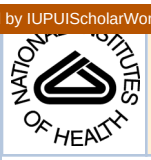

Published in final edited form as:
Bone. 2013 March ; 53(1): 301–305. doi:10.1016/j.bone.2012.12.015.

Reference-Point Indentation Correlates with Bone Toughness Assessed Using Whole-Bone Traditional Mechanical Testing

Maxime A. Gallant, **Drew M. Brown**, **Jason M. Organ**, **Matthew R. Allen**, and **David B. Burr**
Department of Anatomy and Cell Biology, Indiana University School of Medicine, 635 Barnhill Dr, MS-5035, Indianapolis, IN, 46202

Maxime A. Gallant: maxgalla@iupui.edu; Drew M. Brown: drebrown@iupui.edu; Jason M. Organ: jorgan@iupui.edu; Matthew R. Allen: matallen@iupui.edu; David B. Burr: dburr@iupui.edu

Abstract

Traditional bone mechanical testing techniques require excised bone and destructive sample preparation. Recently, a cyclic-microindentation technique, reference-point indentation (RPI), was described that allows bone to be tested in a clinical setting, permitting the analysis of changes to bone material properties over time. Because this is a new technique, it has not been clear how the measurements generated by RPI are related to the material properties of bone measured by standard techniques. In this paper, we describe our experience with the RPI technique, and correlate the results obtained by RPI with those of traditional mechanical testing, namely 3-point bending and axial compression. Using different animal models, we report that apparent bone material toughness obtained from 3-point bending and axial compression is inversely correlated with the indentation distance increase (IDI) obtained from RPI with r^2 values ranging from 0.50 to 0.57. We also show that conditions or treatments previously shown to cause differences in toughness, including diabetes and bisphosphonate treatment, had significantly different IDI values compared to controls. Collectively these results provide a starting point for understanding how RPI relates to traditional mechanical testing results.

Keywords

Bone; mechanical testing; material properties; reference-point indentation; toughness

1. Introduction

Mechanical testing of bones has become a standard outcome when investigating the effect of genetic manipulation or pharmacological intervention on bone properties. Many factors influence the outcome of mechanical testing of a bone sample: geometry, architecture, degree of mineralization, properties of the organic matrix, and hydration among those. Whole bone mechanical testing is routinely performed on rodent samples while microbeam testing and nanoindentation are mostly performed on smaller samples taken from larger animals. Whole bone mechanics can be transformed into material properties using standard

Corresponding author: David B. Burr, Department of Anatomy and Cell Biology, Indiana University School of Medicine, 635 Barnhill Dr, MS-5035, Indianapolis, IN, 46202, Tel: 317-274-7434, FAX: 317-278-2040.

The authors declare no other conflict of interest.

equations accounting for size and shape [1]. These tissue properties provide intrinsic estimates of strength (stress), elasticity (modulus) and energy to fracture (toughness) (Figure 1a). However, one of the downsides of mechanical testing is that whether it is 3 or 4-pt bending of whole bones or machined specimens, axial tension/compression or torsion testing, these tests can only be performed *ex vivo* and are destructive.

Recently, reference-point indentation (RPI) was introduced as a new way to test bone material properties using cyclic micro-indentation [2–4]. An advantage of this technique is that it does not critically damage the samples and theoretically can be performed repeatedly over time to sample longitudinal changes. RPI's main outcome is Indentation Distance Increase (IDI) [2], which is the absolute penetration depth increase from the 1st to the last cycle of each testing session (Fig. 1b). Other outcomes of interest are the 1st cycle indentation distance (ID), the total indentation distance (TID) and the 1st cycle unloading slope (US), an indicator of material stiffness. Because this is a relatively new technique, however, it is not clear how these variables are related to the standard material properties that define strength, modulus and toughness.

In this report, we investigate the relationship between RPI and traditional mechanical testing techniques using two animal models previously shown by our lab to have altered mechanical properties. Our results show that RPI measurements show group differences consistent with traditional mechanical testing techniques and significantly correlate to the apparent tissue-level properties measured by these standard techniques.

