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ORIGINAL ARTICLE

The added value of synovial fluid centrifugation for monosodium urate and calcium pyrophosphate crystal detection

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Abstract The aim of the study was to assess the added value of synovial fluid (SF) centrifugation for microscopic monosodium urate (MSU) and calcium pyrophosphate (CPP) crystal detection in patients with arthritis. This is a prospective observational study using SF samples from joints of patients undergoing joint arthrocentesis. Two blinded observers assessed the SF smears by polarized light microscopy for the presence of crystals before as well as after centrifugation. SF samples were collected from 98 patients with arthritis. After exclusion, 87 samples were eligible for inclusion. Of each sample, 2 smears before and after centrifugation were prepared and microscopically examined, resulting in 348 smears per observer. Observer 1 identified MSU crystals in 18.4% and CPP in 9.2% of the smears before as well as after centrifugation. No extra MSU crystal-positive smears were identified after centrifugation. However, centrifugation yielded 4 additional CPP crystal-positive smears. Observer 2 identified MSU crystals in 15.5% and CPP crystals in 6.3% of the smears before as well as after centrifugation. Centrifugation yielded 2 additional MSU crystal-positive smears and 4 CPP crystal-positive smears. Monosodium urate crystals were well recognized without centrifugation. Centrifugation of SF had limited additional value for increasing the amount of MSU-positive smears. However, CPP crystals were identified in a higher number of smears after centrifugation than before. Therefore, centrifugation may be of additional value in selected patients with suspected calcium pyrophosphate deposition disease and to a lesser extent for gout.

Keywords Calcium pyrophosphate deposition disease · Crystal arthropathies · Diagnostic tests · EDTA and polarized microscopy · Gout · Synovial fluid centrifugation

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Introduction

Crystal-induced arthritis like gout and calcium pyrophosphate deposition (CPPD) disease are common rheumatic disorders characterized by respectively deposition of monosodium urate (MSU) or calcium pyrophosphate (CPP) crystals in joints and other tissues. The diagnosis can be confirmed by demonstrating the presence of characteristic crystals by polarized light microscopy of synovial fluids (SF) collected by arthrocentesis. However, sensitivity and specificity of this microscopic investigation has challenges: first, CPP crystals are harder to detect than MSU crystals because of their overall lower concentrations and small and varying size. Also, only a minority of the CPP crystals appear as positive birefringent [1–4]. Third, correct microscopic identification of MSU and CPP crystals by professionals requires experience and is therefore no sinecure. Berendsen et al. used an online test to assess the performance of crystal identification by 110 professionals involved in examining synovial fluid in routine care [5]. The test consisted of numerous images comprising the whole spectrum of crystals which can be identified at polarized light microscopy. Their outcome was the correct identification of all MSU and non-MSU slides as such. Only 39% correctly

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identified all the images as MSU and non-MSU. MSU and CPP images were identified correctly by 81 and 68% professionals, respectively. Incorrect identification of non-MSU crystals occurred frequently, instead MSU was well identified [5]. Fourth, in SF with a low leukocyte count, the crystal load is also frequently low [6, 7]. This results in lower sensitivity of microscopic examination for CPP (12–67%) compared to MSU (78–79%) crystals [2, 4, 8]. Unfortunately, negative examination by polarized light microscopy does not exclude gout and especially CPPD disease. A negative report could always be a false negative. Also, microscope quality-related factors should be taken into account. The specificity problem can be improved by using state of the art equipment, specific training, and quality control exercise initiatives [4]. In order to increase the sensitivity of polarized light microscopy for both MSU and CPP crystals, techniques such as crystal extraction from synovial fluid and analytical electron microscopy could be used. However, these techniques are very complex and impractical [4]. An alternative is to stain and cytocentrifuge SF (cytospin) as suggested by Robier et al. [6, 7, 9, 10]. With this technique (monolayers of), leukocytes and crystals are placed in increasing numbers onto a glass slide. As a result of this, crystals may be identified easier [6, 7, 9–11]. However, an important disadvantage of this method is that it is more time consuming and expensive than polarized light microscopy of SF smears. A potential alternative to the cytospin is the use of a conventional laboratory centrifuge. We hypothesized that the detection rate of MSU and CPP crystals identified by polarized light microscopy, in SF smears of sediments derived by conventional laboratory centrifugation, is increased compared to their detection rate in routine SF smears. The aim of our study was to assess the added value of SF centrifugation for microscopic MSU and CPP crystal detection in patients with arthritis.

