Effects of drying on the mechanical properties of bovine femur measured by nanoindentation

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Effects of drying on the measurement of mechanical properties of bone by nanoindentation methods have been examined. Tests were conducted to measure the elastic modulus and hardness of two cross-sectional cortical specimens obtained from adjacent areas of bovine femur. One specimen was thoroughly dried in air prior to testing while the other was stored in deionized water. The properties of osteons and interstitial lamellae showed statistically significant differences ($p < 0.0001$) and were therefore investigated separately. Drying was found to increase the elastic modulus by 9.7% for interstitial lamellae and 15.4% for osteons. The hardness was also found to increase by 12.2% for interstitial lamellae and 17.6% for osteons.

1. Introduction

Nanoindentation has recently been shown to be a useful tool for characterizing the mechanical properties of the microstructural components of bone, such as individual osteons and trabeculae [1]. In this technique, micrometer or submicrometer hardness impressions are made in the microstructural feature of interest, and its mechanical properties are derived from analyses of high resolution load–displacement data obtained as the indenter is driven into and withdrawn from the specimen [2–4]. Nanoindentation can be used to probe a bone's surface and map its properties on a spatially resolved basis, often with a resolution of better than 1 μm. Among the properties that can be measured are Young's modulus, E, and the hardness, H [2–4], and by using special indenters that promote cracking during indentation, the fracture toughness of small microstructural features can also be estimated [5, 6].

Because of experimental complications associated with testing in liquid environments and keeping specimens wet during testing, most nanoindentation work to date has focused on dried bone [1]. It is well documented, however, that the mechanical properties of bone show notable changes after dehydration [7–12]. In general, drying increases the Young's modulus of bone, decreases its toughness and reduces the strain to fracture. An important question thus arises as to the degree to which nanoindentation mechanical property measurements are affected by drying. To this end, a series of tests was conducted on wet and dry bovine femur using a special testing fixture in which specimens could be tested fully immersed in a liquid. Results for wet and dry bone are presented, which indicate the extent to which drying affects nanoindentation mechanical property measurements.

2. Materials and methods

All tests were conducted on a fresh bovine femur obtained from a local slaughterhouse. After acquisition, the femur was taken immediately to the laboratory, and a section 30 mm long was cut from the mid-diaphysis of the bone. The section was then cut transversely into two pieces, with the two mating faces representing the surfaces on which nanoindentation testing was performed (Fig. 1). Test specimens were cut from the anterior aspect of each subsection at exactly the same location, and the mating cross-sectional surfaces were metallographically polished to produce the smooth surfaces needed for nanoindentation testing. Surface preparation consisted of coarse grinding with silicon carbide abrasive papers under deionized water, followed by fine polishing using microcloths (TEXMET®, Buehler, Lake Bluff, IL) with successively finer alumina powder to 0.05 μm. As a final step in the surface preparation, the specimens were polished on a plain microcloth with deionized water. After thorough ultrasonic cleaning to remove foreign substances from the surface, the wet specimen was kept hydrated in deionized water containing 0.001% thymol [12], while the dry specimen was dehydrated at 20 °C for 14 days in air. The wet specimen was maintained in deionized water rather than saturated mineral water to prevent unwanted crystal growth and crystal film formation on the specimen surface. Some mineral dissolution might be expected during storage, but the magnitude and effect are masked by the inherent variability in composition, structure and mechanical properties of the specimens [12].

Nanoindentation testing was conducted using a nanoindentation system at the Oak Ridge National Laboratory and a specially designed test fixture in which wet specimens could be tested while fully

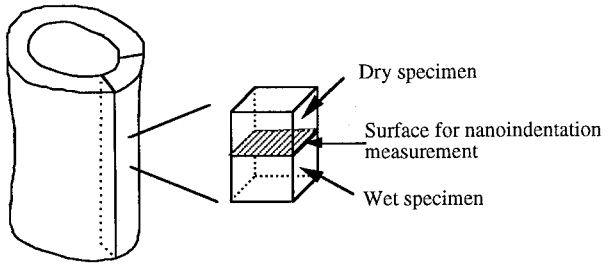

Figure 1 Schematic representation of specimen location for wet and dry testing.

