Measuring the Mechanical Properties of Bone by Instrumented Indentation

Application Note

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Introduction

The adaptive ability of living tissue implies a reciprocal relationship between physical properties and function. That is, properties affect function, but function can also affect properties by means of adaptation. Being able to measure physical properties is critical to understanding this interrelatedness. Thus, the purpose of this work was to use instrumented indentation to measure the elastic modulus (E) and hardness (H) of bone at the level of individual osteons. This work has been published in more detail elsewhere [1], and builds upon previous efforts to use instrumented indentation to characterize bone [2-5], dentin [6], and enamel [7].

Osteons are the primary structural units of compact bone. A polished cross-section of an osteon is shown in Figure 1. An individual osteon is roughly cylindrical; it is several millimeters long and has a diameter of about 200 microns. Each osteon is comprised of a *Haversian canal* and concentric layers of lamellar bone. The Haversian canal runs through the axis of each osteon and contains small blood vessels which deliver nutrients to individual bone cells (*osteocytes*) [8]. In living bone, new osteons are generated to replace older osteons; this process is called *bone remodeling*. As osteons age, they become more and more mineralized. Thus, the goal of this work was to measure the mechanical properties of osteons with varying degrees of mineralization. We hypothesized that the older (more mineralized) osteons would have higher elastic modulus and hardness. These types of measurements are necessary for understanding the relationship between mineralization, properties, and function in compact bone.

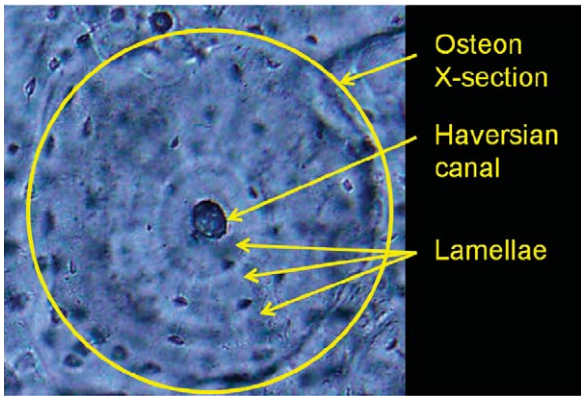

Figure 1. Polished cross-section of bone highlighting an individual osteon and its parts.

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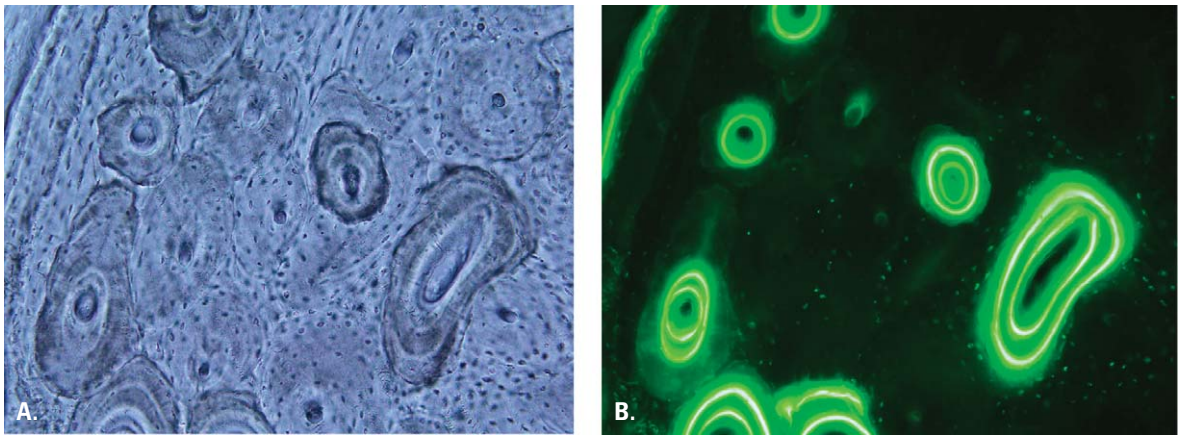

Figure 2. Complementary pair of typical images used to identify osteons for testing. Image A is bright-field and image B is epifluorescent. In image B, the young osteons glow (i.e. are "labeled") due to the calcein injections administered prior to euthanasia.

