The Utility of Alizarin Red S Staining in Calcium Pyrophosphate Dihydrate Crystal Deposition Disease

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ABSTRACT. ***Objective.*** To determine the most suitable staining method for preservation and detection of calcium pyrophosphate dihydrate (CPPD) crystals in histological sections of patients with CPPD crystal deposition disease.

Methods. Paraffin sections of CPPD crystal-bearing tissues of 31 patients were stained with hematoxylin and eosin (H&E) and Alizarin red S (ARS). For H&E, the sections were treated with Mayer's hematoxylin (pH 2.3) for 5 min and with eosin alcohol (pH 4.1) for 1 min. For ARS, 1% ARS dissolved in distilled water was adjusted to pH 6.4 by adding 0.1% ammonia solution drop by drop while stirring. As controls, unstained sections were soaked in 1% citric acid monohydrate solution (CAMS, pH 2.3) for 5 or 10 min. The histological preparations were examined under a compensated polarized light using a first-order red compensator. We counted the number of weakly positive birefringent CPPD crystals in 3 high power fields (HPF, 0.272 mm^2).

Results. CPPD crystals were seen clearly in most specimens stained with ARS, but were markedly reduced in tissue sections stained with H&E or CAMS. The number of CPPD crystals detected in sections stained by ARS (1723 ± 683 per 3 HPF, mean ± standard deviation) was significantly higher compared with H&E, CAMS (5 min), and CAMS (10 min) (401 ± 374, 1022 ± 616, and 494 ± 636 per 3 HPF, respectively; $p < 0.001$, each).

Conclusion. Standard H&E staining reduces the number of visible CPPD crystals, probably due to the strong acidity of both hematoxylin and eosin solutions, whereas the ARS stain seems to preserve a large number of CPPD crystals. The utility of ARS staining may improve the identification of CPPD crystals and contribute to a correct diagnosis of CPPD crystal deposition. (J Rheumatol 2003;30:1032–5)

Key Indexing Terms:

CALCIUM PYROPHOSPHATE DIHYDRATE CRYSTAL DEPOSITION DISEASE
ALIZARIN RED S STAINING
HEMATOXYLIN AND EOSIN
CITRIC ACID MONOHYDRATE SOLUTION
DIAGNOSIS

Calcium pyrophosphate dihydrate crystal (CPPD) deposition disease is diagnosed by confirming both the radiological evidence of articular chondrocalcinosis and the presence of weakly positive birefringent crystals[1]. When the characteristic crystals cannot be detected in the synovial fluid (SF), the histological identification of crystals in affected tissues becomes crucial for establishing the diagnosis of CPPD crystal deposition disease. As a routine procedure, the histological specimens are treated with hematoxylin and eosin (H&E)[2]. However, the H&E sections of CPPD deposition disease lesions occasionally show no evidence of crystals[3,4]. This false negative result is thought to be caused by decalcification of CPPD deposits through the strong acidity of the hematoxylin solution[5].

Our study was carried out to examine the effects of Mayer's hematoxylin staining procedure on the number of CPPD crystals and to determine the usefulness of Alizarin red S (ARS) staining in tissue sections.

MATERIALS AND METHODS

We examined 31 CPPD lesions including the meniscus (n = 12), synovium (n = 9), labrum (n = 2), tendon (n = 1), ligament (n = 1), intervertebral disc (n = 1), and soft tissue mass (n = 5) by surgery from 31 patients (9 male and 22 female, median age 73 yrs) who fulfilled the Ryan and McCarty criteria of definite CPPD crystal deposition disease[1]. The identification of CPPD crystals in the articular tissues of 8 lesions was performed by transmission electron microscopy. The tissue specimens were fixed with 20% formalin, embedded in paraffin, and cut into 3–6 µm sections. After deparaffinization, the sections were stained with H&E and ARS. For H&E, the sections were treated with Mayer's hematoxylin (pH 2.3) for 5 min and with eosin alcohol (pH 4.1)

