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RESEARCH ARTICLE

EVALUATION OF THE EFFICACY OF VARIOUS XYLENE SUBSTITUTES AS A CLEARING AGENT IN TISSUE PROCESSING: A COMPARATIVE STUDY

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Abstract

Conventional tissue processing remains the gold standard. Tissue processing is a physical process that involves chemical solutions reacting with biological specimens. The main purpose of tissue processing is to embed the tissue in a solid medium firm enough to support the tissue and give it sufficient rigidity to enable thin sections to be cut, yet soft enough not to damage the knife or tissue. For any tissue specimen to undergo diagnosis, it must follow procedural steps such as fixation, dehydration, clearing, impregnation, embedding, sectioning and staining. Clearing is the process of replacing the dehydrating solution (which is not miscible with the embedding medium) with a substance that will be miscible with alcohol and the embedding medium. The most used clearing agent is xylene, which has excellent clearing capabilities. However, it can cause unwelcome effects on various systems of the human body, such as the skin, eyes, nervous system, blood, liver and kidneys as reported by Kandyala et al. In the present study, we evaluate the efficacy of three materials - Kerosene, Turpentine and Olive oil as substitutes for xylene in tissue processing and observe their effects on staining characteristics, comparing them with xylene. Promoting the elimination of xylene from the histology laboratory is a duty of those in decision-making positions. Since our study had a limited sample size and there are very few studies comparing all four chemicals as clearing agents, it is recommended to conduct this study on a larger scale, with the addition of different substitutes and analyze it with multiple observers.

Introduction: -

Conventional tissue processing remains the gold standard. Tissue processing is a physical process that involves chemical solutions reacting with biological specimens. The main purpose of tissue processing is to embed the tissue in a solid medium, firm enough to support the tissue and give it sufficient rigidity to enable thin sections to be cut, yet soft enough not to damage the knife or tissue. For any tissue specimen to undergo diagnosis, it must follow procedural steps such as fixation, dehydration, clearing, impregnation, embedding, sectioning and staining. Fixation is a process by which the cells and the tissues are fixed with various reagents without significant loss, distortion or decomposition. This step is followed by dehydration where the water content in the tissue is remove[1,3]. The next step is clearing which is the process of replacing the dehydrating solution (which is not miscible with the embedding

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medium) with a substance that will be miscible with alcohol and the embedding medium[2,3]. The commonly used clearing agent is xylene. It is an aromatic chemical hydrocarbon, a colorless liquid[4]. It has excellent clearing capabilities. It rapidly removes alcohol from the tissues, making the tissue transparent enough to permit light[3]. However, it causes unwelcome effects on the various systems of the human body, such as the skin, eyes, nervous system, blood, liver and kidneys as reported by Kandyala et al[5]. Kerosene is a combustible hydrocarbon, a thin clear liquid. It is non-carcinogenic, non-teratogenic and economical[2]. Turpentine is a hydrocarbon, a clear liquid that is easily available and non-hazardous[1]. Olive oil is one of the commonly used vegetable oils and available throughout the tropical world. It is non-toxic, heat stable, slow to oxidize and a non-biohazardous substitute[6]. In the present study, we evaluate the efficacy of three materials, other than xylene, which serve as substitutes for a clearing agent in tissue processing. We observe their effect on staining characteristics and compare them with xylene.

Aim & Objectives: -

To determine the efficacy of kerosene, turpentine and olive oil as a clearing agent as an alternative for xylene in routine tissue processing and to compare the staining of xylene cleared tissues with the staining of tissues cleared with kerosene, turpentine and olive oil.

Materials And Methods: -

Soft tissue specimens of the human tongue were received in the Department of Oral and Maxillofacial Pathology and Microbiology at our college**.** The tissues were fixed in 10% formalin, grossed and labeled. 50 tissue samples were randomly divided into 4 groups, named - **Group-A**, **Group-B**, **Group C** and **Group-D**. After dehydration with 70%, 80%, 90%, 100% and 100% alcohol for 1 hour each, the clearing step was initiated. **Group-A**: Tissue samples were processed using xylene as a clearing agent (two changes of xylene for 1 hour each) followed by H & E staining. **Group-B**: Tissue samples were processed using kerosene as a clearing agent (two changes of kerosene for 2 hours each) followed by H & E staining. **Group-C**: Tissue samples were processed using turpentine as a clearing agent (two changes of turpentine for 1 hour each) followed by H & E staining. **Group-D**: Tissue samples were processed using olive oil as a clearing agent (two changes of olive oil for 1 hour each) followed by H & E staining. The tissue samples were then embedded in paraffin wax and sectioned. Subsequently, the sections were stained with routine H and E stain. The slides were assessed for nuclear staining, cytoplasmic staining, intensity, clarity and uniformity of staining by three independent observers under 10x and 40x magnification using a light microscope. The observers evaluated the slides blindly to prevent inter-observer bias. After evaluating all the slides, the collected data were subjected to statistical analysis.

