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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 relationship between 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.

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Keywords

Biodent; toughness; bending; material properties

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 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.

Methods

All experiments utilized prismatic beams machined from cadaveric bone. Fresh-frozen long bones (femora) were collected from four cadavers (3 male & 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 x ~5 mm x ~2 mm. Bones were then distributed to two different experiments.

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.

Experiment 1: Variation in RPI measures—Beams (n=6) were tested along the length of each specimen at five locations (~ 6mm 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).

Experiment 2: Effects of material manipulations on RPI and 4 point bending properties—Beams were subjected to 6 RPI measurements at one end (Figure 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 hours (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 hours (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).

Following each intervention, RPI measurements were made on the opposite side of each of the beams (n=6 indents). The beams were then tested to failure under four-point bending.

Four-point bending

Structural mechanical properties of the beams were determined by four-point bending. The surface was placed on two lower supports with a span length of 12 mm and an upper span length of 4 mm. Specimens were loaded to failure at a rate of 2 mm/min, producing a force-displacement curve for each sample. Structural mechanical properties were obtained directly from these curves, whereas apparent material properties were derived from the force-displacement curves, cross-sectional moments of inertia, and the distances from the centroid to the tensile surface using standard beam-bending equations for four-point bending [1] incorporated in a custom MATLAB program.

Statistics

All analyses were performed using the Statistics Toolbox in MATLAB software. A one-way ANOVA was utilized to compare the different locations in experiment one. Paired Student t-tests were utilized to compare RPI measurements before and after interventions. Comparisons of mechanical testing data between groups were made with unpaired Student's t-tests within the individual experiments. The bivariate relationship between IDI and toughness data was evaluated using the Pearson's product-moment correlation algorithm. *A priori* α-levels were set at 0.05 to determine significance.

Results

Variation of RPI measurements along the length of a beam

Our main experimental design utilized pre/post measures on beams that were subjected to varying interventions. To assist in our interpretation, we wanted to understand the variability in properties across the length of the prismatic beam specimen. There was no significant difference in IDI or TID among the five locations (Table 1 and supplementary table 1). More specific analysis between the two sites tested pre- and post-intervention in experiment 2 also showed no significant difference among properties.

Effects of interventions on RPI and traditional mechanical properties

Removal of bone water (drying) or water plus organic material (ashing) caused a significant reduction in both IDI and TID compared to baseline measures (Table 2). Both interventions also showed significantly lower mechanical properties, most notably post-yield properties, compared to control beams (Tables 2, 3 and Figure 2).

Demineralization, both for 8hrs and 24hrs, resulted in increased IDI and TID compared to baseline measures (Table 4). Mechanical testing of the demineralized beams showed no significant change in toughness for either treatment relative to control beams (Table 3 and Figure 2).

Soaking in raloxifene resulted in no difference in RPI measures between RAL and control tissue (no pre-soaking RPI measures were made on these beams) (Table 5). Toughness of RAL-soaked beams was non-significantly higher compared to controls (p=0.3777) (Table 3).

Across the drying and demineralization experiments, there was a significant positive linear correlation between IDI and toughness of all beams (r = 0.5840, p<0.001). When ashed beams were excluded – the relationship was no longer significant (r = 0.2174, p=0.196). (Figure 3).

Discussion

Over the past decade, a significant amount of work has gone into understanding reference point indentation. The technique shows promise, given its ability in numerous studies to differentiate either clinical populations (see review [4]) or preclinical interventions [5]. One limitation to more widespread acceptance is that questions exist concerning what the parameters generated by RPI mean in the context of traditional mechanical properties. To this end, several studies have worked to compare RPI to both bending tests and fracture toughness tests. These studies have shown that RPI parameters (mainly IDI and TID) are either inversely related [10,11,13,14,21] or not related [11,22] to traditional mechanical variables. Despite these studies being well designed and conducted, no study to date has investigated how manipulation of the tissue, in ways that have known effects on traditional mechanical properties, affects RPI outcomes. The current study fills this void and provides clear data showing that traditional mechanical properties and RPI parameters can be differentially changed in various conditions and that the relationship between the two testing methods is complex.

