ORIGINAL RESEARCH

Comparison of Rubeanic acid and Rhodanine staining for detection of copper in liver with copper quantitation by Inductively Coupled Plasma Mass Spectrometry (ICPMS) Method

Asha Raby N[1], Dr. Ajas SN[2]

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

Corresponding author
Dr. Ajas SN
Senior Resident, Department of Community Medicine, Dr SMCSI Medical College &Hospital, Karakonam, Trivandrum, Kerala, India

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ABSTRACT

Background: Copper is an essential trace element that plays vital roles in various physiological processes. However, excessive accumulation of copper in tissues, particularly in the liver, can lead to severe health complications, including liver damage and neurological disorders. Wilson's disease, characterized by abnormal copper metabolism, underscores the importance of accurate methods for detecting copper deposits in liver tissues. Several staining techniques have been developed to visualize copper deposits in histological sections. Among these, Rubeanic Acid and Rhodanine staining methods have gained prominence for their ability to selectively bind to copper ions, producing distinctive colour changes. While both methods have been used for detecting copper in liver tissues, their comparative efficacy remain unclear. **Aim and Objective:** To compare two different histochemical stains ie, Rubeanic acid and Rhodanine for detection of copper in liver with copper quantitation by Inductively Coupled Plasma Mass Spectrometry (ICPMS) Method. **Materials and Methods**: Between 2016 and 2021, a total of 33 patients diagnosed with Wilson's disease or suspected cases were included in the study. Cell blocks from these patients were gathered and subjected to standardized Rhodanine staining. Subsequently, both Rubeanic acid and Rhodanine staining procedures were conducted on all collected samples. The comparison of the histochemical stains with copper quantitation using ICPMS method was then carried out for each stain individually. **Results:** The research involved 33 cases, consisting of 26 males and 7 females, aged between 4 and 93 years, with mean age of 46.70 and a standard deviation of 32.95.Out of these cases, after employing the copper quantitation test and reviewing records from clinical and other biochemical tests, 22 cases were definitively diagnosed as Wilson's disease. The remaining 11 cases included diagnoses such as Alcoholic Liver Disease (ALD), Non-Alcoholic Fatty Liver Disease (NAFLD), Autoimmune Hepatitis (AIH), and Drug-Induced Liver Injury (DILI). Rubeanic acid staining revealed that among the 22 cases diagnosed with Wilson's disease, 10 exhibited positive staining: 5 cases at 1+, 4 at 2+, and 1 at 3+. Rhodanine staining, on the other hand, showed positive results for 7 cases: 3 at 1+, 3 at 2+, and 1 at 3+. The remaining cases in both groups were graded as 0. While Rubeanic acid staining identified 10 cases compared to Rhodanine, no statistically significant association was found between the two methods ($p = <0.07$). Additionally, a moderate positive correlation was observed between Rubeanic acid staining and copper quantitation ($r = 0.365$, $p < 0.001$), suggesting that Rubeanic acid may be a more reliable indicator of copper levels than Rhodanine staining. **Conclusion:** Rubeanic acid outperformed Rhodanine in detecting liver copper accumulation, but larger tissue sections showed staining variability. Negative staining in small biopsies does not rule out Wilson's disease. Some cases with elevated copper levels showed negative staining, indicating early-stage accumulation. So, to conclude, Histochemical stains can support diagnosis alongside clinical factors, but copper quantitation remains the gold standard.

Keywords: Rhodanine,Rubenaic acid, Copper quantitation

INTRODUCTION

Copper is an essential trace element vital for various physiological processes in the human body. However, its dysregulation can lead to severe health complications, particularly in conditions such as Wilson's disease, where copper accumulation in the liver poses a significant risk.(1). Accurate and sensitive methods for detecting copper in biological

