Deparaffination time: a crucial point in histochemical detection of tissue copper

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The search for a sensitive histochemical method for revealing tissue copper has been the object of many workers in the past. In spite of multiple methods available, the occurrence in clinical practice of negative histochemical stains, even in cases with high copper levels demonstrated by quantitative methods is very high. This study was aimed at verifying the role of technical variations in the sensitivity of the Timm method and, in particular, the role of the dewaxing time of paraffin sections. To this end, 15 liver specimens, 10 from patients affected by Wilson's disease and 5 newborn livers were fixed in 10% formalin, paraffin embedded and routinaly processed. Four 4-micron sections from each case were rinsed in xylene for 10, 20, 60 min, and for 24 hrs. All sections were stained with Timm's method.
In 13 out of the 15 liver biopsies utilized in this study, the sensitivity of Timm's method in revealing copper deposits in liver cells appeared to be dependent on the dewaxing time. In two other cases, reactivity of copper granules to Timm solution did not change significantly with the different deparaffination times. The best results were obtained by rinsing sections in xylene for 24 hrs, the worst in sections treated with xylen for 10 minutes. In particular, in five cases of Wilson's disease, Timm stain applied to sections following ten minutes of xylene were completely negative, while copper granules were clearly evidenced in the same section following an overnight bath in xylene. Our data show that an overnight bath of paraffin sections in xylene may completely change the sensitivity of Timm stain in revealing copper deposits in the liver, relaunching copper histochemistry in the diagnosis of copper-related liver diseases.

Key words: Histochemistry, copper, liver.

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The search for a sensitive histochemical method for the demonstration of copper overload in different tissues and cell types has been the objective of many workers in the past years. Although a number of sensitive methods are presently available, none of these can be considered as entirely satisfactory to detect copper deposits for diagnostical purposes (Lecca *et al.*, 1998; Lefkowitch, 2006). Conflicting results have been published on the diagnostic accuracy of orcein (Sipponen, 1976; Henwood, 2003), rhodanine (Okamoto *et al.*, 1937), rubeanic acid (Uzman, 1956), and Timm's silver stain (von Timm, 1961) in the detection of liver copper overload in physiology (Faa *et al.*, 1987) and in Wilson's disease (Goldfischer and Sternlieb, 1968; Pilloni *et al.*, 1998). The histochemical detection of copper deposits has been utilized, during the years, in the diagnosis of several other liver disease, such as Indian Childhood Cirrhosis (Lefkowitch *et al.*, 1982), Endemic Tyrolean Infantile Cirrhosis (Muller *et al.*, 1996), Primary Biliary Cirrhosis (Epstein *et al.*, 1981) and hepatocellular carcinoma (Guigui *et al.*, 1988). Moreover, copper overload has been histochemically evidenced in other organs, such as kidney (Crisponi *et al.*, 2000) and brain (Faa *et al.*, 2001). Among the different methods proposed over the years, von Timm's silver stain, in our hands, proved to be the most sensitive in revealing copper granules in liver biopsies from a large series of patients affected by Wilson's disease (Lecca *et al.*, 1998).

In spite of the multiple methods available for tissue copper histochemistry, in clinical practice the occurrence of negative stains in patients affected by Wilson's disease or by other copper-related liver diseases (Lefkowitch, 1995; Tanner, 1998; Muller *et al.*, 1998; Barresford *et al.*, 1980) is very frequent, even in cases in which high copper levels have been demonstrated by atomic absorption spectroscopy (Pilloni *et al.*, 1998).

False negative histochemical results could be due to the uneven copper distribution in liver, both in the newborn normal liver (Faa *et al.*, 1987) and in Wilson's disease (Faa *et al.*, 2001). On the other hand, the large number of cases in which the histochemical demonstration of copper has failed remains, to the best of our knowledge, unknown.

The role of variations in staining performance following modifications in the technical procedure has been only occasionally reported. A study from our group first evidenced the role of microwave treatment in making the histochemical staining technique for copper by rubeanic acid far more rapid and effective in revealing tissue copper deposits (Lecca *et al.*, 2001). A recent study from Henwood A (Henwood, 2003) lays stress on the influence of dye batch variation and aging of dye solution on the efficacy of orcein in revealing the accumulation of copper-associated proteins in liver biopsies. Here we report the preliminary results of a study aimed at verifying the role of technical variations on the sensitivity of von Timm silver stain, and in particular, the role of the deparaffination time.

