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FEASIBILITY OF A TETRACYCLINE BINDING METHOD FOR DETECTING SYNOVIAL FLUID BASIC CALCIUM PHOSPHATE CRYSTALS

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Abstract

Objective—Basic calcium phosphate (BCP) crystals are common components of osteoarthritic synovial fluids. Progress in understanding the role of these bioactive particles in clinical osteoarthritis has been hampered by difficulties in their identification. Tetracyclines stain calcium phosphate mineral in bone. We hypothesized that tetracycline staining might be an additional or alternative method for identifying BCP crystals in synovial fluids.

Methods—A drop of oxytetracycline was mixed with a drop of fluid containing synthetic or native BCP, calcium pyrophosphate dihydrate (CPPD) or monosodium urate (MSU) crystals and placed on a microscope slide. Stained and unstained crystals were examined by light microscopy with and without a portable broad spectrum UV pen light. A small set of characterized synovial fluid samples were compared with alizarin red S and oxytetracycline staining. Synthetic BCP crystals in synovial fluid were quantified fluorimetrically using oxytetracycline.

Results—Synthetic and native BCP crystals appeared as fluorescent amorphous aggregates under UV light after oxytetracycline staining. Oxytetracycline did not stain CPPD or MSU crystals or other particulates. Oxytetracycline staining had fewer false positive tests than alizarin red S staining, and could estimate quantities of synthetic BCP crystals in synovial fluid.

Conclusions—With further validation, oxytetracycline staining may prove to be a useful adjunct or alternative to currently available methods for identifying BCP crystals in synovial fluids.

Keywords

Apatite; osteoarthritis; synovial fluid

Basic calcium phosphate (BCP) crystals are common components of osteoarthritic synovial fluid (1, 2). They are also associated with a small group of less common musculoskeletal syndromes including Milwaukee Shoulder syndrome (3) and hydroxyapatite pseudopodagra (4). The term “BCP crystals” encompasses three different forms of calcium phosphate crystals: octacalcium phosphate, tricalcium phosphate and carbonate-substituted hydroxyapatite. Similar crystals are found in calcific deposits in tendon, skin and muscle.

BCP crystals are bioactive. They are mitogenic for synovial fibroblasts (5), and cause the release of catabolic cytokines and growth factors from connective tissue cells (6). While they are often found in a non-inflammatory milieu (7), BCP crystal can also initiate an inflammatory response (8). An understanding of the role of these crystals in osteoarthritis has been hampered by the paucity of easy and accurate BCP assays.

Currently, there are several methods of detecting BCP crystals in clinical specimens. Unlike other pathologic crystals, BCP crystals have no distinct morphologic features that render them identifiable by plain light microscopy. They are not birefringent, and thus, are not visible with polarized light microscopy. Alizarin red S staining is perhaps the most widely used stain for their presence. Alizarin red S stains calcium-containing particulates in fluids and tissues. Calcium salts appear deep orange or red under plain or compensated polarized light microscopy (9). This assay is often difficult to interpret, as the background is typically strongly colored and both CPPD and BCP crystals stain. A high rate of false positives has been noted (10). In contrast to alizarin red S staining, the (^{14}C) ethane-1-hydroxyl 1,1-diphosphonate (EHDP) binding assay is very specific for BCP crystals (3). Unfortunately, it requires radiolabeled (^{14}C) EHDP, which is not commercially available. Fluids must be extensively prepared prior to the EHDP binding assay and specialized equipment is required. Other definitive techniques such as x-ray diffraction, analytical electron microscopy, and Fourier Transform Infrared (FTIR) spectroscopy are far too cumbersome and expensive to be practical in the clinic (11).

Tetracyclines are commonly used antibiotics that bind to hydroxyapatite mineral. Their avidity for bone mineral as well as their fluorescent properties explains their use as labels of mineralization in bones and teeth (12). Tetracyclines do not interfere with mineral deposition, and are frequently incorporated into growing bones and teeth. This property precludes the use of the older tetracycline drugs in growing children, as staining of teeth may be problematic. It has been employed in clinical specimens to differentiate healthy from diseased or dead bone (12).

