Multi-scalar Mechanical Testing of the Calcified Cartilage and Subchondral Bone Comparing Healthy versus Early Degenerative states

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Introduction: The calcified cartilage layer is thought to be integral to force transmission between the compliant cartilage and stiff bone [1] by providing an intermediate stiffness [2], which can theoretically minimise the shear stress at the boundary [3]. A previous study on healthy bovine cartilage [2] had shown that the zone of calcified cartilage (ZCC) is about 100 times stiffer than the overlying hyaline cartilage, and 10 times less stiff than the underlying subchondral bone (SB); however how this stiffness gradient changes with joint degeneration is not known. Further, as this region of the cartilage-bone junction is notoriously difficult to access and test, there has been very little reported in the literature, with some recent studies utilising micro and nano scale testing methods which may or may not have relevance to macroscale level joint loading. We thus sought to determine if early degeneration of the joint tissues would influence the structural and mechanical properties of the ZCC and SB, and how different scalar levels of testing are correlated.

Methods: Using our established bovine model for joint degeneration and early osteoarthritis [4] sixteen bovine patellae displaying a range of tissue states, from healthy to moderately degenerate, were used for this study. Cartilage surfaces were stained with India ink and graded macroscopically using the Outerbridge grading system [5]. Samples were also obtained for osteoarthritis scoring using the OARSI grading system [6]. From each patella (n=16), one 16 × 20 mm block was sawn from the distal lateral region, the prevalent site of degeneration in this animal model, and divided along its length into eight samples, each about 2-mm-thick. One of the eight samples from each patella was used for microstructural imaging using Differential Interference Contrast optical microscopy (DIC) and histological staining, and the remainder seven for mechanical testing. Macroscopic three-point bending required five samples from each patella, tested at 3 heights resulting in 15 tests for each sample; microhardness indentation was performed on one sample per patella, at five locations and at five depths from the overlying cartilage, resulting in 25 tests per sample; nanoindentation was performed on one sample per patella at 20 sites.

Results: From the Outerbridge grading, the 16 patellae were sorted as six healthy-intact (group G0), four mildly degenerate (G1), and six moderately degenerate (G2). There was a significant (p < 0.05, r² = 0.65) correlation between Outerbridge grades and OARSI scores across the patellae which ranged from 0 to 4.5. The morphometric measurements obtained from DIC showed that there were no significant differences in total cartilage thickness between groups. There was also no difference in ZCC thickness between groups, while there was a significant increase in the number of duplicate tidemarks with increasing degeneration (Figure 1). Macroscopic three-point bending showed that the mean ratio of SB modulus to ZCC modulus (ESB/EZCC) changed significantly from 10 times to 5 times and back up to 7 times with increasing levels of degeneration (Figure 2). Microhardness testing revealed that there was a monotonic increase in the mean modulus from the ZCC to the trabecular bone (Figure 3). The moduli
obtained from the nanoindentation tests indicate that the SB was stiffer by about two times than the ZCC, not changing significantly with degeneration. Comparing data across macro to micro to nano scale tests, there were significant differences in the moduli obtained such that, for all groups, there was a significant decrease in the moduli obtained from tests done at increasing scalar levels.

**Discussion:** To counter the effects of experimental limitations with each mechanical testing procedure, the present study has relied on carrying out the tests in a consistent manner with an extensive degree of repetition. For the 16 patellae, the number of tests performed on each sample result in nearly 1,000 individual tests. With the repeat testing and detailed structural characterisation of the materials we hope that the effects of tissue and structural inhomogeneities may be reduced.

The decrease in modulus with larger scale testing may result from the increased likelihood of larger samples to contain a defect that allows for deformation when a load is applied. These defects can include the inhomogeneities, holes and voids, irregularities of the cement line, and imperfections that may act as stress concentrations. Three point bending modulus is affected by all of these stress concentrations while the precision of micro and nano indentation can avoid many of them. At some scale, the pattern of increasing stiffness at smaller scales observed in this study will break down when individual microscopic components of the material are being tested and porosity is no longer influential. Nanoindentation has a lateral length scale on the order of 1 µm, which is considerably larger than the length scale of a collagen fibres and mineral platelets.

The non-significant differences in thickness of the ZCC between G0, G1 and G2 may be due to the advancement of calcification into the articular cartilage with paired advancement of the subchondral bone at the cement line. This paired advancement of ZCC and SB would explain why the ratio of the modulus ESB/EZCC decreased from G0 to G1. We believe that in the G1 state, the ZCC development results in a relatively stiffer matrix, while new bone spicule (see yellow arrows in Figure 1) and SB development results is an overall less stiff bone matrix. In the G2 group, where the degeneration is more advanced, the bone matrix has been allowed to develop further and hence the restoration, to some extent, of the original ESB/EZCC modulus ratio at the healthy level. The combination of tidemark duplication with no increase in ZCC thickness, the presence of bony spicules at the cement line, and the significant difference in modulus ratios between groups suggest that the bone cement line may be advancing in the early stages of joint degeneration.

**Significance:** This study hopes to provide new insight into the mechanical and microstructural factors associated with early joint degeneration. Further, the data from multiscale testing indicates to us that complex biomechanical models should consider scalar effects on interpretations of joint loading and osteoarthritis initiation and progression.
Figure 1. Typical microimages of the zone of calcified cartilage, indicated by the yellow bar, in (a) G0 sample and (b) G2 sample. Yellow arrows point to bone spicules that emerge from the bone cement line. Scale bar indicates approximately 100 μm.

Figure 2. Data obtained from macro 3-point bending beam tests. Ratios of Young’s Modulus for subchondral bone (E_{sb}) to zone of calcified cartilage (E_{zcc}), in healthy (G0), mildly degenerate (G1) and moderately degenerate (G2) tissue samples.
Figure 3. Young’s Modulus calculated from microhardness testing. ZCC = Zone of Calcified Cartilage, SB = Subchondral bone. Trabecular bone is that below the subchondral bone plate.