Synovial fluid analysis

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ABSTRACT

At present, the study of the synovial fluid (SF) is a tool that is used frequently in specialized laboratories because it allows the establishment of diagnosis of crystal associated arthropathies, supports the diagnosis of septic arthritis and helps establish other rheumatologic diagnoses such as monoarthritis or joint effusion. The complete study of the SF includes the following analyses: 1. Macroscopic; 2. Microscopic; and 3. Specific stains. Each one provides information of the joint’s state and helps in the establishment of diagnosis and treatment. The characteristics that must be described in the macroscopic analysis are: color, volume and viscosity. Microscopic analysis of the SF confirms the presence of inflammatory or infectious processes and allows for the detection and identification of crystals. Polarized light microscope is a fundamental tool for the analysis of SF and for the identification of the crystals present in the samples, which not only depend on the form, but also of their birefringence. It is important to mention that in the microscopic analysis, artifacts can confuse the inexperienced observer. A suitable interpretation of the analysis of SF requires the observation by at least two experienced observers. The information that the analysis of SF provides to the clinicians gives them the necessary elements to establish the diagnosis and to decide on treatment. Specific stains in the analysis of SF help in the identification of non-birefringent crystals as those of calcium hydroxypatite. In SF analysis, new fields are being explored that include quantification of cytokines, chemokines, immunoglobulins and the characterization of cell lineages.

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Análisis de líquido sinovial

En la actualidad el estudio del líquido sinovial (LS) es una herramienta que se utiliza con frecuencia en los laboratorios especializados y que permite establecer el diagnóstico de artropatías por cristales, apoya el diagnóstico de las artritis sépticas y ayuda a establecer otros diagnósticos reumatológicos como la monoartritis o los derrames articulares. El estudio completo del LS incluye los siguientes análisis: 1) macroscópico, 2) microscópico y 3) uso de tinciones específicas. Cada uno de los estudios proporciona información del estado de la articulación y ayuda a establecer el diagnóstico y tratamiento. Las características que se deben describir en el análisis macroscópico son: color, volumen y viscosidad. El estudio microscópico confirma la existencia de un proceso inflamatorio, infeccioso y la presencia de cristales. El microscopio de luz polarizada es una herramienta fundamental para el estudio del LS y la diferenciación de los cristales, la cual no sólo depende de la forma, sino también de la birefringencia. Es importante mencionar que en el análisis microscópico los artefactos pueden confundir al observador inexperto. Una adecuada interpretación del análisis del LS requiere de la observación por lo menos 2 observadores capacitados. La información que proporciona el análisis del LS al clínico da los elementos necesarios para establecer el diagnóstico del paciente así como el tratamiento del mismo. Las tinciones en el análisis del LS ayudan a a la identificación de cristales no birefringentes, como son los de hidroxiapatita de calcio. Actualmente, en el estudio del LS se están explorando nuevos campos que incluyen cuantificación de citocinas, quimiocinas e inmunoglobulinas y la caracterización de las estirpes celulares.

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Introduction

The study of synovial fluid (SF) through polarised light microscopy started in 1961, with the work of Daniel J. McCarty and Joseph Lee Hollander. In their studies, they identified monosodium urate crystals in patients with gout and calcium phosphate crystals in patients with pseudo-gout. As any study involves taking a sample using an invasive procedure, its use in diagnosis is low because it may cause harm, even though the analysis provides elements that allow for the diagnosis of gout or other crystal arthropathies. The procedure has been modified in the last few years and we now know that, given that the joint is the micro-environment where inflammation develops, with all its effects, SF study provides important information in establishing the diagnosis and, if applicable, starting treatment.

Study of SF has made it possible to establish criteria for diagnosing gout. In 2009, Malik and his associates carried out a study in which they concluded that monosodium urate crystal identification continues to be the gold standard for definitive gout diagnosis. Analysis of SF is a simple test whose main complication is having a polarised light microscope, reagents, and trained staff.

In 1995, the American College of Rheumatology set up the criteria for SF analysis. With regards to methodology, SF analysis is composed of 3 basic analyses: 1) macroscopic, which allows the definition of the physical characteristics of the sample (e.g., volume, colour, and viscosity); 2) microscopic, which includes a complete leukocyte count plus crystal screening and definition through polarised light; and 3) the use of differential dyes (e.g., Gram, Wright, Red Alizarin, and Sudan Black, among others).