One of us (PS) proposed the use of tetracycline eye drops as a rapid bedside test for BCP crystals in synovial fluid. Although these eye drops are no longer clinically available, we were intrigued by the potential of this method. We set out to determine if tetracycline staining might be used to visualize BCP crystals in synovial fluid.

MATERIALS AND METHODS

Materials

All reagents were from Sigma Chemical Co., (St. Louis, MO), unless otherwise specified. The UV penlight was obtained from Carquest.com.

Tetracycline

Multiple forms of tetracycline are available and were initially tried in this assay. Many were deemed unsuitable because they were poorly soluble or required a very low pH to dissolve. Acid conditions favor dissolution of calcium-containing crystals. Oxytetracycline proved the easiest form of tetracycline to use, and was readily soluble in a slightly basic solution. Interestingly, this is the form of tetracycline that had been used in eye drop formulations. A solution of 3.5 mg/ml oxytetracycline dihydrate and 1.5 mg oxytetracycline hydrochloride in 0.1 N NaOH was titrated with 6 M HCl to a pH of 7.0. This mixture was found to be the most stable and did not immediately precipitate when neutralized; however, precipitate did form with storage, necessitating fresh preparations for each assay.

Synthetic crystals

Synthetic BCP and MSU crystals were kind gifts from Dr. Neil Mandel, PhD and the National VA Crystallographic Center, are used as standards. BCP crystals were largely pure hydroxyapatite. CPPD crystals were monoclinic and were synthesized in our laboratory using a modification of the method of Brown et. al.(13). All standard crystal preparations were examined by FTIR spectroscopy to ensure their identity and purity. (Data not shown).

Porcine Synovial Fluids

Hind legs were obtained from 300-500 lb adult pigs from a local slaughter house (Johnsonville Foods, Inc., Watertown, WI). Synovial fluid was removed from the knee joint with a 20 gauge needle and syringe and stored at 4 ° C. Fluids were used within 48 hours of procurement.

Human Synovial Fluids

Fluids obtained for therapeutic or diagnostic purposes were used in accordance with the instructions and approval of the Institutional Review Board of the Medical College of Wisconsin and Froedtert Memorial Lutheran Hospital. Two synovial fluids were collected from shoulder joints from patients with clinical Milwaukee Shoulder Syndrome. Four other synovial fluids were collected from knee joints of patients with clinical osteoarthritis or pseudogout. Samples were examined for crystals using compensated polarizing light microscopy by an experienced examiner (LD), and for the presence of BCP crystals by FTIR.

Oxytetracycline staining of synthetic crystals

One drop of a freshly vortexed solution containing 50 ug/ml BCP, CPPD or MSU crystals in water was placed in a small tube for 15 minutes with one drop of 5 mg/ml oxytetracycline. It was then placed on a microscope slide. A coverslip was placed over the fluid. The samples

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were examined under light microscopy with and without a broad spectrum UV pen light. After focusing the microscope on the plane of the fluid at 20 or 40x, the light source on the microscope was turned off. A fluorescent pen light was directed onto the microscope field from below the stage and stabilized with a clamp on a ring stand (Figure 1). Controls of plain oxytetracycline solution and unstained crystals were similarly examined. Synthetic crystals added to porcine synovial fluids were also examined to ensure that crystals could be visualized in their “native” environment. Controls included porcine synovial fluid with no added crystals, crystal-spiked fluids with no added oxytetracycline, and oxytetracycline alone.

Oxytetracycline staining of native synovial fluid crystals

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One drop of fresh, unprepared synovial fluid was added to a small tube along with one drop of freshly prepared oxytetracycline. After 15 minutes, the mixture was placed on a microscope slide. A coverslip was placed on the fluid, and it was examined under plain light microscopy and with a UV pen light as described above.

Alizarin Red S staining

One drop of freshly prepared filtered 2 % alizarin red S was added to one drop of synovial fluid directly on a microscope slide. Samples were examined under plain light microscopy.