Staining Criteria

Nuclear staining -
For nuclear details, distinct chromatin condensation, prominent nuclear membrane and crisp nucleus staining as score 1 for adequate. Indistinct smudging and pyknosis of nuclei as score 0 for inadequate.

Cytoplasmic staining -
For cytoplasmic details, distinct architecture and good nuclear-cytoplasmic contrast as score 1 for adequate. Indistinct/blurred nuclear - cytoplasmic contrast as score 0 for inadequate.

Intensity -
Overall nuclear and cytoplasm adequate intensity as score 1 and inadequate intensity as score 0.

The same scoring rules as intensity apply for **clarity** and **uniformity**[1,6,7].

Result: -

In our study, we utilized four different clearing agents - xylene, kerosene, turpentine and olive oil. Data analysis was conducted using the Statistical Package for Social Sciences (SPSS) and the summarized data were presented in tables. Group comparisons were performed using an Anova test, with a significance level set at $P < 0.05$. Nuclear staining was found to be satisfactory in Group D ($48 \pm SD = 2.49$) as shown in Table 1. Cytoplasmic staining, on the

other hand, was deemed satisfactory in Group B (49 ± SD =2.49), also presented in Table 1. When comparing the intensity, clarity, uniformity and total scores of staining among the four groups, Group B was found to be satisfactory, as indicated in Table 2 and Table 3.

Table 1: -

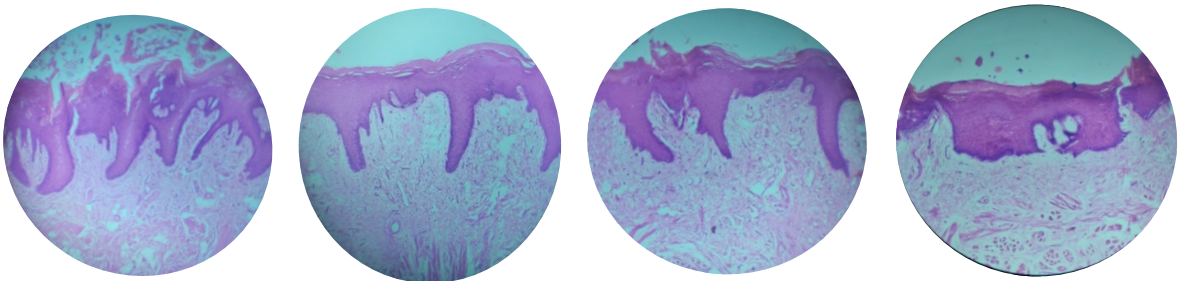
Nuclear Staining	Mean	SD	P value	Significance	Cytoplasmic Staining	Mean	SD	P value	Significance
GROUP-A (Xylene)	50	2.50	0.001	Significant	GROUP-A (Xylene)	50	2.50	0.001	Significant
GROUP-B (Kerosene)	43	2.20	0.001	Significant	GROUP-B (Kerosene)	49	2.49	0.001	Significant
GROUP-C (Turpentine)	46	2.26	0.001	Significant	GROUP-C (Turpentine)	45	2.25	0.001	Significant
GROUP-D (Olive oil)	48	2.49	0.001	Significant	GROUP-D (Olive oil)	30	1.50	0.001	Significant

Table 2: -

Intensity	Mean	SD	P value	Significance	Clarity	Mean	SD	P value	Significance
GROUP-A (Xylene)	34	1.56	0.001	Significant	GROUP-A (Xylene)	50	2.50	0.001	Significant
GROUP-B (Kerosene)	33	1.09	0.001	Significant	GROUP-B (Kerosene)	48	2.49	0.001	Significant
GROUP-C (Turpentine)	10	0.51	0.001	Significant	GROUP-C (Turpentine)	17	0.85	0.001	Significant
GROUP-D (Olive oil)	18	0.78	0.001	Significant	GROUP-D (Olive oil)	16	0.80	0.001	Significant

