Jurnal Ilmu Kedokteran (Journal of Medical Science), Jilid 19, Nomor 1, Maret 2025, Hal. 44-48
https://doi.org/10.26891/JIK.v19i1.2025. 44-48

Diluted Liquid Dishwashing Soap Effectively Displace Xylol in The Deparaffinization Process of Hematoxylin Eosin Staining

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ABSTRACT

This study aimed to determine the optimal concentration of Liquid dishwashing soap (LDS) in the deparaffinization process. The study analyzed 27FFPE of liver from Wistar rats (*Rattus norvegicus*) divided into three groups. Xylol as a control (C), LDS concentrations of 1.7% (P1) and 2% (P2) as treatments. The quality of hematoxylin Eosin staining results was evaluated using scoring, analyzed using Kruskal-Wallis and Mann-Whitney statistical tests (SPSS 26.0). The results showed a significant difference between xylol and 2% LDS concentration but no significant difference with 1.7% LDS concentration. Surfactant bind tightly with both water and paraffin to dissolve paraffin out. However, the lower viscosity solution made pulling paraffin inside the tissue easier. The study found that a lower concentration of LDS (1.7%) was more effective than a higher concentration (2%) in removing paraffin from tissue samples. Therefore, it is recommended to use 1.7% LDS as an alternative deparaffinization agent to xylol.

Keywords: Deparaffinization, Diluted, HE Staining, Liquid Dishwashing Soap, Xylene/Xylol

Xylene, a highly flammable, volatile, and toxic organic solvent, is excellent at clearing and dissolving paraffin, rendering it a viable option as a deparaffinization agent for optimal tissue staining.[1] However, xylene has certain drawbacks, including its toxicity, relative expense, flammability, volatility, and tendency to cause tissue shrinkage if soaked for prolonged durations.[2,3] Xylene contains harmful ingredients, so researchers must find excellent xylene substitutes that dissolve paraffin without affecting the stained image's quality and do not threaten human health or the environment.[4,5]

Liquid dishwashing soap (LDS) has been identified as a potential substitute for xylol due to its lower toxicity and ability to replace it. Xylol is a mixture of three isomers of xylene.[6] According to prior study,[7] when diluted to about 2%, this LDS can be used for household purposes with a lower likelihood of toxic effects than xylol. Furthermore, Pandey et al.[8] showed that 2% LDS at 90°C can be used instead of xylol. In a separate study, Ganesan et al.[9] found that 1.7% liquid dish soap at 90°C is more effective than at 65°C and 75°C. Similarly, it has been demonstrated that the efficacy of 1.7% LDS is a good deparaffinization agent.[2,10] However, another study reported contradictory results using a 2.5% concentration at 90°C[11]. However, there has been no previous comparison of the use of two optimal concentrations, 1.7% and 2.0%, to achieve the best results at 90°C.

Further research is needed to determine the optimal concentration at which diluted LDS can effectively displace xylene as a deparaffinization agent in histology staining procedures.[4] This study compares the two optimal concentrations of LDS as a substitute for xylol in the deparaffinization procedure.

METHODS

Research design

The study utilized a analytical method validation design. All research procedures and the usage of experimental animals have obtained ethical clearance from Prof. Dr. Hamka's Muhammadiyah University with the number 02/23.05/02536. Three Wistar rats (*Rattus norvegicus*) were used as research subjects and divided into three treatment groups. The control group, slides from Wistar rat liver,

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was administered xylol (C), and two experimental groups were given liquid dish soap concentrations of 1.7% (P1) and 2% (P2) in the deparaffinization process. The rat was used due to its lack of human sample tissue for the study. The total of sample was 27 according to Federer's formula as shown.

$(t-1)(n-1)$	$\geq$	15	t = number of groups
$(3-1)(n-1)$	$\geq$	15	n = number of samples required in each group
$2(n-1)$	$\geq$	15	
$2n-2$	$\geq$	15	
$2n$	$\geq$	$15+2$	10% added samples = 2,4 ≈ 3 samples
n	$\geq$	$7,5 \approx 8$	Total samples were 24 + 3 = 27 samples

To validate the method, Wistar rats, sourced from iRatco and Bogor and identified for strain purity, were used in the study due to its similarity to human. The rats were housed in individual cages and allowed one week for acclimatization, with ad libitum access to standard feeds and drinking water. A light-dark cycle of 24 hours was established. Rats were terminated with chloroform until they passed out and then subjected to abdominal surgery to retrieve liver tissue. Liver was used as it contains various cell to represent the effect of LDS and xylol treatment in the deparaffinization process.

