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The roles of architecture and estrogen depletion in microdamage risk in trabecular bone

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ABSTRACT

Bone quantity, or density, has insufficient power to discriminate fracture risk in individuals. Additional measures of bone quality, such as microarchitectural characteristics and bone tissue properties, including the presence of damage, may improve the diagnosis of fracture risk. Microdamage and microarchitecture are two aspects of trabecular bone quality that are interdependent, with several microarchitectural changes strongly correlated to damage risk after compensating for bone density. This study aimed to delineate the effects of microarchitecture and estrogen depletion on microdamage susceptibility in trabecular bone using an ovariectomized sheep model to mimic post-menopausal osteoporosis. The propensity for microdamage formation in trabecular bone of the distal femur was studied using a sequence of compressive and torsional overloads. Ovariectomy had only minor effects on the microarchitecture at this anatomic site. Microdamage was correlated to bone volume fraction and structure model index (SMI), and ovariectomy increased the sensitivity to these parameters. The latter may be due to either increased resorption cavities acting as stress concentrations or to altered bone tissue properties. Pre-existing damage was also correlated to new damage formation. However, sequential loading primarily generated new cracks as opposed to propagating existing cracks, suggesting that pre-existing microdamage contributes to further damage of bone by shifting load bearing to previously undamaged trabeculae, which are subsequently damaged. The transition from plate-like to rod-like trabeculae, indicated by SMI, dictates this shift, and may be a hallmark of bone that is already predisposed to accruing greater levels of damage through compromised microarchitecture.

1. Introduction

Osteoporosis is a bone disease characterized by a loss of bone mass or degradation of architecture that predisposes an individual to fracture, even in the absence of a traumatic event (WHO, 1993). Approximately 10 million Americans currently have osteoporosis, with another 34 million at risk (USDHHS, 2004). Bone mineral density (BMD) as measured by dual energy X-ray absorptiometry (DEXA) or quantitative computed tomography (qCT) is the standard diagnostic measure for osteoporosis. However, it is neither specific nor sensitive enough to be the sole determinant for increased osteoporotic fracture risk (Schuit et al., 2004; Seeman and Delmas, 2006). For example, older patients can have a 10-fold increase in fracture risk when compared to BMD-matched young patients (Hui et al., 1988). As such, factors other than density must also affect bone strength and play a role in bone's mechanical integrity.

Bone quality refers to structural features and intrinsic tissue properties that can determine differences in mechanical behavior not explained by quantity as measured by mineral density (Seeman and Delmas, 2006). In trabecular bone, quality includes microarchitectural characteristics such as structure model index (SMI), trabecular thickness (Tb.Th.), and slenderness, that degrade with aging and are associated with increased fracture risk (Liu et al., 2012; Sornay-Rendu et al., 2006). Microdamage is also a factor in bone quality. Microdamage presents in the form of cracking or diffuse damage (Schaffler et al., 1995) that can affect mechanical properties at both the tissue and apparent level (Lambers et al., 2013; Wu et al., 2013). The presence of microdamage can compromise mechanical integrity, and has been

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correlated to reductions of bone modulus and strength (Garrison et al., 2011; Lambers et al., 2013; Wu et al., 2013).

Microdamage burden increases with age (Arlot et al., 2008), consistent with *ex vivo* experiments demonstrating damage formation in response to fatigue loading at physiologic levels (Wenzel et al., 1996). The increase in microdamage with age coincides with microarchitectural degradation (Garrison et al., 2009) and changes in tissue mineralization related to aging and disease (Zioupos et al., 2008), although it is not clear that this correlation is causal. These factors can be difficult to investigate systematically using human cadaver bone, where many variables are outside of experimental control. However, for experimental purposes, similar structures and densities of microdamage can be induced by monotonic overloading (Moore and Gibson, 2002), and animal models employing ovariectomy can mimic some of the changes that occur in aging bone (Brennan et al., 2011; Holland et al., 2013; Kennedy et al., 2008).

