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Utility of dimethylsulfoxide to preserve synovial fluid samples for microcrystal detection and identification

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Abstract

Aims: To study whether the addition of dimethylsulfoxide (DMSO) to synovial fluid (SF) samples could be helpful to store frozen samples to improve the rates of detection and identification of crystals.

Methods: Cross-sectional study of samples of SF consecutively obtained. Three aliquots were generated: one for immediate observation by a senior observer, and 2 to be frozen, one with 10% DMSO (DMSO+) and one without DMSO (DMSO–). Each aliquot was randomly allocated and blinded for further observation when once the samples were unfrozen 3 months afterward. Variables included for analysis were total leucocyte count, detection of crystals, identification of present crystals as monosodium urate (MSU) or calcium pyrophosphate (CPP), number of fields to the first crystal observation, and number of crystals per field. The vitality of leucocytes was evaluated using a trypan blue stain. All samples were examined using ordinary light and polarized light with a red compensator, and unfrozen samples by both senior and junior observers.

Results: In the 30 reference samples of SF studied, the mean leucocyte count was 13.1 × 10^9/L, and 18/30 samples showed crystals (8 MSU, 10 CPP). Once unfrozen, leucocyte counts were 58% lower in DMSO aliquots *vs.* 22% in DMSO+ aliquots, with vitality (> 50% cells) reduced from 100% in the reference sample to 76.6% in the DMSO+ aliquots to none in the DMSO– aliquots. Agreement in the detection of crystals was much better in DMSO+ aliquots than DMSO– (kappa 1.00 *vs.* 0.69 and 0.65 *vs.* 0.11 for the senior and junior observers respectively). Moreover, 4/5 false-negative crystal detection in DMSO– aliquots showed CPP in the reference simple, even though a high density of crystals was observed in the reference sample.

Conclusions: The addition of 10% DMSO to SF samples allows freezing and storage with a small loss of leucocyte counts and excellent agreement in the detection and identification of crystals. Cellular lysis may account for the false negative results in aliquots without DMSO, especially in the case of CPP, non-refringent crystals.

Keywords

Crystals, dimethylsulfoxide, urate, pyrophosphate, microscopy, storage, freezing

Introduction

Microscopy examination of synovial fluid (SF) samples is the standard tool to evaluate synovial effusions [1]. Light microscopy is the gold-standard technique for the diagnosis of crystal-induced arthritis, allowing the detection and identification of monosodium urate (MSU) crystals in gout [2], and calcium pyrophosphate (CPP) microcrystals in CPP deposition disease (CPPD) [3]. In addition, it allows evaluation of the presence and amount of inflammation in SF samples via leucocyte counts.

Indeed, observation of smears of fresh samples of SF allows the detection and identification of crystals in most cases. Avoiding delay from joint aspiration to microscopy observation is key to preventing leucocyte apoptosis and crystal degradation, especially in the case of CPP crystals, which may undergo degradation *ex vivo* via pyrophosphatases present in neutrophils [4].

Cooling or freezing SF samples are usual methods to preserve SF samples when immediate observation is not at hand, such as in primary care or emergency room, but even despite storage in a cold environment, the samples may get degraded and biased results [5]. A suggestion to use dimethylsulfoxide (DMSO) for storing frozen SF samples has been made [6], but not based on a formal study.

DMSO is a chemical compound commonly used in biology to cryopreserve cells [7], prevent damage by intracellular ice microcrystals that may damage the cell membrane, and induce cytolysis during subsequent unfreezing [8]. It is the most common agent used to cryopreserve cells and tissues [9].

This study aims to assess whether the addition of DMSO to SF samples helps maintain crystal and cellular morphology when comparing frozen versus unfrozen SF storage samples.

Materials and methods

This is an observational, cross-sectional study with prospective and consecutive recruitment using excess SF samples from clinical practice in a 3rd level university hospital.

Samples

Consecutive SF samples were obtained from exceeds of SF over 3 mL during clinical practice from 1st December 2020 to 28th February 2021. All patients visited a crystal-induced-arthritis clinic and signed consent to use excess SF for clinical investigation after all usual clinical proceedings for SF samples had been performed. The local ethics committee approved the use of excess SF samples. Three aliquots of 1 mL each were obtained from each sample: aliquot 0 (reference, for immediate analysis), aliquot DMSO+ (with 10% DMSO), and aliquot DMSO– (without DMSO). DMSO+ and DMSO– were frozen and stored for a second analysis after unfreezing. After analysis of the reference aliquot, a random number was assigned to each aliquot using a random sequence generated by the statistics program and assignment concealed to avoid bias for further blinded observation of frozen aliquots.