tissues are crucial for diagnosis and monitoring of such disorders. Among the numerous techniques available for copper detection, staining methods utilizing chelating agents like Rubeanic acid and Rhodanine have gained prominence for their simplicity, cost-effectiveness, and sensitivity. These staining methods rely on the formation of distinctive coloured complexes with copper ions, facilitating visual detection of copper deposits within tissue samples. Despite their widespread use, discrepancies in the sensitivity and specificity of staining methods have been reported, necessitating comparative evaluations to determine their efficacy in detecting copper accumulation accurately(2). Moreover, while staining methods provide qualitative assessments of copper presence, quantitative analysis is often required for precise determination of copper levels, especially in cases requiring meticulous monitoring or intervention. In this study, we compared the performance of two commonly employed staining techniques, Rubeanic acid, and Rhodanine, for the detection of copper in liver tissue samples. Additionally, wecorrelated the staining results with quantitative measurements obtained through Inductively Coupled Plasma Mass Spectrometry (ICPMS), a highly sensitive and precise analytical method capable of quantifying trace elements like copper in biological matrices. By systematically evaluating the staining methods against the gold standard of ICPMS, we seek to elucidate their relative advantages, limitations, and overall suitability for copper detection in liver tissue. Such comparative analyses are indispensable for enhancing diagnostic accuracy, guiding therapeutic decisions, and advancing our understanding of copper-related disorders.

OBJECTIVE

To compare two different histochemical stains ie, Rubeanic acid and Rhodanine for detection of copper in liver with copper quantitation by Inductively Coupled Plasma Mass Spectrometry (ICPMS) Method

MATERIALS AND METHODS

A prospective-retrospective study was conducted at a tertiary level teaching hospital in Kerala, focusing on 33 patients diagnosed or suspected of having Wilson's disease from 2016 to 2021, utilizing a convenient sampling technique. A self-administered, semi-structured questionnaire was employed for data collection. The study included patients diagnosed/suspected with Wilson's disease and those with tissue exhibiting Rubeanic acid positivity in liver biopsy/explant, while patients not meeting these criteria were excluded. Cell blocks from these patients were obtained and subjected to standardized Rhodanine staining following established protocols and guidelines.Modified Rhodanine and Rubeanic acid techniques were applied to assess copper deposition in all cases. Liver tissue copper levels (dry weight copper estimation) were determined using an Inductively Coupled Plasma Mass Spectrophotometer (ICPMS) method, capable of detecting metals and non-metals at concentrations as low as parts per billion with minimal interference from background isotopes. Subsequently, a comparison of histochemical stains with copper quantitation was performed for each stain individually. The collected data were inputted into Microsoft Excel and analyzed using a combination of Microsoft Excel and SPSS (trial version 25.0). Statistical analysis involved calculating percentages for qualitative data and determining mean values ± standard deviation (SD) for quantitative data. Spearman rank correlation coefficient was computed to assess the relationship between Rubeanic acid stain value, Rhodanine stain value, and copper quantitation in the liver. Fisher–Z-test transformation with Z-test was utilized to compare the correlation coefficients of the two histochemical stains with copper quantitation.

RESULTS

In our research, 33 participants took part, comprising 26 males (78.8%) and 7 females (21.2%), with a mean age of 46.70 and a standard deviation of 32.95. The majority fell within the 0-20 age group (39.4%), followed by the 41-60 age group (30.3%).

Among a total of 33 cases, 22 received a diagnosis of Wilson's disease, remaining 11 cases involved diagnoses such as Alcoholic Liver Disease (ALD), Non-Alcoholic Fatty Liver Disease (NAFLD), Autoimmune Hepatitis (AIH), and Drug-Induced Liver Injury (DILI) which was determined through the results of the Copper quantitation test, in addition to records obtained from clinical examinations and other biochemical tests. Rubeanic acid and Rhodanine staining were performed on all 33 cases regardless of diagnosis and compared with ICPMS copper quantitation method.

Table 1: GRADING SYSTEM FOR STAINING

Grading	Copper Staining Pattern
0	Absent
1+	Few Granules
2+	Moderate Granules
3+	High Granules

Among the 22 cases diagnosed with Wilson's disease, Rubeanic acid staining yielded positive results in 10 cases: 5 cases at 1+, 4 at 2+, and 1 at 3+. In contrast, Rhodanine staining showed positive results in 7 cases: 3

cases at 1+, 3 at 2+, and 1 at 3+. All other cases in both groups received a grade of 0. The grading system for staining was specified in Table 1. Despite Rubeanic acid staining identifying 10 cases when compared with Rhodanine, no statistically significant association was found between the two methods (p = <0.07). Furthermore, the staining quality was noted to be superior with Rubeanic acid.(Figure 1&2)