Patients and methods

Design and patient selection

This prospective observational study assessed the added value of SF centrifugation for microscopic MSU and CPP crystal detection using SF samples derived from joints of consecutive patients with any form of arthritis undergoing joint arthrocentesis with SF aspiration. The study was performed at the outpatient clinic of Arthritis Centre Twente, Medisch Spectrum Twente Hospital, Enschede, The Netherlands. Synovial fluid samples of any joint were eligible for inclusion. Two experienced observers independently assessed the SF smears by polarized light microscopy for MSU and CPP crystals before as well as after centrifugation with a conventional laboratory centrifuge. Both observers, one rheumatologist (MH) and one rheumatology resident (DB), were blinded to the patients' diagnosis.

Inclusion/exclusion criteria

Inclusion criteria were (1) patient with age 18 years or over, (2) arthritis of any joint, and (3) at least 4 ml of SF available at arthrocentesis. The age of 18 years was chosen pragmatically as we wanted to have as much eligible patients as possible. Also, especially gout can occur at younger ages. Exclusion criteria were (1) the presence of hemarthrosis or (2) septic arthritis. We excluded these two patient categories from our study because respectively their erythrocyte and leukocyte counts before centrifugation are already very high, resulting in a dense and multilayer smear which would be hard to examine. After centrifugation, the sediments would even contain a higher number of cells limiting microscopy and especially the identification of crystals.

Patient and sample characteristics

Demographic, clinical, and laboratory data were obtained from the medical records of all participating patients. Medical records were verified for their physicians' diagnosis. This could be established clinically, using imaging, diagnostic/classification criteria and in cases of gout and CPPD disease most commonly by crystal identification using polarized light microscopy. However, there were no special requirements set for the physicians regarding "how to make diagnosis and how to register," as their role was exclusively to provide synovial fluids for the study. Data of the physicians' diagnosis were therefore less reliable and not suitable or used for statistical purposes.

Handling procedure of SF samples and preparation of smears

After arthrocentesis, each SF sample was processed: an EDTA tube was filled with 3 ml and, subsequently, partially divided over two separate tubes without additional anticoagulants. Both tubes, filled with 1 ml of SF, were stored in a refrigerator at 4 °C awaiting microscopy [6, 12–17]. SF smears were microscopically examined before and after centrifugation, as soon as possible, within 5 days as target:

(1) SF smears before centrifugation: the tubes were shaken and a drop of SF obtained from each of the two tubes was pipetted onto separate glass microscope slides.

(2) The remaining SF in both tubes was centrifuged for 10 min at 700 rpm in a Hettich Rotofix 32 centrifuge to provide a sediment.
(3) SF smear after centrifugation: the cell sediments from both tubes were also pipetted onto two separate glass microscope slides.
(4) Two observers, blinded for patient data, independently performed polarized light microscopy of the 2 SF smears before as well as the 2 after centrifugation, scoring the presence of leukocytes and MSU or CPP crystals. The samples were assessed systematically according to a fixed protocol using an Olympus BX 41 light microscope (with a 6 V 30W halogen bulb); first, a magnification of ×100 was used for orientation, positioning, and global assessment of the presence, morphology (i.e., rhomboid/flat or needle shaped), and aspect (i.e., (weak) positive or negative birefringent under polarized light) of the crystals. If (intra- or extracellular) crystals were identified, they were confirmed at ×400. Also, 25 visual fields (5 × 5) were scanned to exclude the co-existence of other crystals. If no crystals were identified at ×100, a magnification of ×400 was used to structurally scan 25 (5 × 5) visual fields for the presence of crystals. The results of both observers were noted separately on standardized case record forms. Roughly 5 min was maximally required for each smear to be fully assessed according to this protocol.