Materials and Methods

We studied 10 consecutive liver needle biopsies from patients affected by Wilson's disease from the island of Sardinia (Italy), and 5 newborn liver specimens obtained at autopsy from patients who died in the perinatal period affected by respiratory distress syndrome. All liver specimens were fixed in 10% formalin, paraffin-embedded and processed routinely. Multiple four micron serial sections of each liver specimen were made. Four sections from each case were deparaffined by rinsing them in xylene for different times: 10, 20, 60 minutes and 24 hours. Copper deposits were stained using the modified Timm's method involving trichloroacetic acid (Lecca *et al.*, 2001). In brief, sections were first treated with 0.3% sodium sulphide solution for 30 minutes, washed in deionized water for 30 minutes, placed in 15%Trichloroacetic acid for 15 min, then washed with water for 10 min. Sections were stained by Timm's solution (25 mL of arabic gum additioned by 0.3 mL of 10% HgNo3, 3 mL of a solution constituted by 3 g hydroquinone and 5 g citric acid in 10 mL of deionized water) for 45 min. The rinsing bath containing Timm's solution was placed in a dark room. Nuclear staining was obtained rinsing sections in hematoxylin for 10 minutes. Sections were then washed with distilled water, dehydrated in graded alcohol, cleared in the changes of xylene and mounted by Biomount.

Results

The evaluation of copper staining in the 15 cases analyzed was independently evaluated by two authors (NS and FG). Copper deposits appeared as intracytoplasmic small dark granules in the perinuclear region or at the biliary pole of hepatocytes in cases with mild deposition, and as coarse granules occupying the whole cytoplasm in areas of heavy deposition. In cases with mild positivity, Timm-positive granules were mainly detected in the hepatocytes of the acinar zone 1 (periportal hepatocytes). With the increasing positivity for Timm's silver stain, even hepatocytes of the other zones of the acinus were progressively recruited for copper storage. In 13 out of the 15 liver biopsies utilized in this study, 8 cases of Wilson's disease and 5 newborn livers, the sensitivity of the Timm method in revealing copper deposits in liver cells appear to be dependent on the dewaxing time. In two other cases, reactivity of copper granules to Timm's solution did not change significantly with the different times of xylene immersion. The best results were obtained by rinsing sections in xylene for 24 hrs, the worst in sections treated with xylene for 10 minutes (Figure 1, Figure 2); intermediate degrees of positivity were observed in the intermediate intervals utilized.

In particular, in five cases of Wilson's disease, Timm's stain applied to sections following ten minutes of xylene were completely negative, while copper granules were clearly evidenced in the same section following an overnight bath in xylene.

Discussion

In spite of the large number of histochemical stains available for staining of tissue copper, a review of the literature shows that none of the stains in use is highly sensitive and specific (Goldfischer *et al.*, 1980; Fuentalba *et al.*, 1987; Sato *et al.*, 1989; Davies *et al.*, 1989; Blasco *et al.*, 1992). Moreover, the practical experience of false