2. Material and methods

2.1 Bone samples

Bone samples were collected from previous studies conducted in our lab. All bones were kept frozen (−20°C) in saline-soaked gauze until testing. Femora (n=7–11/group), and 3rd and 4th lumbar vertebrae (n=11–12/group) were used from type 2 diabetic ZDSD male rats (PreClinOmics, Indianapolis, IN) and from control CD male rats (CD® IGS, Charles Rivers). The ZDSD and CD rats were fed a high fat diet (start at 20 weeks of age) and only the ZDSD rats were clinically diabetic for 10 weeks. ZDSD rats and CD rats were sacrificed at 32 wk of age. We also used the 9th left ribs (n=9–10/group) from skeletally mature female beagles that had been treated daily with saline (1 ml/kg) or alendronate (1.0 mg/kg) for three years before sacrifice [5]. The rib samples were kept at −20°C in saline-soaked gauze for ~3.5 yrs (time of sacrifice to time of RPI testing). All animal experiments were approved by our university's Animal Care and Use Committee.

2.2 Mechanical testing

Rat femoral mid-diaphyseal mechanical properties were measured via a three-point bending test using standard methods [1]. Briefly, bones were equilibrated to room temperature and placed posterior side down on the bottom support (18 mm span) of a servohydraulic test system (MTS Bionix II Test System, MTS Systems, Eden Prairie, MN). Bones were loaded centrally using a cross-head speed of 2 mm/min, and force vs. displacement data were collected at 10 Hz. The force-displacement points were converted to stress-strain data using standard beam bending equations [1], and material bone properties (ultimate stress, modulus, and modulus of toughness) were calculated using midshaft geometry from microCT scans.

LV_4 vertebral bodies were tested by axial compression loading to failure. Prior to testing, the LVs had their processes and end-plates removed (parallel cuts), leaving an intact vertebral body (± 3 mm in height). Testing was performed at a rate of 0.5 mm/min, and the load-displacement data were used to directly measure the structural properties similar to

those of the femoral diaphysis and analyzed up to the point of ultimate load. Material properties (ultimate stress, modulus, and modulus of toughness) were calculated using standard equations allowing for correction of bone volume (obtained by μCT) in the samples [1].

Mechanical testing of the dogs' 11^{th} left ribs was performed previously by 3-pt bending [5].

2.3 microCT

Rat femurs midshafts and lumbar vertebrae bodies were scanned with a high-resolution μCT system (Skyscan 1172, Belgium) using 60 kV and 120 μA and 0.7° rotation steps, and then isotropic volume elements were reconstructed at 8 μm resolution. The scanning region was defined as a 1 mm region located at the femoral midshaft (determined using calipers between the inter-condyle region and the femoral neck) and the whole vertebral body prior to mechanical testing. Scans were reconstructed and analyzed using NRecon and CTAn, respectively (Skyscan). Outcome parameters from the 3D analyses included moment of inertia (I_x, mm^4) and cortical thickness (Ct.Th, mm) for the femur and bone volume (BV, mm^3) for the LV bodies.

2.4 Reference point indentation

Tissue mechanical properties of the anterior mid-diaphysis of the rat femur and the canine ribs were analyzed by cyclic micro-indentation using the BioDent 1000™ Reference Point Indentation instrument (Active Life Scientific, Inc., Santa Barbara, CA) using BP1 probes. Following published protocols [6], a series of pre-conditioning cycles (5 cycles, 2 N) was applied to the anterior mid-diaphysis of the rat femur, followed by 20 indentation cycles performed at 2 Hz, with a maximum force of 10 N. The left 9^{th} rib of the dogs was cut into 4 cm section at the point of largest curvature and RPI testing was performed on the anterior side using the same protocol as described above. For the 3^{rd} lumbar vertebral cortical shells, a newer RPI instrument, the BioDent H and concentric BP3 probes were used. The left and right anterior regions of the LV_3 body were tested using a similar protocol as for the femur, but with reduced force (5N for 10 cycles at 2 Hz) as the cortex is thinner at this location. The primary outcome measures are shown in Figure 1b, and further described by Diez-Perez et al. [6]. The first cycle indentation distance is similar to a measurement obtained from a standard microindentation test, and is related to hardness, which will have a high correlation to density and tissue mineralization. The slope of the unloading portion of the first cycle (US) is considered to be a measure of elastic modulus. The increase in indentation distance (IDI) over the entire set of cycles has been shown to be related to the modulus of toughness [6]. Measurements were repeated 5 times per femur and rib and 4 times (2 on each lateral side) for the vertebrae and separated by 1–2 mm. Bones were kept wet prior to testing (wrapped in saline-soak gauze) and a saline drop was deposited at the test site. Prior to testing, probes were tested on a PMMA block according to manufacturer's indication to ensure proper function. Replicates were averaged for each sample and used to calculate the mean of each group. For the main outcome, IDI, the variance within a sample was less than 8%.