immersed in deionized water. A schematic representation of the test fixture is shown in Fig. 2. The specimen was held securely in place by two set screws with the polished surface extending slightly above the aluminum mount. Deionized water was added to the test fixture until the specimen was completely immersed, but to avoid problems caused by water rising too far up the indenter shaft, the level was carefully controlled so that water extended only 50–100 μm above the specimen surface. At this level, the indenter tip was immersed during testing, but water did not rise high enough up the indenter shaft to damage the testing system. The proper water level was determined by focusing separately on the specimen surface and the water surface using the optical microscope in the nanoindentation system and noting when the two focal planes were separated by 50–100 μm. Reducing the surface tension by adding a drop of liquid dishwashing detergent to the water (the total water volume was about 0.5 liters) aided in keeping water on the specimen surface and reduced the force needed for the indenter to penetrate the water surface at the beginning of a test. The latter effect helped in identifying the point of first surface contact during indenter approach to the surface at the beginning of a test.

All nanoindentation tests were conducted with a Berkovich diamond indenter in load control using the load–time sequence shown in Fig. 3. As seen in the plot, the indenter was loaded and unloaded three times, with two intervening periods during which the load was held constant. The multiple loading scheme was used to examine the extent to which the load–displacement data were influenced by viscoelastic deformation. Fig. 4 presents a typical set of load–displacement data for the wet specimen. After the initial loading, in which most of the plastic deformation occurs and the hardness impression is formed, there is a hysteresis in the data, which indicates that a non-negligible portion of the indentation displacement is viscoelastic. Viscoelastic deformation is also apparent in the creep displacements observed during the constant load hold period at peak

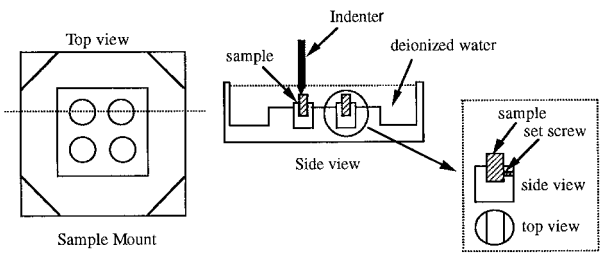

Figure 2 Mounting stage used for testing wet specimens.

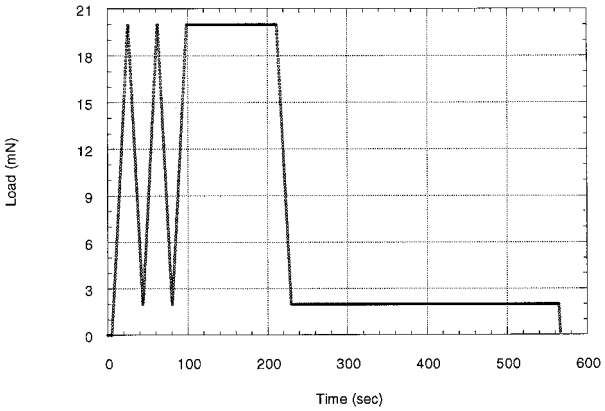

Figure 3 The indenter load–time sequence used for all nanoindentation testing.

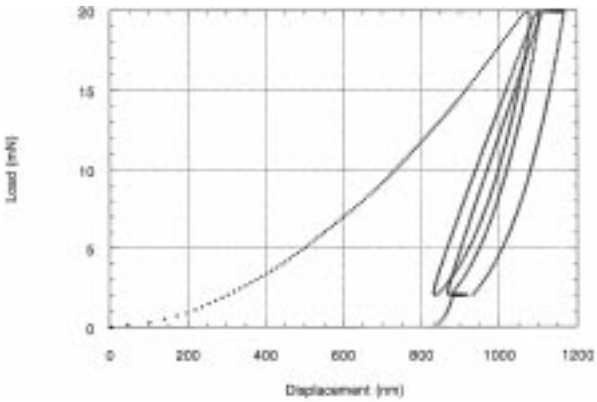

Figure 4 Typical load–displacement data for a wet specimen (tested in an osteon).

load. Viscoelastic deformation is important because analysis procedures used to obtain mechanical properties from the nanoindentation load–displacement data are premised on the notion that the upper portion of the unloading curve is dominated by elastic rather than viscoelastic recovery. Thus, to minimize the effects of viscoelasticity and creep on property measurements, a relatively long constant load hold period was inserted prior to the final unloading, during which the viscoelastic deformation diminished to a negligible rate. Similar but slightly smaller viscoelastic effects were observed for the dried bone.

