Abstract: We report on responses of hydrated and dehydrated cortical bone tissues to mechanical loading applied by a Vickers indenter to understand the influence of water on mechanical properties of bone. The Vickers indentations were section-scanned using confocal laser scanning microscopy to understand the deformation and damage mechanisms of bone tissues. The observation of confocal laser scanning microscopy shows the fundamental indentation responses for both the hydrated and dehydrated bone tissues were plastic deformation. No visible fracture was observed in the Vickers indentation patterns in the hydrated bone tissue, while microcracks occurred in the dehydrated bone tissue. This indicates that the dehydration resulted in increased brittleness of the bone tissue. The Vickers hardness values of dehydrated bone tissue were significantly higher than those of hydrated bone tissue at any applied loads (p<0.05).

Keywords: Cortical bone, microcracks, deformation, microindentation, microhardness.

1 Introduction

Cortical bone has compact, mineralised connective tissue with a porosity formed from the vascular network and osteocytes of approximately 5% [1]. Long bones are mostly cortical bone, which represents approximately 80% of the skeletal mass [2]. Bone contains approximately 60% ceramic nanoparticles of inorganic carbonated hydroxyapatite, 10% water and 30% fibrous polymer matrix of organic collagen fibers by weight [2]. Because bone is a hierarchical composite with multiple linked length scales [3], the nature and type of damage in bone could also be hierarchical at multiple length scales [4,5].

Most investigations of bone mechanical properties have been reported on dry samples or semi-dry samples [6]. However, water in bone tissue is an important contributor to bone strength [7,8], but the role of water in the mechanics of bone is little known [9]. Water removal was found to influence the strength and toughness of cortical bone [10]. In bone studies, it is very difficult to keep bone tissues hydrated. This is because conventional techniques for imaging bone tissue necessarily involve dehydration [11], which in turn, results in microstructural change. Confocal laser scanning microscopy (CLSM) enables observation of biological samples in their natural states without dehydration [12]. It also enables production of in-focus images of thick specimens via optical sectioning and reconstruction of three-dimensional images for topologically-complex objects. CLSM has been used successfully to study microdamage in trabecular bone [12], the morphology of in vitro microcracks in compressive and bending testing of cortical bone [13], and dense arrays of ultra-microcracks in human tibiae [14]. Tensile microdamage was also examined using CSLM in beam specimens of bovine, equine and human long bone loaded in vitro and whole specimens of rat ulnae loaded in vivo [15].

Indentation has been employed to probe the mechanical behavior of materials for a wide range of engineering applications. It can be simply conducted with minimal specimen preparation and mounting requirements. It can also be performed several times on a single specimen and can probe different volumes of materials via appropriate choice of load and tip geometry [16]. More importantly, indentation response is tied to specific aspects of material behavior in bone. The purpose of this paper is to study the Vickers indentation behavior of the hydrated and dehydrated cortical bone tissues using...
CLSM. We conducted the Vickers indentation testing on both the hydrated and dehydrated cortical bone tissues at loads 0.245–9.8 N. CLSM was applied to section-scan the indentation patterns to understand the microstructural deformation and damage of bone.

2 Experimental Procedure

2.1. Preparation of Bone Samples

The bone samples used in this investigation were lamb femurs from industrially raised, 6 month-old lambs. They were stored in a refrigerator at –20 °C before all joints were cut off using a diamond saw machine. These femurs were macerated in a solution which contained 40 g Biozet laundry powder (KAO, Australia) and 2 l water for 5 days at room temperature in a fume cupboard. Biozet laundry powder contains two types of enzymes for biological active cleaning, anionic and nonionic surfactant for lifting dirt from clothes, sodium perborate monohydrate for oxygen bleach, sodium alumino silicate for softening water, sodium carbonate for breaking up fatty soils, fluorescers for brightening fabric, soil suspending agent and perfume (KAO, Australia). The pH value of the solution was 10.5. After 5 days, the soft tissues of the cortical bone samples were manually removed. After the soft tissues were cleaned, the bone samples were stored in a phosphate buffered saline (PBS) solution with 0.2% sodium azide as preservative at room temperature.
Transverse-section samples of 10 mm thickness were cut from the central femurs using a diamond saw machine at a low rotary speed. During the cutting process, alcohol was utilized as coolant. The samples were washed to remove any residual abrasives from the cutting and they were then polished using metallographic polishing techniques. The initial polishing was performed on a series of silicon carbide papers of grit sizes 60 µm, 40 µm, 15 µm, and 9 µm. Fine polishing was performed using diamond suspension slurries with grades 6 µm, 3 µm, 1 µm, and 0.25 µm on polishing cloth. The samples were cleaned before proceeding to the next finer level of polishing. After the final polishing, the hydrated bone samples were stored in the PBS solution with 0.2% sodium azide as preservative at room temperature, while the dehydrated samples were stored in a fume cupboard at room temperature for two weeks before indentation.

