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Reference point indentation is insufficient for detecting alterations in traditional mechanical properties of bone under common experimental conditions

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ABSTRACT

Reference point indentation (RPI) was developed as a novel method to assess mechanical properties of bone *in vivo*, yet it remains unclear what aspects of bone dictate changes/differences in RPI-based parameters. The main RPI parameter, indentation distance increase (IDI), has been proposed to be inversely related to the ability of bone to form/tolerate damage. The goal of this work was to explore the relationshipre-intervention RPI measurebetween RPI parameters and traditional mechanical properties under varying experimental conditions (drying and ashing bones to increase brittleness, demineralizing bones and soaking in raloxifene to decrease brittleness). Beams were machined from cadaveric bone, pre-tested with RPI, subjected to experimental manipulation, post-tested with RPI, and then subjected to four-point bending to failure. Drying and ashing significantly reduced RPI's IDI, as well as ultimate load (UL), and energy absorption measured from bending tests. Demineralization increased IDI with minimal change to bending properties. *Ex vivo* soaking in raloxifene had no effect on IDI but tended to enhance post-yield behavior at the structural level. These data challenge the paradigm of an inverse relationship between IDI and bone toughness, both through correlation analyses and in the individual experiments where divergent patterns of altered IDI and mechanical properties were noted. Based on these results, we conclude that RPI measurements alone, as compared to bending tests, are insufficient to reach conclusions regarding mechanical properties of bone. This proves problematic for the potential clinical use of RPI measurements in determining fracture risk for a single patient, as it is not currently clear that there is an IDI, or even a trend of IDI, that can determine clinically relevant changes in tissue properties that may contribute to whole bone fracture resistance.

1. Introduction

Assessment of bone mechanical properties is an essential part of determining skeletal fracture resistance. While a number of techniques exist to measure bone mechanical properties in preclinical studies [1], clinical assessment of bone fracture resistance/mechanical properties presents challenges. Reference point indentation (RPI) was developed as a novel method to assess mechanical properties of bone *in vivo* [2,3]. This technique has been utilized in both patients (for review see [4]) and in several pre-clinical models [5–7], as well as multiple *ex vivo* studies [8–12] and has demonstrated the ability to separate disparate group means across varying conditions. However, it remains unclear what mechanical and/or morphological aspects of bone dictate changes/differences in RPI-based parameters.

Initial studies with Biodent, the early generation RPI device, documented an inverse relationship between bone toughness (either from fracture toughness tests or estimated from bending tests) and indentation distance increase (IDI), one of the main RPI variables [11,13,14] Based on traditional mechanical tests, lower fracture toughness is indicative of a material in which cracks propagate more easily [15–18] and thus a high IDI has been suggested to represent a scenario where cracks more easily propagate with the subsequent cyclic loading of the probe apparatus. Surprisingly little RPI data exist for conditions that have known effects on material properties of bone.

The goal of this work was to explore the relationship between RPI parameters and traditional mechanical properties under varying experimental conditions. Specifically, we chose interventions expected to embrittle (dehydration and ashing) and toughen (demineralization and

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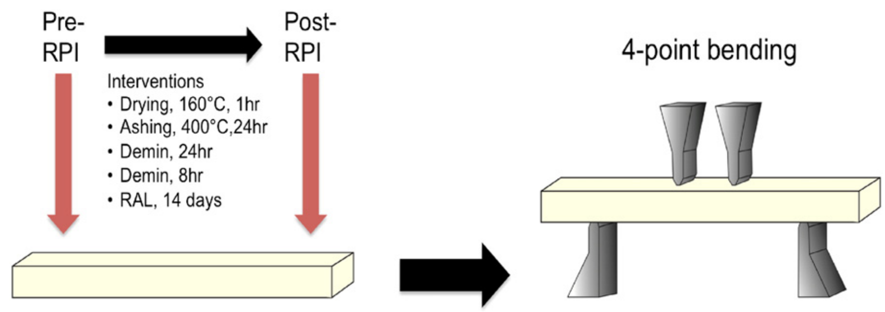

Fig. 1. Experimental schematic of RPI testing and beam manipulation.

Table 1
Variation of RPI measurements along the length of each of 6 beams at 5 locations (3 RPI measurements at each location).