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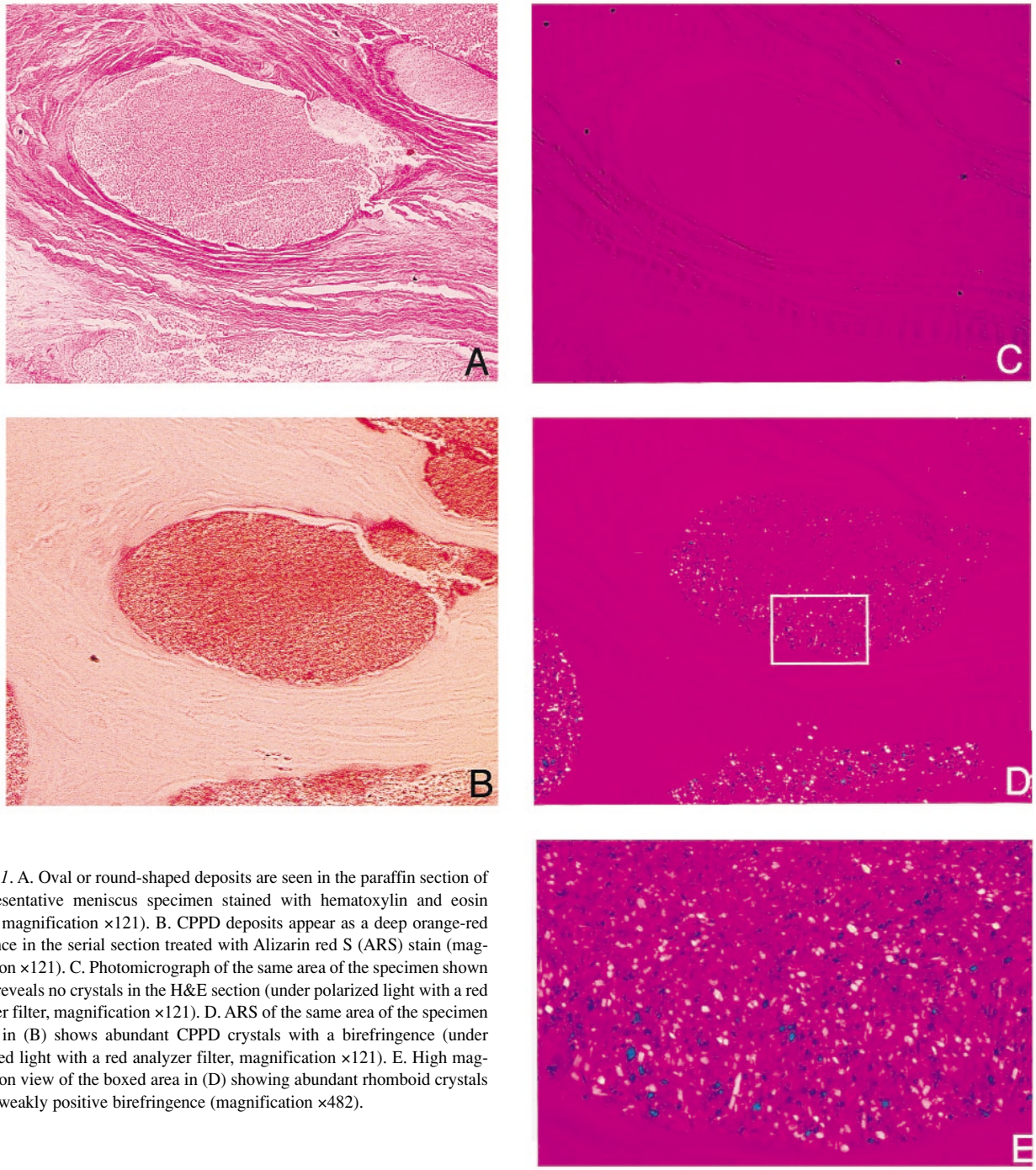

Figure 1. A. Oval or round-shaped deposits are seen in the paraffin section of a representative meniscus specimen stained with hematoxylin and eosin (H&E, magnification ×121). B. CPPD deposits appear as a deep orange-red substance in the serial section treated with Alizarin red S (ARS) stain (magnification ×121). C. Photomicrograph of the same area of the specimen shown in (A) reveals no crystals in the H&E section (under polarized light with a red analyzer filter, magnification ×121). D. ARS of the same area of the specimen shown in (B) shows abundant CPPD crystals with a birefringence (under polarized light with a red analyzer filter, magnification ×121). E. High magnification view of the boxed area in (D) showing abundant rhomboid crystals with a weakly positive birefringence (magnification ×482).