Figure 1: Rubeanic Acid Staining Results

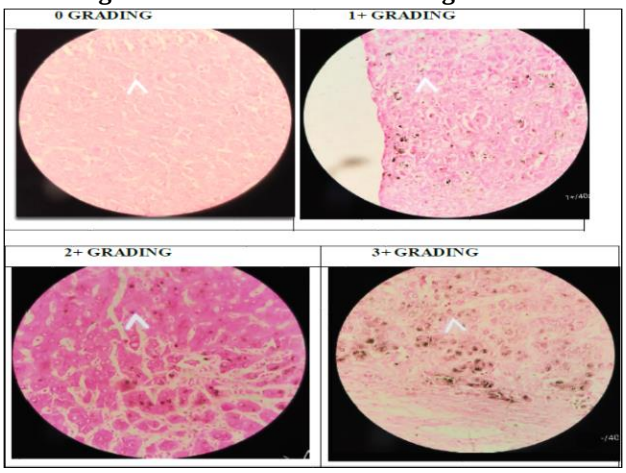
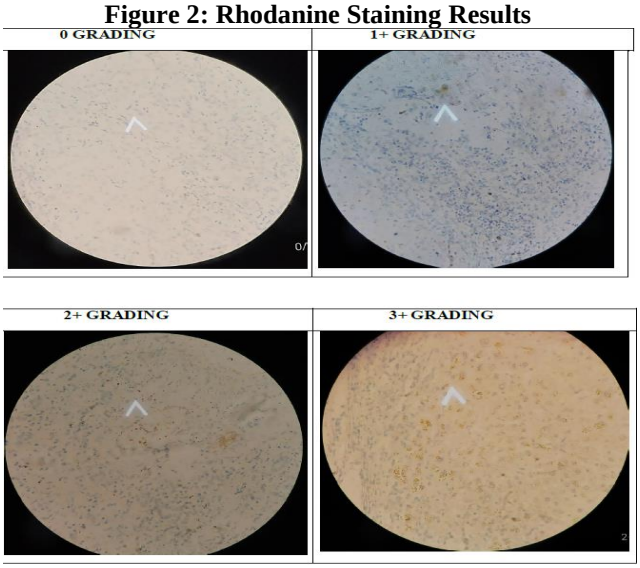


Figure 2: Rhodanine Staining Results



Concerning cholestasis, among the 33 participants, 3 cases were graded as 2+ for Rhodanine staining, while the remaining 30 cases were graded as 0. For Rubeanic acid staining, 6 cases exhibited cholestasis, with 5 cases graded as 1+ and 1 case graded as 3+, whereas the remaining 26 cases were graded as 0. (Figure 3&4)

Figure 3: Rhodanine Grading And Cholestasis

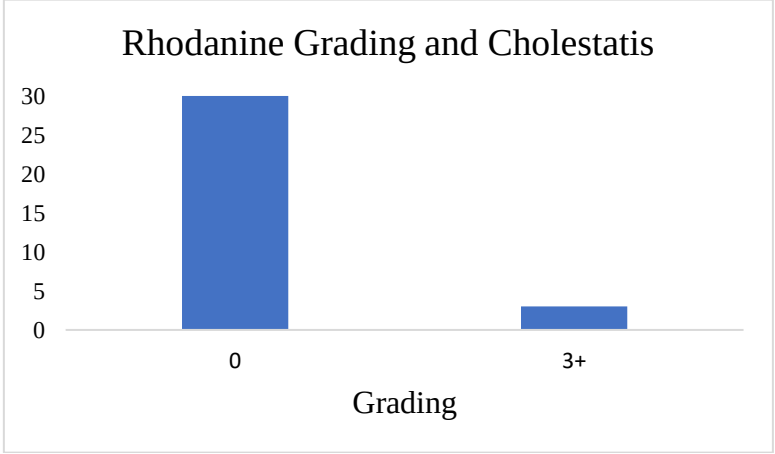
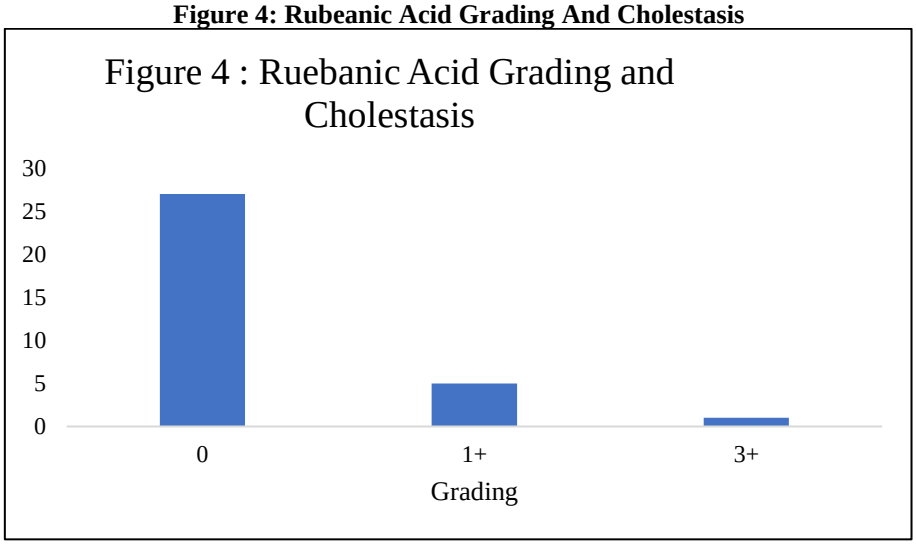