Indentation distance increase (IDI) has previously been shown to correlate inversely with mechanical properties, namely toughness, by both our group and others. The explanation suggested is that when tissue is more brittle (less tough) it is easier to propagate damage generated during RPI testing. In the current study, we used drying and ashing of bone, two techniques known to embrittle the tissue and for which we clearly showed reductions in toughness with bending tests. The expectation was that this would increase IDI and TID. Interestingly, the effects on RPI properties were opposite of what the above explanation would predict as both IDI and TID were reduced. These results also differ from those of earlier work with dryed bone [2], although that study used an early generation device, bovine bone (which is typically plexiform instead of osteonal) and tested the bones submerged thus at least partially offsetting the effects of drying. We hypothesize that this observation is related to the inability of the system to generate enough force to generate damage in the tissue. Although we acknowledge that the ashing group is an extreme example of altered tissue properties that is non-physiological, changes in bone hydration are known to occur *in vivo* [23,24] and thus could be relevant to clinical/experimental conditions.

Our second experimental condition utilized demineralization to alter tissue properties with the expectation that RPI properties would be reduced. Using a low concentration of EDTA for times sufficient to primarily alter surface properties, we were able to produce modest alterations to post-yield properties of whole bone mechanical tests. Yet because the predominant region affected by the demineralization was the surface, we saw significant increases in RPI properties. As noted above, the physiological relevance of this demineralization is not directly clear but we propose that this could be the type of response one would see if RPI was applied to a bone surface that was actively forming and therefore consists of osteoid/low mineralized bone.

Finally, we studied bone that was soaked in raloxifene. Our group has shown that raloxifene modulates hydration (positively) when cortical bone beams are exposed in vitro [20]. In the current work, we reproduce these data (although the difference was not statistically significant), thus resulting in tissue that would be less likely to be damaged (suggesting reduced IDI and TID). Yet RPI results showed no difference from pre-tests. These data are interesting given our recent work showing that raloxifene increases bone hydration *in vivo* [25] and reduces IDI *in vivo* [5]. Although these data were collected in two separate cohorts of animals, we hypothesized the results were linked. These new controlled *in vitro* tests (current study) suggest that our interpretation of the *in vivo* RPI results was oversimplified and could possibly be due to some other aspect, perhaps alterations in surface mineralization or other properties.

A key aspect of the experiments described herein is that we show an opposite relationship between IDI and traditional mechanical properties as compared to other recent studies. Whereas the previous work suggests that a decrease in IDI indicates an increase in material toughness, this work shows that a material with lower toughness can have a lower IDI. These data highlight the already prevalent idea that RPI measurements are complex with respect to translating to traditional mechanical properties and that we still do not understand the tissue-level properties that affect RPI values [4]. It has been hypothesized that increased bone fragility translates to a higher IDI. This current study shows that bone fragility is not the only factor that can dictate results of RPI measurements and that a high IDI can exist in the context of bone with normal mechanical properties. This proves problematic for the potential clinical use of RPI measurements in determining fracture risk for a single patient as it remains unclear if it can determine clinically relevant changes in tissue properties that may contribute to whole bone fracture resistance. Whether or not further work will show clear trends in specific disease states, or if pairing RPI measurements with other tests (i.e. imaging) will improve RPI measurement interpretation is yet unclear.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Turner CH, Burr DB. Basic biomechanical measurements of bone: a tutorial. Bone. 1993; 14:595–608. [PubMed: 8274302]
2. Hansma PK, Turner PJ, Fantner GE. Bone diagnostic instrument. Review of Scientific …. 2006; 77:075105.10.1063/1.2221506
3. Hansma P, Turner P, Drake B, Yurtsev E, Proctor A, Mathews P, et al. The bone diagnostic instrument II: Indentation distance increase. Rev Sci Instrum. 2008; 79:064303–064303–8.10.1063/1.2937199 [PubMed: 18601422]
4. Allen MR, McNerny EM, Organ JM, Wallace JM. True Gold or Pyrite: A Review of Reference Point Indentation for Assessing Bone Mechanical Properties In Vivo. J Bone Miner Res. 2015; 30:1539–1550.10.1002/jbmr.2603 [PubMed: 26235703]
5. Aref M, Gallant MA, Organ JM, Wallace JM, Newman CL, Burr DB, et al. In vivo reference point indentation reveals positive effects of raloxifene on mechanical properties following 6 months of treatment in skeletally mature beagle dogs. Bone. 2013; 56:449–453.10.1016/j.bone.2013.07.009 [PubMed: 23871851]
6. Allen MR, Newman CL, Smith E, Brown DM, Organ JM. Variability of in vivo reference point indentation in skeletally mature inbred rats. Journal of Biomechanics. 2014; 47:2504–2507.10.1016/j.jbiomech.2014.04.035 [PubMed: 24856912]
7. Srisuwananukorn A, Allen MR, Brown DM, Wallace JM, Organ JM. In vivo reference point indentation measurement variability in skeletally mature inbred mice. BoneKEy Reports. 2015; 4:1–5.10.1038/bonekey.2015.81

