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Reference point indentation study of age-related changes in porcine femoral cortical bone

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ABSTRACT

The reference point indentation (RPI) method is a microindentation technique involving successive indentation cycles. We employed RPI to measure average stiffness (Ave US), indentation distance increase (IDI), total indentation distance (TID), average energy dissipated (Ave ED), and creep indentation distance (CID) of swine femoral cortical bone (mid-diaphysis) as a function of age (1, 3.5, 6, 14.5, 24, and 48 months) and loading directions (longitudinal and transverse). The Ave US increases with animal age, while the IDI, TID, Ave ED, and CID decrease with age, for both longitudinal (transverse surface) and transverse (periosteal surface) loading directions. Longitudinal measurements generally give higher Ave US and lower IDI and TID values compared to transverse measurements. The RPI measurements show similar trends to those obtained using nanoindentation test, and ash and water content tests.

1. Introduction

Bone mechanical properties are affected by diverse factors including age, diet, disease, and exercise. Numerous studies have reported age-related changes in bone density (Sherk et al., 2009; Banu et al., 2002; Cheng et al., 2007; Pothuaud et al., 2004; Inui et al., 2004; Iida and Fukuda, 2002; Steiger et al., 1992), elastic modulus (Hoffler et al., 2000; Ding et al., 1997; Ding et al., 2002; Burstein et al., 1976), strength (Zioupos and Currey, 1998; Wall et al., 1979; Martin and Atkinson, 1977; Courtney et al., 1995; Willinghamm et al., 2010), and fracture toughness (Nalla et al., 2004; Wang et al., 2002; Bonfield et al., 1985; Zioupos et al., 1999; Yeni and Norman, 2000). These studies show that bone properties deteriorate with aging, underlying the importance of assessing bone's fracture susceptibility as a function of patient age (Launey et al., 2010).

Bone mineral density (BMD) is measured clinically to assess bone fracture-resistance (Marshall et al, 1996). However, there is increasing evidence that BMD data alone are not sufficient to accurately predict bone fracture risk (Ager et al., 2006; Heaney, 2003; Miller et al., 2005; Bouxsein, 2003; Cefalu, 2004).

Mechanical properties of bone such as strength and fracture toughness provide direct information about bone's ability to resist failure (Currey, 1979). Various testing methods have been used in vitro to measure bone mechanical properties including tensile (Feng and Jasiuk, 2010; McCalden et al., 1993; Kotha and Guzelsu, 2007; Braidotti et al., 2000), compression (Hsu et al., 2008; George and Vashishth, 2005), bending (Barak et al., 2009; Sadeghi-Mehr and Hosseini-Mansoob, 2007; Draper and Goodship, 2003) and torsion (Taylor et al., 2003; Nazarian et al., 2009; Jepsen et al., 1999) tests, which give properties at the tissue or whole-bone levels. Nanoindentation (Rho et al., 1997; Hengsberger et al., 2002; Lewis and Nyman, 2008; Tjhia et al. 2012) and microindentation (Coats et al., 2003; Johnson and Rapoff 2007; Zwierzak et al., 2009) methods have been developed to provide bone mechanical properties at lower material scales.

Reference point indentation (RPI), a new microindentation method involving successive indents, was recently developed by Hansma and coworkers to measure bone material properties (Hansma et al., 2006, 2008, 2009; Randall et al., 2009). The RPI technique is so named because the probe assembly consists of a reference probe, which rests on the bone surface, and a test probe, which moves relative to the reference probe during testing. The RPI method is minimally invasive and can be used even if bone is covered by skin. This technique has already been applied in vivo to assess bone physical parameters in living patients (Diez-Perez et al., 2010). However, correlation between RPI data and bone mechanical properties is still not well-established, which provided motivation for conducting the current study.

In this report, we used the RPI technique to characterize properties of porcine cortical femoral bone as a function of age and loading direction, and compared the RPI results to those obtained using tensile, nanoindentation, and ash and water content tests. We selected swine bone because we have already characterized its structure, composition, and mechanical properties (Ambekar et al., 2012;

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Feng et al., 2012; Feng and Jasiuk ,2011). Although many studies have focused on aging bone, our work addressed developing bone. The animal ages studied ranged from 1 to 48 months, which represent very young to mature animals (Tumbleson, 1996). Our objective was to assess whether connections exist between RPI data and bone mechanical properties and composition. Such knowledge will further advance RPI methodology and facilitate its application in clinical settings.