Histological staining

The histological preparation technique is based on prior studies,[12] with adjustments based on the previously mentioned treatment. The obtained liver was immersed in Neutral buffer formaline (NBF) 10% for 21 hours. The process continued with tissue preparation process by immersion in a dehydration solution. After dehydration, the tissue is soaked in xylol I and II to clarify it, followed by paraffin infiltration and embedding. Tissue preparation was done by tissue processor (Leica TP 1020, Leica Biosystems).

Slides were deparaffinized with xylol and LDS solution according to each treatment group, as shown in table 1, followed by rehydration and followed with HE staining procedure. To optimize the best result, 90°C temperature was used in the LDS treatment. The routine staining of the Hematoxylin-Eosin (HE) was performed to examine the status of the tissue, and the staining results were graded to compare each treatment that has been applied.

Table 1. Groups deparaffinization treatment

No	Groups	Note
1	Control xylol (C)	Groups with xylol solution for the deparaffinization process
2	LDS 1.7% (P1)	Groups with 1.7% of liquid dishwashing soap (LDS) solution for the deparaffinization process
3	LDS 2.0% (P2)	Groups with 2.0% of liquid dishwashing soap (LDS) solution for the deparaffinization process

Statistical analysis

The staining results were presented as scoring as described in a previous study[13] in tables and figures with descriptive explanations. The data were analyzed both descriptively and analytically. Bivariate analysis was performed using Kruskal-Wallis and Mann-Whitney tests because of its non-parametric data characteristic (data distribution test with Saphiro-Wilk, $p<0.05$) to compare the scores.

RESULTS

Deparaffinization is crucial for removing paraffin so the tissue can bind to the stain. In the deparaffinization process, liquid dishwashing soap (LDS) has been used as a replacement for xylol. The current study used 1.7% (P1) and 2.0% (P2) of LDS, as well as xylol control (C), to determine the best treatment for Hematoxylin-eosin staining of rat hepatic tissue. The staining results of the treatment and control groups are presented as scoring according to the prior study[13] in Figure 1. All scoring was statistically tested using the Kruskal-Wallis and Mann-Whitney tests (SPSS 26.0) to distinguish the scoring system among groups, as shown in Table 2.

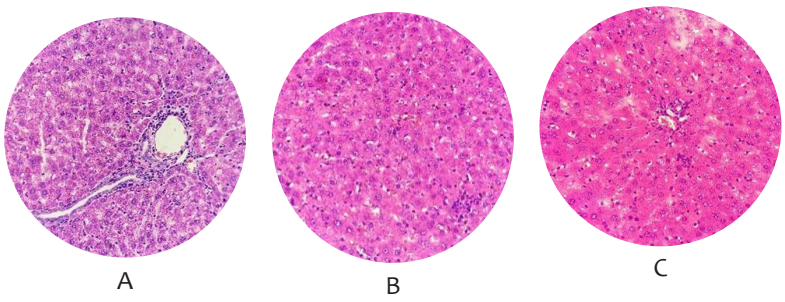

Figure 1. A comparison was made between the microscopic images of Wistar White Rat liver tissue Hematoxylin-Eosin (HE) staining results in groups control xylol (C), 1.7% (P1) of LDS and 2.0% (P2) of LDS in 400X magnification. Hematoxylin was shown in the nuclei, and Eosin was stained in the cytoplasm. The control group showed HE staining results with a score of 3; group P1 showed HE staining results with a score of 3; group P2 showed HE staining results with a score of 1.

Table 2. Statistical analysis of all groups staining scoring

No	Groups	n	Median (Min – Max)	p value
1	Control xylol (C)	9	3 (2 – 3)[a]	p < 0.05
2	LDS 1.7% (P1)	9	2 (1 – 3)	
3	LDS 2.0% (P2)	9	1 (1 – 3)[a]	

Data distribution test with Saphiro-Wilk, p<0.05. Data showed in median (minimum-maximum); Kruskal-Wallis tests significant, p<0.05. Mann-Whitney test with significant results is marked with a superscript notation; a,b,c different subscript value in the same column indicates significant differences (p<0.05).

