Revisiting the Topic of Histochemically Detectable Copper in Various Liver Diseases With Special Focus on Venous Outflow Impairment

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Key Words: Rhodanine; Copper; Venous outflow impairment; Chronic biliary disease; Chronic liver disease; Cirrhosis

DOI: 10.1309/AJCPDZR4OHDQNG3L

Abstract

We surveyed histochemically detectable copper in various liver diseases with emphasis on chronic biliary disease (CBD) and venous outflow impairment. Using rhodanine, we graded copper accumulation in 298 liver specimens: venous outflow impairment (n = 64), CBD (n = 123), Wilson disease (WD) (n = 12), chronic hepatitis C (n = 32), steatohepatitis (n = 28), sarcoidosis (n = 15), cholestatic hepatitis (n = 12), and acute large bile duct obstruction (n = 12). Copper was detected in 39% of specimens; all had chronic liver disease. Copper increased with increasing fibrosis. CBD accumulated copper more frequently than other chronic diseases (except WD), both in early (61% vs 3%) and late (94% vs 59%) stages and in larger amounts. Rhodanine was positive in 73% of livers with CBD, 20% with sarcoidosis, 9% with chronic hepatitis C, and 7% with steatohepatitis. Copper was detected in 14% of chronic venous outflow impairment specimens; with 1 exception, stainable copper was absent in early stages but detected in 38% of cirrhotic livers. In conclusion, rhodanine helps differentiate CBD from other conditions, including venous outflow impairment; in the absence of advanced fibrosis, rhodanine positivity strongly favors CBD. In contrast, rhodanine positivity is nonspecific in cirrhosis, but the absence of copper in that setting excludes CBD.

The liver plays a central role in storing, mobilizing, and excreting copper.1,2 After intestinal absorption and uptake by hepatocytes, 80% of absorbed copper is packaged in lysosomes for excretion in bile, and a small fraction is incorporated into ceruloplasmin for release into blood.2 Copper accumulates in hepatocytes in some physiologic conditions (fetal and neonatal periods)3,4 when there are defects of copper metabolism (Wilson disease [WD])1,5 or when copper excretion is impaired (chronic biliary injury).2,4,6-13 Cytoplasmic copper is toxic to hepatocytes,14,15 acting by damaging the cellular plasma membrane, mitochondrial membrane, and tubulin2,16 and eventually triggering apoptosis via upregulation of Bax.17 In contrast, lysosomal copper is not toxic5; insoluble protein-bound copper complexes stored in lysosomes offer a cellular defense mechanism in conditions of chronic copper accumulation.14,18,19 Histochemical detection of excess copper accumulated in such complexes by various stains, including rhodanine,20,21 rubeanic acid,22,23 Timm silver stain,5 and orcein,4,11,13,24,25 can play a role in distinguishing chronic biliary disease (CBD) from other conditions,4,11,22 but this method is not entirely specific, as histochemically demonstrable copper has been described in many other forms of hepatic injury.4,10-13,21,22,25-29

Paradoxical as it may seem, venous outflow impairment can mimic CBD. Liver biochemistry profiles in venous outflow impairment often resemble those of chronic biliary injury, featuring mildly elevated transaminases in two-thirds of patients and elevated alkaline phosphatase in nearly all, with values often exceeding 500 U/L.30 In patients with Budd-Chiari syndrome, endoscopic retrograde cholangiopancreatography may show biliary abnormalities such as crowding, elongation, and dilation of bile ducts.31 Histologic changes...
mimicking CBD, including portal expansion, portal inflammation, bile ductular proliferation, and even focal lymphocytic cholangitis, are commonly seen in biopsy specimens from patients with venous outflow impairment.30

Copper levels in the noncirrhotic liver of patients with cardiovascular disease are reportedly comparable to those of healthy adults,6 but copper accumulation in livers with venous outflow impairment has not been studied in detail. The objective of this study is to use the rhodanine stain to survey hepatic copper contents in a variety of liver diseases, with emphasis on CBD and venous outflow impairment. We aim to evaluate the role of rhodanine in differentiating between CBD, venous outflow impairment, and other liver conditions, as well as investigate the relationship between histochemical positivity for copper and disease progression.

Materials and Methods

Patients

Using our electronic pathology database, we selected 298 liver specimens (246 liver biopsy specimens and 52 explanted livers) obtained from adults between 1987 and 2005. The study set was chosen to represent a spectrum of acute and chronic liver disease but was intentionally skewed toward venous outflow impairment and CBD. Cases were included from various disease stages in chronic conditions (except WD). We reviewed histologic slides and medical records to confirm all diagnoses.