Macroscopic analysis

Normal SF is straw-coloured. If placed in a test tube, you should be able to read writing through it. It has viscosity similar to that of an egg white. This fluid is included among the non-Newtonian fluids and as a pseudoplastic fluid in these. A practical, easy way of measuring SF viscosity is by placing a drop on a slide and lifting it slowly with a wooden spatula. Normal SF should form a strand of about 3–6 cm in length. This property is known as stranding. Stranding is defined as the ability of mucosity to extend itself until strands are formed. The SF strand size decreases with inflammatory processes. More exact measurements can be made by using a viscometer, in which case normal SF viscosity value is $\eta=45\pm8$ Pa.$^s$

Microscopy analysis

Cell count. The cell count must be carried out within two hours after the sample is taken, given that later readings affect the results due to cell fragility once the cells are removed from the joint. The cell count must be done manually in a Neubauer chamber, diluting the sample with a hypotonic sodium chloride solution (0.03%), as automated equipment can give incorrect values from the viscosity of the liquid or the presence of artefacts. Dilution of SF is performed with a Thoma pipette for white blood cells, which has two inflow markers; the first one is at 0.05, which corresponds to 20 $\mu$L, the point it must be filled to with SF, and the second one at 11, which corresponds to 200 $\mu$L, the point it must be filled to with saline solution. It is mixed by gently shaking for about a minute and is later placed in the Neubauer chamber. The four quadrants are counted for leukocytes under the microscope. The SF leukocyte count should be less than 200 cells/$\mu$L (Table). The formula used to establish the number of cells is: $4N=50\cdot$cells/$\mu$L.

Where:

$4N=$ number of white blood cells counted in the 4 quadrants
$50=$ dilution factor

Crystal identification. Microscopic analysis includes the search for crystals and the characterisation of their refringence, using a polarised light microscope. Polarised light is generated using a polarised lens over a visible light source. The polarised lens, or polariser, filters the light allowing only the one on a single plane through; a second polariser (also known as an analyser) located on the first filter than steers the light perpendicularly to this, blocking the light and making the background seen through the eyepiece dark. If a birefringent crystal is placed between the polarisers, the light is separated into short and long wavelengths. Some of these rays go through the second polariser and make the crystals appear bright against the dark background. Luminosity is a characteristic of birefringent materials. Generally, a red compensator is used to eliminate green light, which produces a pink background instead of a dark one. The red compensator has a 540 nm wavelength. When the light plane generated by a crystal is parallel to the compensator plane, it causes certain crystals to appear blue; if this same crystal is turned 90°, its light plane will be parallel to the analyser and will be yellow. When the crystal is positioned parallel to the compensator and a blue light is seen, it is said to have positive elongation. When the crystal is positioned parallel to the compensator and a yellow light is seen, it is said to have negative elongation. Another of the characteristics of a true birefringent crystal is that these crystals have an extinction angle. This is a phenomenon that is seen when the microscope plate is turned to position the crystals perpendicular to the compensator, which makes them lose their yellow and blue colour and has an effect that makes the crystals being examined seem to disappear. The extinction angle varies according to the nature of the crystals.

Microscopy analysis of SF should include a description of the shape (needle, rhomboid, square with a notch, cigar-shaped, bi-pyramidal, Maltese cross, etc.) birefringence, location (intracellular or extracellular), and quantity (scarce or plentiful) of the crystals observed.

Because the cost of polarised light microscopes is high, there is an alternative that can help convert an optical microscope into a polarised light microscope. This is done by placing two filters with a wavelength greater than 600 nm, one directly over the light source and another between the sample and the observer. A slide should also be placed over the first filter, to which a long piece of transparent adhesive tape should be affixed, which gives a wavelength of between 250 and 350

| Table  
| Synovial fluid classification |
|---|---|---|
| Normal | Inflammatory | Septic |
| **Volume, mL** | <3.5 | >3.5 | >3.5 |
| **Viscosity, cm (Stranding)** | 3 to 6 | <3 | <3 |
| **Colour** | Straw yellow | Opaque yellow | Milky |
| **Leukocytes/$\mu$L** | 50–2,000 | 2,000–7,500 | >7,500 |
nm. With this “home-made” system one cannot determine the type of birefringence, but using crystals of a known structure as a control, through comparison the type of crystal observed can be established.

Crystal identification using dyes

Staining with red alizarin. Calcium hydroxyapatite crystals (Ca_{10}[PO_{4}]_{6}[(OH)_{2}]) and other calcium phosphate crystals are found as small clusters or large pleomorphisms that can measure between 0.5-10 μm. They are not generally birefringent and can be seen on an optic microscope. They have great avidity for the dye red alizarin, which unites with calcium and other cations. The alizarin stain can detect up to 0.005 μg/ml of hydroxyapatite in SF but is not very specific, so it is used only for an initial screening. The stain is made by placing a drop of 2% red alizarin and a drop of SF on a clean slide, homogenising the mixture, and observing it under a normal optic microscope. One should look for either clusters of red stained particles or small round particles, similar in size to leukocytes. The staining should be performed regardless of whether or not birefringent crystals are present.