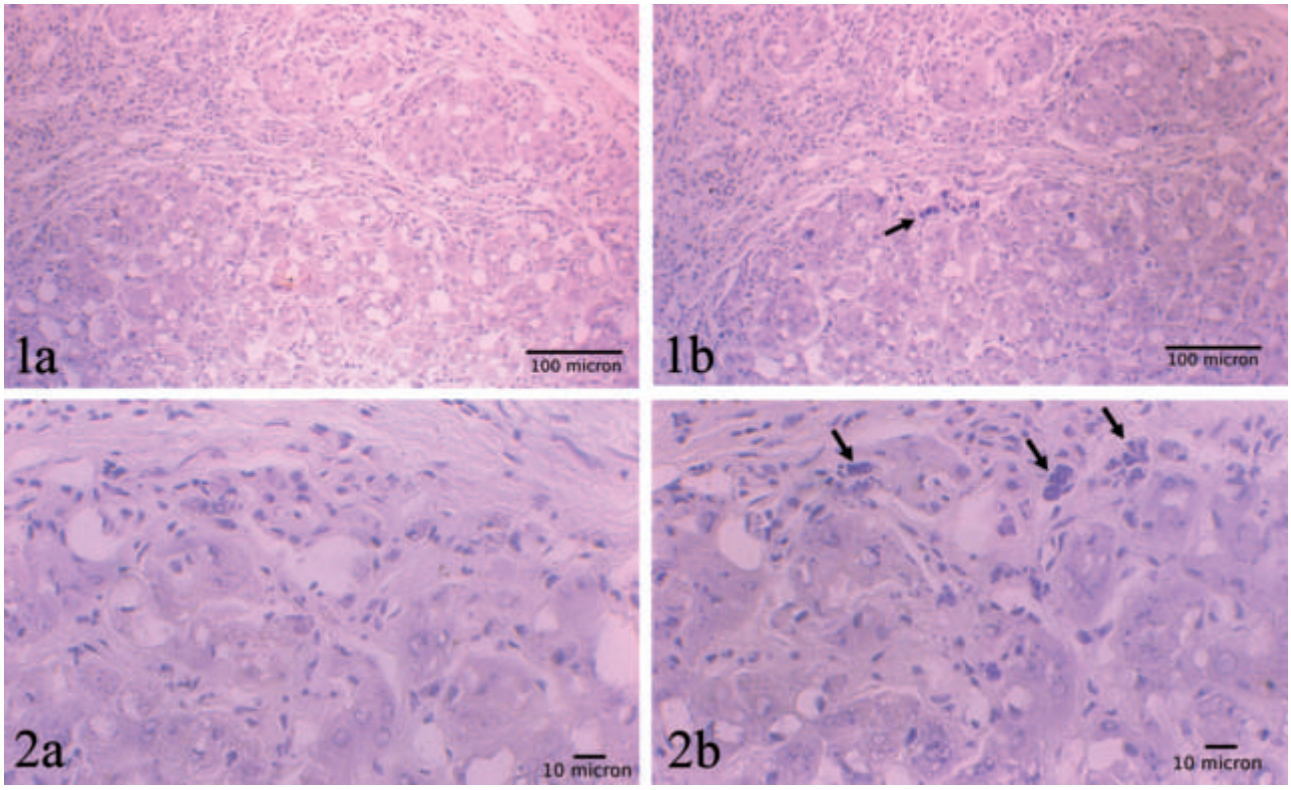

Figure 1. Liver biopsy from a patient affected by Wilson's disease. Original magnification x 250. a. Timm's stain after 10 minutes of deparaffination. b. Timm's stain after 24 h of deparaffination (arrows indicate copper deposits).
Figure 2. Liver biopsy from a patient affected by Wilson's disease. Original magnification x 400. a. Timm's stain after 10 minutes of deparaffination. b. Timm's stain after 24 h of deparaffination (arrows indicate copper deposits).

negative stains for copper in over 50% of liver biopsies from patients with certain diagnosis of Wilson disease (Pilloni *et al.*, 1998), has forced the majority of pathologists to abandon copper histochemistry for quantitative methods. In doing so, they are no longer able to obtain useful data on the distribution of copper deposits in different acinar zones of the liver.

Our data evidence that, in order to obtain useful results by Timm silver stain, all the steps of the procedure related to the treatment of tissue samples, before starting the histochemical procedure, should be accurately checked. According with the results of this study, increasing the time of xylene immersion of paraffin sections, from the usual ten minutes up to 24 hours, appears as a simple and inexpensive trick for lowering the percentage of false negative cases. A long rinsing time in xylene proved to be a critical point in the final results of histochemical detection of copper. In particular, the negativity in five cases of Wilson's disease for copper stain after a short xylene immersion and the positivity after a long stay of the same liver sections in xylene, lays stress on the role of this simple step in the sensitivity of this histochemical method. The reason for this finding is not completely clear: we may speculate that a prolonged action of xylene on liver sections could completely remove paraffin, rendering copper deposits more reactant with Timm's solution. Since a long deparrafination time was able, in this study, to clearly amplify copper deposits even in the newborn liver, we suggest the use of a section of neonatal liver, dewaxed overnight, as a positive control in every histochemical reaction performed for clinical purposes.

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References

Barresford PA, Sunten JP, Harrison V, Lesna M. Histochemical demonstration and frequency of intrahepatocytic copper in patients suffering from alcoholic liver disease. Histopathology 1980; 4:637-43.

Blasco A, Dominguez P, Colina F, Castellano G. Wilson's disease. A histological review of 7 patients and the value of histological copper positivity in relation to other hepatopathies. Med Clin (Barc) 1992; 98:207-11.