8. Setters A, Jasiuk I. Towards a standardized reference point indentation testing procedure. Journal of the Mechanical Behavior of Biomedical Materials. 2014; 34:57–65.10.1016/j.jmbbm.2014.01.012 [PubMed: 24556325]

9. Milovanovic P, Rakocevic Z, Djonic D, Zivkovic V, Hahn M, Nikolic S, et al. Nano-structural, compositional and micro-architectural signs of cortical bone fragility at the superolateral femoral neck in elderly hip fracture patients vs. healthy aged controls. Experimental Gerontology. 2014; 55:19–28.10.1016/j.exger.2014.03.001 [PubMed: 24614625]

10. Granke M, Coulmier A, Uppuganti S, Gaddy JA, Does MD, Nyman JS. Insights into reference point indentation involving human cortical bone_ Sensitivity to tissue anisotropy and mechanical behavior. Journal of the Mechanical Behavior of Biomedical Materials. 2014; 37:174–185.10.1016/j.jmbbm.2014.05.016 [PubMed: 24929851]

11. Granke M, Makowski AJ, Uppuganti S, Does MD, Nyman JS. Identifying novel clinical surrogates to assess human bone fracture toughness. J Bone Miner Res. 2015:n/a–n/a.10.1002/jbmr.2452

12. Karim L, Van Vliet M, Bouxsein ML. Comparison of cyclic and impact-based reference point indentation measurements in human cadaveric tibia. Bone. 2015:1–6.10.1016/j.bone.2015.03.021

13. Gallant MA, Brown DM, Organ JM, Allen MR, Burr DB. Reference-point indentation correlates with bone toughness assessed using whole-bone traditional mechanical testing. Bone. 2013; 53:301–305.10.1016/j.bone.2012.12.015 [PubMed: 23274349]

14. Katsamenis OL, Jenkins T, Thurner PJ. Toughness and damage susceptibility in human cortical bone is proportional to mechanical inhomogeneity at the osteonal-level. Bone. 2015; 76:158–168.10.1016/j.bone.2015.03.020 [PubMed: 25863123]

15. Bonfield W, Datta PK. Fracture toughness of compact bone. Journal of Biomechanics. 1976; 9:131–134. [PubMed: 1254608]

16. Currey JD. Bone strength: what are we trying to measure? Calcif Tissue Int. 2001; 68:205–210.10.1007/s002230020040 [PubMed: 11353945]

17. Currey JD. How Well Are Bones Designed to Resist Fracture? J Bone Miner Res. 2003; 18:591–598.10.1359/jbmr.2003.18.4.591 [PubMed: 12674319]

18. Burr DB. Why bones bend but don't break. J Musculoskelet Neuronal Interact. 2011; 11

19. Wang X, Bank RA, Tekoppele JM, Agrawal CM. The role of collagen in determining bone mechanical properties. J Orthop Res. 2001; 19:1021–1026.10.1016/S0736-0266(01)00047-X [PubMed: 11781000]

20. Gallant MA, Brown DM, Hammond M, Wallace JM, Du J, Deymier-Black AC, et al. Bone cell-independent benefits of raloxifene on the skeleton: A novel mechanism for improving bone material properties. Bone. 2014; 61:191–200.10.1016/j.bone.2014.01.009 [PubMed: 24468719]

21. Diez-Perez A, Güerri R, Nogues X, Cáceres E, Peña MJ, Mellibovsky L, et al. Microindentation for in vivo measurement of bone tissue mechanical properties in humans. J Bone Miner Res. 2010; 25:1877–1885.10.1002/jbmr.73 [PubMed: 20200991]

