VISUALISATION AND QUANTIFICATION OF FATIGUE INDUCED MICRODAMAGE IN BONE: HISTOLOGY AND RADIOLOGY

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ABSTRACT

Fatigue induced microdamage in bone accumulates with age and in osteoporosis, compromising bone strength and leading to fracture failure. Such microdamage must be visualised and quantified in order to understand its role and aid in the prediction and prevention of fractures. Histological dyes have been used singly and in sequence to achieve this. Basic fuchsin, detected using transmitted light and epifluorescence microscopy, has been used to label microdamage and quantify crack density. Dye sequences have been used to identify cracks at intervals during fatigue testing and to label propagation. PET sensors have shown that damaged bone provides chelation sites, while SEM studies indicate that the damaged, porous lattice facilitates dye binding. For *in vivo* usage, a non-invasive, radiological detection method is required. Preliminary studies with iodinated chelating agents have permitted detection in the solid state, but a liquid form is required for intravenous administration.

1. INTRODUCTION

Bone is a composite material in which cells lie in a matrix of their own creation, which subsequently becomes mineralised. The bone forming cells, or osteoblasts, secrete a collagen rich matrix, osteoid, in which hydroxyapatite crystals are laid down. Some of the cells become trapped in the matrix and are termed osteocytes, but they continue to communicate with each other, and with surface cells, by cellular processes running in canaliculi. These communications enable nutrients to reach the cells from blood vessels and also help form a mechanosensory network within mineralised bone. Fatigue loading causes microdamage in the mineralised matrix which may injure these cellular processes and may be the means by which such damage is detected and repair initiated (Taylor et al. [1]). Whatever the cellular transducer, the link between microdamage and bone repair is well established (Prendergast and Taylor [2]; Mori and Burr [3]; Lee et al. [4]). If successful, repair leads to adaptive bone remodelling and, if unsuccessful, microdamage accumulates leading to bone fracture.

Osteoporosis is defined as a skeletal disorder characterized by compromised bone strength predisposing a person to an increased risk of fracture. Bone strength reflects the integration of two main features: bone density and bone quality (NIH [5]). Loss of bone strength leads to 700,000 vertebral, 280,000 hip and 250,000 Colles fractures annually in the US at a cost of $7-$10 billion, while hip fractures are associated with 10-20% mortality within 6 months (Riggs & Melton [6]). Bone quantity accounts for up to 60% of the variance in elastic modulus and bone strength (Goldstein [7]; Rice et al.,[8]). Bone quality includes parameters such as trabecular and cortical microarchitecture, the degree of mineralization of bone matrix, and the amount of damage accumulation (Bouxsein [9]). Hence there is a need to visualise and quantify bone microdamage.

2. HISTOLOGY

Single stains

Histology is the study of the structure of biological material and involves the study of thin sections of tissue under the microscope. Bone poses particular problems, as it is difficult to cut and, with microdamage, the question arises as to whether it was sustained during life, *in vivo*, or the result of the preparation and cutting procedure – artefactual damage. This problem was addressed by Frost [10] who bulk-stained blocks of bone for several weeks in an ethanol-soluble dye, basic fuchsin, which penetrated microcracks and other microstructural features such as blood vessels, cell lacunae and canaliculi. The bone was then rehydrated and cut in an aqueous medium. As fuchsin is insoluble in water, any new, artefactual cracks would be unstained, hence any fuchsin stained cracks were sustained *in vivo*. One flaw remained, could alcohol dehydration have caused shrinkage and artefactual cracking? Burr and Stafford [11] found no difference in crack counts between specimens prepared by this method and others cut and ground in water before fuchsin staining and so validated Frost's technique. This technique has been used to identify microdamage and to quantify it by measuring the numerical crack density (number of cracks per mm^2), crack length (μm) and crack surface density (μm/ mm^2) (Mori and Burr [3]).