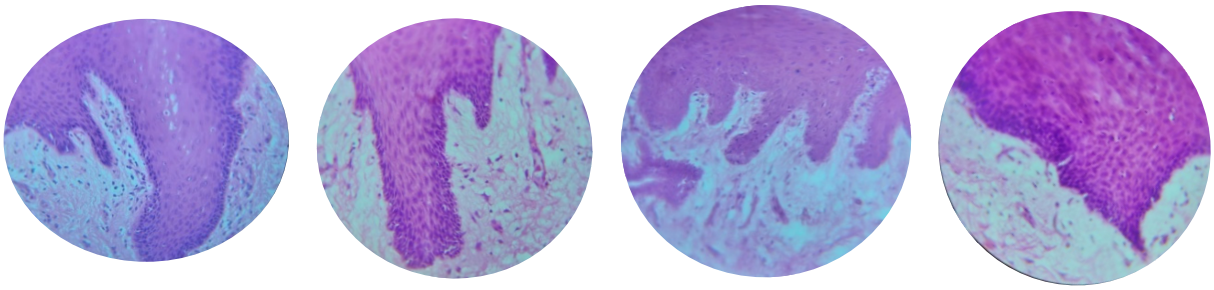
Table 3: -

Uniformity	Mean	SD	P value	Significance	Total scores	Mean	SD	P value	Significance
GROUP-A (Xylene)	41	2.01	0.001	Significant	GROUP-A (Xylene)	225	10.21	0.001	Significant
GROUP-B (Kerosene)	30	1.50	0.001	Significant	GROUP-B (Kerosene)	202	9.76	0.001	Significant
GROUP-C (Turpentine)	18	0.78	0.001	Significant	GROUP-C (Turpentine)	136	8.42	0.001	Significant
GROUPD (Olive oil)	14	0.59	0.001	Significant	GROUP-D (Olive oil)	126	7.98	0.001	Significant



A B C D

Photomicrographs showing H & E-stained sections using Xylene (A), Kerosene (B), Turpentine (C) and Olive oil (D) as a clearing agent (10X)



E F G H

Photomicrographs showing H & E-stained sections using Xylene (E), Kerosene (F), Turpentine (G) and Olive oil (H) as a clearing agent (40X)

Discussion: -

Xylene offers numerous advantages, including its stability as a fluid, rapid removal of alcohol and dehydrating agents, easy removal of molten wax, minimal tissue damage and cost-effectiveness[8]. However, it can penetrate garments and laboratory safety accessories such as gloves and boots causing skin blistering, charring and edematous changes[5]. Few studies have addressed the potential carcinogenic effects of xylene[9].

Our study revealed that xylene performed as the standard and was preferred by all groups of observers. An ideal clearing agent should be completely miscible with alcohol and paraffin wax[7]. Olive oil, being a nonpolar liquid, demonstrated miscibility with alcohol (a semipolar substance) and paraffin wax (a nonpolar substance)[6]. Similar results were reported in studies conducted by **Sermadi Z M et al[6]** and **Tsamiya R I et al[10]** where olive oil preserved cellular details and displayed normal nuclear features because of above mentioned reason. However, **Bruun Rasmussen et al[11]** found incomplete impregnation when using a mixture of coconut oil and olive oil as a clearing agent, contrasting our findings.

Mineral oil like kerosene has a density (0.83 g/ml) closer to the average density of human fat (0.92-0.97 g/ml). This allows mineral oil to remove tissue fat through displacement, unlike xylene, which dissolves it[7]. This may explain our study's results regarding cytoplasmic staining.

Clearing is a process that renders tissues transparent. When the refractive index of the clearing agent matches the tissue protein, the tissue becomes transparent[3]. Kerosene's refractive index (1.43) is like tissue protein's refractive index (1.33-1.40), so reducing light scattering properties and enhances optical clearance, making tissues more transparent[12]. This may explain our findings related to staining intensity.

The rapid penetration of a clearing agent into tissue is essential for through clearing. The viscosity of a solution plays a critical role in achieving this, as less viscous solutions penetrate more rapidly than highly viscous ones[13]. The low viscosity of kerosene may explain the favorable results regarding staining clarity observed in our study.

Dineshshankar J et al[14] found that kerosene exhibited equal clearing and staining properties without altering tissue morphology or cellular details making it a superior alternative to xylene for cytoplasmic staining, clarity and uniformity in routine H and E staining. Similarly, **Singh A et al[2]** reported excellent cytoplasmic staining, clarity and uniformity in kerosene sections, consistent with our finding. However, **Ofusori DA et al[15]** suggested that a mixture of xylene and kerosene at various ratios like 70:30 and 50:50 can effectively replace absolute xylene in histology. **Shah AA[1]** recommended using either xylene, a mixture of kerosene and xylene in the ratio 50:50 and 70:30, all of which provided good morphology of the nucleus, cytoplasm, uniformity and clarity of staining.

Conclusion: -

There is a strong case for eliminating xylene from histology laboratories. However, given the limitations of our study, including a small sample size and limited comparative data on all four clearing agents, we recommend conducting larger-scale studies that incorporate different substitutes and involve multiple observers for a more

comprehensive assessment. The responsibility for this decision lies with those in positions of authority within the field of histology.

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