22. Carriero A, Bruse JL, Oldknow KJ, Millán JL, Farquharson C, Shefelbine SJ. Reference point indentation is not indicative of whole mouse bone measures of stress intensity fracture toughness. Bone. 2014; 69:174–179.10.1016/j.bone.2014.09.020 [PubMed: 25280470]

23. Granke M, Does MD, Nyman JS. The Role of Water Compartments in the Material Properties of Cortical Bone. Calcif Tissue Int. 2015; 97:292–307.10.1007/s00223-015-9977-5 [PubMed: 25783011]

24. Manhard MK, Horch RA, Gochberg DF, Nyman JS, Does MD. In Vivo Quantitative MR Imaging of Bound and Pore Water in Cortical Bone. Radiology. 2015:140336–9.10.1148/radiol.2015140336

25. Allen MR, Territo PR, Lin C, Persohn S, Jiang L, Riley AA, et al. In Vivo UTE-MRI Reveals Positive Effects of Raloxifene on Skeletal-Bound Water in Skeletally Mature Beagle Dogs. J Bone Miner Res. 2015; 30:1441–1444.10.1002/jbmr.2470 [PubMed: 25644867]

Highlights

1. The relationship between Reference point indentation and traditional mechanical properties was explored under varying experimental conditions.
2. Drying and ashing significantly reduced RPI's indentation distance increase (IDI), and energy absorption from bending
3. Partial demineralization increased IDI with minimal change to bending properties.
4. *Ex vivo* soaking in raloxifene had no effect on IDI but tended to enhance post-bending properties
5. These data suggest that RPI, as compared to bending tests, is insufficient to determine mechanical properties of bone.

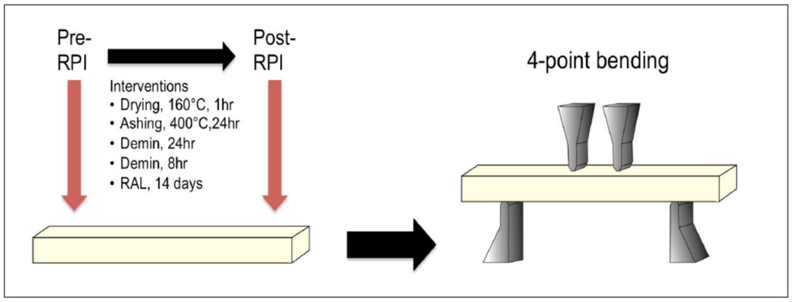

Figure 1.

Experimental schematic of RPI testing and beam manipulation.

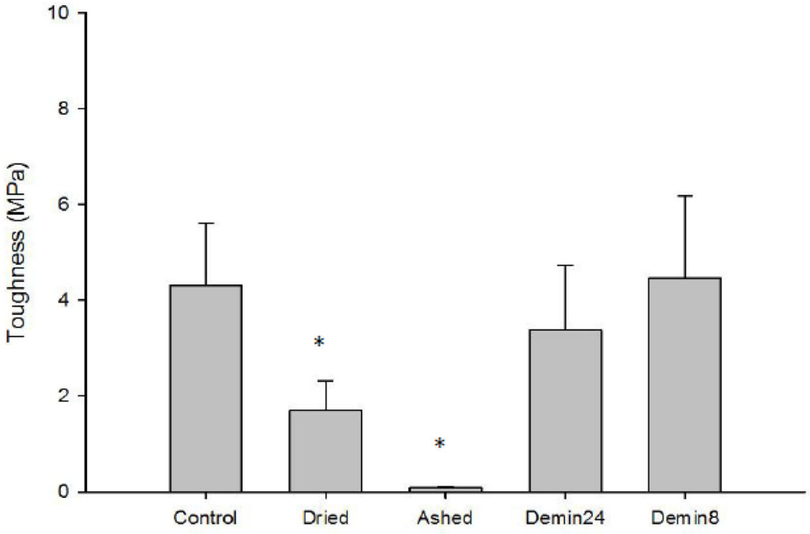

Figure 2.