There were 64 examples of venous outflow impairment. The diagnosis required congestion and sinusoidal dilatation in zone 3, liver cell plate atrophy, and red blood cell extravasation, with clinical and radiologic confirmation of the diagnosis. Twenty-six patients had veno-occlusive disease, 21 had Budd-Chiari syndrome, 9 had congestive heart failure, and 8 had cardiac amyloidosis. Venous outflow impairment was classified as acute (acute clinical presentation [<6 months' duration], and absence of fibrosis) or chronic. Because standardized staging systems of fibrosis in venous outflow impairment do not exist, fibrosis was evaluated using the Batts-Ludwig system.34 Steatohepatitis (28 patients) was staged according to Brunt et al.35 Chronic hepatitis C (32 patients) was staged using the Batts-Ludwig criteria.32,33 Chronic hepatitis B (10 patients) was staged according to Guarascio et al.11 Staging criteria for sarcoidosis do not exist, fibrosis was evaluated using the Batts-Ludwig system34 (for chronic hepatitis) or the Ludwic criteria32,33 (for CBD) depending on the predominant histologic pattern of injury (hepatitic vs biliary).

All 12 cases of cholestatic hepatitis had an acute presentation (<6 months' duration), lacked significant fibrosis, and were either proven or suspected to be medication/toxin induced. A diagnosis of acute large bile duct obstruction (12 cases) required radiologic confirmation, characteristic morphologic features (portal edema, ductular reaction, and cholestasis), and lack of fibrosis. All 12 patients with WD had decreased serum ceruloplasmin levels, increased hepatic copper determined by quantitative analysis (>250 µg/g dry weight), and compatible histologic features including steatosis (10), steatohepatitis (5), prominent glycogenated nuclei (4), panacinar hepatitis (1), and submassive necrosis (2). Kayser-Fleischer rings were identified in 4 patients, and neuropsychiatric symptoms were present in 3 patients. WD was staged based on the predominant histologic pattern (steatohepatitis vs chronic hepatitis).

Histochemistry

Tissue copper was assessed by rhodanine stain using 10-µm sections cut from archived formalin-fixed, paraffin-embedded tissue blocks. After being deparaffinized and hydrated in distilled water, sections were placed in rhodanine working solution and heated for 45 seconds in a microwave set on medium heat. Heating was continued at 10-second intervals on medium heat until the control colored. Sections were removed from the microwave and kept in the hot solution for 2 minutes and then rinsed in distilled water, counterstained with hematoxylin for 30 seconds, and washed in running tap water. Finally, sections were placed in saturated lithium carbonate for 15 seconds, washed in running tap water, dehydrated in 95% alcohol and absolute alcohol, and cleaned in xylene. Positive staining was characterized by rusty red granules in the hepatocellular cytoplasm. We used the system developed by Ludwig et al10 and adapted it with minor modifications by Guarascio et al11 and Miyamura et al12 to grade copper accumulation. Hence, rhodanine-stained slides were reviewed and graded by 2 pathologists (T.M.
and T.C.S.) as follows: negative, no rusty red cytoplasmic granules in hepatocytes; grade 1, isolated periportal cells containing small sparse granules (not seen at low magnification and involving less than 2 lobules/nodules); grade 2, moderate to numerous periportal hepatocytes containing copper granules (seen at low magnification, mainly in zone 1); and grade 3, widespread and heavy deposition of granules throughout the lobule.

**Results**

The patients ranged from 19 to 73 years of age; there were 161 men and 137 women. Overall, 115 of the 298 specimens (39%) had copper detected by rhodanine stain. Table 1 shows the distribution of cases with demonstrable copper, separated by diagnosis and subdivided by stage of disease. Samples from patients with acute liver diseases (cholestatic hepatitis, acute large bile duct obstruction, and

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**Table 1**

Copper Grading of 298 Rhodanine-Stained Liver Specimens Using the Modified Ludwig Stainable Copper Grading System

<table>
<thead>
<tr>
<th>Liver Disease</th>
<th>Rhodanine Staining Results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td>Acute venous outflow impairment</td>
<td>13</td>
</tr>
<tr>
<td>Chronic venous outflow impairment</td>
<td>44</td>
</tr>
<tr>
<td>Wilson disease</td>
<td>2</td>
</tr>
<tr>
<td>PBC</td>
<td>16</td>
</tr>
<tr>
<td>PSC</td>
<td>17</td>
</tr>
<tr>
<td>Hepatitis C</td>
<td>29</td>
</tr>
<tr>
<td>Cholestatic hepatitis</td>
<td>12</td>
</tr>
<tr>
<td>Acute large bile duct obstruction</td>
<td>12</td>
</tr>
<tr>
<td>Steatohepatitis</td>
<td>26</td>
</tr>
<tr>
<td>Sarcoidosis</td>
<td>12</td>
</tr>
<tr>
<td>Total</td>
<td>183</td>
</tr>
</tbody>
</table>

PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis.