Differential stain with Wright dye. This stain makes it possible to identify the cells present in SF samples. It should be carried out on fluids with cell counts greater than 1,000/μl. The methodology consists of spreading a drop of fluid on a slide, which is fixed through open air evaporation and is later stained with the Wright dye. The staining times should be adapted and standardised according to the methodology suggested by the dye manufacturer.

Staining with Sudan black. When lipid structures or intracellular inclusions are seen on the SF sample, staining with Sudan black should be performed, turning the lipids into pleomorphic accumulations or black inclusions. The stain is made by placing 7% Sudan black and a drop of SF on a clean slide, gently mixing it, and placing it under a slide cover. Finally, the slide is incubated for 1 minute at room temperature and observed under the microscope.

Gram staining. It is important to carry out the staining when the number of cells is greater than or equal to 75,000/μl with the aim of finding bacteria. With this cell count, it is highly likely that the fluid will be septic. The Gram staining is carried out according to the manufacturer’s instructions. As in all laboratory tests, it is important to take into account the patient’s clinical manifestations; if septic arthritis is suspected, sample cultures must also be made.

Staining with Congo red. Positive staining with Congo red supports the diagnosis of amyloidosis, which is a heterogeneous group of diseases characterised by extracellular deposits of fibrillar protein material. The characteristic molecular structure inherent to type A amyloid substance is what is responsible for the insolubility of the amyloid deposits and their resistance to proteolytic digestion. As a consequence of the molecular stability of the protein, the amyloid deposit provokes the substitution and destruction of the affected organ parenchyma, causing different functional changes according to the location and intensity of the deposit. The current histological criteria most used in amyloidosis diagnosis are the affinity of the protein to Congo red, also known as congophilia. Clusters of characteristic apple-green refringence are seen under the microscope. The staining should be carried out in a cool place, placing a drop of SF and a drop of solution saturated with Congo red (0.019% Congo red in ethanol) on a slide. The mixture is gently homogenised and is observed under a polarised light microscope. The presence of amyloid is seen as apple-green clusters formed by the amyloid and dye.

Crystal identification

Monosodium urate crystals (MSU). The presence of MSU crystals in joints and connective tissue is the laboratory test that confirms the diagnosis of gout. MSU crystals are needle-shaped and show negative birefringence when seen under a polarised light microscope (Figure 1). Their extinction angle is 0-45º, and their size is about 3-40 μm. They can be inside or outside the white cells series in the SF. In severe gout attacks, there can be a cell count of 2,000-100,000 cells/μl with a predominance of polymorphonuclears. It is important to rule out infectious processes during microscopy analysis, as both pathologies can coexist. In gout tophi, the crystals are plentiful and large; however, in chronic gout, the cell count can be below 2,000/μl and MSU crystals can be scarce, making their identification difficult, which is why careful screening must be undertaken even when the cell count is normal. It is also possible to see partially digested crystals inside the cells, which can cause confusion as they do not present characteristic morphology. MSU crystals can be present together with other crystals, such as calcium pyrophosphate, cholesterol, or even Maltese cross. Studies show that after a severe mono- or oligoarticular gout attack, some patients can develop chronic deforming synovitis, which can confuse the clinician into diagnosing rheumatoid arthritis.

Calcium pyrophosphate dihydrate (CPPD) crystals. These are present in the joints of patients with pseudo-gout syndrome or diseases with CPPD deposits. Because of its radiological characteristics, the illness was initially described as joint chondrocalsinosis, due to the appearance of calcium deposits in the joint areas. CPPD crystals are pleomorphic; they typically form short sticks, rectangles, or small squares. On a polarised light microscope, they are seen as weak positive birefringent crystals, with a size of about 2-20 μm (Figure 2A). The observer must be experienced to identify them, because they can be go unnoticed due to their weak birefringence. It is important to screen the interior of the cells, constantly turning the plate, because, depending on their position, they sometimes do not appear to have birefringence. In severe pseudo-gout attacks, the cell count can be 2,000-80,000/μl, predominating polymorphonuclear cells with intracellular crystals (Figure 2B). In patients with chronic arthritis or those recuperating, the leukocyte count can be less than 2,000/μl with a predominance of mononuclear cells with intra- and extracellular crystals. Crystal identification does not exclude other joint inflammation causes such as infections, gout, oxalosis, or rheumatoid arthritis because these illnesses can present themselves together.

