Synovial fluid hydroxyapatite crystals: detection thresholds of two methods

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SUMMARY A method of synovial fluid preparation giving optimal hydroxyapatite detection as well as definitions of the threshold masses of hydroxyapatite in viscous synovial fluid detectable by *x* ray diffraction and scanning electron microscopy with energy dispersive analysis is reported. Use of an equal volume of 100% hydrazine with the synovial fluid optimised detection of hydroxyapatite. By *x* ray diffraction the threshold mass of hydroxyapatite was 500 µg and by scanning electron microscopy with associated energy dispersive analysis 5 µg.

Hydroxyapatite crystals have been identified in synovial fluids from many conditions, particularly in apatite associated destructive arthropathy,¹ osteoarthritis,^{2,3} and calcium pyrophosphate arthropathy.^{2,4} Detection rates show considerable differences, which may reflect the variety of techniques of synovial fluid preparation and the different sensitivities and specificities of the detection methods. Only one of these methods is reported as semiquantitative with an established detection threshold.⁵ Thus useful comparison of studies and diseases for the presence of hydroxyapatite is very limited.

The role, if any, of hydroxyapatite in joint destruction remains undefined. Certainly, in vitro, hydroxyapatite releases potential mediators of articular damage from a variety of cells in a dose dependent manner.^{6–8} The relevance of in vitro studies will remain unknown until synovial fluid and membrane hydroxyapatite concentrations, as well as the thresholds of the detection methods, are determined.

In this study we report a method of synovial fluid preparation giving optimal hydroxyapatite detection as well as definitions of the threshold masses of hydroxyapatite in viscous synovial fluid detectable by *x* ray diffraction and scanning electron microscopy with energy dispersive analysis.

Methods, materials, and results

TEST SYNOVIAL FLUIDS

Viscous synovial fluid from a patient with osteoarthritis of the knee, in which hydroxyapatite was not detected by alizarin red staining and microprobe analysis, was used to prepare a series of synovial fluid samples with various hydroxyapatite concentrations by the addition of increasing masses of synthetic hydroxyapatite crystals comparable with international standards with particle size of less than 37 µm.

A constant sample volume of 2.5 ml was chosen to enable future study of small osteoarthritis effusions.

TREATMENT OF ORGANIC CONTAMINATION

Although previous studies have used enzymatic methods (hyaluronidase, trypsin) to reduce organic contamination which hinders hydroxyapatite detection, we preferred to use 100% hydrazine; a technique with the advantage of reducing possible crystal transformation which may occur during the aqueous phase digestion of the other methods.⁹ Hydrazine (100%, 2.5 ml) was added to one of the paired samples of 2.5 ml synovial fluid with 1 mg synthetic hydroxyapatite. After mixing for 15 minutes and incubation for 12 hours the paired samples were centrifuged. The samples were dried and analysed by *x* ray diffraction (Guinier camera). We found that the use of an equal volume of 100% hydrazine clearly optimised the detection of hydroxyapatite as the characteristic bands of hydroxyapatite were only visible in the hydrazine treated samples.

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Table 1 Hydroxyapatite detection*

Hydroxyapatite		x Ray diffraction	Scanning EM† crystal morphology	Energy dispersive analysis Ca, P
µg/2.5 ml	µg/ml			
500	200	+++	+++	+++
50	20	—	+++	+++
5	2	—	+++	+++
0.5	0.2	—	—	+
0.05	0.02	—	—	+
0.005	0.002	—	—	±

*Hydroxyapatite masses and corresponding hydroxyapatite concentrations in 2.5 ml synovial fluid detected in blinded studies using the techniques of x ray diffraction (Guinier camera) and scanning electron microscopy with energy dispersive analysis.

†EM=electron microscopy.

CENTRIFUGATION

As the variable viscosities of synovial fluids could be expected to influence the sedimentation of hydroxyapatite the effect of different centrifugation rates of 750 g or 11 150 g on crystal yield were compared using 2.5 ml samples of synovial fluid with 1 mg synthetic hydroxyapatite treated as above and analysed by x ray diffraction. The intensity of the diffraction patterns of hydroxyapatite was equal for the samples centrifuged at both speeds.

STORAGE

The possibility of artefactual development of hydroxyapatite in stored specimens was investigated. To one of two volumes of synovial fluid was added 200 µg amorphous calcium phosphate prepared according to Eanes *et al.*¹⁰ Both were stored at 4°C for 48 hours, then treated with hydrazine and centrifuged at 750 g. The diffraction pattern of apatite developed only in the sample to which amorphous calcium phosphate had been added.

