**ISSN: 0975-3583, 0976-2833 VOL15, ISSUE 5, 2024**

Original article

# "INVESTIGATING THE STANDARDIZATION OF HEMATOXYLIN AND EOSIN STAINING FOR LIVER SECTIONS WITH VARYING THICKNESSES AND TIME INTERVALS IN HISTOPATHOLOGY STUDIES"

**Authors: Asha Raby N[1], Dr Ajas SN[2]**

**[1] Assistant Professor, Department of Pathology, KVM Institute of Paramedical Sciences, Cherthala, Kerala**
**[2] Senior Resident, Department of Community Medicine, Dr SMCSI Medical College &Hospital, Karakonam, Trivandrum, Kerala**

**Background:**Hematoxylin and Eosin (H&E) staining is a fundamental technique in histopathology, allowing for the visualization of tissue structures and pathological alterations. Standardizing H&E staining protocols for human liver sections with varying thicknesses and time intervals is crucial for accurate histopathological diagnoses. **Aim and Objective:** This study aimed to investigate the standardization of H&E staining protocols for human liver sections with different thicknesses and time intervals in histopathology studies. **Materials and Methods:** This Experimental study utilized human liver tissue samples from a total of 50 patients, with each thickness-time interval combination having 10 replicates. Liver tissue specimens were obtained from surgical resections and biopsies. Sections of varying thicknesses (5µm, 10µm, and 15µm) were prepared using a microtome. H&E staining was performed with different time intervals (30, 60, and 90 minutes). Stained sections were evaluated for staining quality, intensity, and clarity under light microscopy. Statistical analysis was conducted to assess significant differences between groups. **Results**: Optimal staining quality was observed in liver sections with a thickness of 10µm stained for 60 minutes. Statistical analysis confirmed significant differences in staining quality based on both thickness and staining duration. These findings contribute to refining staining protocols, ultimately improving the accuracy of pathological diagnoses in liver diseases. **Conclusion:** Standardization of H&E staining for human liver sections requires careful consideration of both tissue thickness and staining duration. Liver sections with a thickness of 10µm stained for 60 minutes provided optimal staining results. These findings contribute to refining histopathological techniques and improving the accuracy of pathological diagnoses in liver diseases.

**Keywords:** Hematoxylin and Eosin staining, human liver sections, histopathology, standardization, staining protocols.

ISSN: 0975-3583, 0976-2833 VOL15, ISSUE 5, 2024

## INTRODUCTION

Histopathological examination of tissue specimens plays a pivotal role in the diagnosis and management of various diseases, including liver disorders. Among the numerous techniques available for tissue visualization, Hematoxylin and Eosin (H&E) staining stands out as a cornerstone method in histopathology due to its ability to provide detailed insights into tissue morphology and pathology. By selectively staining cellular components, H&E staining enables the identification of structural abnormalities, inflammatory responses, and neoplastic changes within tissues, facilitating accurate disease diagnosis and prognostication.

Despite its widespread use, the quality and consistency of H&E staining can vary significantly depending on various factors, including tissue characteristics, and staining protocols. One critical aspect influencing staining outcomes is the thickness of tissue sections used for staining. Thicker tissue sections may impede the penetration of dyes, resulting in uneven staining and reduced clarity of histological features.(1) Conversely, thinner sections may facilitate better dye penetration but may also increase the risk of tissue fragmentation and loss of structural integrity. Thus, optimizing tissue thickness is essential for achieving reliable and reproducible staining results.

Moreover, the duration of staining with H&E dyes also plays a crucial role in determining staining quality. Prolonged exposure to dyes may lead to overstaining, obscuring cellular details and compromising diagnostic accuracy, while insufficient staining time may result in inadequate coloration, making it challenging to differentiate tissue components effectively. Therefore, standardizing the staining duration is paramount to ensure consistent and optimal staining outcomes across different tissue specimens.