2. Materials and methods

2.1. Microindentation sample preparation

We studied six animal age groups: 1 month ($n=10$), 3.5 months ($n=2$), 6 months ($n=10$), 14.5 months ($n=2$), 24 months ($n=2$), and 48 months ($n=2$). Bone samples were taken from the mid-diaphysis of swine femur. Femurs were obtained from the Department of Animal Sciences at the University of Illinois following Institutional Animal Care and Use Committee protocols. The femurs were wrapped in a phosphate-buffered saline (PBS) soaked gauze after harvesting and were stored at -20 °C until analysis. For sample preparation, femurs were first cleaned of remnant soft tissue. Cylindrical 25-mm-long mid-diaphysis samples were cut from each femur (Fig. 1) using a band saw. Indents were made in the longitudinal (transverse surface) and transverse (periosteal surface) directions. Bone section transverse surfaces were polished using a silicon carbide abrasive paper (180- and 1200-grit sizes) to smooth out cut surface irregularities from sectioning. Samples were wrapped in PBS-soaked gauze and stored at 4 °C until testing time. All samples were tested within 24 h of thawing.

2.2. Reference point indentation instrument

The RPI measurements were done using a BioDent™ Hfc instrument (Active Life Scientific, Inc., Santa Barbara, CA) (Hansma et al., 2006, 2008, 2009; Randall et al., 2009). This apparatus consists of a reference probe that rests on the bone surface and a test probe that indents onto the bone during testing. Displacements are measured as the distance between the test and reference probes. The test probe has a diameter of 375 μm with a 90° conical end and a tip radius of 2.5 μm. This technique involves successive indentation cycles, in which the test probe advances deeper into the bone with each cycle. The load function has a trapezoidal shape, involving a linear increase followed by a hold time and a linear decrease. The purpose of the hold is to monitor creep effects. The indentations give rise to load-displacement curves shown schematically in Fig. 2.

Several quantities can be computed from the RPI-generated load-displacement plots. The indentation distance increase (IDI) is measured as the increase in an indentation distance from the first to the last cycle. The total indentation distance (TID) is measured from the initial touchdown distance to the maximum indentation distance in the last cycle. The average energy dissipated (Ave ED) is the average of an integrated area between the loading and unloading curves. The average

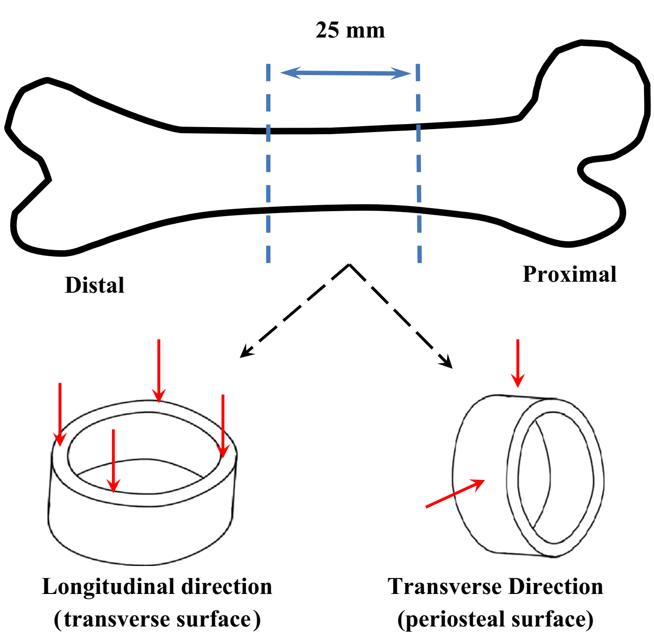

Fig. 1. Porcine femur sample preparation and surface nomenclature.