Mean toughness for each the groups. Mean + SD *p<0.05 compared to control group

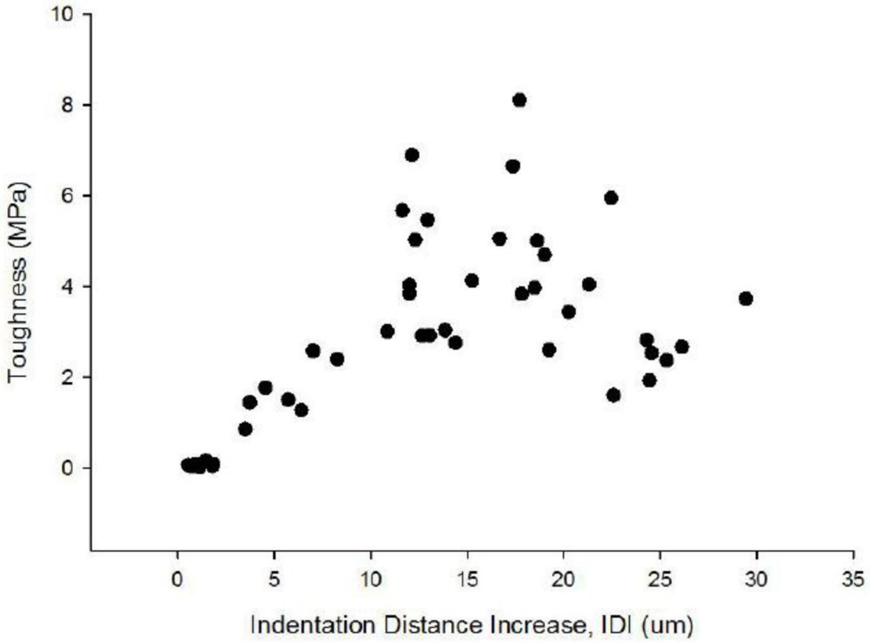

Figure 3.

Linear relationship between IDI and toughness. Across all experiments experiments, there was a significant positive linear correlation between IDI and toughness of all beams (r = 0.5840, p<0.001). When ashed beams were excluded – the relationship was no longer significant (r = 0.2174, p=0.196). Note – the cloud of data points near an IDI and toughness of zero represent exclusively the ashed beams (n=12)

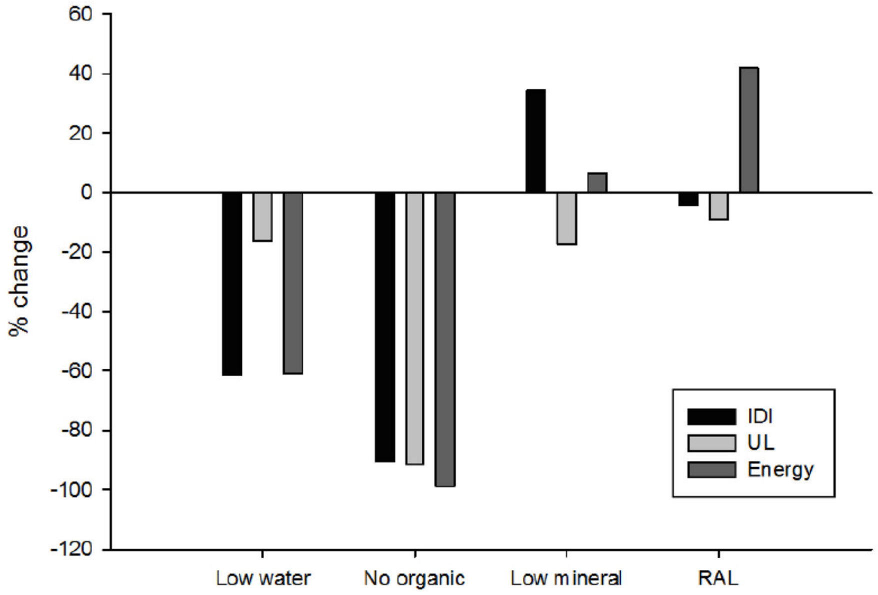

Figure 4.

Summary of experimental results for indentation distance increase (IDI), ultimate load (UL) and energy absorption. IDI displayed as percent change from pre to post-treatment. UL and Energy are displayed as percent change from four-point bending data in appropriate control group.