Statistical analysis

Descriptive statistics were used for demographic, clinical, and laboratory data. Cross tables were used to compare the results of polarized light microscopy before and after centrifugation. The interobserver agreement (kappa, $p < 0.05$) for crystal detection was calculated between both observers. A kappa >0.80 was considered sufficient agreement. Statistical analyses were performed using IBM SPSS software, version 20.0.

Results

Between April 2014 and April 2015, in total, 98 SF samples were collected from different joints of 98 individual patients with arthritis. Three SF samples eventually did not meet the inclusion criteria: hemarthrosis (1) and bursitis instead of arthritis (2). Eight other samples were not correctly processed, and had to be excluded, resulting in 87 eligible SF samples (Table 1). Two smears were prepared of each SF sample before and after centrifugation. As result, each of the two observers assessed 4 smears per SF sample, in total 348 smears per observer.

Table 1 Demographic and clinical characteristics of the patients/samples

		Patients/samples
Demographics	Total number	87
	Age, year (range)	60 (21–92)
	Patients <40 years, *n (%)*	8 (9.2)
	Male, *n (%)*	60 (69.0)
Aspirated joint, *n* (%)	Knee	81 (93.1)
	Other joints	6 (6.9)
Physicians' diagnosis, *n* (%)	Gout	20 (23.0)
	CPPD disease	7 (8.1)
	Gout and CPPD disease	1 (1.1)
	Rheumatoid arthritis	8 (9.2)
	Spondyloarthritis	8 (9.2)
	Osteoarthritis	10 (11.5)
	Other causes of arthritis	33 (37.9)
Microscopy performed by physician, *n* (%)		78 (89.7)
History of gout, *n* (%)		14 (16.1)
Comorbid disease, *n* (%)	Hypertension	35 (40.2)
	Diabetes mellitus	11 (12.6)
	Chronic renal failure (known or MDRD <60 ml/min)	14 (16.1)
Current medication, *n* (%)	NSAIDs	24 (27.6)
	Colchicine	9 (10.3)
	Steroids	10 (11.5)
	Allopurinol	10 (11.5)
	Diuretics	21 (24.1)
	ACE-inhibitor or ARB drugs	26 (29.9)
Mean serum UA, mmol/l (±SD) (calculated using available samples)		0.35 (0.12)
Mean serum creatinine, mmol/l (±SD)		94.1 (88.7)
Mean serum MDRD clearance, ml/min (±SD)		75.6 (18.9)

NSAIDs nonsteroidal anti-inflammatory drugs, *ACE* angiotensin-converting enzyme, *ARB* angiotensin II receptor blocker, *UA* uric-acid, *MDRD* modification of diet in renal disease

Patient and sample characteristics

Patient and sample demographics are shown in Table 1. Mean age was 60 and nearly 70% were men. The knee was the most common aspirated joint (93.1%). Gout (23.0%) and other causes of arthritis (37.9%) were the most frequent physicians' diagnosis of arthritis.

The mean duration between joint arthrocentesis and microscopy of the smears was 1.9 days. Thirty SF samples

(34.5%) were examined the same day and 73 (83.9%) within 3 days.

Crystal detection before and after centrifugation

The microscopy results for the presence of MSU and CPP crystals of the 87 patient samples (174 smears in total) are shown in Table 2. In none of the smears, MSU and CPP crystals were identified simultaneously.

Observer 1 identified MSU crystals in 18.4% (32 of 174) and CPP in 9.2% (16 of 174) of the smears before as well as after centrifugation. Centrifugation provided no extra MSU crystal-positive smears. However, centrifugation yielded 4 additional CPP crystal-positive smears (20.0% of the final number CPP crystal-positive smears). In 70.1% (122 of 174) of the smears, no crystals were identified before and after centrifugation.