Using transmitted light, variations in light intensity, depth of focus and magnification are necessary to distinguish fully-stained microcracks generated *in vivo* from partially-stained or unstained artefactual cracks caused by cutting and machining. Such microscopic techniques are both difficult and time consuming. However, basic fuchsin is a mixture of three dyes, pararosanilin, rosanilin and magenta II, which appears purple under transmitted light but also fluoresces under both green and ultraviolet incident light (Rost [12]). This fluorescent property was used by both Lee et al. [13] and Huja et al. [14] to facilitate microdamage identification. Lee et al. [13] studied human rib sections which had been bulk-stained in basic fuchsin to label *in vivo* microdamage. Using green epifluorescence (λ = 546 nm), only microcracks containing fuchsin fluoresced orange against the darkfiled background, thus unstained artefactual cracks could be screened out. Using UV epifluorescence (λ = 365 nm), microcracks stained through the full depth of the section (100 μm) fluoresced purple (Fig. 1) while partially stained cracks did not fluoresce and were screened out. They found no significant difference in crack number, length or density between the transmitted light and epifluorescence methods, but the latter was simpler and quicker. Huja et al. [14] compared the two methods using canine femurs tested in four-point cyclic bending and detected significantly more microdamage using epifluorescence. While the studies differ in the use of *in vivo* or *in vitro* microdamage, bone type and microscope, both indicate the usefulness of the fluorescence technique.

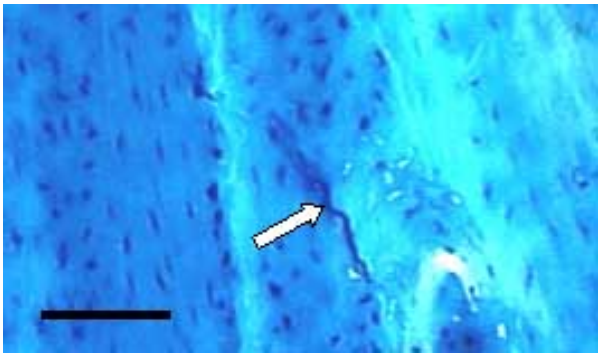

Figure 1. Microcrack labelled with basic fuchsin viewed under UV epifluorescence. Scalebar= 50 μm.

Stain sequences

A single dye can label microdamage at a single time point, but a series of dyes is required to identify damage incurred at different times during fatigue testing and to label crack propagation. In 1961, Frost et al. [15] showed that, *in vivo*, the fluorescent antibiotic, tetracycline, is deposited where bone or cartilage is mineralising and its pattern is the same as that of radiolabelled calcium deposition. From this observation, he developed a method of intermittent administration of tetracyclines which enabled bone formation rates to be measured (Frost [16]). All tetracyclines are yellow-green in colour, so the advent of other fluorescent agents such as alizarin (red), calcein blue and xylenol orange enabled easier interpretation and have been successfully used to quantify bone growth and adaptation (Lanyon et al. [17]; Lee and Taylor [18]). Stover et al. [19] noted that microcracks in equine bone, identified using post mortem fuchsin staining, were also labelled by calcein which had been given *in vivo*. So could these agents be used to label microdamage during mechanical testing?

Lee et al. [20] randomly assigned human rib sections to bulk staining in basic fuchsin, alizarin complexone, calcein, calcein blue, oxytetracycline and xylenol orange and found no difference in crack numerical density or length. These dyes were then used in sequence to label bovine trabecular bone specimens undergoing fatigue testing in compression. Oxytetracycine was used to label preexisting damage incurred *in vivo* or during specimen preparation. The waisted specimens were then immersed in calcein blue for the first 75% of the test, washed with distilled water and then placed in xylenol orange for the final 25% of the test. Some microcracks were stained blue, and so were sustained in the first part of the test, some orange, and so originated in the final quarter, and some with both dyes, indicating crack growth. However, the transition points were not clearly defined and dyes ‘painted over’ each other at the machined edges.

O’Brien et al. [21] used ion chromatograpy to measure the affinity for each agent for free calcium and then applied them, in decreasing order of affinity, to a bone crack model – a surface scratch. By varying the concentrations and immersion times, he developed an optimal labelling protocol: alizarin complexone, xylenol orange, calcein (all at 0.5 mM) and calcein blue (at 0.1 mM). This provided clear definition between dyes and avoided ‘over painting’. This technique was then successfully applied to identify microcrack initiation at intervals during fatigue testing of bovine bone in compression and to label crack propagation (O’Brien et al., [22]).

Parkesh et al. [23] studied the mechanism by which dyes label the scratch rather than the polished bone surface surrounding them. The inorganic matrix of bone is composed of impure hydroxyapatite containing carbonates, citrates, magnesium fluoride and strontium within its crystal lattice. Scratching breaks this lattice and reveals free lattice vacancies for bound elements that can bind to suitable receptors. Scanning electron microscopy (SEM) was used to show the porosity of scratches Fig.2).