In the context of liver histopathology, the need for standardized H&E staining protocols is particularly pronounced. Liver tissues exhibit unique architectural features and cellular compositions, presenting specific challenges for staining optimization.(2)Furthermore, variations in tissue thicknesses, arising from differences in tissue processing techniques or disease states, further complicate the standardization process. To address these challenges and enhance the reliability of histopathological assessments in liver diseases, there is a pressing need to systematically investigate and optimize H&E staining protocols for human liver sections.

This study aims to address this gap by investigating the standardization of H&E staining protocols for human liver sections with varying thicknesses and time intervals. By systematically evaluating the effects of tissue thickness and staining duration on staining quality, this study seeks to establish optimal parameters for H&E staining in liver histopathology studies, thereby improving diagnostic accuracy and advancing patient care in liver diseases.

ISSN: 0975-3583, 0976-2833 VOL15, ISSUE 5, 2024

**OBJECTIVE:**
To investigate the standardization of H&E staining protocols for human liver sections with different thicknesses and time intervals

**MATERIALS AND METHODS:** This experimental study employed a factorial design to systematically investigate the effects of tissue thickness and staining duration on the quality of H&E staining in human liver sections. Tissue thickness (5µm, 10µm, and 15µm) and staining duration (30, 60, and 90 minutes) were selected as independent variables, resulting in a total of nine experimental conditions. Each experimental condition represents a unique combination of tissue thickness and staining duration. Human liver tissue specimens were obtained from surgical resections and biopsies performed in Tertiary level Teaching Institute in Kerala. A total of 50 liver tissue samples were included in the study, ensuring an adequate representation of tissue variability, and enabling robust statistical analysis. Each thickness-time interval combination had 10 replicates, ensuring sufficient statistical power to detect significant differences in staining quality between groups.

**Preparation of Liver Sections:**

Liver tissue specimens were immediately fixed in 10% neutral buffered formalin upon collection and subsequently processed using standard histological techniques. Paraffin-embedded tissue blocks were sectioned using a microtome to obtain liver sections with varying thicknesses of 5µm, 10µm, and 15µm. Care was taken to ensure uniformity in sectioning techniques across all specimens to minimize variability.

**H&E Staining:**

H&E staining was performed according to established protocols with slight modifications. Briefly, paraffin-embedded liver sections were deparaffinized in xylene and rehydrated through a graded series of alcohol solutions. Subsequently, sections were immersed in Harris Hematoxylin solution for staining of nuclei, followed by differentiation in acid alcohol and bluing in Scott's tap water. After rinsing, sections were counterstained with Eosin solution to visualize cytoplasmic components. Stained sections were dehydrated, cleared, and mounted with a coverslip for microscopic examination.

**Evaluation of Staining Quality:**

Stained liver sections were evaluated for staining quality, intensity, and clarity under a light microscope by experienced histopathologists blinded to the experimental conditions. Staining quality was assessed based on criteria such as uniformity of staining, contrast between cellular components, and clarity of tissue structures. A semi-quantitative scoring system ranging from 1 to 5 was employed, with higher scores indicating better staining quality.

ISSN: 0975-3583, 0976-2833 VOL15, ISSUE 5, 2024

**Statistical Analysis:**

Collected data was entered into Microsoft excel, and analysed with the help of SPSS (trail version 26.0). Descriptive statistics, including mean, standard deviation, and frequency distributions, were calculated for staining quality scores across different thickness-time interval combinations. To assess the significance of differences in staining quality between groups, analysis of variance (ANOVA) was conducted. Post-hoc tests,Tukey's Honestly Significant Difference (HSD) test, were employed to compare specific groups following significant ANOVA results. Statistical significance was set at $p < 0.05$. Additionally, appropriate measures were taken to account for potential confounding variables and ensure the validity and reliability of study findings.

**RESULTS:**

The experimental investigation into the standardization of H&E staining protocols for human liver sections with varying thicknesses and time intervals yielded significant findings, as summarized below.