	Location 1		Location 2		Location 3		Location 4		Location 5	
	TID (μm)	IDI (μm)	TID (μm)	IDI (μm)	TID (μm)	IDI (μm)	TID (μm)	IDI (μm)	TID (μm)	IDI (μm)
Specimen 1	88.97 ± 2.95	11.13 ± 1.79	99.73 ± 8.13	12.79 ± 1.59	92.49 ± 3.32	12.16 ± 1.21	93.43 ± 15.07	13.01 ± 2.54	96.12 ± 8.93	12.66 ± 1.12
Specimen 2	92.48 ± 1.42	12.83 ± 0.06	106.38 ± 8.74	11.35 ± 2.65	102.96 ± 16.42	13.23 ± 2.64	102.04 ± 10.40	12.00 ± 0.91	98.47 ± 6.19	13.11 ± 3.51
Specimen 3	95.33 ± 12.73	11.61 ± 4.01	101.66 ± 5.56	11.71 ± 2.01	87.47 ± 1.37	10.90 ± 1.90	90.34 ± 4.62	10.83 ± 1.20	85.78 ± 0.37	11.18 ± 1.13
Specimen 4	99.94 ± 5.85	12.56 ± 0.24	93.17 ± 8.62	12.03 ± 1.09	105.60 ± 9.50	13.73 ± 3.57	97.98 ± 4.43	10.25 ± 1.87	99.47 ± 5.45	9.93 ± 1.19
Specimen 5	88.49 ± 2.81	11.24 ± 0.07	105.40 ± 10.65	11.11 ± 0.76	90.96 ± 3.45	12.06 ± 1.32	95.06 ± 8.50	11.13 ± 1.66	95.39 ± 6.55	13.23 ± 3.01
Specimen 6	92.10 ± 5.52	13.09 ± 2.69	104.50 ± 4.59	12.59 ± 6.19	94.18 ± 8.75	12.19 ± 3.84	107.74 ± 21.81	12.32 ± 0.65	85.12 ± 1.33	11.36 ± 1.56
Average	93.24 ± 3.94	12.09 ± 0.79	101.81 ± 4.47	11.93 ± 0.61	95.61 ± 6.50	12.38 ± 0.91	97.76 ± 5.76	11.59 ± 0.94	93.39 ± 5.78	11.91 ± 1.19

Data presented as mean ± SD. There was no significant different across the five locations. TID, total indentation distance, IDI, indentation distance increase.

raloxifene) the tissue. Our working hypothesis was that conditions exist in which RPI variables and toughness, as estimated by bending tests, were not inversely related.

2. Methods

All experiments utilized prismatic beams machined from cadaveric bone. Fresh-frozen long bones (femora) were collected from four cadavers (3 males & 1 female aged 76–85) donated through the Indiana University body donation program. Bones were processed, by low speed saw (Buehler) and milling (Sherline) under constant irrigation, to final dimensions of ~35 mm × ~5 mm × ~2 mm. Bones were then distributed to two different experiments.

2.1. Reference point indentation (RPI)

The surface tissue mechanical properties of the rectangular beams were assessed using Reference Point Indentation or RPI (Biodent Hfc, Active Life Scientific, Santa Barbara, CA). Each RPI measurement was performed as a series of 10 testing cycles at 10 N and 2 Hz. Although several different test settings have been used in the literature, these parameters generally matched those previously published. From the resulting force-displacement curves, we used a custom MATLAB program to calculate the total indentation distance (TID) and the indentation distance increase (IDI), as described previously [5–7]. These parameters were chosen as the focus as they are the most prominently discussed parameters in the RPI literature. When multiple tests were done in a certain location (pre or post intervention), each test was performed at least a millimeter away from all the other tests to avoid the overlap of damage fields generated by testing. The data within each location was averaged to get a single representative value. For all experiments where interventions were used, pre-intervention tests were done at one end while post-interventions tests were done on the opposite ends. All RPI tests were done outside of the bending support fixtures so as to not interfere with the bending tests.

2.2. Experiment 1: variation in RPI measures

Beams (n = 6) were tested along the length of each specimen at five locations (~6 mm apart). Three RPI measurements, each a series of 10 testing cycles at 10 N and 2 Hz, were conducted at each location. Means and standard deviation were calculated to evaluate the variability of RPI tests across the length of the beam. This experiment was necessary to determine if our setup for experiment two was valid (i.e. properties are assumed to be uniform across the beams).

2.3. Experiment 2: effects of material manipulations on RPI and 4 point bending properties

Beams were subjected to 6 RPI measurements at one end (Fig. 1). The beams were then subjected to one of the following manipulations:

1) Dried in oven at 160 °C for one hour (n = 12) or 800 °C for 24 h (n = 12) in order to remove water [19] or water plus all organic material.
2) Placed in 14% EDTA buffered to pH 7.4 on a rocker at room temperature for 8 (n = 12) or 24 h (n = 12).
3) Soaked in PBS-raloxifene solution (2 μm dissolved in DMSO with 1% penicillin–streptomycin) at 37 °C for 14 days (n = 6) [20]. A separate set of controls (n = 8) that were soaked in control solution (PBS-DMSO) were used for comparison of this intervention.
4) Control beams in which no intervention was used (n = 8).

Table 2
Effects of drying and ashing on RPI parameters.

	Total indentation distance (TID)—μm	Indentation distance increase (IDI)—μm
Pre-drying	100.29 ± 11.99	13.79 ± 2.21
Post-drying	53.10 ± 6.16*	5.34 ± 1.60*
Pre-ashing	85.32 ± 7.68	12.11 ± 1.50
Post-ashing	21.46 ± 2.06*	1.15 ± 0.45*

* $p < 0.05$ versus pre-intervention RPI measurements.