Storage and unfreezing

Aliquots DMSO+ and DMSO– were frozen within a period not exceeding 30 min from joint aspiration to storing and stored in a –20°C freezer, with a permanent external monitor of temperature used to store samples for clinical trials and therefore monitored by the bioengineering department.

Samples were unfrozen after 3-month storage. Unfreezing was performed at 37°C for 10 min, and data from microscopy observation was blindly retrieved in the following 10 min.

Analysis of samples

A reference senior observer (Fernando Pérez-Ruiz), credited through a validation process to participate as an investigator in the Study for Updated Gout ClAssification CRiteria (SUGAR) study for the classification of gout, evaluated all reference samples for detection and identification of crystals, with the search starting at the center of the slide. Those samples not showing crystals after 3 × 3 field scanning (9 fields clockwise) were considered negative. The average number of crystals observed was recorded as a semi-quantitative variable (< 1/field, 1–5/field, 6–10/filed, > 10/field), and the number of fields observed to find the first crystal was also in a semi-quantitative variable (1–3 fields, 3–6 fields, 6–9 fields). The viability of cells was evaluated in each aliquot only by the reference observer using trypan blue staining, commonly used as a marker of cellular membrane damage [10] as a semi-quantitative variable: < 25%, 25–49%, 50–75% and > 75% of present viable cells.

A junior observer, an MD student, instructed for 2 h/day during 5 days of microscopy experience (Elsa Lopez-Bardón), evaluated each aliquot simultaneously with the senior observer, in separate rooms, and blinded for reference results.

Both observers used a Nikon Eclipse50i microscope at 400× with both ordinary light and polarized light with a first-order red compensator to observe the presence of refringence and elongation properties of the crystals.

Statistical analysis

We used an institutional, licensed package of the IBM SPSS.23 statistical program for analysis of the data and comparisons: paired *t*-test ($P < 0.05$ to be considered significant), a Cronbach's alpha and intra-class correlation for leucocyte counts comparisons, and kappa index for agreement for crystal detection and crystal identification, these last three analysis showing best results close to 1.00, good over 0.60.

Results

Thirty SF samples, 90 aliquots, were available. As for the reference observation of aliquots 0, the mean leukocyte count was 13.14 × 10^9 cells/L (median 9.0 × 10^9 cells/L, interquartile range 3.37 × 10^9–20.00 × 10^9 cells/L). Eighteen (60%) samples showed crystals (8 MSU and 10 CPP), and in 12 (40%), crystals were not observed. In 17/18 (94%) samples, crystals were found in fields 1 to 3, and in 13/18 (72%), over 10 crystals per field were counted.

Aliquots with and without DMSO were observed after 3 months by both senior (observer 1) and junior (observer 2) observers; due to severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) pandemic issues, the junior observer could only evaluate 22 out of 30 samples.

A loss of the leucocyte counts was apparent in frozen samples, but the addition of DMSO significantly reduced the effect from 58% in DMSO– aliquots to 22% in DMSO+ aliquots (Table 1).

Table 1. Paired *t*-test for leucocyte counts before (aliquot 0 as reference) and after unfreezing with and without DMSO

Leucocyte count	*N*	Mean (× 10^9/L)	SD
Observer 1	30	13.14	10.67
Observer 1/DMSO+	30	10.20*	7.74
Observer 1/DMSO–	30	5.50**	6.10
Observer 2	22	6.73*	4.10
Observer 2/DMSO+	22	3.19**	2.69
Observer 2/DMSO–	22	1.87	11.27

N: number; SD: standard deviation. * $P < 0.01$, ** $P < 0.001$

Cronbach's alpha test for accuracy of leucocyte count showed the senior to perform best in DMSO+ aliquots (0.91, 0.81–0.96 *vs.* 0.43, 0.35–0.76), but accuracy was reduced for both observers once again in DMSO– aliquots (0.79, 0.56–0.90 *vs.* 0.28, 0.69–0.70) the accuracy in leucocyte count. The kappa index for agreement in the detection of any crystal in DMSO+ aliquots was 1.00 and 0.62, and in DMSO– aliquots 0.61 and 0.14 for observers 1 and 2, respectively.

It is shown that agreement for observer 1 (senior) regarding crystal identification is better than for the junior observer. A reduction of 0.31 in kappa (from 1.00 to 0.69) was observed comparing DMSO+ with DMSO– aliquots for the senior observer, and from 0.84 to 0.11 for the junior. Most (4/5) false-negative samples for the senior observer in the DMSO– aliquots contained CPP in the reference aliquot, and forty percent (4/10) of the CPP samples were false-negative in DMSO– aliquots compared to 1/8 of the MSU samples (Table 2).

