An improved labelling technique for monitoring microcrack growth in compact bone.

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TECHNICAL NOTE

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Abstract

Fatigue-induced damage plays an important role in bone remodelling and in the formation of stress and fragility fractures. Recently a technique has been developed (Lee et al, 2000a) which allows microcrack growth in trabecular bone to be monitored by the application of a series of chelating fluorochromes, however some limitations were identified with the process. The aims of this study were to refine the method of detection using these agents in order to determine the optimal sequence of application and the optimal concentrations which allowed all the agents to fluoresce equally brightly using UV epifluorescence. A chemical analysis process, ion chromatography, followed by validation tests on bone samples showed that the optimal sequence of application and concentration of each agent was alizarin complexone (0.0005M) followed by xylenol orange (0.0005M), calcein (0.0005M) and calcein blue (0.0001M). A fifth agent, oxytetracycline was excluded from the study after recurring problems were found with its ability to chelate exposed calcium when applied in sequence with the other agents. This work has developed a sequential labelling technique, which allows for microcrack propagation during fatigue testing of bone specimens to be monitored without the problem of chelating agent substitution occurring.

1. Introduction

Fatigue-induced microdamage acts as a stimulus for bone remodelling (Martin and Burr, 1982; Burr et al, 1985; Burr and Martin, 1993; Mori and Burr, 1993; Prendergast and Taylor, 1994; Martin, 2000) and contributes to fragility fractures (Schaffler et al, 1995; Lee et al, 2000b). Microcracks have been stained using basic fuchsin (Frost, 1960; Burr and Stafford, 1990; Burr and Hooser, 1995; Lee et al, 1998; Huja et al, 1999), but a series of stains is required to label microcracks at various stages during their growth. We have demonstrated microcrack growth *in vitro* using chelating fluorochromes in sequence (Lee et al, 2000a), but substitution of one agent by another made measurement imprecise (O'Brien et al, 1998). The aims of this study were to measure the affinity of each of five chelating agents for Ca^{2+} ions and to determine the optimal sequence and concentration of these agents for labelling the growth of surface scratches in compact bone.

2. Material and Methods

Optimal Sequence

The levels of free calcium in a solution of calcium chloride before and after the introduction of each chelating agent were measured using ion chromatography (Haddad and Jackson, 1990). Samples were separated on a Dionex system which incorporates a pump, cation-trap pre-column and a cation micro membrane suppressor (Dionex, Sunnyvale, Ca). The separation of calcium cations was accomplished in a 15 minute analysis run with a Dionex IonPac CS-14 column (4 mm x 250 mm) using 1 x 10^{-2}M methane sulphonic acid as mobile phase and suppressed conductivity detection. The sample loop size was 50 μL and the flow rate was 1.0 ml/minute. Calcium chloride

concentration was 1 x 10^{-3} M and each of the five chelating agents, alizarin complexone (A), calcein blue (B), xylenol orange (X) (all from Aldrich Chemical Co., Milwaukee, Wi., USA), calcein (C) (Sigma Chemical Co., St. Louis, Mo., USA) and oxytetracycline (O) (Bimeda Ltd, Dublin) was injected separately at a concentration of 5 x 10^{-4} M. The chelating agents were ranked in order of decreasing affinity for calcium and this sequence was then tested on bone specimens.

Fresh bovine tibiae were obtained from a meat wholesalers, soft tissue was removed and the bone stored at -20^{o}C until required. Longitudinal sections of cortical bone from the mid-diaphysis were cut into beams using a band saw and polished using emery paper. A 5 mm line was scratched on the surface of each beam using a ruler and compass point and the specimen was immersed in a vial containing a 5 x 10^{-4} M aqueous solution of a single chelating agent and placed in a vacuum desiccator for 4 hours to ensure maximum penetration of the surface scratches by each chelating agent. The specimen was then washed in de-ionised water, a second 5mm line scratched parallel to the first and the specimen immersed in 5 x 10^{-4} M aqueous solution of a second chelating agent in the vacuum desiccator for 4 hours. This protocol was repeated using two, three, four and five agent sequences. Five tests were carried out using each sequence. The bone specimens were then analysed using UV (λ=365 nm) epifluorescence (Rahn, 1977) and green (λ=568 nm) epifluorescence at X50 and X125 magnifications (Nikon. Optiphot, Japan) (Lee et al, 2000a). Images were captured using a CCD colour video camera (Optronics, Goleta CA) and HP PIII personal computer (Hewlett Packard, France) and measured using Scion Image software (Scion Corp., MA). The length of scratch over which dye

substitution occurred was measured and then graded as follows: 0 (nil); 1 (1-20% - the second dye displaced 1-20% of the first); 2 (21-40%); 3 (41-60%); 4 (61-80%); 5 (81-100%).