Observer 2 identified crystals in MSU in 15.5% (27 of 174) and CPP crystals in 6.3% (11 of 174) of the smears before as well as after centrifugation. Centrifugation yielded 2 additional MSU crystal-positive smears (6.9% of the final number MSU crystal-positive smears) and 4 additional CPP crystal-positive smears (26.7% of the final number CPP crystal-positive smears). In 74.7% (130 of 174) of the smears, no crystals were identified before and after centrifugation.

Overall, the observers reported that MSU and CPP crystals were identified more easily and faster in smears after centrifugation than before, as illustrated by Fig. 1.

Discussion

In this study, centrifugation of SF slightly (0.0–6.9%) increased the number of MSU crystal-positive smears. In other words, the detection rate did improve compared to routine microscopic assessment, however, not as much as we expected concerning our hypothesis. Centrifugation had limited advantage, probably because MSU crystals are commonly well recognized. However, centrifugation did enhance CPP crystal detection to a much larger degree. After centrifugation, the observers identified CPP crystals in an additional 20.0–26.7% of the total number of CPP-positive smears.

Although centrifugation of SF prior to or following microscopic evaluation of smears is not routine practice, our data support that it may specifically be useful in suspected CPPD disease and to a lesser extent for gout. This is reinforced by the knowledge that CPP crystals are harder to detect than MSU crystals, because of their overall lower concentration and smaller, variable and often non-birefringent appearance [1–3]. Several previous studies by Robier et al. supported our view that a form of centrifugation, either their cytospin (cytocentrifugation) or the technique we used, could be useful [6, 7, 9]. In an outpatient clinic setting, a conventional centrifuge would probably be more desirable than the cytospin because of its wide availability, lower cost, and lack of need for trained personal. However, in accordance with the recommendations regarding the cytospin used by Robier et al., it is likely that this technique is mainly beneficial in selected patients. As centrifugation is more expensive, time consuming, and labor intensive that usual care, it should be preserved for situations when the smear is (repeatedly) negative for crystals while the suspicion of crystal-induced arthritis remains high, the SF is hypocellular, microscopy is postponed to a later date, or when there is a low grade of experience by the physician [6, 7, 10].

As expected, our observers reported that crystals were identified easier and faster in smears after centrifugation than before, because of their increased numbers per microscopic field (Fig. 1). Although this observation was subjective and not the aim of our study, it is supported by findings of Robier et al. In two studies, they showed that the cytospin is superior to routine microscopic smear assessment in the detection of crystals in low white blood cell count SF. The crystal count per high power field and detection increased significantly after cytocentrifugation [6, 7]. Gordon et al. showed that after centrifugation, CPP crystals were more commonly detected than MSU [18]. Another study highlighted the high sensitivity and specificity of stained cytospin preparations for CPP crystal detection [9]. Repeated examination of the same SF 24 h after arthrocenthesis was also useful to increase the yield of crystal identification [13, 14].

Our study had several limitations:

Firstly, the relative small sample size of crystal-positive smears could impede the validity of our study. Therefore, a higher number of crystal-positive samples as well as independent observers could probably have increased the robustness of our results.

Secondly, the SF storage technique and delayed microscopy, on average 2 days after arthrocentesis, may have influenced the SF quality and therefore the chance of correctly identifying crystals. In MSU, the temperature appears to play a role in crystal formation through effects on urate solubility.

Table 2 Microscopy results: number of monosodium urate crystals (MSU) and calcium pyrophosphate crystals (CPP)-positive smears of 87 patient samples (174 smears in total) before and after centrifugation

	Centrifugation, *n*		Additional number of positive smears as result of centrifugation, *n* (%)
Total number of smears	Before 174	After 174	
Observer 1			
MSU positive smears	32	32	0 (0.0%)
CPP positive smears	16	20	4 (20.0%)
Observer 2			
MSU positive smears	27	29	2 (6.9%)
CPP positive smears	11	15	4 (26.7%)