Experimental Method

Specimen Source

Bone samples were obtained from five skeletally mature male dogs which were sacrificed for other studies. In order to highlight newly formed osteons, two doses of Calcein (Sigma-Aldrich, St. Louis, MO) were administered to the dogs intravenously at 17 days and 3 days prior to euthanasia. Calcein is a fluorescent dye that preferentially labels newly forming bone surfaces. The femurs were harvested immediately after euthanasia and frozen in saline soaked gauze at -20° C.

Specimen Preparation

Two mid-femoral cross sections, each about 3 mm thick, were obtained from each dog for a total of 10 samples. The test surfaces were lightly polished according to the following procedure. The bone block was glued into a well of a custom-made polycarbonate specimen holder. The mounting was verified on a certified level stage to ensure parallelism. The sectioned specimens were wet-polished on a rotary wheel (Ecomet, Buehler, Lake Bluff, IL) at 120 rpm with 2,400 grit SiC papers. Additional polishing was done on a napless cloth (OP-Chem, Struers A/S, Rodovre, Denmark) with diluted 0.3 µm and 0.05 µm alumina oxide pastes (Micropolish C alpha Alumina, Buehler, Lake Bluff, IL). The specimens were sonicated for 2 mins. After polishing, surface roughness was less than 30 nm.

Test-site Selection

Next, we identified and mapped the coordinates of the labeled and unlabeled osteons under an epifluorescent microscope (Olympus BX 51, Tokyo, Japan). This was essential, because labeled osteons cannot be identified under the optics of the indenter system. Multiple perpendicular lines were scribed into the surface of the polished bone specimen with a surgical blade. The exact location (x, y coordinates) of the central Haversian canal of the labeled osteon relative to two orthogonal scribe lines was measured in microns using a linear microscope eyepiece of the epifluorescent microscope. In addition, photomicrographs aided in documenting the unique cross sectional morphology of each labeled osteon site and its neighboring osteons and other structures such as blood vessels.

After the bone slice was mounted in the indenter (Nano Indenter XP, Agilent Technologies, Inc., Chandler, AZ), the photographic map produced from the epifluorescent microscope was referenced. A specific labeled osteon was located using the intersection of nearest two perpendicular lines from the osteon. The sample was moved so that the intersection of the two perpendicular lines was visible in the indenter optics. From this position, the sample was moved so that the labeled osteon was visible. The location of the labeled osteon, in the reference frame of the indenter, was recorded. The photographic map confirmed the specific osteon of interest and a neighboring unlabeled osteon (Figure 2).

After all osteons that we desired to examine were located, we programmed the software to place indents on the osteons. The indents were located approximately half the distance between the central canal and outer border for all the osteons. We made 5-6 indents on each osteon, both labeled and unlabeled. A total of 610 indents were made on 147 osteons (labeled = 35; unlabeled =112).

Testing

During testing, a hydration system, was used to keep the specimens moist for the entire test. The hydration fluid was a mixture of distilled water and 0.5 mg/ml of gentamicin sulphate (Sigma Chemical Company, St. Louis, MO).

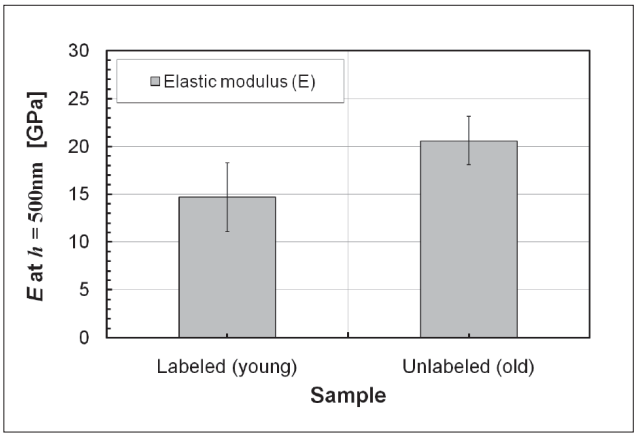

Figure 3. Comparison of elastic modulus for labeled and unlabeled osteons. Difference is directly related to the degree of mineralization.