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

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

Table 3

Summary of mechanical property results from bending tests

	Yield Force (N)	Ultimate Force (N)	Displacement to Yield (mm)	Postyield Displacement (mm)	Total Displacment (mm)	Stiffness (N/mm)	Work to Yield (mJ)	Postyield Work (mJ)	Total Work (mJ)	Yield Stress (MPa)	Ultimate Stress (MPa)	Strain to Yield (me)	Total Strain (me)	Modulus (GPa)	Resilience (MPa)	Toughness (MPa)
Control	231.89 ± 28.08	318.28 ± 51.85	329.87 ± 18.58	206.84 ± 78.40	536.70 ± 79.42	834.19 ± 58.39	38.51 ± 6.47	59.02 ± 29.37	97.524 ± 31.63	135.96 ± 23.88	185.43 ± 29.094	25097 ± 1458	40971 ± 7067	6.45 ± 1.11	1.713 ± 0.327	4.306 ± 1.31
Dried	266.76 ± 54.41	266.76 ± 54.41	311.68 ± 54.41	0*	311.68 ± 54.41*	962.13 ± 52.70*	38.20 ± 13.28	0*	38.20 ± 13.28*	155.97 ± 37.24	155.969 ± 37.235	23716 ± 3021	23716 ± 3021*	7.37 ± 0.93	1.70 ± 0.62	1.70 ± 0.62*
Ashed	27.57 ± 9.43*	27.57 ± 9.43*	66.33 ± 15.53*	0*	66.33 ± 15.53*	404.09 ± 91.04*	0.93 ± 0.57*	0*	0.93 ± 0.57*	41.46 ± 9.50*	41.46 ± 9.50*	3682 ± 1192*	3682 ± 1192*	11.55 ± 3.38*	0.08 ± 0.04*	0.08 ± 0.04*
Demin - 24hr	141.12 ± 47.80*	175.44 ± 66.27*	477.53 ± 53.00*	266.44 ± 108.01	743.98 ± 120.83*	372.36 ± 138.51*	31.19 ± 9.91	43.46 ± 25.51	74.64 ± 32.79	84.82 ± 23.90*	105.45 ± 33.77*	35798 ± 5365*	55606 ± 9907*	3.01 ± 0.95*	1.41 ± 0.40*	3.37 ± 1.37
Demin - 8hr	223.43 ± 55.22	263.36 ± 79.47	496.47 ± 32.44*	189.90 ± 92.68	686.37 ± 116.29*	515.10 ± 98.18*	54.23 ± 16.23*	49.58 ± 33.62	103.81 ± 46.53	122.52 ± 12.94	143.49 ± 21.08*	39144 ± 5905*	54264 ± 12712*	3.65 ± 0.40*	2.34 ± 0.50*	4.46 ± 1.72
Control - VEH	52.32 ± 24.85	73.04 ± 31.35	293.26 ± 24.79	647.58 ± 234.97	940.84 ± 236.75	202.43 ± 84.65	8.59 ± 4.25	39.03 ± 11.50	47.622 ± 15.330	65.52 ± 31.68	91.30 ± 39.35	14747 ± 3778	45474 ± 6682	4.86 ± 1.23	0.58 ± 0.36	3.11 ± 1.52
RAL	43.12 ± 11.61	66.271 ± 21.51	293.50 ± 28.15	983.69 ± 185.92#	1277.20 ± 191.23#	175.94 ± 58.54	6.96 ± 1.59	60.57 ± 26.03	67.529 ± 27.332	54.72 ± 13.12	84.05 ± 24.85	13567 ± 1208	59718 ± 13418	4.69 ± 0.86	0.42 ± 0.12	4.12 ± 2.00

*p<0.05 versus Control

#p<0.05 versus Control-VEH

Table 4

Effects of surface demineralization (8hr and 24hr) on RPI parameters, pre-drying and post-drying RPI measurements

	Total Indentation Distance (TID) - µm	**Indentation Distance Increase (IDI) - µm**
Pre-demineralization (8hr)	102.06 ± 8.01	13.03 ± 1.50
Post-demineralization (8hr)	142.31 ± 19.49 *	17.49 ± 3.59 *
Pre-demineralization (24hr)	86.63 ± 23.36	13.62 ± 3.83
Post-demineralization (24hr)	192.57 ± 40.79 *	21.88 ± 5.09 *

* p<0.05 versus pre-intervention RPI measurements

Table 5

Effects of soaking beams in raloxifene for 14 days on RPI parameters

	Total Indentation Distance (TID) - μm	**Indentation Distance Increase (IDI) - μm**
Control/Vehicle	11.61 ± 1.42	90.48 ± 9.75
Raloxifene	11.12 ± 3.59	94.05 ± 5.25