Table 2. Agreement in the identification of microcrystals (kappa index) compared with reference with and without DMSO for both observers (disagreement shown in bold and underlined)

Reference	DMSO+ observer 1			DMSO+ observer 2			DMSO– observer 1			Observer 2		
	Negative	MSU	CPP	Negative	MSU	CPP	Negative	MSU	CPP	Negative	MSU	CPP
Negative	12	0	0	2	1	1	11	0	1	0	0	0
MSU	0	8	0	0	8	0	1	7	0	5	1	0
CPP	0	0	10	0	0	9	4	0	6	2	1	2

Agreement kappa: 1.00 for reference *vs.* DMSO+ observer 1; kappa: 0.845 for reference *vs.* DMSO+ observer 2; kappa: 0.691 for reference *vs.* DMSO– observer 2; kappa: 0.111 for reference *vs.* DMSO– observer 2

In most of the samples, crystals were identified in the very first fields (17/18) in the DMOS+ aliquots, 13/13 in the DMSO– (5 samples in the DMSO– considered negative). Interestingly, 3 DMSO– aliquots containing CPP in the 3 first fields in the reference sample were considered negative (Table 3). Moreover, in 3/4 CPP of the false-negative samples in the DMSO– aliquots, the reference sample had shown abundance (> 10 crystals per field).

Table 3. Agreement in the identification of microcrystals for the senior observer in samples **with** crystals depending on the number of fields needed in the reference sample (disagreement shown in bold and underlined)

Reference		DMSO+			DMSO–		
		Crystal negative	MSU	CPP	Crystal negative	MSU	CPP
1–3 fields	MSU	0	8	0	**1**	7	0
	CPP	0	0	9	**3**	0	6
> 6 fields	CPP	0	0	1	**1**	0	0

Agreement kappa: 1.00 for detection of crystals in DMSO+ simples; kappa: 0.62 for detection of crystals in DMSO– samples

Finally, trypan blue staining showed that all of the reference aliquots were classified as > 50% vital cells, compared to 76.6% of DMSO+ and none of the DMSO– aliquots.

Discussion

The detection and identification of microcrystals (are there any, and if present, what kind of crystals, respectively) are considered to date as the gold standard for diagnosis of gout [2] and CPP arthritis [3].

The availability of light microscopy at the local healthcare providers' clinics may delay the examination of SF samples [11]. Although some authors recognize that a short, 3-day delay may not compromise the reliability of the sample, cooling or freezing SF samples may improve the reliability of results more than room temperature storage [12]. Indeed, freezing samples may render better results [12] than cooling [4].

Nevertheless, the formation of intra-cytoplasm water crystals may render massive cell apoptosis after unfreezing. This may create some sluggishness, especially in smears with high leucocyte counts, and jeopardize the detection of crystals. Hypothetically, it would be expected that MSU crystals known to exhibit intense refringence would be more easily detected in this setting than CPP crystals, which show mild or no refringence [13].

The quality of SF samples can deteriorate before an exam. Therefore, if long-term storage is desired (for academic purposes), or needed (delayed examination for clinical purposes), avoiding apoptosis of leucocytes in frozen samples of SF would be helpful. CPP crystals in SF keep present for prolonged periods by freezing [12]. Maintaining the viability of cells after freezing and unfreezing using DMSO has been performed for over 50 years [8], and is a current method for cell cryopreservation [9]. Our results are not best regarding the rate of vitality after unfreezing samples, as would be expected in a laboratory setting; managing samples at the office during clinical activity is the most plausible cause.

To our knowledge, this method has not been tested nor published regarding cryopreserving SF samples in order to store samples long-term, thus, avoiding a reduction in the reliability of detecting and identifying MSU and CPP crystals. The fact that trypan blue staining of the unfrozen aliquots DMSO– showed no acceptable viability compared to two-thirds of DMSO+ aliquots supports the hypothesis that apoptosis is probably one main cause of false-negative results, especially in the case of CPP.

Our results neatly show that the addition of DMSO to SF samples is associated with a reduction in the loss of leucocyte counts, and improves the detection and identification of MSU and CPP crystals. As expected, samples with CPP crystals and no addition of DMSO comprised most of the false negative results after unfreezing. It has been recently shown that the addition of 10% DMSO to SF samples may preserve cellular morphology and avoid artifacts and cellular clumping [14], but no specific study on crystal detection or identification was reported.

Nevertheless, it is important to note that the results were only applicable to the experienced observer, therefore suggesting that short training may not be enough to be reliable, contrary to previous publications [15].

