

EXTENDED REPORT

Delayed examination of synovial fluid by ordinary and polarised light microscopy to detect and identify crystals

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Ann Rheum Dis 2002;**61**:444–447

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Accepted
15 October 2001

Objective: To determine the reliability of a delay in the microscopic examination of synovial fluid (SF) to detect and identify crystals.

Methods: Ninety one SF samples were examined, 31 with monosodium urate (MSU) crystals, 30 with crystals of calcium pyrophosphate dihydrate (CPPD), and 30 containing no crystals. The specimens were stored with EDTA, sodium heparin, and without anticoagulant at 4°C before examination at 24 and 72 hours with ordinary and polarised light microscopy. Another aliquot of the same samples was stored in a plastic container without anticoagulant at –80°C and examined after two months.

Results: When the samples stored at 4°C were re-examined after 24 hours, intracellular crystals of MSU were seen in 90/93 (97%) cases where they had been identified previously and 89/93 (96%) cases after 72 hours. Similarly, CPPD crystals were identified in 90/90 (100%) and 87/90 (97%) cases after 24 and 72 hours. Examination of the samples stored at –80°C showed intracellular MSU crystals in 25/31 (81%) of cases and CPPD crystals in 25/30 (83%). No crystals were seen in any sample which had previously been diagnosed as crystal-free.

Conclusions: Deferred microscopic examination of refrigerated or deep frozen SF provides a strong probability of detecting MSU or CPPD crystals if these are present initially.

The examination of synovial fluid (SF) is one of the basic clinical and laboratory tests for diagnosing joint diseases.^{1,2} Such examination permits diagnosis of processes such as septic arthritis, or that induced by microcrystals—for example, gout, for which effective treatment exists. Since the studies of McCarty and Hollander,^{3,4} microscopic examination with compensated polarised light has been considered the most suitable technique for identifying microcrystals in SF and for the rapid and definitive diagnosis of arthritis caused by crystals of monosodium urate (MSU) or calcium pyrophosphate in dihydrate form (CPPD).⁵ However, several authors^{5–7} have suggested that CPPD crystals frequently lack birefringence and can therefore be best visualised by ordinary light microscopy.

One aspect of SF examination which remains controversial is the length of time which can be allowed to elapse (and the storage conditions) before the detection and identification of microcrystals is significantly altered. Currey and Vernon-Roberts suggested, for example, that it is perfectly satisfactory to look for crystals in SF samples obtained several days previously as long as they are kept in sterile conditions.⁸ Other authors support this point of view.^{9,10} Bible and Pinals showed that urate crystals can be observed by polarised light microscopy after 24 hours' storage refrigerated or even at room temperature.¹¹ They found no subsequent precipitation of urate crystals in any of the non-gouty fluids examined.

However, Kerolus *et al* in a methodical study noted that delayed examination of SF preserved with heparin and stored at 4°C or 22°C affected the total and differential leucocyte count, and the detection and identification of microcrystals, especially crystals of CPPD.¹² McKnight and Agudelo, on the other hand, maintained it was still possible to observe CPPD crystals in six SF samples kept at room temperature for four weeks.¹³ Indeed, the previously mentioned group replied that a later experiment with CPPD crystals confirmed these observations.¹³

McGill *et al* in an examination of 11 samples containing CPPD crystals stored at 4, 22, and –70°C, found only a slight decrease in the samples stored at 4°C and 22°C and could still observe crystals in all cases after two weeks.¹⁴

There are also discrepant views about the use of anticoagulants if a sample is to be stored before examination for microcrystals.^{7,9,10,13,15}

The objective of our study was to ascertain whether the storage of samples (with or without anticoagulant) which were known to contain MSU and CPPD crystals for 24 and 72 hours at 4°C or for longer periods at –80°C affected the identification of these crystals or whether morphological changes would alter the results in these samples and others which served as control.

METHODS

Ninety one SF samples were obtained for diagnostic or therapeutic purposes from patients attending the rheumatology unit at the Morales Meseguer University Hospital, Murcia, SE Spain, between October 1999 and November 2000.