Crisponi G, Ambu R, Caria MP, Lisci M, Cristiani F, Nurchi VM, *et al.* Renal copper content and distribution in Wilson's disease. Journal of Urologic Pathology 2000; 13:23-30.

Davies SE, Williams R, Portmann B. Hepatic morphology and histochemistry of Wilson's disease presenting as fulminant hepatic fealure: a study of 11 cases. Histopathology 1989;15:385-94.

Epstein O, Jain S, Lee RG, Cook DG, Boss AM, Scheuer PJ *et al.* D-penicillamine treatment improves survival in primari biliary cirrhosis. Lancet 1981;1:1275-7.

Faa G, Liguori C, Columbano A, Diaz G. Uneven copper distribution in the newborn liver. Hepatology 1987;7:838-42.

Faa G, Nurchi V, Demelia L, Ambu R, Parodo G, Congiu T, *et al.* Uneven hepatic copper distribution in Wilson's disease. J Hepatol 1995;22:303-8.

Faa G, Lisci M, Caria MP, Ambu R, Sciot R, Nurchi VM, *et al.* Brain copper, iron, magnesium, zinc, calcium, sulfur and phosphorus storage in Wilson's disease. J Trace Elem Med Biol 2001;15:155-60.

Fuentalba IC, Haywood S, Traford J. Evaluation of histochemical methods for the detection of copper overload in rat lever. Liver 1987;7:277-88.

Goldfischer S, Sternlieb I. Changes in the distribution of hepatic copper in relation to the progression of Wilson's disease (hepatolenticular degeneration). Am J Pathol 1968;53:883-94.

Goldfischer S, Popper H, Sternlieb I. The significance of variations in the distribution of copper in liver disease. Am J Pathol 1980;99:715-30.

Guigui B, Mavier P, Lescs MC, Pinaudeau Y, Dhumeaux D, Zafrani ES. Copper and copper-binding protein in liver tumors. Cancer 1988;61:1155-8.

Henwood A. Current applications of orcein in histochemistry. A brief review with some new observations concerning influence of dye batch variations and aging of dye solutions on staining. Biotech Histochem 2003;78:303-8.

Lecca S, Pilloni L, Ambu R, Flore C, Callea F, Faa G. The use of multiple histochemical methods in the study of Wilson's disease. A study of 74 cases and a review of the literature. Pathologica 1998;90:771-5.

Lecca S, Pilloni L, Faa G. A quick microwave histochemical stain for copper. Eur J Morphol 2001;39:145-7.

Lefkowitch JH, Honig CL, King ME, Hagstrom JWC. Hepatic copper overload and features of Indian childhood cirrhosis in an American sibship. N Engl J Med 1982;307:271-7.

Lefkowitch JH. Liver biopsy in the diagnosis of inherited metabolic disease. In: Proceedings of the American Association for the Study of Liver Disease, Chicago 3-4 November 1995;185-91.

Lefkowitch JH. Special stains in diagnostic liver pathology. Semin Diagn Pathol 2006;23:190-8.

Muller T, Feichtinger H, Berger H, Muller W. Endemic Tyrolean infantile cirrhosis:an ecogenetic disorder. Lancet 1996;347:877-80.

Muller T, Muller W, Feichtinger H. Idiopathic copper toxicosis. Am J Clin Nutr 1998;67:S1082-6.

Okamoto K, Utamura M. Biologische untersuchungen des kupfers. I. Histochemische kupfer nachweismethode. Acta Scholae Med Univ Imp in Kioto 1937;20:573-80.

Pilloni L, Lecca S, Van Eyken P, Flore C, Demelia L, Pilleri G, *et al.* Value of histochemical stains for copper in the diagnosis of Wilson's disease. Histopathology 1998;33:28-33.

Sato H, Kobori K, Haratake J. Histochemistry of copper and copper binding protein in liver with a comparison between histologic sections and biochemical evaluation. Sangyo Ika Daigaku Zasshi 1989;11:327-32.

Sipponen P. Orcein-positive hepatocellular material in long-standing biliary diseases. I. Histochemical characteristics. Scand J Gastroenterol 1976;11:545-52.

Tanner MS. Role of copper in indian childhood cirrhosis. Am J Clin Nutr 1998;67:S1074-81.

Uzman LL. Histochemical localization of copper with rubeanic acid. Lab Invest 1956;5:299-305.

von Timm F. Der histochemische nachweis des kupfers in gehirn. Histochemie 1961;2:332-41.