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**Figure 1** Distribution of copper in 298 rhodanine-stained liver specimens by disease stage and rhodanine staining grade. NA, not applicable; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; WD, Wilson disease.
acute venous outflow impairment) did not have stainable copper. Copper was detected in only a few patients with chronic hepatitis C (3 of 32) and steatohepatitis (2 of 28) and was very much a function of disease stage; 4 of 5 copper-positive samples in these 2 patient groups were cirrhotic. Three of 15 sarcoidosis biopsy specimens had stainable copper, 1 with portal fibrosis (stage 1) and 2 with bridging (septal) fibrosis (stage 3). As expected, WD and CBD accumulated copper in higher grades than other chronic liver diseases. Copper was detected in all cases of WD in which fibrosis (of any degree) had developed. On the other hand, 2 cases of WD without fibrosis had no detectable copper. In addition, the grade of copper deposition increased with disease progression (Figure 1). Both in early-stage and late-stage disease, stainable copper was found more frequently in CBD than in other chronic liver diseases (except WD) (stages 1 and 2: 61% vs 3%, stages 3 and 4: 94% vs 59%, respectively) (Figure 1). Overall, 69% (35 of 51) of the PBC samples had stainable copper; in PSC, the rate was 76% (55 of 72). As shown in Figure 1, both the prevalence and the grade of copper deposition increased with increasing stage; 38% of biopsy specimens with stage 1 CBD had stainable copper compared with 78% in stage 2, 91% in stage 3, and 100% in cirrhosis. Whereas all livers with biliary cirrhosis accumulated copper, stainable copper was detected in 36% (9 of 25) of livers with nonbiliary cirrhosis (excluding WD).

The 64 patients classified as having venous outflow impairment tended to have cholestatic biochemical profiles. Alkaline phosphatase was elevated in 92% of patients and exceeded 500 U/L in 34%. Serum bilirubin was increased in 78% of patients. Transaminases were increased in 64% of patients but did not exceed 180 U/L in any patient. Despite the biochemical findings, none of 13 livers with acute venous outflow impairment and only 7 of 51 (14%) with chronic venous outflow impairment had stainable copper. Except for a single liver explant from a patient with stage 2 Budd-Chiari syndrome that had focal subcapsular copper deposition, no other liver with venous outflow impairment had stainable copper.

Liver explant with cirrhosis in a patient with Wilson disease. A rhodanine stain shows widespread and heavy deposition of copper granules throughout the cirrhotic nodule. This was interpreted as a positive result (grade 3) (rhodanine, ×200).

A liver biopsy specimen from a patient with primary biliary cirrhosis, stage 1. A, Histologic sections demonstrate a dense lymphoplasmacytic portal inflammatory infiltrate with a poorly formed noncaseating granulomatous component and lymphocytic cholangitis (H&E, ×40). B, A few isolated copper-positive cells containing small sparse granules are observed in zone 1. This was interpreted as a positive result (grade 1) (rhodanine, ×400).
deposition in hepatocytes and multinucleated giant cells [Image 3], early stages (1 and 2) of venous outflow impairment did not show stainable copper. In contrast, copper was detected in 5 of 13 (38%) specimens with stage 4 disease [Image 4].

Discussion

Histochemical detection of copper can be accomplished with orcein, which reacts with disulphide groups in lysosomal copper complexes. Orcein, however, is not specific for copper; it binds to a lysosomal substance that may or may not contain copper. More specific histochemical copper detection can be achieved with Timm silver stain, rhodanine, and rubeanic acid. Although orcein and Timm silver stain have high sensitivity, rhodamine is considered the most reliable among the aforementioned stains, as it gives the most reproducible results and displays a linear relationship with tissue copper levels as determined by atomic absorption spectroscopy. In contrast to Timm stain, rhodamine is also technically simple and fast. We use rhodamine in routine clinical practice and chose it for this study.

Venous outflow impairment sometimes masquerades clinically as CBD. Liver biopsy can point to the correct diagnosis in such instances, but histologic overlap with biliary features (portal inflammation and fibrosis, ductular proliferation, and focal lymphocytic cholangitis) can cause diagnostic difficulty.