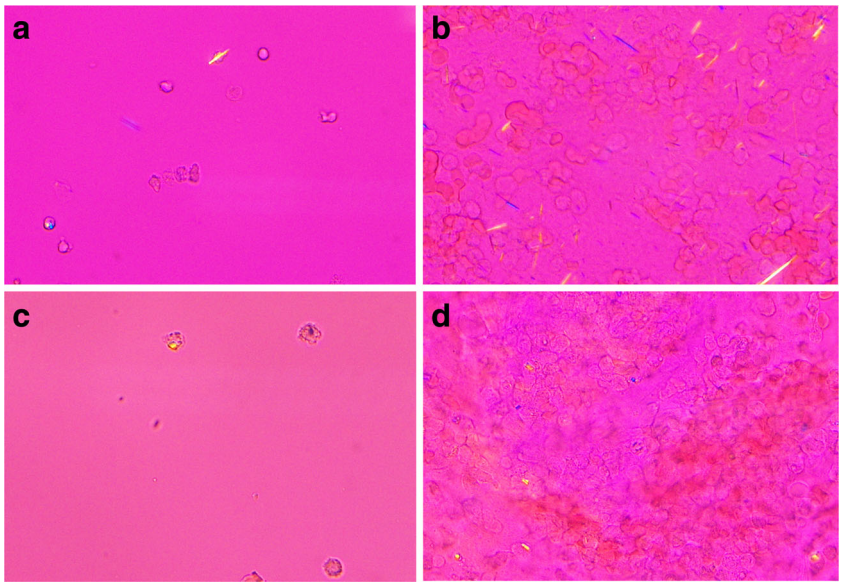

Fig. 1 Microscopy of typical SF smears showing leukocytes and crystals before and after centrifugation. **a** MSU crystals before centrifugation,. **b** MSU crystals after centrifugation. **c** CPP crystals before centrifugation. **d** CPP crystals after centrifugation

In vitro studies suggest that a reduction of even 2 °C is sufficient to lower the solubility point [19]. Although, our preservation method, in a refrigerator at 4 °C, is supported by a handful of small studies which assessed the impact of storage handling on crystals recognition [6, 12–17]. Synovial fluids were maintained for at least 48 h without morphological change. Even after one to several weeks of storage at 4 °C, the crystal and leukocyte concentrations only tend to be minimally reduced [12, 14–16, 20–22]. Therefore, we think that our storage handling had none or minimal influence on crystal recognition. This probably would also apply for the usage of EDTA tubes for storage. However, studies on this issue are scarce. Tausche et al. assessed the detectability of MSU and CPP crystals in 75 SF samples over a period of 3 days and showed that the crystal counts nor pH appeared dependent on whether the samples were sent in native (plain, sterile tubes) or anticoagulated tubes with EDTA. Also, neither significant cell decay between day 0 and 3 was evident nor secondary formation of crystals in EDTA-treated samples was observed [17]. Another study on 100 SF samples showed that the white blood cell (WBC) counts, performed after preserving SF for 24 and 48 h in EDTA tubes at 4 °C, were very close to the values obtained just after arthrocentesis [16]. They stated that the use of EDTA as anticoagulant probably was important for the preservation of the fluids. A remarkable finding was that their results differed from other cited studies in which heparin was used [16]. In these studies, a pronounced decrease in the WBC counts was observed. To explore this difference, Salinas et al. also performed a small pilot study on 14 SF samples which were kept in both heparin and EDTA tubes. The decrease in WBC counts in the EDTA tubes was significantly lower than that in heparin tubes ($p < 0.001$). Therefore, they suggest that EDTA tubes were much more suitable as preservative than heparin for the performance of SF counts [16]. Important remark is that they did not explore the suitability of EDTA in relation to crystal recognition. Instead, Gálvez et al. was interested in the question whether different storage techniques of SF samples (with or without anticoagulants) affected the identification of crystals and whether morphological changes would alter the results in these samples [14]. In their study, they used 91 SF samples taken from patients with gout ($n = 31$), CPPD disease ($n = 30$), and a control group with several arthropathies ($n = 30$). Among others, they found that the number of crystals was the same in samples preserved with or without anticoagulant (EDTA and sodium heparin). They concluded that SF analysis for the detection of MSU or CPP crystals could be deferred up to 72 h, when samples were stored at 4 °C with or without EDTA or sodium heparin as anticoagulant [14]. As far as we could find, there were no more recent studies on the subject. Based on the limited available evidence, EDTA preservation for several days seems comparable with instantaneous performed polarized light microscopy [14, 16, 17].