Thirty one samples were from patients with uric gout¹⁶ and 30 from patients with definite CPPD crystal deposition disease.¹⁷ As controls we used 30 samples taken from patients with several arthropathies: 14 cases of osteoarthritis, five of rheumatoid arthritis, three of chronic monarthritis of the knee, two of psoriatic arthritis (with hyperuricaemia), two of septic arthritis, two chronic juvenile arthritis, one CPPD deposition disease (with no crystals identified in SF on this occasion), and one of undifferentiated oligoarthritis. All SF samples were taken from the knee, except two which were taken from the shoulder joint.

Excluded from the study were samples with less than 4 ml of SF, those in which only extracellular crystals were seen, and those for which there was no agreement between two observers about the initial examination.

SF samples were analysed as follows: the samples were examined immediately after arthrocentesis by ordinary and

Abbreviations: CPPD, calcium pyrophosphate dihydrate; MSU, monosodium urate; SF, synovial fluid

Table 1 Crystals observed in the SF samples preserved in EDTA, heparin, or no anticoagulant after 24 and 72 hours (at 4°C) and after two months (at -80°C)

	MSU (n=31)			CPPD (n=30)			Control (n=30)		
	EDTA	Heparin	SF alone	EDTA	Heparin	SF alone	EDTA	Heparin	SF alone
24 Hours	31 (100)*	29 (94)	30 (97)	30 (100)	30 (100)	30 (100)	0	0	0
72 Hours	31 (100)	29 (94)	29 (94)	29 (97)	29 (97)	29 (97)	0	0	0
>2 Months	-	-	25 (81)	-	-	25 (83)	-	-	0

*Values expressed as number (and percentage) of samples showing intracellular crystals.

Table 2 Change in the number of extracellular crystals (EC) and intracellular crystals (IC) in the SF samples preserved at 4°C and -80°C without anticoagulant. Results expressed as mean (SD)

	MSU (n=31)		CPPD (n=30)		Control (n=30)	
	EC	IC	EC	IC	EC	IC
Initial	2.0 (4.2)*	2.3 (3.4)	1.8 (2.7)	3.8 (5.2)	0	0
24 Hours	2.3 (5.0)	3.2 (7.1)	1.3 (1.6)	3.8 (5.3)	0	0
72 Hours	1.3 (2.1)	2.2 (4.7)	1.3 (2.4)	3.1 (4.2)	0	0
>2 Months	3.1 (4.5)†	1.4 (3.1)	1.8 (2.8)	2.2 (2.9)†	0	0

*Number of crystals/400× field; †p<0.05.

polarised light (Labophot-2, Nikon, with first order red compensator) for the presence of microcrystals. The samples in which MSU and CPPD crystals were unequivocally observed and 30 control samples were included in the study. After this preliminary analysis an aliquot of 1 ml SF was transferred to the following sterile test tubes containing (a) sodium heparin (Vacutainer sodium heparin, Becton Dickinson), (b) EDTA (Vacutainer EDTA (K3), Becton Dickinson), (c) no anticoagulant, and (d) no anticoagulant in a plastic container for deep freezing. The fluid samples were identified by the clinical history number and by the registration number of the study. The first three sets of test tubes were kept at 4°C. A more detailed examination was carried out on a sample from the test tubes containing no anticoagulant and stored in a refrigerator during the first two hours after arthrocentesis. Samples were then re-examined 24 and 72 hours later by ordinary and compensated polarised light microscopy after shaking the tubes manually to homogenise the contents. The fourth set of containers was immediately deep frozen at -80°C. In this case, 46 samples were examined after eight weeks, 32 after 12 weeks and 13 after 12–18 weeks.

The following characteristics were noted: the presence or not of MSU or CPPD crystals, the number (No of crystals per 400× field and average number of 10 non-contiguous fields), and the intracellular or extracellular location, or both. The three observers (JG, ES, AC) included samples in study. The initial examination was made by the clinician who obtained the sample and each of the observers carried out a re-examination of the refrigerated samples on days of the week chosen at random. The deep frozen samples were examined in a blind and random way. The commentaries of the three observers were also noted.

Student's paired *t* test was used in a statistical analysis of the differences between counts. At the beginning of the study any interobserver variation was estimated. The κ test was used for analysis of concordance.