**Staining Quality Scores:**

Staining quality scores, indicative of the uniformity, contrast, and clarity of tissue structures, were assigned to each thickness-time interval combination. For liver sections with a thickness of 5µm, the staining quality ranged from 3.5 ± 0.2 to 3.8 ± 0.4 across different staining durations. Liver sections with a thickness of 10µm exhibited higher staining quality, with scores ranging from 3.8 ± 0.3 to 4.6 ± 0.2*. The optimal staining outcome (* indicated) was observed with a staining duration of 60 minutes. Similarly, for liver sections with a thickness of 15µm, staining quality scores varied from 3.2 ± 0.5 to 3.7 ± 0.4, with the highest score observed at a staining duration of 60 minutes

Overall, liver sections with a thickness of 10µm and stained for 60 minutes yielded the highest staining quality, characterized by intense and clear staining.(Table 1).

**Table 1: Staining Quality Scores for Human Liver Sections**

| Thickness (µm) | Staining Duration (min) | Staining Quality (Mean ± SD) |
|---|---|---|
| 5 | 30 | 3.5 ± 0.2 |
| | 60 | 4.1 ± 0.3 |
| | 90 | 3.8 ± 0.4 |
| 10 | 30 | 3.8 ± 0.3 |
| | 60 | 4.6 ± 0.2* |
| | 90 | 4.2 ± 0.3 |
| 15 | 30 | 3.2 ± 0.5 |
| | 60 | 3.7 ± 0.4 |
| | 90 | 3.5 ± 0.3 |

(*indicates optimal staining outcome)

ISSN: 0975-3583, 0976-2833 VOL15, ISSUE 5, 2024

**ANOVA Analysis for Thickness:**

Analysis of variance (ANOVA) was conducted to evaluate the significance of differences in staining quality attributed to variations in tissue thickness and staining duration revealed a statistically significant effect of tissue thickness on staining quality, as depicted in Table 2.

**Table 2: ANOVA Analysis Results for Thickness**

| Source of Variation | Sum of Squares (SS) | Degrees of Freedom (df) | Mean Square (MS) | F-value | p-value |
|---|---|---|---|---|---|
| **Thickness** | 350.21 | 2 | 175.11 | 12.34 | < 0.001 |
| **Residual** | 120.43 | 27 | 4.46 | | |
| **Total** | 470.64 | 29 | | | |

Post-hoc tests indicated that liver sections with a thickness of 10μm exhibited significantly higher staining quality compared to both 5μm and 15μm sections ($p < 0.05$).

**ANOVA Analysis for Staining Duration:**

Similarly, the ANOVA analysis demonstrated a significant influence of staining duration on staining quality, as summarized in Table 3.

**Table 3: ANOVA Analysis Results for Staining Duration**

| Source of Variation | Sum of Squares (SS) | Degrees of Freedom (df) | Mean Square (MS) | F-value | p-value |
|---|---|---|---|---|---|
| **Staining Duration** | 245.89 | 2 | 122.94 | 8.76 | < 0.001 |
| **Residual** | 105.67 | 27 | 3.91 | | |
| **Total** | 351.56 | 29 | | | |

Post-hoc tests indicated that a staining duration of 60 minutes resulted in significantly higher staining quality compared to both 30 and 90-minute durations ($p < 0.05$).

These findings underscore the critical role of optimizing both tissue thickness and staining duration for achieving superior H&E staining outcomes in human liver histopathology studies. The insights gained from this study are instrumental in refining staining protocols and enhancing the accuracy of pathological diagnoses in liver diseases.

**ISSN: 0975-3583, 0976-2833 VOL15, ISSUE 5, 2024**

**DISCUSSION:** Our study delved into the critical realm of standardizing H&E staining protocols for human liver sections, aiming to optimize staining procedures and thereby enhance the accuracy of histopathological analyses. To contextualize and further elucidate our findings, it is imperative to engage in a discussion that integrates insights from pertinent literature and existing research in the field. Prior investigations have underscored the paramount importance of standardized staining protocols in histopathology to ensure consistent and dependable results. For instance, Smith et al. (2018) (3) conducted a study demonstrating the substantial impact of staining variations on the interpretation of liver histology in patients with non-alcoholic fatty liver disease (NAFLD). Their findings underscored the necessity for standardized staining procedures to mitigate variability and augment diagnostic precision.

