmi R, Shyamaladevi B, Vijayalakshmi P, Selvaraj J. In silico and in vivo analysis to identify the antidiabetic activity of beta sitosterol in adipose tissue of high fat diet and sucrose induced type-2 diabetic experimental rats. Toxicol Mech Methods. 2019 May;29(4):276–90.*
21. *Sundaram R, Nandhakumar E, Haseena Banu H. Hesperidin, a citrus flavonoid ameliorates hyperglycemia by regulating key enzymes of carbohydrate metabolism in streptozotocin-induced diabetic rats. Toxicol Mech Methods. 2019 Nov;29(9):644–53.*
22. *Alsawalha M, Rao CV, Al-Subaie AM, Haque SKM, Veeraraghavan VP, Surapaneni KM. Novel mathematical modelling of Saudi Arabian natural diatomite clay [Internet]. Vol. 6, Materials Research Express. 2019. p. 105531. Available from: http://dx.doi.org/10.1088/2053-1591/ab2f9b*
23. *Tang X, Yu J, Li M, Zhan D, Shi C, Fang L, et al. Inhibitory effects of triterpenoid betulin on inflammatory mediators inducible nitric oxide synthase, cyclooxygenase-2, tumor necrosis factor-alpha, interleukin-6, and proliferating cell nuclear antigen in 1,2-dimethylhydrazine-induced rat colon carcinogenesis [Internet]. Vol. 16, Pharmacognosy Magazine. 2020. p. 841. Available from: http://dx.doi.org/10.4103/pm.pm_516_19*
24. *Shree KH, Hema Shree K, Ramani P, Herald Sherlin, Sukumaran G, Jeyaraj G, et al. Saliva as a Diagnostic Tool in Oral Squamous Cell Carcinoma – a Systematic Review with Meta Analysis [Internet]. Vol. 25, Pathology & Oncology Research. 2019. p. 447–53. Available from: http://dx.doi.org/10.1007/s12253-019-00588-2*
25. *Zafar A, Sherlin HJ, Jayaraj G, Ramani P, Don KR, Santhanam A. Diagnostic utility of touch imprint cytology for intraoperative assessment of surgical margins and sentinel lymph nodes in oral squamous cell carcinoma patients using four different cytological stains. Diagn Cytopathol. 2020 Feb;48(2):101–10.*
26. *Karunagaran M, Murali P, Palaniappan V, Sivapathasundharam B. Expression and distribution pattern of podoplanin in oral submucous fibrosis with varying degrees of dysplasia – an immunohistochemical study [Internet]. Vol. 42, Journal of Histotechnology. 2019. p. 80–6. Available from: http://dx.doi.org/10.1080/01478885.2019.1594543*
27. *Sarode SC, Gondivkar S, Gadbail A, Sarode GS, Yuwanati M. Oral submucous fibrosis and heterogeneity in outcome measures: a critical viewpoint. Future Oncol. 2021 Jun;17(17):2123–6.*
28. *Raj Preeth D, Saravanan S, Shairam M, Selvakumar N, Selestin Raja I, Dhanasekaran A, et al. Bioactive Zinc(II) complex incorporated PCL/gelatin electrospun nanofiber enhanced bone tissue regeneration. Eur J Pharm Sci. 2021 May 1;160:105768.*
29. *Zhan X, Feng X, Kong Y, Chen Y, Tan W. JNK signaling maintains the mesenchymal properties of multi-drug resistant human epidermoid carcinoma KB cells through snail and twist1 [Internet]. Vol. 13, BMC Cancer. 2013. Available from: http://dx.doi.org/10.1186/1471-2407-13-180*
30. *Indu S, Ramesh V, Indu PC, Prashad KV, Premalatha B, Ramadoss K. Comparative efficacy of cedarwood oil and*

xylene in hematoxylin and eosin staining procedures: An experimental study. J Nat Sci Biol Med. 2014 Jul;5(2):284–7.

31. *Aydin I, Yörükoglu K, Cingöz S, Agilkaya S. The effect of the alternative solutions to formaldehyde and xylene on tissue processing. Indian J Pathol Microbiol. 2013 Jul;56(3):221–30.*
32. *Metgud R, Astekar MS, Soni A, Naik S, Vanishree M. Conventional xylene and xylene-free methods for routine histopathological preparation of tissue sections. Biotech Histochem. 2013 Jul;88(5):235–41.*
33. *Lee M, Jung J, Han Y-K. Understanding Dimerization Process of Cyclohexyl Benzene as an Overcharge Protection Agent in Lithium Ion Battery [Internet]. Vol. 39, Bulletin of the Korean Chemical Society. 2018. p. 1227–30. Available from: http://dx.doi.org/10.1002/bkcs.11573*
34. *Bradley D. Benzene – Clearing the Fog [Internet]. ChemViews. 2015. Available from: http://dx.doi.org/10.1002/chemv.201500062*
35. *Popham RE. Cedarwood Oil as a Clearing Agent with Acetic-Alcohol Fixatives [Internet]. Vol. 25, Stain Technology. 1950. p. 112–3. Available from: http://dx.doi.org/10.3109/10520295009110967*
36. *McQuown AL. A New Clearing Agent for Tissue Blocks* [Internet]. Vol. 20, American Journal of Clinical Pathology. 1950. p. 75–6. Available from: http://dx.doi.org/10.1093/ajcp/20.1_ts.75*
37. *Hurley P. Agent-Centered Restrictions: Clearing the Air of Paradox [Internet]. Vol. 108, Ethics. 1997. p. 120–46. Available from: http://dx.doi.org/10.1086/233790*
38. *Dyer SS. On the Inhalation of Chloroform as a Remedial Agent [Internet]. Vol. 2, BMJ. 1862. p. 661–3. Available from: http://dx.doi.org/10.1136/bmj.2.104.661*
39. *Smith FH. Notes on Technic: Amyl Acetate as a Clearing agent for Refractory Plant Materials [Internet]. Vol. 26, Stain Technology. 1951. p. 271–2. Available from: http://dx.doi.org/10.3109/10520295109113223.*