DETECTABLE THRESHOLD MASS OF HYDROXYAPATITE

Several series of concentrations of synthetic hydroxyapatite in 2.5 ml viscous synovial fluid varying from 200 µg hydroxyapatite/ml to 0.002 µg hydroxyapatite/ml were prepared. Coded samples were analysed by x ray diffraction and by scanning electron microscopy with energy dispersive analysis (Jeol/link system with electron probe diameter 100 Å). Interpretation of the electron microscopy images was performed without knowledge of the sample concentrations.

By x ray diffraction the threshold mass of hydroxyapatite was 500 µg corresponding to a concentration of 200 µg hydroxyapatite/ml in the synovial fluid samples of 2.5 ml. With scanning electron microscopy the morphology of hydroxyapatite crystals was

identified with certainty to a mass of 5 µg, but the interpretation introduced a degree of subjectivity. Associated energy dispersive analysis detected high calcium and phosphate signals to 5 µg with Ca/P weight ratios generally compatible but not diagnostic of apatite. This threshold corresponds to a concentration of 2 µg hydroxyapatite/ml in a sample of 2.5 ml (Table 1).

SAMPLE SYNOVIAL FLUIDS

A series of 20 synovial fluids was analysed by scanning electron microscopy with energy dispersive analysis using 2.5 ml samples treating according to the above protocol. The diagnoses of the synovial fluids studied (four in each case) were as follows: osteoarthritis; Ca/P deposition disease and osteoarthritis; rheumatoid arthritis, psoriatic arthritis and reactive arthritis, idiopathic monoarthritis. Hydroxyapatite was detected in only three samples from the following conditions: osteoarthritis of the shoulder following radionecrosis of the humeral head, destructive osteoarthritis of the shoulder, and advanced rheumatoid arthritis of the knee.

Discussion

In this study we have attempted to analyse possible methodological causes for the variable detection rates^{2,3} of hydroxyapatite in synovial fluid and to establish approximate threshold masses of hydroxyapatite necessary for detection by two commonly used methods: x ray microdiffraction and scanning electron microscopy with energy dispersive analysis. One obvious but remarkably poorly reported factor which accounts in part for the variability in hydroxyapatite detection is the volume of fluid analysed. Simply, the larger the synovial fluid volume the greater the mass of hydroxyapatite for the same hydroxyapatite concentration. This may also be a factor in the ease of detecting hydroxyapatite in destructive arthropathy of the shoulder, which is characterised by large synovial fluid volumes.

Hydrazine proved useful in increasing the detection of hydroxyapatite by reducing organic contamination. The results of this study indicate that considerable differences in centrifugation (750–11 150 g) have little effect on the detection rate of hydroxyapatite. Storage of synovial fluid specimens at 4°C, as often occurs in practice, does not appear to allow the formation of hydroxyapatite, though caution is necessary as newly formed hydroxyapatite is detected in the synovial fluid containing amorphous calcium phosphate. Whether calcium phosphate is present naturally in synovial fluids remains controversial but our studies of native synovial fluids provide little support for this possibility.

The threshold sensitivity of scanning electron microscopy with energy dispersive analysis is of the same order as that of the ethane-1-hydroxy-1,1-diphosphonate assay,⁵ but the limited specificity (lack of differentiation of hydroxyapatite from octacalcium phosphate, calcium phosphate, or whitlockite) and the inability to measure the hydroxyapatite detected are disadvantages of the former method. The specificity of x ray diffraction is excellent but unfortunately the threshold mass of hydroxyapatite is about 100 times greater than for the preceding methods.

The detection threshold mass of 5 µg hydroxyapatite by scanning electron microscopy corresponds to a concentration of 2.0 µg hydroxyapatite/ml in our synovial fluid samples of 2.5 ml. In 20 synovial fluids we detected hydroxyapatite in only three samples from joints with advanced destruction. Hydroxyapatite concentrations in the other synovial fluids would be less than 2.0 µg/ml. Hydroxyapatite concentrations of 10–200 µg/ml are necessary for in vitro cell activation and release of potential mediators of joint destruction.^{6–8} Therefore the inability to detect hydroxyapatite in 2.5 ml synovial fluid by this method argues against a role (at least non-mechanical) for hydroxyapatite in damage to the joint under study even if hydroxyapatite is present in subthreshold quantities. Other factors, however, such as protein coating, may alter the destructive potential of hydroxyapatite, and localised concentrated intrasynovial deposits may be capable

of cell activation whereas synovial fluid concentrations are insufficient.

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