Calcium hydroxyapatite crystals. The majority of hydroxyapatite deposits are found in soft periarticular and articular tissue, their presence being associated with symptomatic inflammation. The
leukocyte count in the SF of patients with hydroxyapatite crystals is generally low and in some joints destructive arthritis is visible. When they are dyed with red alizarin dye, one can see under an optical microscope large or small clusters, which are either irregular or shaped like a Chinese coin (Figure 3). Their size is about 0.05-10 μm; when observed under the polarised light microscope, they are generally not birefringent. When observed under an electronic microscope, the individual crystals measure between 50-200 Å in diameter and have a needle or stick shape. As previously mentioned, hydroxyapatite crystals are visible once they are mixed with red alizarin dye. The method is sensitive but not very specific. Their identity is confirmed through electronic microscopy, X-ray diffraction, or elemental analysis.

Cholesterol crystals. Cholesterol crystals are found in inflammatory SF and in fluids drained from bursas of patients with rheumatoid arthritis, generalised erythematous lupus, and seronegative spondyloarthropathy. They are rare in the SF of patients with osteoarthritis and gout. They are generally large square crystals with a notch on one of the corners (Figure 4). They measure about 8-100 μm and can have either positive or negative birefringence. From a side view, they look like large rectangles or curved needles.

Lipid crystals. These crystals are found in the SF of patients with acute monoarthritis, chronic polyarthritis, and pigmented villonodular synovitis. They are also known as Maltese crosses, from the shape that they present when observed under a polarised light microscope. They have positive birefringence and measure about 2-8 μm in diameter. These crystals are formed by multiple layers of phospholipids, cholesterol, and water. Under an optical microscope, they are seen as small greenish bubbles.

Calcium oxalate crystals. Calcium oxalate crystals are almost exclusively seen in patients with renal damage and oxalosis. They are square bi-pyramidal, irregular square, short stick or oval in shape (Figure 5). When they are plentiful, they form clusters; they measure about 5-30 μm. Under the polarised light microscope, the majority of these crystals show strong birefringence with positive elongation, although some may show no birefringence. The clinical manifestations of patients who have calcium oxalate crystals in SF appear similar to those of patients with gout, pseudo-gout, or illnesses caused by hydroxyapatite crystal deposits.

Charcot-Leyden crystals. These crystals are not very common, and are seen in the SF of patients with eosinophilic synovitis and eosinophilia associated to vasculitis. They are cigar-shaped and their birefringence can be positive or negative. They measure...
between 17-25 μm approximately (Figure 6) and are formed by lysophospholipase or phospholipase B. They can deposit themselves on joints or the kidney.\textsuperscript{24}

Hematoxin crystals. Hematoxin crystals can be found in the SF of patients with haemarthrosis. They are a product of haemoglobin degradation. They have a rhomboid or rectangular shape and measure between 8-10 μm approximately.\textsuperscript{4} They are seen as coffee- or gold-coloured under an optical microscope; when they are influenced by polarised light, they show positive or negative birefringence (Figure 7). They can be mistaken for calcium pyrophosphate crystals. It is important to emphasise that SF must be first observed under an optical microscope (Figure 7A) and later with one with polarised light (Figure 7B).

Other crystals that can be identified in SF samples are steroids. Corticosteroid crystals are pleomorphic with strongly positive or negative birefringent. Betamethasone crystals are needle-shaped and show negative birefringent. They can be confused with MSU crystals, in which case, the observer’s experience plays an important role in identifying which type of crystals are present in the sample.

Synovial fluid analysis is a useful tool that, properly used, is a help to the rheumatologist in diagnosing arthropathies through crystals. Some new determinations have now been brought into SF analysis, such as protein detection (e.g., gamma globulin and β2-microglobulin), enzyme activities (e.g., aldolase-dehydrogenase-phosphatase), glucose, and hyaluronic acid, among others. These new determinations make it possible to detect changes such as articular inflammation, sepsis, articular activity, and trauma suffered by the joints of patients with pathologies such as rheumatoid arthritis, arthrosis, hyperthyroidism, tumours, or generalised lupus erythematosus.

Conclusions

Synovial fluid is a tool that helps in the diagnosis and treatment of arthropathies. The range of studies that can be carried out is enormous. It is necessary to define the macroscopic characteristics and cell count before proceeding with crystal screening and, if necessary, crystal culture. The analysis of this fluid establishes the basic identification of crystals, bacteria, and cells, and chemical and physical changes. It also provides the means to carry out a relevant diagnosis and suitable follow-up in a wide range of illnesses.

Investigation on SF changes in patients with arthropathies due to crystals has gained greater importance because new techniques are being applied to quantify cytokines, activation molecules, immunoglobulins, and cell stocks, all to understand what is happening to the affected joints in patients.
References