Optimal Concentration

Sequence testing was conducted using 5 x 10^{-4} M solutions, the lowest concentration at which all agents fluoresced clearly and chromatograms could be obtained. Using the optimal four-agent sequence, a second series of scratch tests was carried out to compare the 5 x 10^{-4} M solutions with an *in vivo* dosage regime devised to ensure that all agents appeared equally bright using ultraviolet epifluorescence microscopy (Rahn, 1977) (Table 1). Five scratch tests using each regime were compared and the degree of substitution and clarity of each agent assessed.

3. Results

Figure 1 shows the free calcium peaks obtained in a chromatogram before and after the injection of alizarin into calcium chloride solution. Table 2 summarises the average chromatogram peak height and peak area, which are a measure of strength of the signal for the calcium chloride standard solution, and in the presence of each chelating agent. The calcium affinity of the chelating agents is inversely related to the height and area of the second peak. From this we deduced that the order of affinity for calcium is (from greatest to least): A-X-B-C-O.

Scratch tests were carried out using chelating agents applied in this order. With A and X present, both agents fluoresced red and were difficult to differentiate. However when C was applied, it fluoresced green and X now fluoresced bright orange and was clearly distinct from A. Calcein blue followed by calcein resulted in greater substitution (Grade 3) than C followed by B (Grade 1). The concentration of B was then reduced in stages, firstly to 2.5 x 10^{-4} M and then to 1 x 10^{-4} M until the degree of substitution was negligible. Oxytetracycline was problematic as there was Grade 4 substitution of B and Grade 1 substitution of X and C by this agent. This could not be rectified by altering the sequence or concentration and so O was excluded from the study. The revised four stain protocol A-X-C-B, using 5 x 10^{-4} M concentrations of A, X and C and B at 1 x 10^{-4} M, is shown in Figure 2. All four scratched regions can be clearly distinguished from each other and from the surrounding bone matrix and substitution is negligible. When this revised sequence, A-X-C-B, was used with the *in vivo* dosage regime (Rahn, 1977), A and X were difficult to distinguish from each other and calcein blue showed Grade 1 substitution of both A and C.

4. Discussion

This study has established a sequence of application for the chelating agents which minimises substitution of one agent by a subsequent one. The results obtained from ion chromatography tests provide a sequence of chelating agents based on affinity for free calcium in a solution of calcium chloride. This sequence was then applied to bone, using surface scratches as a model for microcracks. This regime was more effective in

sequentially labelling surface scratches than the concentrations recommended by Rahn (1977) (Table 1) for *in vivo* labelling of bone formation. This labelling regime can be applied in sequence to bone samples undergoing mechanical testing to monitor microcrack propagation at different intervals during testing. Figure 3 shows an example of a propagating compressive fatigue microcrack stained with X and C. This image is shown at different exposure times and colour levels, which allows the agents to be distinguished from each other.

This study refines a technique for sequentially labelling microcrack growth in bone (Lee et al, 2000a; O'Brien et al, 2000; Arthur and Gibson, 2000). By applying the agents at different intervals during a mechanical fatigue test, it may be possible to learn more about microcracks, their effect on the fatigue behaviour of bone, their interaction with the bone's microstructure and the processes by which they initiate and grow.

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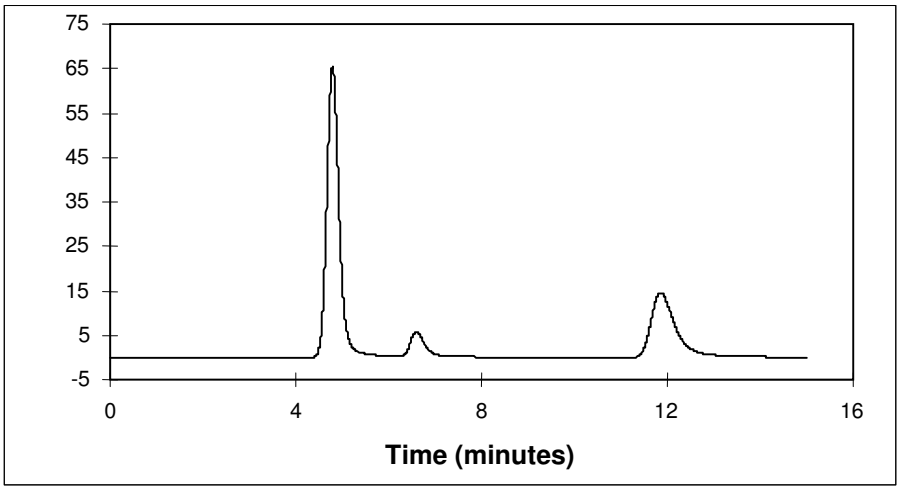

Fig. 1 Chromatogram obtained on injection of alizarin complexone (5 x 10^{-4}M) into a calcium chloride concentration of 1 x 10^{-3}M. The first peak in the chromatogram (5 mins) is known as the solute retention time and is the time it takes for the components to travel along the columns. It displays the amount of free calcium present in the solution before chelation has taken place. The second peak (11.7 mins) is the time it takes for the calcium peak to elute following chelation. It shows the maximum amount of free calcium left after chelation has occurred. Measurements of both peak height and peak area were carried out.

