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Characterization of damage mechanisms associated with reference point indentation in human bone

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ABSTRACT

Measurement of bone mineral density (BMD) is the clinical gold standard in cases of compromised skeletal integrity, such as with osteoporosis. While BMD is a useful measurement to index skeletal health, it is also limited since it cannot directly assess any mechanical properties. The ability to directly assess mechanical properties of bone tissue would be clinically important. Reference point indentation (RPI) is a technology that has been designed to try and achieve this goal. While RPI has been shown to detect altered bone tissue properties, the underlying physical mechanism of these measurements has not been characterized. Thus, we designed a study whereby the contribution of (1) test cycle number and (2) test load level to RPI test-induced sub-surface damage was characterized and quantified. Standardized specimens were prepared from cadaveric human tibiae ($n = 6$), such that 12 replicates of each testing condition could be carried out. A custom rig was fabricated to accurately position and map indentation sites. One set of tests was carried out with 1, 5, 10, 15 and 20 cycles (Max Load: 8 N, Freq: 2 Hz), and a second set of tests was carried out with Load levels of 2, 4, 6, 8 or 10 N (Cycle number: 20, Freq: 2 Hz). The RPI parameter Loading Slope (LS) was cycle dependent at 5, 10, 15 and 20 cycles ($p < 0.05$). First Cycle Indentation Distance (ID 1st), Total Indentation Distance (TID), Mean Energy Dissipation (ED), First Cycle Unloading Slope (US 1st), Mean Unloading Slope (US) and LS were significantly different at 6, 8 and 10 N compared to 2 N ($p < 0.05$). From the histomorphometric measurements, damage zone span was significantly different after 5, 10, 15 and 20 cycles compared with 1 cycle while indent profile width and indent profile depth were significantly different at 10, 15 and 20 cycles ($p < 0.05$). With the load varying protocol, each of these parameters differed significantly at each increased load level (4, 6, 8, 10 N) compared with the basal level of 2 N ($p < 0.05$). The damage area parameter in both protocols was significantly different from baseline at the three upper levels tested (i.e. 10, 15, 20 cycles and 6, 8, 10 N, in cycle and load variant protocols, respectively). Specimens were scanned by micro-computed tomography, which showed no material or microstructural differences between samples, and processed for histological analyses and damage quantification. Consistent microdamage patterns were present with evidence of damage via compaction at the indented regions. While damage in the direction of loading was established early, the damage area then increased radially with cycle number. These data help to further understand the physical manifestations of RPI parameters and will help to further facilitate its use as a clinical diagnostic tool.

Introduction

Bone strength is influenced by both tissue mass and its quality [1,2]. Bone mineral density (BMD) is often used clinically as a surrogate for bone strength, and is most commonly measured by dual-energy X-ray absorptiometry (DEXA). BMD can be a highly useful measurement due to its relationship to bone strength and stiffness. However, despite its practicality and ubiquity, DEXA remains an indirect method of quantifying bone material properties, and thus is limited [3]. In a typical load-versus-deformation curve from a mechanical test of bone to failure, the curve can be separated into the pre- and post-yield regions for analytical purposes. BMD has quite good predictive ability in the pre-yield region, where it has been shown to correlate well with bone stiffness and strength [4,5]. However, the predictive precision of BMD for determining a change in fracture risk is less straightforward since there are other factors, such as tissue material properties, that contribute to fracture risk [6–8]. Thus, the ability to directly index material properties of bone in patients would be clinically important and would represent a significant advance in the field.

Gaining physical access to bone tissue for mechanical testing purposes in the clinical setting has traditionally been impossible, as traditional methods ultimately destroy the sample; hence, the popularity of indirect imaging techniques such as DEXA. Thus, there is a need for a better method of directly collecting mechanical information from bone; ideally these data would relate in some way to fracture toughness

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at the tissue level. Reference point indentation (RPI) is a recently developed technique [9,10] which is designed to achieve that goal in a relatively minimally-invasive manner by employing a test that is carried out trans-dermally, using a standard gauge needle, under local anesthesia [11]. Promising results have been reported in both human and animal models [11–15], where RPI measurement parameters appear to detect altered tissue properties, meaning it may be used as a diagnostic tool [16]. While a standardized testing method has not yet been established, various groups have been working towards optimizing the approach [13,17,18]. Although the parameters generated by the RPI method may be related to standard material properties, the underlying physical damage mechanisms on which these measurements are based have not yet been characterized. It has been shown that a physical impression of the probe tip can been seen, using electron microscopy, at the bone surface after RPI testing [11,14], suggesting that some kind of non-recoverable deformation or crack damage has occurred. However, details of the damage type that is produced have not yet been investigated. These details will be important in order to better understand and optimize the use of RPI in the future. Thus, we tested the hypothesis that sub-surface microdamage is caused beneath the probe tip during RPI testing, and that cycle number and load level contribute differentially to that resultant damage.