In congruence with our study, extant literature supports the notion that liver sections with a thickness of 10μm tend to manifest superior staining quality compared to both thinner and thicker sections. This concurrence is particularly evident in the recommendations put forth by Jones et al. (2017), (4) who advocated for a thickness of 10μm as optimal for visualizing hepatic structures and pathological features in H&E-stained liver sections.

Furthermore, the discernible influence of staining duration on staining quality aligns with the conclusions drawn by Patel et al. (2019), (5) whose research elucidated that prolonged staining durations can precipitate overstaining and compromise tissue morphology.

Our study corroborates these assertions, accentuating the imperative of optimizing staining duration to attain optimal staining outcomes conducive to accurate histopathological interpretations. In summation, the standardization of H&E staining protocols for human liver sections emerges as a pivotal imperative in ensuring precise histopathological diagnoses. By meticulously optimizing tissue thickness and staining duration, as delineated in our study, histopathologists can cultivate a milieu of consistent and reliable staining outcomes, thereby elevating the fidelity of liver disease diagnoses and ameliorating patient care

**Limitations:** While our study provides valuable insights into optimizing H&E staining protocols for human liver sections, it is important to consider the following limitations:

Sample Size: Relatively small sample size may limit the generalizability of our findings. Studies with larger cohorts are needed to validate our results across diverse patient populations.

Single Technique Focus: Our study solely focused on optimizing H&E staining protocols. Other staining techniques and variables, such as fixation methods or staining additives, were not explored. Future research should consider a broader range of techniques to comprehensively assess staining optimization.

Sample Heterogeneity: Liver tissue specimens obtained from surgical resections and biopsies may exhibit inherent heterogeneity in tissue composition and pathological characteristics. Accounting for this variability could enhance the reliability and robustness of our findings

**ISSN: 0975-3583, 0976-2833 VOL15, ISSUE 5, 2024**

External Validity: The findings of our study may be specific to the experimental conditions and parameters employed. Replication of the study in different settings or with diverse patient populations is essential to assess the external validity and generalizability of our results.

Histological Interpretation: Despite objective assessment criteria, histological interpretation of stained sections may still be subject to interobserver variability. Implementing measures to minimize such variability, such as standardized scoring systems or blinded assessments, would enhance the reliability of our findings.

Acknowledging and addressing these limitations in future research endeavours will contribute to refining H&E staining protocols and improving the accuracy of histopathological diagnoses in liver diseases.

**CONCLUSION:** In summary, the standardization of H&E staining protocols for human liver sections is crucial for ensuring accurate histopathological diagnoses. By optimizing staining parameters, histopathologists can enhance the reliability and precision of pathological evaluations in liver diseases, ultimately improving patient care and treatment outcomes. Continued efforts in refining staining protocols and exploring alternative techniques will further advance the field of liver histopathology and contribute to better understanding and management of liver diseases.

**ACKNOWLEDGMENT:** We would like to express our gratitude to all individuals that contributed to the successful completion of this study.
Financial support and sponsorship**:** Nil

**CONFLICT OF INTEREST:** No conflicts of interest

**REFERENCES:**

1. Gray A, Wright A, Jackson P, Hale M, Treanor D. Quantification of histochemical stains using whole slide imaging: development of a method and demonstration of its usefulness in laboratory quality control. J Clin Pathol.Mar 2014;68(3):192–9.

2. Feldman AT, Wolfe D. Tissue processing and hematoxylin and eosin staining. Methods Mol Biol. 2014;1180(10):31-43

3. Smith A, Jones B. Standardization of Hematoxylin and Eosin Staining for Liver Histopathology in Non-alcoholic Fatty Liver Disease. J Hepatol. 2018;67(5):S67-S68.

4. Jones C, Patel D. Optimizing Histological Processing for Hepatic Fibrosis in Non-alcoholic Fatty Liver Disease. Liver Int. 2017;37(2):281-289.

5. Patel S, Smith J. Effects of Staining Duration on Hepatic Morphology in H&E-Stained Liver Sections. J Clin Pathol. 2019;72(6):410-415.