Figure 4: Rubeanic Acid Grading And Cholestasis



Statistical Analysisofcomparison Of Rubeanic Acid And Rhodanine With Copper Quantitation

A moderate positive correlation was noted between Rubeanic acid staining and copper quantitation ($r = 0.365$, $p < 0.001$), indicating that Rubeanic acid might serve as a more dependable indicator of copper levels compared to Rhodanine staining.

DISCUSSION

A literature review examining the histochemical demonstration of tissue copper indicates that none of the stains currently in use achieves 100% sensitivity or specificity. The objective of this study was to compare histochemical staining with copper quantitation in liver tissue using the ICPMS method. Among the histochemical stains assessed, Rubeanic acid demonstrated superiority over Rhodanine in detecting copper accumulation in the liver. Discrepancies were observed in larger tissue sections, revealing variations in copper staining. Therefore, a lack of positive histochemical staining in small biopsies does not rule out the diagnosis of Wilson's disease. In our series, there were cases that tested negative for both histochemical stains despite having elevated liver copper values. This discrepancy may be attributed to the type of copper accumulation in early stages, highlighting once more that histochemical negativity does not preclude the diagnosis of Wilson's disease. S. Jain et al. compared histochemical staining for copper using Rubeanic acid, Rhodanine, and Orcein in 35 biopsies from patients with chronic liver disease, including 17 from patients with Wilson's disease (WD). They concluded that histochemical stains may be useful for identifying copper deposits. All 17 biopsy specimens from patients with Wilson's disease exhibited high liver copper concentrations, but only nine showed positive staining for copper. (3) Nese Karadag et al. investigated the effect of copper staining in Wilson's disease through a liver explant study. Among 33 patients meeting the examination criteria, two copper stains, Timm silver sulphide and Rhodanine, were performed in all cases, while Orcein staining was conducted in 25 cases. The positivity rates for these copper stains were 85%, 82%, and 36%, respectively.(4)Blasco et al. conducted a study on Wilson's disease, performing a histological review of seven patients and assessing the value of histological copper positivity compared to other hepatopathies. They utilized the Rhodanine histochemical technique to demonstrate copper in the mentioned samples and in three other types of liver diseases. The sensitivity of the histochemical demonstration of copper with Rhodanine was high (6/7), but its specificity was low, being positive in 68% of primary biliary cirrhosis cases and 19% of other cholestatic hepatopathies.(5) In contrast, our study found that Rubeanic acid performed effectively. In summary, our study findings suggest that histochemical stains are not reliable indicators for supporting the diagnosis of Wilson's disease, as the presence of staining in suspected cases does not necessarily align with clinical and morphological perspectives. Therefore, the gold standard for diagnosis continues to be copper quantitation.

LIMITATIONS

Due to limited case availability and financial constraints, our study lacked enough cases for comparison with the gold standard, copper quantitation. Additionally, the study was hindered by the absence of more than two categories for detecting sensitivity and specificity of stains, as we only had two stains available. Therefore, our study faced challenges in assessing sensitivity and specificity.

CONCLUSION

Gold standard for diagnosis remains copper quantitation when compared to staining techniques. However, further research is necessary in this field to explore and validate the efficacy of different diagnostic methods.

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Conflict Of Interest: No conflicts of interest

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