The goal of this study was to define the contributions of trabecular microarchitecture and ovariectomy status in microdamage formation and propagation in trabecular bone. Ovariectomized sheep were used as a model of post-menopausal osteoporosis (Turner, 2001). This model results in significant degradation of BMD and trabecular microarchitecture in the vertebral body (Giavaresi et al., 2001), iliac crest (Newton et al., 2004), and femoral neck (Wu et al., 2008) within one year of ovariectomy (Sigrist et al., 2007). In addition, ovine bones are large enough to provide samples that are suitable for mechanical testing (Lill et al., 2002). In this study, (1) microdamage was mechanically induced in trabecular bone from the distal femur through sequential compressive and torsional overloads; (2) relationships between damage, ovariectomy status, and microarchitectural parameters were quantified; and (3) the effects of existing damage on the risk of further damage were quantified.

2. Materials and methods

2.1. Sample preparation

Twelve female sheep underwent ovariectomy (OVX) under general anesthesia. The sheep were then returned to pasture until they were sacrificed two years following the OVX. Seven sheep of similar age sacrificed for other studies or due to age were used as controls. Bones were harvested immediately, stripped of soft tissue, and stored at −20 °C until they were prepared for testing. The animal care was reviewed and approved by the Institutional Animal Care and Use Committees of Colorado State University and the University of Notre Dame.

Thirty-seven cylindrical cores were prepared from the medial and lateral femoral condyles of the control sheep, and thirty-nine cores were prepared from the femoral condyles of the ovariectomized sheep. The cores were aligned with the principal axes of the trabecular bone using a previously reported protocol (Wang et al., 2004). Briefly, parallelepipeds were cut from the samples and scanned using micro-computed tomography (μ-CT80, Scanco, Brattiselen, Switzerland) at 30 μm resolution while submerged in phosphate buffered saline (PBS). The images were converted into micro-finite element models, which were solved to determine the principal mechanical axis. A custom jig was used to align the sample to the calculated axes, and a diamond coring drill (Starlite Industries, Bryn Mawr, PA) was used to prepare a cylindrical sample with a nominal diameter of 8 mm and length of 30 mm.

The prepared specimens were scanned prior to mechanical testing using μ-CT to determine trabecular microarchitecture. A 7.4 mm long region at the center of each sample was scanned at 20 μm isotropic resolution while submerged in PBS. Architecture was quantified using a model free method (Scanco Image Processing Language, Version 4.3) (Table 1). Mineral density levels were quantified using the scanner's calibration (Kazakia et al., 2008).

2.2. Mechanical testing

Specimens were embedded in brass endcaps to facilitate gripping during microdamage induction (Keaveny et al., 1994). The marrow was removed from the pore space using a water jet while the sample was submerged in water. The ends of the specimens were briefly soaked in ethanol to dry the surface prior to fixing in the endcaps using cyanoacrylate glue (Prism 401, Loctite, Newington, CT).

Specimens were subjected to sequential compressive and torsional overloads to induce microdamage (Fig. 1). The elastic and shear modulus were first measured in uniaxial compression to 0.4% strain and torsion to 0.7% shear strain, respectively. Microdamage was then induced by a compressive overload to 2% strain followed by a torsional overload to 4% shear strain at the cylinder surface. Hence, the principal compressive strain for both load cases was the same. Microdamage was labeled before and after each overload as described below. Mechanical tests were performed at room temperature at a strain rate of 0.5% s^{-1}. Compressive strains to 2% and shear strains below 0.7% were measured using a biaxial extensometer (Epsilon, Jackson, WY). The shear overloading exceeded the shear strain limits on the extensometer, and, as such, angular rotation data were acquired from the RVDT during this phase of the tests. Specimens were kept hydrated using saline-saturated gauze wrapped around the exposed length of the specimen. Data were collected at 200 Hz and were filtered using a low-pass filter (GCVSPL) to remove high-frequency noise. The elastic and shear moduli were measured prior to and following each overload. However, due to drift in the load cell, we were unable to obtain accurate post-damage mechanical properties, or to calculate the modulus loss to compare to the original properties as is normally done (Lambers et al., 2013; Wu et al., 2013).