This study's strengths are that samples were randomized, anonymized, blinded, and simultaneously examined. There are also some limitations to this study. The study was limited in the number of samples because it needed to be completed within one academic year, including the time needed for collection, freezing, and unfreezing. Therefore, further studies sustaining our initial results would be welcome. The samples were obtained in the clinic during clinical care, a fact that may induce some deleterious effects on the quality of the samples. In addition, the impact of the coronavirus disease 2019 (COVID-19) pandemic during 2020 and the disease sequentially suffered by FPR and ELB even more limited the availability of these investigators. Also, a study after long-term storage would be desirable.

In conclusion, our results show that the use of DMSO added to SF samples is useful at least for mid-term storage contributes to the maintenance of cellular morphology in SF when stored in frozen conditions, and is especially useful to avoid false-negative results in the case of CPP crystals.

Abbreviations

CPP: calcium pyrophosphate

DMSO: dimethylsulfoxide

MSU: monosodium urate

SF: synovial fluid

Declarations

Author contributions

FPR, ELB, FL, NS, TU, and JJMM: Conceptualization, Writing—review & editing. FPR and ELB: Data curation, Writing—original draft. FPR, ELB, FL, NS, TU, and JJMM read and approved the submitted version.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Ethical approval

The study was included within a prospective inception gout cohort study approved by Cruces University Hospital Ethics and Clinical Investigation Committee (CEIC Cruces).

Consent to participate

Written informed consent was obtained and signed by patients and investigators to use exceeds of biological samples, and approved by the CEIC Cruces.

Consent to publication

Not applicable.

Availability of data and materials

Data can be obtained by contacting Fernando Pérez-Ruiz (fernando.perezruiz@osakidetza.eus).

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References

1. Pascual E, Sivera F, Andrés M. Synovial fluid analysis for crystals. Curr Opin Rheumatol. 2011;23:161–9.
2. Richette P, Doherty M, Pascual E, Barskova V, Becce F, Castaneda J, et al. 2018 updated European league against rheumatism evidence-based recommendations for the diagnosis of gout. Ann Rheum Dis. 2020;79:31–8.
3. Zhang W, Doherty M, Bardin T, Barskova V, Guerne PA, Jansen TL, et al. European league against rheumatism recommendations for calcium pyrophosphate deposition. Part I: terminology and diagnosis. Ann Rheum Dis. 2011;70:563–70.
4. Kienhorst LB, Janssens HJ, Eijgelaar RS, Radstake TR, van Riel PL, Janssen M. The detection of monosodium urate crystals in synovial fluid after long-term and varying storage conditions. Joint Bone Spine. 2015;82:470–1.
5. Pastor S, Bernal JA, Caño R, Gómez-Sabater S, Borras F, Andrés M. Persistence of crystals in stored synovial fluid samples. J Rheumatol. 2020;47:1416–23.
6. Perez-Ruiz F, Castillo E, Chinchilla SP, Herrero-Beites AM. Clinical manifestations and diagnosis of gout. Rheum Dis Clin North Am. 2014;40:193–206.
7. Hornberger K, Yu G, McKenna D, Hubel A. Cryopreservation of hematopoietic stem cells: emerging assays, cryoprotectant agents, and technology to improve outcomes. Transfus Med Hemother. 2019;46:188–96.
8. Lovelock JE, Bishop MW. Prevention of freezing damage to living cells by dimethyl sulphoxide. Nature. 1959;183:1394–5.
9. Li R, Johnson R, Yu G, McKenna DH, Hubel A. Preservation of cell-based immunotherapies for clinical trials. Cytotherapy. 2019;21:943–57.
10. Kim MY, Yoon HY, Lee S. The advantage of the supercooling storage method for transplantable sources: human umbilical vessel endothelial cells and mouse skin grafts. Transplant Proc. 2021;53:1756–61.
11. Tausche AK, Gehrisch S, Panzner I, Winzer M, Range U, Bornstein SR, et al. A 3-day delay in synovial fluid crystal identification did not hinder the reliable detection of monosodium urate and calcium pyrophosphate crystals. J Clin Rheumatol. 2013;19:241–5.
12. McGill NW, Swan A, Dieppe PA. Survival of calcium pyrophosphate crystals in stored synovial fluids. Ann Rheum Dis. 1991;50:939–41.

13. Perez-Ruiz F, Modesto-Caballero MC. Crystal-associated arthritis: gout. In: Mandell BF, editor. Synovial fluid analysis and the evaluation of patients with arthritis. Springer, Cham; 2022. pp. 91–100.
14. Pavic K, McGill V, D'Souza M, McGill N. The preservation of synovial fluid using dimethyl sulfoxide. Ann Clin Biochem. 2022;59:296–301.
15. Lumbreras B, Pascual E, Frasquet J, González-Salinas J, Rodríguez E, Hernández-Aguado I. Analysis for crystals in synovial fluid: training of the analysts results in high consistency. Ann Rheum Dis. 2005;64:612–5.