Comparison of Alizarin Red S and oxytetracycline assays

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Six synovial fluids that were fully characterized in regards to the crystals they contained, were read with oxytetracycline and alizarin red S stains by three observers, blinded to the crystal contents of the synovial fluids. Two fluids contained CPPD crystals; two contained native BCP crystals: two were from osteoarthritic joints and contained no identifiable crystals/ Two samples consisted of these same osteoarthritic synovial fluids spiked with synthetic BCP crystals. The observers included an experienced rheumatologist (AKR), a rheumatology fellow (TB) and a laboratory technician (MF). Each fluid was read on 4 separate occasions, two with alizarin red S staining and two with oxytetracycline staining.

Quantification of synthetic BCP crystals

Synthetic BCP crystals were added to crystal-free human synovial fluid at concentrations of 10 -50 µg/ml. One hundred µl of sample were added to a well of a 96 well black fluorimeter plate. Twenty-five µl of 5 mg/ml oxytetracycline were added to each sample. Samples were read in a Biotek® Synergy™ HT plate reader with excitation at 450/50 nm and emission at 540/35 nm.

Synchrotron FTIR

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The presence of BCP crystals in human synovial fluids was confirmed by synchrotron FTIR. Fresh synovial fluids were placed on IR reflective Kevley slides. They were examined with a Thermo Fisher Continuum FTIR Microscope at the Synchrotron Radiation Center in Stoughton, WI and compared with standard FTIR spectra for BCP crystals.

RESULTS

Oxytetracycline staining of synthetic crystals in water

We stained solutions of 50-500 µg/ml BCP crystals in water by adding a drop of oxytetracycline to a drop of the BCP solution, and placing this on a microscope slide. Stained and unstained BCP crystals could not be distinguished from background debris under plain light microscopy. However, with a broad spectrum UV pen light, stained BCP crystals were clearly visible as fluorescent amorphous aggregates (Figure 2A). Oxytetracycline solution should always be examined alone to ensure that crystals of oxytetracycline have not precipitated and are not being mistaken for BCP crystals. Similar sized and shaped crystals were seen with Alizarin red S staining of synthetic BCP crystals in water (Data not shown). CPPD crystals were considerably smaller than the BCP crystal aggregates and were not clearly fluorescent with oxytetracycline exposure (Data not shown). Similarly, needle-shaped MSU crystals were clearly morphologically distinguishable (Data not shown). We also examined common particulates seen in synovial fluid, such as type II collagen and fibrin strands, using this procedure. No fluorescence was seen. (Data not shown).

Oxytetracycline staining of synthetic crystals in porcine synovial fluid

To ensure that oxytetracycline staining could identify BCP crystals in the high protein, particulate dense milieu of synovial fluid, we incubated synthetic BCP, MSU or CPPD crystals with porcine synovial fluids for 5 minutes. The porcine fluids were from normal knee joints of adult pigs with no signs of disease, and were examined under polarizing light for any native crystals before synthetic crystals were added. As shown in Figure 2B, synthetic BCP crystals in synovial fluids appeared similar to those in water. Longer incubation times, including overnight incubation in synovial fluid at 37 ° C did not change the characteristic appearance of BCP crystals. (Data not shown).

Oxytetracycline staining of “native” synovial fluid crystals

We next explored the ability of this assay to detect BCP crystals in fresh un-prepared synovial fluids. We incubated a drop of fresh synovial fluid with a drop of oxytetracycline for 15 minutes in a small tube, and then examined it under UV light. Figure 2C shows the typical appearance of oxytetracycline-stained BCP crystals from a patient with Milwaukee Shoulder Syndrome with FTIR-proven BCP crystals.

Oxytetracycline based measurements of synthetic BCP crystals in human synovial fluid

The quantitative method described above using synthetic BCP crystals in synovial fluid generated a linear standard curve with an r^2 value of 0.98, over concentrations of 10-50 µg/ml of crystal (Figure 3).

Comparison of oxytetracycline staining to alizarin red S staining

We performed a pilot study directly comparing Alizarin red S and oxytetracycline staining in a small number of well-characterized human synovial fluids and with a limited number of

observations. False positives with oxytetracycline were 35.7%, compared to 58.3% with alizarin red S ($p < 0.01$).