The second constant load hold period, near the end of the test at 90% of the peak load, was used to establish the rate of thermal expansion or contraction of the testing apparatus to correct the displacement data for thermal drift. An example of the displacements observed in the second hold period is given in Fig. 5. Careful examination showed that viscoelastic recovery is significant during the initial portion of the hold period, but after about 200 s, the viscoelastic effects become relatively small compared to the thermal effects. Consequently, data from the last 100 s of the second hold period were used to establish the thermal drift rate and correct the displacement data. Data corrected for thermal drift are also included in Fig. 5. Care was taken to let the system thermally equilibrate for an extended period of time prior to testing. Consequently, thermal drift rates were very small, and it was generally found

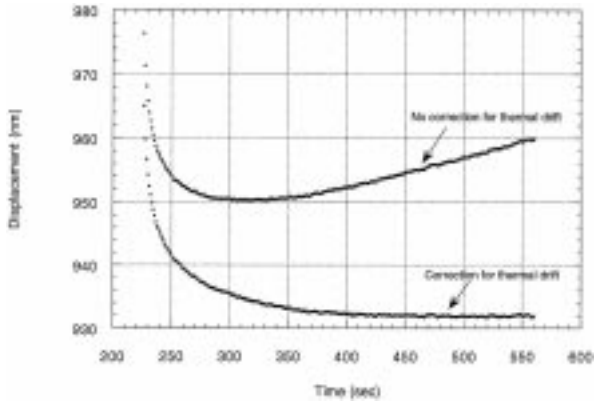

Figure 5 Typical displacement–time behavior during the 300-s hold period near the end of the test from which the thermal drift rate was established. Data after correction for thermal drift are also shown.

that correcting the data for drift changed the measured mechanical properties by less than 1%.

Each nanoindentation test was conducted to a maximum load of 20 mN, which produced hardness impressions with depths of about 1 μm. To minimize the effects of spatial variation in bone properties, all measurements were made within 1 mm of the same area on the two mating surfaces of the wet and dry specimens. The hardness and elastic modulus for each indentation were determined using the method of Oliver and Pharr from data obtained in the third unloading [3].

During the course of the investigation, it was discovered that there was a measurable difference in the properties of osteons and interstitial lamellae, as previously observed in nanoindentation studies of dried human tibiae [1]. Consequently, an effort was made to test individual osteons and interstitial lamellae with equal frequency and separate the results into two groups. Two indentations were thus made in each of 30 osteons and 30 interstitial lamellae giving a total of 120 indentations for the two specimens. Significant differences in the mechanical properties of dry and wet osteons and interstitial lamellae were analyzed using one-way analysis of variance (ANOVA). Scheffé's test was then employed to find differences between the mechanical properties in the dry and wet conditions. All differences were considered significant at a probability level of 95% ($p < 0.05$).

3. Results

A summary of the elastic moduli, E, and hardnesses, H, for the wet and dry bovine femur as measured by nanoindentation is presented in Table I. For wet bone, the elastic modulus of interstitial lamellae is 15.7% higher than for osteons ($E_{\text{interstitial}} = 25.1 \pm 1.6$ GPa versus $E_{\text{osteon}} = 21.1 \pm 2.0$ GPa), and the hardness is 20.8% higher ($H_{\text{interstitial}} = 0.730 \pm 0.048$ GPa versus $H_{\text{osteon}} = 0.578 \pm 0.052 GPa$). For dry bone, the same trends are observed, with the elastic modulus of interstitial lamellae being 11.2% higher ($E_{\text{interstitial}} = 27.5 \pm 1.2$ GPa versus $E_{\text{osteon}} = 24.4 \pm 2.2$ GPa), and the hardness 16.9% higher ($H_{\text{interstitial}} = 0.818 \pm 0.049$ versus $H_{\text{osteon}} = 0.680 \pm 0.102$). The elastic modulus of interstitial lamellae and osteons is increased by drying by 9.7 and 15.4%, respectively, and the hardness by 12.2 and 17.6%, respectively. ANOVA showed that the mean values of E and H are statistically significantly different ($p < 0.0001$) for both osteon and interstitial lamellae.

4. Discussion

The water content of bone, which typically ranges from 10 and 20% [8], plays an important role in determining its mechanical properties. Results of this study demonstrate that both the hardness and elastic modulus as measured by nanoindentation techniques depend on whether the bone is wet or dry and that drying increases both properties by a measurable amount.