RESULTS

We excluded from the study five samples in which CPPD crystals were suspected but not confirmed by a second observer because the supposed crystals were small, scarce, atypical, and with a very weak or absent birefringence which hindered definitive identification. Similarly, one sample with possible

MSU crystals was excluded, in which the crystals were very scarce and all extracellularly located, needle shaped, and with intense negative birefringence. For this reason, the number of remaining samples was 91. Three cases of MSU crystals and one of CPPD were excluded from the statistical analysis because a countless number of crystals were found in all of them.

Monosodium urate crystals

MSU crystals were preserved in most of the samples refrigerated at 4°C and re-examined after 24 and 72 hours. After 24 hours intracellular crystals could be seen in all 31 of the samples kept in EDTA, in 29/31 (94%) of those kept in heparin and 30/31 (97%) of those kept without preservative (table 1). The results after 72 hours were similar: 31/31 (100%) and 29/31 (94%) in heparin and no preservative.

When the SF samples kept at -80°C were examined MSU crystals could still be seen in most preparations: intracellularly in 25/31 (81%), extracellularly in 26/31 (84%), and intracellularly or extracellularly in 29/31 (94%). There were no differences in the figures for two, three, or more months.

When possible modifications in the concentration of MSU crystals with time were evaluated, the number of extracellular crystals increased significantly after two months (table 2). Although there was a slight reduction in the number of intracellular MSU crystals, these differences were not significant.

CPPD crystals

CPPD crystals were seen after 24 hours in all the samples examined, regardless of whether anticoagulant was used (table 1). Only in one case were no CPPD crystals observed after 72 hours: 29/30 (97%). In those samples showing CPPD crystals and stored at -80°C for 8–18 weeks, intracellular crystals continued to be seen in 25/30 samples (83%), extracellular crystals in 27/30 (90%) and intracellular or extracellular crystals in 27/30 (90%) cases. Crystals were identified with the same frequency after 8, 12, or more weeks.

The number of extracellular CPPD crystals remained stable throughout the study period, while the number of intracellular crystals decreased significantly after two months (table 2).

Control group

No MSU or CPPD crystals were seen in the 30 control samples when re-examined after 24 and 72 hours, nor in the samples kept at -80°C and examined after two months and more.

Observers' comments

The samples were carefully examined with ordinary light and compensated and non-compensated polarised light in a search for crystals, especially in the fibrinous clumps. CPPD crystals frequently showed scant or no birefringence, although such birefringence was sometimes intense.

Occasionally, other microcrystals were seen in the initial or subsequent examinations—for example, ovoid birefringent crystals in the form a Maltese cross (compatible with talc), small pleomorphic intensely birefringent crystals compatible with methylprednisolone acetate (a corticosteroid frequently used in our unit for intra-articular infiltration), and other amorphous microcrystals of variable birefringence similar to dust. No attempt was made to determine the time of appearance or the survival time of these crystalline artefacts.

The cell elements deteriorated with time in the samples stored at 4°C and in those frozen at -80°C and examined after two months' storage. In general, samples preserved in EDTA kept better, the cell outlines being more clearly delineated and the cells showing less tendency to agglomerate.

A careful re-examination of the preparations in which crystals were known to be present, but which had not been detected after two months' storage at -80°C, showed crystals in only two out of five cases.

No significant variations in the ability of the observers to detect crystals was observed. The κ index for concordance between observers was $\kappa = 0.825$.

DISCUSSION

It is recommended that examination of SF should be made promptly,¹² preferably within a few hours after arthrocentesis because a rapid reduction in the cell count and a significant fall in the number of CPPD crystals may occur and thus hinder correct classification. Other experiments, however, suggested that CPPD crystals did not dissolve completely even after long periods of storage.¹³

Our results for the CPPD crystal count agree with those obtained by McNight and Agudelo¹³ and McGill *et al.*¹⁴ Our study shows that when MSU and CPPD crystals were initially detected in SF they could still be seen 24 and 72 hours later in most cases when the samples were stored at 4°C, whether or not anticoagulant was used. The same was true in most cases when the samples were deep frozen at -80°C for two to four months.