2.5 Statistical analysis

Statistical analyses were performed using GraphPad Prism v5.04 (San Diego, CA) and SYSTAT v11 (Richmond, CA). Data were found to be of Gaussian distribution, therefore *Student T-tests* were used to compare groups, and correlations were made using the *Pearson* product moment algorithm. Backward stepwise multiple regression models were constructed to explore the efficacy of predicting standard material properties obtained through traditional mechanical testing (i.e., toughness, post-yield toughness, and ultimate stress) from reference-point indentation material property data (i.e., IDI, ID, TID, and US). For all

statistical tests, significance was set at α=0.05, and for the multiple regression significance criteria to enter and remove predictor variables were set at α_E=0.15 and α_R=0.15.

3. Results

3.1 Rat type 2 diabetes model

The diabetic status of the rats was confirmed by blood glucose levels and glycated hemoglobin (HbA_{1c}), as described elsewhere [7]. The ZDSD rats had higher blood glucose levels (629.6 ± 22.8 vs. 142.1 ± 2.8 mg/dl, $p< 0.001$) and HbA_{1c} (8.90 ± 0.21 vs. 3.38 ± 0.08 %, $p< 0.001$) compared to the control CD rats. Mechanical testing of the rat's femurs and fourth lumbar vertebrae revealed that diabetic ZDSD rats have significantly reduced bone material properties compared to control CD rats (Table 1). Diabetic rats have lower femoral cortical bone ultimate stress, toughness and post-yield toughness, and lower lumbar vertebrae strength and toughness than control rats. As 3-pt bending and axial compression are destructive testing techniques, reference-point indentation was performed on the contralateral femur and on a different lumbar vertebra (LV_3).

As shown in Figure 2a, the femurs of the diabetic ZDSD rats have higher cortical bone IDI (14.3 ± 1.3 vs. 11.3 ± 1.6 μm, $p< 0.0001$), indicating a greater increase in tissue penetration over 20 cycles by the testing probe in the diabetic bones compared to the controls. Diabetic rats also showed lower 1st cycle unloading slope (0.86 ± 0.1 vs. 0.77 ± 0.1 N/μm, $p< 0.05$) than control CD rats. In order to see associations between traditional mechanical testing and RPI, we sought to determine if toughness from 3-pt bending and IDI were related. Figure 2b shows a strong relationship between these two measurements ($r^2 = 0.56$; p = 0.0004). The IDI also was highly correlated with extrinsic strength ($r^2 = 0.49$, p < 0.001) and energy to failure ($r^2 = 0.56$, p < 0.001) (Table 2). A weaker correlation was found between IDI and ultimate stress ($r^2 = 0.30$, p < 0.05). In the backward stepwise multiple regression models, femoral IDI was the single significant predictor of femoral toughness ($R^2 = 0.42$, p < 0.001; β = − 0.22, p < 0.001) and post-yield toughness ($R^2 = 0.39$, p < 0.001; β = −0.20, p < 0.001), whereas the combination of ID (β = 0.40, p = 0.007) and IDI (β = −2.03, p = 0.001) significantly predicted ultimate stress ($R^2 = 0.59$, p < 0.001).