Thirdly, we used a conventional laboratory centrifuge, in contrast to other studies which used cytocentrifuges [6, 7, 9, 10, 23–26]. Besides some disadvantages, mentioned before, the latter has important advantages: sedimentable particles from liquid suspensions can be directly transferred as a concentrated thin layer of cells onto a microscope slide without additional manual pipetting and it places cells and crystals in the same optical plane [6, 7, 9].

Another possible limitation was that observer-related bias may have been introduced. If the observers recognized crystals in the routine smear, they could have been more focused to seek for them after centrifugation. This probably did not affect the results because if any crystal was present before centrifugation, there were many more thereafter.

Most SF samples were derived from the knee joint. Arthritis of the knee was frequent and large SF volumes, like

required in our study, are evacuated much easier from the knee compared to smaller joints. Therefore, our conclusion probably cannot be generalized to other joints. This would require a study including a higher number of SF samples from other (smaller) joints than the knee.

Identifying crystals correctly is not always as easy as it seems and requires specific training and exposure. This was shown by Berendsen et al. using an online test to assess the performance of crystal identification by 110 professionals [5]. However, it is unlikely that a different degree of experience between both observers could have influenced our results significantly, as the interobserver agreement for crystal recognition between both observers was sufficiently high before (kappa 0.82, p value 0.049) and even higher after centrifugation (kappa 0.92, p value 0.032). Also, given the fact that crystal-based diagnosis by polarized light microscopy is common practice in the Netherlands, a microscope is always available at our clinic and patients with gout and CPPD disease are frequently seen at our department, both observers were experienced in performing this examination on a daily basis before the initiation of the study. This is supported by our findings; in 89.7% of all samples, the patients' physician performed microscopy (Table 1). Unfortunately, our study was not designed to assess the sensitivity or specificity of microscopy (before or after centrifugation) as there was no golden standard to compare to.

A last possible limitation and remarkable finding was that with the currently used centrifuge settings, the supernatant of numerous SF samples was not completely clear after centrifugation. This was not in line with our prior expectation that with our settings, almost all leukocytes would be displaced into sediment. In a small pilot (data not shown) on several SF samples, we also examined the fluids transparency, amount of leukocytes, and crystals which remained in the supernatant after centrifugation. In most supernatant samples, leukocytes and crystals were still present after a 10-min centrifugation at 700 rpm, however, clearly less in numbers than in the sedimentation itself. After centrifugation at a higher speed (1200 rpm during 10 min), the supernatant was clearer, the sedimentation volume increased, and leukocytes and crystals were much sparser in the supernatant. This resulted in higher concentrations in the sediment of both. Nevertheless, it is questionable whether this would also have increased the amount of crystal-positive smears. We already have shown that all samples that were MSU and CPP positive before centrifugation were also positive after. The centrifugation settings we used were chosen to increase comparability to other studies [6, 7, 9, 10, 24–26].

Conclusions

Synovial fluid centrifugation using a conventional laboratory centrifuge is a simple procedure to perform in daily clinical practice and crystals tend to be identified easier and more quickly. Monosodium urate crystals are well recognized without centrifugation. Centrifugation of SF had limited additional value for increasing the amount of MSU-positive smears. However, CPP crystals were obviously identified in a higher number of smears after centrifugation than before. Therefore, centrifugation may specifically be of additional value in selected patients with suspected CPPD disease and to a lesser extent for gout.

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Compliance with ethical standards

Ethics In accordance with Dutch legislation, non-interventional studies, like this study, are not subjected to Ethical approval.

Disclosures None.

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