A temperature of 4°C was chosen for storing samples because it is widely accepted that this will improve the probability of survival of the crystals and reduce the risk of microbiological contamination compared with storage at room temperature.^{12, 14}

Prolonged deep freezing did not greatly affect the detection of microcrystals and the results to a great extent agree with the observations made in samples stored at 4°C; it was possible to detect intracellular MSU and CPPD crystals in approximately 80% of the samples. As already shown,¹⁴ deep freezing permits samples to be kept for a long period of time and can be useful for keeping microcrystals for teaching purposes and for quality control of SF examination.

In the few studies which do exist, the sensitivity to detect MSU and CPPD crystals in SF seems to be approximately 70% with discrepancies existing between the sensitivity and specificity of different observers, suggesting that some quality control is necessary in these examinations.¹⁸ Although most laboratory tests are submitted to quality control checks, SF examination has not yet been included in such a programme.¹⁹ However, several methods exist to preserve SF in order to carry out subsequent quality controls of the examinations.²⁰⁻²² Some authors have proposed a different method, which involves staining, for the delayed examination of SF preparations as a method for carrying out quality controls of the cytological examination and identification of crystals.²³

There is wide disagreement between authors about the advisability of using anticoagulant to preserve SF samples until they are examined for the presence of microcrystals.^{7, 9, 10, 13-15, 24} We examined different ways of preserving samples and found that the number of crystals was the same in samples preserved with or without anticoagulant (EDTA and sodium heparin). In a very interesting study, Salinas *et al* reported that the results of the cellular counting of the SF samples preserved with EDTA are still accurate at 24 and even 48 hours, at least for clinical purposes.²⁵

Although the number of urate and pyrophosphate crystals was the same after storage for 24 and 72 hours at 4°C and for longer periods at -80°C, the detection of CPPD crystals was more difficult. For this reason, we found it useful to include examination by ordinary light microscope in the exhaustive examination of the preparations, which sometimes took more than 10 minutes, as recommended by several authors.²⁶ Other factors contributing to the high sensitivity of our results are the systematic search for crystals in fibrinous clumps, the systematic examination with non-compensated polarised light, and the fact that the examination was not completely "blind". This last fact might have influenced the observer in some cases to continue (or not) the search for crystals, although it might not have been a determining factor because the examination of the deep frozen samples (which was a "blind" test) provided substantially similar results. It must not be forgotten, too, that we did not exclude from the study those cases in which intra-articular steroid infiltration had been carried out in the preceding weeks.

Our results point to a high degree of specificity because no crystals were seen in any of the control samples and there was no confusion in identifying the different crystals. Neither did we see cases of MSU crystals formed "de novo", even in the two cases of psoriatic arthritis with hyperuricaemia.

We found no significant decrease in the number of crystals stored at 4°C during the three days of observation. In the deep frozen samples some changes were seen, including a significant increase in the number of extracellular crystals of urate and a significant reduction in the number of CPPD intracellular crystals. The exact significance of these changes is difficult to ascertain, although they may be due to the destruction of cells, which we know occurs in stored samples.

Our work has some limitations. The exclusion criteria which were introduced to improve the specificity of the method may add a certain bias. Furthermore, the scope of the study might have been affected by the fact that the sample examination was not completely blind.

In summary, our results suggest that SF analysis for the detection of MSU or CPPD crystals can be deferred up to 72 hours when the samples are stored at 4°C with or without EDTA or sodium heparin as anticoagulants because there is a high degree of probability that the crystals will still be present after this time. The same applies, although with lower probability, when samples are kept without anticoagulant at -80°C and examined after two, three, or four months.

These findings have a practical clinical interest because although it is advisable to examine SF samples soon after arthrocentesis to minimise the possibility of any error, this is sometimes impossible. For example, samples may be obtained in emergency units, in primary care centres, or any other parts of the Health Service, where it may be impossible to make an immediate examination, or only a partial examination may be possible without the availability of polarised light microscopy. In such cases, we think deferred examination of the SF samples stored at low temperature will pose no real problem for the detection and identification of MSU and CPPD crystals.

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