Using conventional microhardness testing techniques to test a variety of bone types in an extensive study, Evans *et al.* [13] have shown that bone hardness can be influenced by several factors involved in its storage, preparation and testing. These include embedding the test specimen in resin, which was observed to produce an approximately 4% decrease in hardness, the time of indentation load application, which had significant effects only at times less than 10 s, the storage time of frozen specimens, which had no significant effect, and the recovery time after unloading prior to indentation size measurement, which also had no significant effect. Moreover, Evans *et al.* found that hardness correlates with Young's modulus, specifically, hardness increases with modulus, in a manner which is qualitatively consistent with the current observations on wet and dry specimens.

Effects of water content on bone mechanical properties have also been demonstrated in other studies. Currey [11] showed that rewetting dried bone restores the mechanical properties to values similar to those of fresh wet specimens. Evans and Lebow [7], using conventional macroscopic testing techniques, reported that air-drying at 105 °C increases the elastic modulus by 18% and the hardness by 54%. Dempster and Liddicoat studied the influences of drying in both tension and compression tests, finding that drying produces an increase in elastic modulus of about 26% in compression and an even greater increase, 55%, in tension [14]. Amprino [15] reported that drying causes a significant increase in the microhardness of bone and found that higher drying

TABLE I Elastic moduli and hardnesses measured by nanoindentation for wet and dry interstitial and osteonal bone

	Osteons		Interstitial lamellae	
	Wet	Dry	Wet	Dry
Elastic modulus, GPa	21.1 ± 2.0	24.4 ± 2.2	25.1 ± 1.6	27.5 ± 1.2
Hardness, GPa	0.578 ± 0.052	0.680 ± 0.102	0.730 ± 0.048	0.818 ± 0.049

temperatures produce greater increases in microhardness. Townsend and Rose [10], studying buckling of single human trabeculae, reported an elastic modulus increase due to drying of 24%, as well as a general change in the deformation mode from ductile for wet specimens to brittle for dry ones. The modulus increase observed in each of these studies is qualitatively consistent with the nanoindentation results obtained in this investigation, although the increase in the current results (10–15%) is not quite as large as that observed in the other studies (>20%). The changes in mechanical properties caused by varying the water content are inherently associated with important structural changes. Ten Cate *et al*. [16] observed that upon demineralization, dentine shrinks whether it is fully hydrated or not. This suggests that the mineral phase in hydrated dentine provides a rigid scaffolding that prevents the collagen fibrils from contracting and the dentine from ultimately shrinking (collapsing). Finlay and Hardie [17] reported that vacuum dehydration causes a shrinkage, which is anisotropic in nature, but did not elucidate the effects of hydration on the mechanical properties.

Other effects on bone mechanical property measurements are due to mounting the specimen in polymer resin and chemical fixation of the bone prior to testing. Weaver [18] demonstrated that prolonged formalin fixation causes a 20% increase in microhardness, although Amprino [15] suggested that brief fixation has little or no effect. Amprino [15] has also reported that mounting and infiltration of the bone structure with methylmethacrylate resin increases its microhardness by 30–40%. Since the specimens tested here were not mounted or chemically fixed prior to testing, neither of these effects are important in the current study.

Another important observation from this study is that the elastic modulus and hardness of interstitial lamellae are higher than those of osteons in the same region of the bone. This observation, which is consistent with previous nanoindentation measurements of dried human bone [1], is probably due to the greater degree of mineralization in interstitial bone [19]. It is also interesting to note that drying results in a greater percentage change in the hardness than in the elastic modulus and that drying effects are greater for osteons than for interstitial lamellae.

We postulate that drying leads to contraction of the individual collagen fibrils with the degree of contraction depending on the level of mineralization in the bone. Indeed, Lee and Glimcher [20] directly measured equatorial collagen spacings using neutron diffraction for both wet and dry collagens in the apex region (least mineralized) of intramuscular herring bones as well as in regions of bone in progressively more mature stages of mineralization. The least mineralized region demonstrated an equatorial collagen spacing of 1.491 nm in the wet state and 1.107 nm in the dry state. Regions with larger amounts of mineral produced equatorial spacings of 1.331 nm in the wet state and 1.182 nm in the dry state. These data suggest that drying-induced changes in equatorial distances in collagen decrease as mineralization progresses [20]. Since it has been observed using back scattering scanning electron microscopy that interstitial bone is more mineralized than osteonal bone [19], these same effects may be responsible for the greater mechanical property changes produced by drying in osteons.

Acknowledgments

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