To further confirm our findings, we tested cortical bone from the rat vertebrae. As depicted in Figure 2c, the IDI of the LV_3 of the diabetic rats was higher than the CTL (9.9 ± 1.5 μm compared to 7.3 ± 0.7 μm, $p< 0.0001$), while the unloading slope was reduced (0.56 ± 0.03 vs 0.50 ± 0.03 N/μm, $p< 0.001$). The vertebral IDI was significantly correlated with axial compression toughness ($r^2 = 0.50$, p = 0.0002). As with the femurs, the vertebral IDI also correlated with ultimate stress, ultimate load and energy to failure from axial compression (Table 2). In addition, the vertebral IDI significantly predicted toughness ($R^2 = 0.47$, p < 0.001; β = −2.03, p = 0.001), whereas the combination of US (β = −107.76, p = 0.149) and IDI (β = −6.55, p = 0.003) predicted vertebral ultimate stress ($R^2 = 0.37$, p = 0.004).

3.2 Dog model

Analysis of the 3-pt bending data of the 11th rib showed that the alendronate-treated dogs had lower toughness compared to vehicle-treated animals (19.9 ± 5.0 vs. 26.9 ± 8.2 MJ/m^3, $p<0.05$). BioDent testing revealed that the alendronate-treated dogs had higher IDI than vehicle-treated animals (Fig. 3a; 12.5 ± 0.3 μm compared to 10.6 ± 0.3 μm, $p< 0.001$), whereas no difference was seen in the unloading stiffness (0.86 ± 0.1 vs. 0.92 ± 0.1 N/μm [p=0.39] for VEH and ALN, respectively). The IDIs obtained from the 9th ribs significantly correlated with toughness (Fig. 3b, Table 2) post-yield toughness and to a lesser extent to ultimate stress (Table 2) obtained from 3-pt bending of the left 11th ribs of the same animals. In multiple regression models, the IDI was again the single significant predictor of rib

toughness ($R^2 = 0.55$, $p < 0.001$; $\beta = -0.43$, $p < 0.001$), post-yield toughness ($R^2 = 0.53$, $p < 0.001$; $\beta = -0.394$, $p < 0.001$), and ultimate stress ($R^2 = 0.22$, $p = 0.026$; $\beta = -11.22$, $p = 0.026$).

4. Discussion

Mechanical testing of animal bones represents the gold standard to study the effect of genetic or pharmacologic interventions on bone material properties. While most techniques are destructive, they can provide critical information about bone strength and capacity to resist fracture. Until recently, no technique could provide an estimate of mechanical properties without critically damaging the bone. The goal of this study was to determine how material testing using a newer minimally invasive technique (reference-point indentation) reflects the material properties of bone measured by standard mechanical testing techniques.

The ZDSD rat model develops type 2 diabetes and chronic hyperglycemia which renders bone more brittle and decreases the energy needed to fracture [7]. As expected, standard mechanical testing of long (femur) and axial (lumbar vertebrae) bones showed reduced strength and toughness in the diabetic animals compared to controls. In the femurs, the post-yield portion of the stress-strain curve was longer in the control animals than in the diabetic rats. This demonstrates that bone from this diabetic animal has reduced plasticity (i.e. post-yield) and is more brittle than non-diabetic bone. Likewise, previous experiments from our group using standard mechanical testing have shown that one and three years of bisphosphonate treatment (at a dose equivalent to that used clinically) in skeletally mature dogs reduces energy absorption and material toughness of vertebrae and of cortical bone from the rib and tibia [5, 8, 9]. Therefore we chose these two models for RPI testing.

RPI testing was conducted on frozen specimens collected during previous studies. In the rat model, both femur and vertebral cortical shell of diabetic animals showed higher IDI compared to their respective healthy controls (CD rats). Because IDI correlated inversely to toughness, this result is consistent with the results from standard mechanical tests demonstrating greater fragility in these diabetic animals. Similarly, increased IDI also was observed in the alendronate-treated dogs compared to the vehicle-treated controls. This is again consistent with previous results showing reduced toughness in dogs treated with alendronate for 1–3 years. Indeed, we found that IDI was negatively correlated to whole bone material toughness in different bones under two different experimental conditions. Individually, all these different anatomical sites showed a significant negative correlation ($r^2 > 0.50$, $p < 0.001$) between toughness and IDI. When all the data points are pooled together (Figure 4), the general negative correlation between IDI and toughness persisted among different bones and species, and was further supported by significant negative regression coefficients in all multiple regression models.