for 1 min. ARS staining was performed according to the method described by Dahl[6]. For ARS, 1% Alizarin red S (Wako Pure Chemical Industries, Osaka, Japan) dissolved in distilled water was adjusted to pH 6.4 by adding 0.1% ammonia solution (Katayama Chemical Industries, Osaka, Japan) drop by drop while stirring. As controls, unstained sections were soaked in 1% citric acid monohydrate (Katayama Chemical Industries) solution (CAMS) (pH 2.3) for 10 or 5 min. In order to avoid site/sampling bias, the same histologic sections were examined either unstained (treated with 1% CAMS), or stained with either H&E or ARS.

The histological preparations were examined under a compensated polarized light using a first-order red compensator. We counted the number of weakly positive birefringent CPPD crystals in 3 high power fields (HPF, 0.272 mm^2). Histological slides were examined in a blinded fashion by 2 observers (KY and HI) who were experienced in crystal identification. All data were expressed as mean ± standard deviation (SD). Differences between groups were examined for statistical significance using the paired t test. $p < 0.05$ denoted the presence of a statistically significant difference.

RESULTS

H&E sections showed oval-shaped foci of CPPD deposits, associated with hypertrophic/metaplastic cartilage[7,8] in an

abundant fibrous matrix. In some lesions, very few or no crystals were observed in H&E sections (Figure 1A and 1C) and in sections soaked in 1% CAMS (for 5 or 10 min). Soaking sections in 1% CAMS for 10 min resulted in a more remarkable decrease in the number of CPPD crystals than soaking for 5 min.

In the ARS technique, most lesions exhibited foci of CPPD deposits as aggregates of an orange-red granular substance, showing a sharp contrast to a light pink hue of the surrounding fibrous stroma (Figure 1B). Under polarized light with a red analyzer filter these lesions clearly showed abundant weakly positive birefringent CPPD crystals within the oval-shaped foci (Figure 1D and 1E).

The number of CPPD crystals in H&E, ARS, CAMS (5 min), and CAMS (10 min) were 401 ± 374 (0–1107), 1723 ± 683 (426–2930), 1022 ± 616 (0–1968), and 494 ± 636 (0–1963) per 3 HPF, respectively. Values are mean ± SD (range). ARS significantly improved the recognition of CPPD crystals compared with H&E ($p < 0.001$) and CAMS (5 and 10 min, $p < 0.001$, each). Further, the differences in the number of crystals counted in CAMS (5 min) and CAMS (10 min) and in H&E and CAMS (5 min) were significant ($p < 0.001$, each). The differences between H&E and CAMS (10 min) were not significant (Figure 2).

DISCUSSION

CPPD crystal deposition is often found in destructive osteoarthropathy in the elderly[9], but could easily be over-