2.2. Vickers Indentation

Both the hydrated and dehydrated cortical bone tissues were indented along their longitudinal axes with a Vickers diamond indenter in a microhardness tester (MHT-1, Matsuzawa Seiki, Japan). Five indentation loads of 0.245 N, 0.49 N, 1.96 N, 4.9 N, and 9.8 N were applied for 10 seconds. Six indentations were made at each load on each sample, resulting in a total of 30 indentations in each sample. A distance of at least two times the impression diagonal was kept between the indentations to prevent interaction between neighboring indentations. The indentation diagonals were measured with optical microscopy. Three samples were selected for repeat tests. For the hydrated bone samples, the indentations were completed within 45 min from the time each bone sample was taken out of the PBS solution. After indentation, the indented hydrated samples were stored in PBS solution at room temperature. The indented dehydrated samples were stored in an oven at 42 ºC for 48 hours and then coated with carbon for scanning electron microscopy before they were observed using CLSM. Analysis of variation (ANOVA) at a 5% significant level was applied for statistical analysis of hardness.

2.3. Confocal Laser Scanning Microscopy

Both the indented hydrated and dehydrated bone samples were examined using a CLSM configured in reflection mode (Leica TCS-SP2-UV, Leica Microsystems Heidelberg GmbH, Germany). The confocal scan head was attached to a Leica DM6000 upright research microscope. During the imaging process, water was dripped onto the hydrated bone sample surface for purposes of hydration. The bone tissues were imaged using 488 nm illumination, a RT 30/70 primary dichroic mirror and a 485–490 nm detection window. Image acquisition was performed using a ×20, 0.70 NA, plan apochromat objective lens, corrected for direct dripping water immersion. Image intensity was optimized using detector offset and gain controls to ensure that a full dynamic range image was collected. Scanning depths along the bone longitudinal direction (Z-direction) were chosen to correspond to a full depth profile into the cross-section of the indentation. Whilst this varied from specimen to specimen, depths of 15–30 µm were typical. An optical slice ‘Z step’ spacing of 0.57 µm was utilized. This value was chosen based upon a 2.3 over-sampling of the theoretical Z resolution, as calculated by the Leica Confocal software (LCS version 2.61.1537). By scanning the sections of the indentation patterns, at various focal planes along the indentation direction, a three-dimensional data set was acquired for the sample. From these data sets two-dimensional maximum projection and color-coded depth projection images were obtained, using the LCS software.

3 Results and Discussion

Figure 1 shows a CLSM color-coded depth projection image of the complete indentation pattern for the hydrated bone at load 9.8 N. It demonstrates plastic deformation associated with a consequence of
indentation in the hydrated bone tissue. Figure 2 shows a CLSM color-coded depth projection image of the indentation patterns for dehydrated bone at the applied load 9.8 N. Although local microchipping was observed, there are no observed radial crack extensions. The indentation is fundamentally plastic deformation. However, there is microchipping as a result of microcracks occurring in the indentation patterns as shown in Figure 2. These microcracks are not seen to extend as radial cracks which are otherwise often observed in brittle ceramic materials. Indentation behaviors of both the hydrated and dehydrated cortical bone reveal ductile/plastic deformation similar to that in ductile/plastic metal materials.

Figure 3 shows the Vickers hardness versus the applied load for the hydrated and dehydrated cortical bone tissues. Each datum is the average with one standard deviation of 18 indentations in 3 samples. For the dehydrated bone tissue, at higher loads larger than 0.49 N, hardness values were found nearly unchanged. At the lowest load of 0.245 N, hardness values are smaller than those at higher loads. For the hydrated bone tissue, hardness values fluctuated. The hardness values for the dehydrated bone were found to be significantly higher than the hydrated bone hardness mean value (ANOVA, $p<0.05$). For instance, at the lowest load of 0.245 N, the dehydrated bone is 25% harder than the hydrated bone. At the highest load of 9.8 N, the dehydrated bone is 38% harder than the hydrated bone.

4 Conclusions
We have investigated the responses of hydrated and dehydrated cortical bone tissues to mechanical loading applied by a Vickers indenter. The Vickers indentations were section-scanned using confocal laser microscopy to understand the deformation and damage mechanisms of bone tissues. The results show that the Vickers hardness values of dehydrated bone tissue were significantly higher than those of hydrated bone tissue at any applied loads (ANOVA, $p<0.05$). The observation of confocal laser scanning microscopy shows the indentation deformations for both the hydrated and dehydrated bone tissues were plastic. No visible fracture was observed in the Vickers indentation patterns in the hydrated bone tissue, while there were microcracks occurring in the dehydrated bone tissue. This indicates that dehydration resulted in hardening of bone tissue in the cortical microstructure, which made it more brittle.

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References