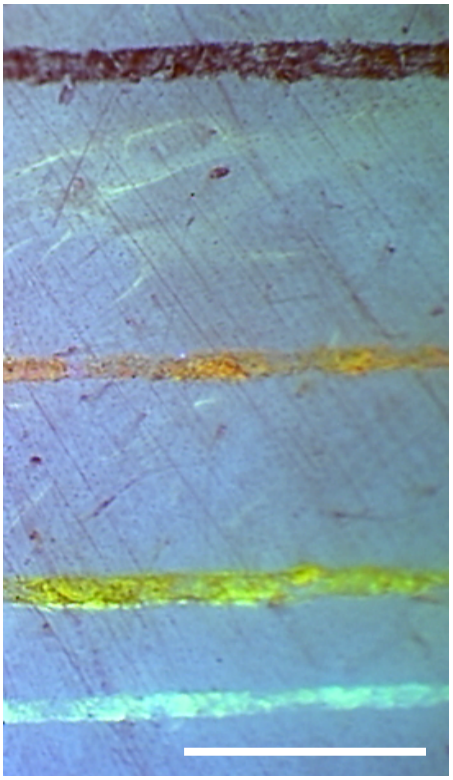

Fig. 2 Most efficient 4 agent sequence, from top to bottom: alizarin complexone (0.0005M), xylenol orange (0.0005M), calcein (0.0005M) followed by calcein blue (0.0001M). All 4 stains are clearly distinct from each other and from the surrounding autofluorescent bone. Scale bar=200 µm.

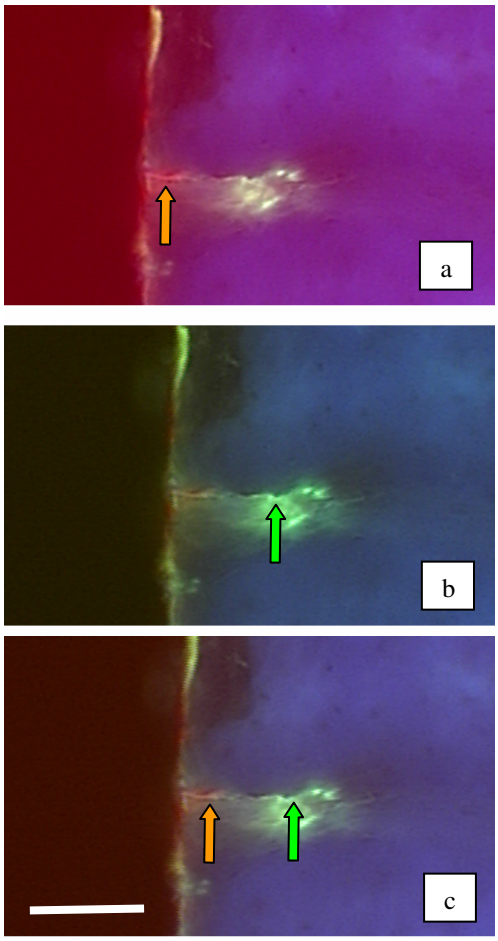

Fig. 3 Example of a propagating microcrack during fatigue testing in compression. It is initially stained with xylenol orange, showing it to have been formed during the first 10,000 cycles, and then stained with calcein which indicates crack growth between 10,000 and 50,000 cycles. Fig 3(a) shows the crack viewed at high red levels in order to show the part stained with xylenol orange more clearly. Fig 3(b) shows it at high green levels to show the calcein-stained part more clearly. Fig 3(c) shows the same image at balanced colour levels. Scale bar=100 µm.

Table 1 Comparison of *in vivo* dosage concentrations (Rahn, 1977) and concentrations used in ion chromatography tests.

	Group R (Rahn, 1977)		Group C
Chelating Agent	DOSAGE (mg/kg)	CONCENTRATION	Ion Chromatography
Calcein blue	30	0.0000712 M	0.0005 M
Xylenol orange	90	0.000016 M	0.0005 M
Calcein (green)	10	0.0000933 M	0.0005 M
Alizarin complexone (red)	30	0.00012 M	0.0005 M
Oxytetracycline (yellow)	30	0.00006 M	0.0005 M

Table 2 The average height and area of the calcium peak for each of the chelating agents investigated along with the calcium standard (1×10^{-3}M).

CHELATING AGENT	Mean Height Of Ca^{2+} Peak	Average Area of Ca^{2+} Peak
Calcium chloride	32.45	13076154543
Alizarin complexone	14.59	549684922
Xylenol orange	28.56	1072856912
Calcein blue	29.73	1154869484
Calcein	30.67	12203869425
Oxytetracycline	31.76	12895983615