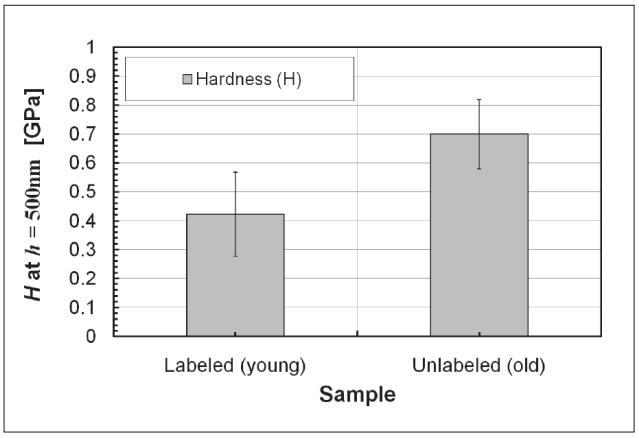

Figure 4. Comparison of hardness for labeled and unlabeled osteons. Difference is directly related to the degree of mineralization.

Each indentation test consisted of the following segments:

0. The indenter approaches the test surface until contact is sensed.

1. The indenter is pressed into contact with the test material at a rate of 10 nm/sec to a peak depth of 500 nm. During this pressing, a small oscillation is superimposed on the quasi-static loading by means of the *continuous stiffness measurement* (CSM) option. The amplitude of the oscillating force, F_O, was continuously adjusted in order to maintain the amplitude of the resulting displacement oscillation at z_O = 2 nm.

2. The force on the indenter is held constant for a dwell time of 30 seconds.

3. The indenter is withdrawn from the sample completely, and the sample is moved into position for the next test.

Post-test Analysis

Contact stiffness was calculated as a continuous function of penetration depth from the amplitude ratio F_O/z_O measured during test segment 1. Elastic modulus (E) and hardness (H) were calculated from this continuous measure of contact stiffness; this analysis is described in detail elsewhere [9, 10]. In order to report scalar properties of each type of osteon (labeled and unlabeled), CSM results for E and H were taken at the maximum displacement of 500 nm.

Results

The results are summarized in Table 1 and plotted in Figures 3-4. Statistically significant differences were found between labeled and unlabeled osteons. Elastic modulus of unlabeled (older) osteons was 40% higher than that of labeled (newer) osteons; hardness of unlabeled osteons was 66% higher than that of labeled osteons. The difference in mechanical properties is directly related to the degree of mineralization.

Osteon Type	$E \pm \sigma(E)$ GPa	$H \pm \sigma(H)$ GPa	E/H
Labeled (young)	14.7 ± 3.58	0.422 ± 0.146	2.87%
Unlabeled (old)	20.6 ± 2.53	0.700 + 0.120	3.40%

Table 1. Summary of results.

Both hardness and elastic modulus were higher for unlabeled osteons, but hardness was more so. The ratio H/E was 2.8% for labeled (newer) osteons and 3.4% for unlabeled (older) osteons. This implies that mineralized osteons will be more resistant to permanent deformation than newer osteons. This finding is consistent with the observation that excessive remodeling, stimulated by micro-cracks, increases the likelihood of stress fracture [11], and that phenomena that result in poor mineralization tend to make bone more vulnerable to excessive deformation and fracture (osteomalacia) [12] . Yet, the characterization of bone should not be over-simplified. As a composite, it is likely that the mechanical properties of compact bone are determined by complex interactions between osteons of differing properties, not simply by a volume-weighted average.

Conclusions

An Agilent NanoIndenter was used to measure the mechanical properties of individual osteons of a femur bone. This study confirms that new and old osteons have significantly different mechanical properties and that those properties are directly related to the degree of mineralization. The testing techniques developed in this work, especially those related to imaging and hydration, should be useful in the testing of other biological tissues.

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