CBD is known to accumulate copper due to impaired secretion in bile. Hepatic copper levels in advanced-stage CBD often exceed 250 mg/100 g of dried liver, sometimes reaching levels higher than those observed in WD. The reported prevalence of histochemically detectable copper in CBD is extremely variable between different studies, ranging from 3% to 100%. In contrast, isolated reports indicate that livers with venous outflow impairment do not have excess copper. Hence, we hypothesized that copper staining could help distinguish between venous outflow impairment and CBD. Our results support this hypothesis in the precirrhotic liver. We found stainable copper in 73% of liver specimens with CBD but detected no copper in acute large bile duct obstruction. We also found a correlation between both the prevalence and the grade of rhodamine positivity and disease stage. This is similar to previous data suggesting such a correlation. CBD was the only condition other than WD in which stainable copper was frequent in early chronic disease. In contrast, acute venous outflow impairment had no stainable copper. Seven of 51 (14%) chronic venous outflow impairment samples were rhodamine positive, but 6 of those had advanced fibrosis, and the 1 exception had a focal and unusual distribution of stainable copper.

Our data support previous reports describing frequent histochemical detectability of copper in WD and its occasional absence despite elevated liver copper contents. In nonfibrotic specimens, copper was not detected by rhodamine, but all specimens with fibrosis had detectable copper. This increased in grade with disease progression. It has been proposed that in early stages of WD, diffuse cytoplasmic localization of non–protein-bound copper evades histochemical detection. In contrast, in later disease stages, copper is stored in lysosomal complexes readily detected by histochemical stains. Our experience here underlines the fact that histochemical staining for copper lacks sensitivity for WD in its early stages.

Our findings in other chronic liver disorders were similar to those in previous reports. We detected copper in 9% of patients with chronic hepatitis C and only in those with advanced disease. Low-stage chronic hepatitis typically shows no significant elevations in copper content. When histochemically detectable copper occurs in this setting, it is usually associated with cholestatic features, possibly representing hepatitis/biliary disease overlap syndrome. Nevertheless, it has been proposed that hepatic injury can cause copper deposition in hepatocytes via disturbed bile secretion. Conversely, copper sequestration in hepatocytes causes oxidative stress, likely contributing to disease progression in chronic active hepatitis.

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Am J Clin Pathol 2013;139:79-86
DOI: 10.1309/AJCPDZR4OHDQNS3L

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Mean copper levels are reportedly normal in noncirrhotic fatty liver disease.\textsuperscript{8,11,28} Our study supports these data—we found no stainable copper in noncirrhotic steatohepatitis specimens. Two of 6 livers (33%) with stage 4 steatohepatitis had stainable copper, a result similar to previous studies (22%-44%).\textsuperscript{21,28}

Except for a single anecdotal report of stainable copper in hepatic sarcoidosis,\textsuperscript{44} there are no detailed studies of copper staining in this condition. We found stainable copper in 20% of liver specimens tested, including one with only portal fibrosis. Destruction of interlobular bile ducts by granulomatous inflammation can occur in sarcoidosis, occasionally producing a chronic cholestatic pattern resembling CBD,\textsuperscript{45} probably accounting for the occasional copper detection in early disease.

A positive stain for copper is not specific for CBD in cirrhotic livers; we found stainable copper in all livers with biliary cirrhosis and in 36% of livers with nonbiliary cirrhosis (excluding WD). This is in keeping with previous data indicating that a cirrhotic liver has 2 times the normal hepatic copper content\textsuperscript{7} and describing detectable copper in cirrhosis from various biliary and nonbiliary causes.\textsuperscript{4,10-13,21,28,29} It is unclear why copper accumulates in cirrhosis, but architectural remodeling may lead to distortion of bile flow, hindering copper excretion in bile.\textsuperscript{11,21} This, however, signifies that a positive stain for copper has no significance in revealing the etiology of cirrhosis.\textsuperscript{29}

In summary, venous outflow impairment can occasionally mimic CBD by laboratory, radiologic, and histologic findings.\textsuperscript{30,31} A rhodanine stain for copper can help with the differential diagnosis. Stainable copper is seen early and often in CBD, and the prevalence and grade of stainable copper increase with increasing disease stage. In contrast, we did not detect copper in acute venous outflow impairment,
and except for a single explanted liver from a patient with Budd-Chiari syndrome with focal subcapsular copper, rhodanine-positive granules were not observed in early chronic disease. We conclude that in the absence of advanced fibrosis (or WD), a positive rhodanine stain for copper argues strongly in favor of CBD and against other liver diseases, including venous outflow impairment. Although stainable copper lacks specificity in cirrhotic liver, its absence in that setting can be helpful since it essentially rules out CBD.

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