DISCUSSION

The oxytetracycline binding assay may provide an additional or alternative method to detect BCP crystals in synovial fluids. The bright fluorescence on the dark blue background with the oxytetracycline staining was readily visualized. UV pen lights are easily acquired and inexpensive, most costing between 20 and 30 dollars. Oxytetracycline is also readily available and easy to use. It does require a weak sodium hydroxide solution and hydrochloric acid to be solubilized. However, these reagents are easy to find, and along with some pH paper, could be kept near the microscope used for examining synovial fluids.

How this assay will perform in the clinic and in comparison to other available assays remains uncertain. We did not attempt to use the oxytetracycline assay quantitatively on native fluids, but it performed well with synthetic crystals. We also directly compared it with alizarin red S in a small trial, and it appeared to perform better. In particular, the false positive rate was considerably lower with oxytetracycline than with alizarin red S, although it was not negligible with oxytetracycline staining. While this may improve with observer experience, further optimization of the procedure may also be warranted. At present, we envision that the oxytetracycline assay might function similarly to the microscopic exams used to diagnose gouty arthritis and CPPD deposition disease. The sensitivity of this assay to very low levels of crystals is unproven, and likely depends on the luck inherent in sampling any fluid, the experience of the examiner, the size of the crystal aggregates, and the time spent searching multiple fields for crystals. While the (^{14}C) EHDP assay can detect BCP crystal levels as low as 2 $\mu\text{g/ml}$, the clinical relevance of very small quantities of crystal is difficult to ascertain. Further work on the sensitivity of this assay and its quantitative adaptation are currently underway in our laboratory.

This assay clearly needs validation in large numbers of synovial fluids with and without BCP crystals and should be checked for accuracy and reproducibility by having blinded observers score large numbers of standard samples. The major crystal species in synthetic BCP preparations is hydroxyapatite and we did not examine the ability of oxytetracycline to bind to other crystal species in native BCP. In addition, it is unclear if preparation or centrifugation of the synovial fluid samples would increase the yield of this assay. The UV pen light can be tricky to focus on the slide, and may require some practice. A small ring-stand and clamp works well for these purposes.

We describe here a simple microscopic assay for synovial fluid BCP crystals that can be done in an office laboratory and requires little extra equipment beyond a light microscope, a broad spectrum UV pen light and a solution of oxytetracycline. We demonstrate that BCP crystals in synovial fluid exposed to oxytetracycline fluoresce when exposed to UV light. Oxytetracycline does not bind to other particulates commonly encountered in joint fluid. Once refined and validated in larger numbers of samples, and tested in the field, this assay should increase our understanding of the role of these crystals in osteoarthritis and expand our diagnostic armamentarium for BCP-related syndromes.

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Figure 1. Apparatus for focusing the UV pen light on the microscope field

This is a photograph of the apparatus used to stabilize the UV pen light under the microscope stage.

Figure 2. BCP crystals stained with oxytetracycline under UV light

A Synthetic BCP crystals in water were incubated for 15 minutes at room temperature with a drop of oxytetracycline, and then placed on a microscope slide. **B.** Synthetic BCP crystals in porcine synovial fluid were incubated for 15 minutes at room temperature with a drop of oxytetracycline, and then placed on a microscope slide. **C.** One drop of unprocessed synovial fluid from a patient with Milwaukee Shoulder Syndrome was incubated with oxytetracycline for 15 minutes at room temperature, and then placed on a microscope slide. All samples were examined with a microscope under broad spectrum UV light and photographed. (Magnification 200 x)

Figure 3. Standard curve of synthetic BCP crystals in human synovial fluid stained with oxytetracycline

Crystal-free synovial fluid from a patient with osteoarthritis was spiked with 10, 20 or 50 $\mu\text{g/ml}$ synthetic BCP crystals. One hundred μl of sample were added to a well of a 96 well black fluorimeter plate. Twenty-five μl of 5 mg/ml oxytetracycline were added to each sample. Samples were read in a Biotek® Synergy™ HT plate reader with excitation at 450/50 nm and emission at 540/35 nm. Background fluorescence in the un-spiked fluid was subtracted from each value.