Methods

Sample preparation and microtomography

One block of bone was extracted initially from each donor ($n = 6$), using a standard band-saw with water irrigation, from the anteromedial aspect of the tibial diaphysis beginning 10 cm distal to the anterior tibial plateau. This site was chosen because it is close to the site used for clinical applications of RPI. Each block was then cut down further into four specimens, along the longitudinal axis of the bone, using a low-speed saw with a diamond-tipped cutting wheel with water-based irrigation (Isomet, Buehler, IL) (total samples, $n = 24$). Each sample was finished to final dimensions of 20 mm (length) × 3 mm (width) × 5 mm (depth). From each donor, two samples were randomly assigned to be tested with a load-variation protocol, while the remaining two were assigned to a cycle-variation protocol. All donors were female with a mean age of 79 years (ranged from 76 to 88 years). While donors had no indications of musculoskeletal pathologies, there was no information available on drug treatments, which may have affected bone properties, and it should be noted that this could be a source of variation in these data. Samples were then scanned by micro computed tomography (MicroCT) with the following settings: 100 kV voltage, 100 μA current, 2 K matrix, 0.4 degree steps, 13.5 μm pixel size (1132 SkyScan, Kontich, Belgium) for assessment of microstructural parameters and tissue mineral density (TMD). A standard global thresholding algorithm was used to separate bone from any soft tissues and the surrounding medium.

Reference point indentation

RPI testing of these specimens was performed using a BioDent™ *Hfc* instrument (Active Life Scientific, Inc., Santa Barbara, CA). The system is comprised of a reference probe, which is a modified hypodermic needle with an internal bore diameter of 380 μm. The end of the reference probe has a beveled tip and therefore can rest on the bone surface without causing damage during testing. However, this design may not always be sufficient to prevent damage to the tissue surface. The second component, called the test probe, is a custom-made cylindrical rod with a 375 μm diameter, a 2.5 μm tip radius, and a 90° conical angle. A custom specimen holder was designed and fabricated for these tests. A hydration bath with internal sample fixtures was secured to a linear XY translation stage (DS25, Newport, Franklin, MA) which had micrometer-based mechanical controls to allow precise and linear movement of the sample between tests. The entire unit was then fixed to the BioDent testing table. Once specimens were secured in the PBS hydration bath, the reference probe was positioned on the midline of the sample using the stage micrometer. In order to control for any potential damage caused by the resting reference probe, the head-unit was allowed to rest on the tissue surface under the same weight (1300 g, required for the maximum load (10 N) in our testing range) for all tests. Furthermore, the reference probe rested on the tissue surface for the same length of time (30 s) in every test. A pre-load protocol of three cycles at 0.5 N and a frequency of 2 Hz was performed to eliminate surface effects (e.g. unevenness, debris and soft-tissue).

Then, five indentations on that specimen (either force or cycle variants) were made along the mid-line with an interval of 1.2 mm between each test site (Fig. 1). This meant that each of the 24 bone samples received indentation tests at 5 equidistant sites along the midline of the sample. One group of samples ($n = 12$) was tested with constant cycle number and frequency (20 cycles, 2 Hz). On each sample, an indentation test was carried out with a maximum load of either 2, 4, 6, 8 or 10 N. Similarly, the other half of the samples were tested with a constant maximum load and frequency (8 N, 2 Hz) and on each sample and indentation test was carried out to a cycle number of 1, 5, 10, 15 and 20. Therefore, in all cases, there were twelve replicates of each testing condition.

The upper limit of 20 cycles was chosen since this was used in the in vivo applications [11] and the load level of 8 N was selected since this load is close to that used in vivo (11 N, [11]), while remaining in the range recommended by others in the literature (4–8 N, [14]). The reference probe was in contact with the bone surface for a total of 30 s per indentation in order to standardize testing and minimize any potential creep effects from the head unit. For the purposes of this study, seven parameters are reported in each test case as follows: First Cycle Indentation Distance (ID 1st), Total Indentation Distance (TID), Indentation Distance Increase (IDI), First Cycle Unloading Slope (US 1st), Mean Unloading Slope (US), Mean Loading Slope (LS) and Mean Energy Dissipation (ED). These parameters were taken directly as calculated from the manufacturer's software (BioDent, ActiveLife, Santa Barbara, CA).

Histological preparation and analyses

Immediately after indentation testing, samples were fixed in 10% neutral buffered formalin (NBF) and then dehydrated and stained en bloc with basic fuchsin in graded alcohol solutions for microdamage characterization and analyses. Once stained and dehydrated, samples were embedded in polymethylmethacrylate (PMMA) using established protocols [19]. Once fully polymerized, PMMA blocks were then trimmed to size and sequentially polished with silicon carbide paper of various grit sizes through the thickness of the sample to expose and study the profile of the indentation sites and any surrounding tissue damage beneath the periosteal surface (Fig. 1, right panel). Fluorescent images were obtained using reflected epifluorescence microscopy at 20× magnification (Olympus IX71, Center Valley, PA) and captured with a digital charge-coupled camera (ORCA-R2 C10600-10B, Hamamatsu Corporation, Middlesex, NJ) to visualize microdamage. Measurements of indentation profiles and damage zone dimensions were made by one user, blinded to groups, using ImageJ software (version 1.48, National Institutes of Health).

Statistical analysis

Initially, data were evaluated with the Kolmogorov–Smirnoff test to assess for normality of distribution. Analysis of variance (ANOVA) testing was utilized for the comparison of continuous variables among two or more groups when the data were normally distributed. Post-hoc Tukey testing was used to identify specific inter-group differences if ANOVA indicated that the null-hypothesis should be rejected. In order to assess associations among our testing factors we constructed and