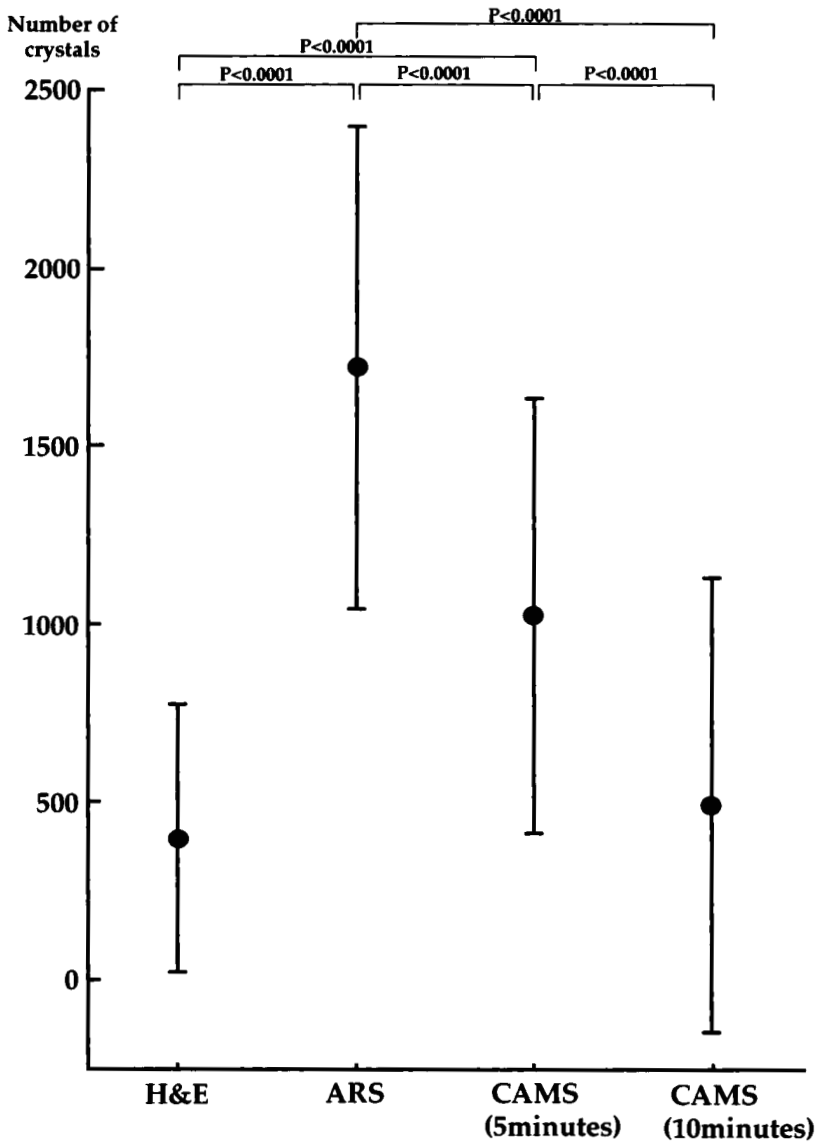

Figure 2. Number of CPPD crystals in tissue sections. Comparison between H&E, ARS, and CAMS. The number of CPPD crystals in sections stained with ARS is significantly higher than in that stained with H&E ($p < 0.0001$, paired t test). Data are the mean ± SD. See Figure 1 for abbreviations.

looked[10]. Although the number of patients with destructive osteoarthropathy of CPPD crystal deposition disease[1] is probably greater than generally considered, several pitfalls in making a correct diagnosis may lead to lack of awareness of CPPD disease. This may be attributable in part to the difficulty in recognizing CPPD crystals in SF[11-13] or in tissue sections stained with H&E[3,4]. The difficulties in detecting CPPD crystals on routine H&E sections led us to investigate the usefulness of ARS as a rapid screening test. ARS has been widely used since 1905 as a reagent for calcium staining of bone[14]. Using the ARS reagent, calcium deposits are stained with an orange-red color, which are easily distinguished from a paler pink background[15]. Paul, *et al*[15] used ARS to examine SF preparations from apatite deposition disease and CPPD crystal deposition disease. To our knowledge, however, there are no studies that have previously examined the usefulness of ARS in detecting CPPD in paraffin sections.

Our results show that staining with H&E or soaking in CAMS could result in a marked loss of CPPD crystals. We speculate that the CPPD crystals might have dissolved due to the strong acidity of Mayer's hematoxylin solution containing citric acid monohydrate. These results are consistent with the previous report suggesting a reduction in the number of crystals due to the acidity of hematoxylin solution[5]. However, since differences were present between H&E and CAMS (5 min) in our study, we think that the negative effects of eosin alcohol solution containing acetic acid could not be ignored.

We think that Alizarin red S is a nonspecific stain of calcium-containing crystals such as CPPD, apatite, or other basic calcium phosphate crystals, and that transmission electron microscopy, x-ray powder diffraction analysis, or other analytical technique are required to confirm the identity of the particular crystal. However, based on our results, we conclude that ARS staining is a rapid, simple, and sensitive staining technique for the detection of CPPD deposits and other calcium compounds. Our results may contribute to the correct diagnosis of future cases of CPPD crystal deposition disease.

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