2.3. Damage quantification

Damage was labeled by a sequence of calcium chelating fluorophores (O'Brien et al., 2003). Specimens were soaked in 0.5 mM alizarin complexone (ICN Biomedicals Inc., Aurora, OH) prior to mechanical testing to label microdamage that was present *in vivo* or during specimen preparation. After the compressive overload, specimens were stained with 0.5 mM xylenol orange (ICN Biomedicals Inc., Aurora, OH). Finally, after torsional overloading, specimens were stained with 0.5 mM calcein (ICN Biomedicals Inc., Aurora, OH). In each case, specimens were stained for 2 h under vacuum, and rinsed with deionized water after staining.

Following testing, specimens were dehydrated in a series of ethanol solutions ending with a 100% ethanol solution for 12 h. They were then embedded in transparent methyl methacrylate (MMA, Aldrich Chemical Company Inc., Milwaukee, WI) under vacuum. Two 200 μm thick sections of each core were cut along the long axis of the specimen using a diamond wire saw (DDK Inc., Wilmington, DE). The sections were mounted on glass slides using Eukitt mounting medium (Sigma-Aldrich Co., St. Louis, MO), and polished to a final thickness of approximately 150 μm starting with 600 grit paper and ending with 1/4 μm diamond paste (Buehler, Lake Bluff, IL).

Microdamage was quantified using epifluorescent microscopy. The sections were imaged at 100x magnification using UV excitation at an excitation wavelength of 365 nm (UV1A filter, Nikon Inc., Melville, NY). Images were captured over an 8×8 mm region in the center of the specimen using a CCD camera (QImaging, Surrey, BC, Canada), and composited into a single image (Photoshop, Adobe Systems, San Jose, CA). The outer 0.4 mm of the samples were excluded from the microdamage quantification to eliminate labeling of the surfaces cut during specimen preparation. The composite image was divided into three longitudinal regions of equal radius about the central axis of the specimen to identify regions of differing shear strain during torsion (Fig. 1) (Wang et al., 2005; Wang and Niebur, 2006; Wu et al., 2013).

The length of microcracks and area of diffuse damage regions were measured using ImageJ software (National Institute of Health, Bethesda, MD) as described previously (Wu et al., 2013). Briefly, bone area (B.Ar.) was found by thresholding the images. Diffuse damage area (Dx.Ar.) was identified as stained regions on the bone that did not present in a linear morphology, and was normalized by B.Ar. Linear microcracks were identified as stain that showed permeation into the bone and distinct edges that presented in a linear morphology (Fig. 1). Cracks in cross-hatched patterns were each counted as separate linear microcracks. The crack density (Cr.Dn.) was defined as the number of cracks normalized by bone area. The mean crack length (Cr.Ln.) for each sample was found by dividing the sum of the lengths of the cracks by the number of cracks. Crack surface density (Cr.S.Dn.), was found by multiplying the Cr.Ln. by the Cr.Dn.

Crack propagation was also quantified. Cracks with only one staining agent were assumed to be newly formed during the preceding load, while cracks showing more than one adjacent staining agent were assumed to have propagated from one loading mode to the next.

2.4. Statistical analysis

Statistical tests were performed using JMP IN 5.1 (SAS Institute Inc., Cary, NC). Parametric tests were applied to the microarchitecture measures, as all parameters were normally distributed and had a large sample size ($n > 30$). Microdamage was not normally distributed. Therefore, a Wilcoxon/Kruskal-Wallis test was used to determine differences between ovariectomy groups and specimen regions. ANCOVA regression was used to assess the effects of microarchitecture and pre-existing damage on quantified damage parameters, with ovariectomy status as a nominal factor. Damage and microarchitecture correlations were calculated for each site and ovariectomy group. A p-value of 0.05 was considered to be significant for all tests.

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