Table 1 showed that liquid dishwashing soap (LDS) 1.7% (P1) was not significantly different from the control group (C), whereas LDS 2.0% showed otherwise. This means that 1.7% of LDS was suitable for deparaffinization.

DISCUSSIONS

Deparaffinization removes any remaining paraffin from the tissue to allow optimal dye absorption[14]. Hematoxylin-Eosin (HE) staining quality can be improved by using surfactant in liquid dishwashing soap (LDS) to remove paraffin from tissue and microscope slides. LDS shares hydrophobic (non-polar) properties similar to xylol, as surfactant, which binds tightly to non-polar material like paraffin. However, it is essential to note that LDS should be validated and optimized for each specific staining protocol to ensure accurate and reliable results.

Moreover, the use of LDS in treatment groups did not require the withdrawal and inclusion of water content, which is typically accomplished using alcohol in the rehydration and dehydration. This process was eliminated because it forms micelles bridging polar and non-polar solutions. When removing dirt and oil, the hydrophobic part of the LDS dissolves in the oil and surrounds the oil dirt, while the hydrophilic part separates from the cleaned surface and disperses in water, allowing it to be washed away.[14,15]

The scoring system from HE stains was performed according to the prior study[13] to distinguish the quality of the deparaffinization process. Some preparations with a score of 1 still leave paraffin residue on the tissue slide. This can cause changes in cell morphology, including alterations in cell size and folded parts. Additionally, the boundaries between cells may need to be clarified, making it difficult to diagnose the preparation (figure 1). Previous studies have also identified preparations with paraffin residue.[2,7-9,11,16-19] The presence of paraffin residue indicates that staining methods without xylol and alcohol are susceptible to temperature, and therefore, temperature stability must be strictly maintained. Using 90°C temperature showed the best practice in accommodating the dewaxing process. Temperature

instability can cause sections of tissue to be washed out of the preparation, leaving behind paraffin residue and resulting in out-of-focus areas. Recent studies have attempted to stabilize the temperature, but it remains challenging to maintain stability even for short periods.

It is important to note that any increase in temperature, no matter how slight, can have this effect. Maintaining a stable temperature throughout preparation is crucial to avoid this issue. Previous studies have suggested that tears or folds in the sections, thick sections, moisture in the cover glass, dirty microscopic lenses, incomplete deparaffinization, and low scoring can cause non-uniform colour and out-of-focus areas (see Figure 1).[8,11,18,20]

The scoring showed that using liquid dishwashing soap at 1.7% could assist the deparaffinization process. This study's findings were consistent with previous research.[8-9,17-18] Liquid dishwashing soap effectively dissolves paraffin, both on tissue and glass preparations. However, the results of group P2 showed a significant difference ($p<0.05$), which contradicts previous research findings that reported no significant difference between the use of 2.0% concentration and the control. This discrepancy may be attributed to using different brands of liquid dish soap and varying levels of active ingredients.[7]

According to Table 2, diluted LDS is more effective when used in low concentrations. As explained, dewaxing depends on concentration and duration, regardless of temperature.[21] Excessive extraction of paraffin due to higher concentrations can result in tissue tearing and perforation, as illustrated in Figure 1. It is essential to use the appropriate concentration to avoid these issues. The effectiveness of LDS in dewaxing processes depends on achieving the right balance between concentration and duration. Choosing the best concentration and duration is also applied in another step of histology preparation, such as fixation, dehydration, etc.

CONCLUSIONS

Diluted liquid dishwashing soap (LDS) of 1.7% assisted the deparaffinization process. However, diluted LDS must maintain the temperature strictly to prevent tissue tearing and paraffin residue. Yet, the duration and concentration of diluted solution need to be considered. Another concentration should be carried out in the following agenda, and the utility of a coated slide should be regarded to prevent the wrinkling and tearing of the tissue.

Acknowledgement

The authors thank Michael Alvian Grey for his assistance in the Cyto-histotechnology laboratory. This research was funded independently by the authors.

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