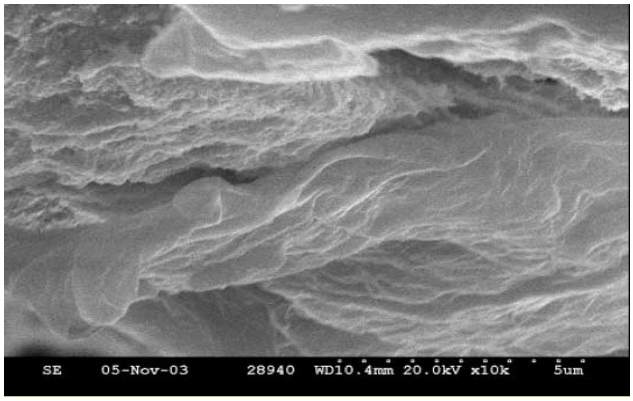

Figure 2. Scanning electron micrograph of scratched region of cortical bone.

SEM analysis specimens stained with Rose Bengal showed crystals forming within scratches, but not on the polished surface, suggesting that the porous, broken lattice induces dye crystallisation. Energy dispersive X-ray analysis showed iodine, a constituent of Rose Bengal, in the scratch. Chemical binding of Rose Bengal to bone is limited to ionic interactions.

Parkesh [24] also investigated the use of photoinduced electron transfer (PET) sensors in detecting microdamage. PET sensors are based on a fluorophore-spacer-receptor format, where fluorescence intensity is switched on when the agent binds to a cation. Aqueous solutions were prepared and applied to scratched bone specimens in order of decreasing binding constant for Ca ions: calcium crimson, calcium orange and fluo-3, each of which has chelating groups organised in three dimensions as their receptor. The dyes were distinct, with no ‘over painting’ and only fluoresced when bound to Ca ions in the scratches.

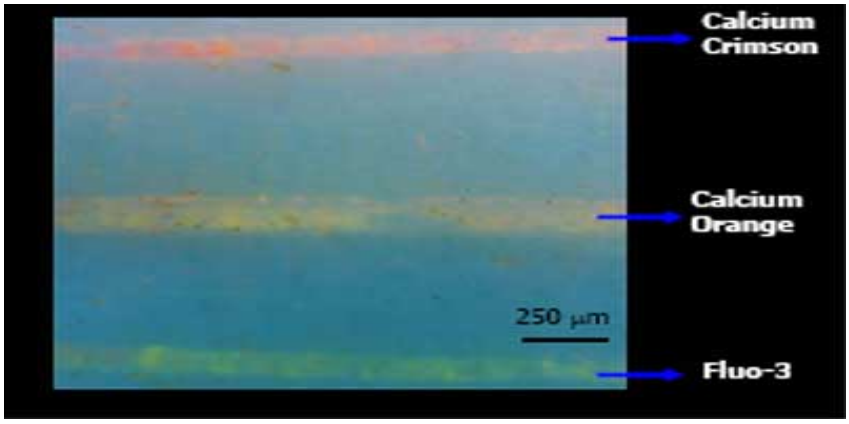

Figure 3. Scratched bone specimens containing dyes added in order of decreasing binding constant for calcium: calcium crimson, calcium orange and fluo-3. All three

dyes are clearly distinct from each other and from the surrounding autofluorescent bone.

4. RADIOLOGY

While histological methods are useful in quantifying microdamage in test specimens, a non-invasive detection method is required for *in vivo* measurements so that microdamage, along with bone mass, can be used in assessing fracture risk in osteoporotic patients (Bouxein, [9]). Having established that dyes can bind to microdamage sites by Ca ion chelation, a chelating phenyliminodiacetate receptor was used and iodine chosen as the radioopaque label (Parkesh et al., [23]). Three iodinated agents were synthesised and examined using a 'cone beam' μ-CT system and it was possible to distinguish the internal architecture of the bone from each agent in the solid state. However, when diluted in water (10^{-3}M), it was not possible to distinguish scratches labelled with the agents from the surrounding bone matrix. The most likely reason for this is 'beam hardening' where the attenuation of the X-ray beam is not homogenous. Further efforts are needed to evaluate the solution phase studies of these contrast agents. However, the synthesis and preliminary μ-CT studies are promising and provide new insights to the non-invasive detection of microdamage.

5. ACKNOWLEDGEMENTS

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