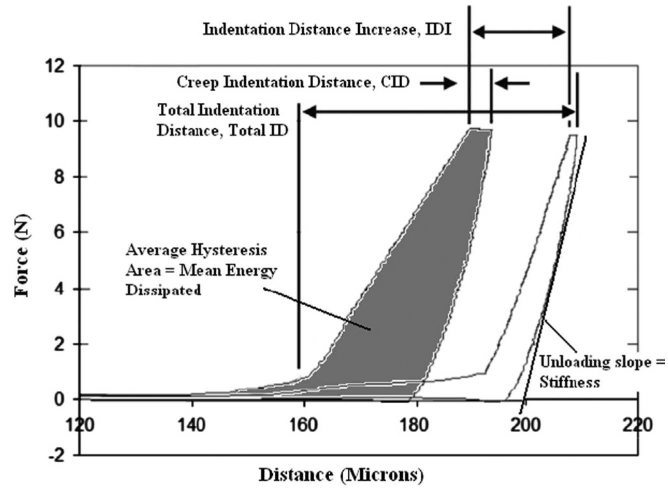

Fig. 2. Representative force-displacement plot obtained from RPI analysis, and definitions of the measured quantities.

stiffness (Ave US) is the average of all slopes calculated from the top 50% of the unloading part of the force-displacement curve. The creep indentation distance (CID) is the distance traversed by the probe during the hold period for the first indentation cycle, and the average creep indentation distance (Ave CID) is the CID averaged over all indentation cycles, whereas the total creep indentation distance (Tot CID) is the sum of the CIDs from all indentation cycles. Definitions of these quantities are shown schematically in Fig. 2.

2.3. Microindentation testing

For each bone sample, two regions were selected for indentation: the proximal transverse surface (longitudinal testing direction) and the periosteal surface (transverse testing direction). On each surface type, 5–6 indentations were performed in each quadrant (anterior, lateral, medial, and posterior) to account for spatial variability of bone mechanical properties. Indents were made 1–2 mm apart and were approximately equally-spaced. In the longitudinal direction (transverse surface) indents were made in a circular fashion in each quadrant, while in the transverse direction (periosteal surface) they were made along the bone length. The measurements obtained from all four quadrants were pooled together since the differences between the results from these different quadrants were statistically insignificant. The averages of measurements taken from all indentations were reported as the RPI values.

For all samples, load-controlled indents were applied with a 6 N force and each indentation involved 10 cycles. Preliminary analysis showed that 10 cycles were sufficient, because no measurement differences were observed using a larger number of cycles. In each cycle, the force was increased linearly for 1/3 of the cycle, held constant for the next 1/3 of the cycle, and then decreased linearly for the remaining 1/3 of the cycle, with each cycle lasting 500 ms following (Hansma et al., 2008).

For indentation in the longitudinal direction, the sample was immobilized on a platform and the probe tip was lowered until it rested on the bone surface. For transverse measurements, a special clamp was used to stabilize the sample and prevent movement. During testing, samples were kept moist by placing few drops of PBS on the bone testing site.

2.4. Statistical analysis

Animal age (1, 3.5, 6, 14.5, 24, and 48 months) and force loading direction (longitudinal and transverse) were the two studied variables. One-way and two-way analysis of variance (ANOVA) were performed to determine the significance of variation of the RPI measurements with age in the longitudinal and transverse directions, using ORIGINPRO v.8.5 statistical analysis and graphing software (OriginLab Corp., Northampton, MA), followed by Tukey tests. A confidence level of 95% ($p<0.05$) was considered statistically significant. For the nanoindentation and water and ash content data, included for comparison with RPI findings, differences were analyzed for statistical significance with two-way and/or one-way ANOVA, as appropriate, followed by Tukey tests. Nanoindentation tests involved 1 month ($n=3$), 3.5 months ($n=2$), 6 months (2), 12 months ($n=2$), 30 months ($n=3$), and 48 months ($n=2$) swine femurs. Water and ash content tests involved four swine femurs from different animals for each of the same six age groups. Additionally, to confirm our results, Pearson correlation values and corresponding one-tailed p-values were calculated between RPI measurements (IDI, TID, CID, Ave CID, Tot CID, Ave ED, Tot ED, Ave US) in the longitudinal direction and results from our nanoindentation study (elastic modulus, hardness, mineral percentage, water percentage). Four common age groups (1, 3.5, 6, and 48) were used for correlation analyses.