Fracture risk is a great concern in numerous populations and RPI testing might be a new technique by which early detection of reduced bone material toughness could be done, allowing for a better management of pharmacological interventions in these patients. Diez-Perez *et al.* recently tested the RPI technique in vivo in a human population of osteoporotic patients and age-matched controls [6]. Using a small sample of cadaveric bones, they also found that IDI was negatively correlated with crack growth toughness, which is the resistance to crack extension. Our finding that IDI correlates with and predicts whole-bone toughness agrees with their data.

Our experiment has some limitations. First, we did not test the same bone using both standard mechanical testing techniques and RPI. However, other data generated in our

laboratory (not shown) suggests that performing RPI on the same bone either before or after mechanical testing could alter the results of the subsequent mechanical test. Therefore, the best test of the relationship between parameters measured by RPI and those measured by standard mechanical testing is to use either a contralateral (femurs and ribs) or adjacent (vertebrae) bone to one that was mechanically tested. This conservative approach will likely lead to somewhat lower correlations than might be found if the same area and bone could be used for both tests. Even so, our data suggest a strong relationship between toughness and IDI. Second, compression tests of the lumbar vertebrae included both cortical and cancellous bone, whereas measurements of the vertebral cortical shell using RPI cannot evaluate the composite structure. Therefore, these correlations may be less meaningful than those of cortical bone that was tested in 3-point bending. Third, because the sample size from each individual study was modest, we combined data from the diabetic ZDSD and control CD rats obtained in our lab to our data from dogs and still showed a strong correlation (Figure 4, $r^2 = 0.51$, $p < 0.0001$). Fourth, there is a possibility that the correlations seen are model dependent, though in our study IDI predicted toughness in all three of the different animal models tested. However, bone which is poorly mineralized or osteomalacic would also be expected to have a high IDI, but in this case the modulus of toughness might also be high as substantial deformation and energy absorption could occur prior to failure. Finally, this is only the first step in the process of validating this novel technique against other standard mechanical measures. It will be important for continued and more complete validation to include in the future in vivo longitudinal measurements in animal models with known material property changes.

5. Conclusion

Our data using appendicular and axial bones from different animal models indicate that the IDI obtained from RPI testing correlates with and predicts both material toughness and with whole bone energy to fracture obtained by traditional mechanical testing. It also provides a rapid and less laborious means to perform assessments of bone material properties from animals without the need to machine standardsized specimens.

Acknowledgments

The authors would like to thank Active Life Scientific for their technical support during this study. Active Life Scientific was made aware of this study but had no control over the outcomes or the content of the manuscript.

References

1. Turner CH, Burr DB. Basic biomechanical measurements of bone: a tutorial. Bone. 1993; 14:595–608. [PubMed: 8274302]
2. Hansma P, Turner P, Drake B, Yurtsev E, Proctor A, Mathews P, Lulejian J, Randall C, Adams J, Jungmann R, Garza-de-Leon F, Fantner G, Mkrtchyan H, Pontin M, Weaver A, Brown MB, Sahar N, Rossello R, Kohn D. The bone diagnostic instrument II: indentation distance increase. Rev Sci Instrum. 2008; 79:064303. [PubMed: 18601422]
3. Hansma P, Yu H, Schultz D, Rodriguez A, Yurtsev EA, Orr J, Tang S, Miller J, Wallace J, Zok F, Li C, Souza R, Proctor A, Brimer D, Nogues-Solan X, Mellbovsky L, Pena MJ, Diez-Ferrer O, Mathews P, Randall C, Kuo A, Chen C, Peters M, Kohn D, Buckley J, Li X, Pruitt L, Diez-Perez A, Alliston T, Weaver V, Lotz J. The tissue diagnostic instrument. Rev Sci Instrum. 2009; 80:054303. [PubMed: 19485522]
4. Randall C, Mathews P, Yurtsev E, Sahar N, Kohn D, Hansma P. The bone diagnostic instrument III: testing mouse femora. Rev Sci Instrum. 2009; 80:065108. [PubMed: 19566227]
5. Allen MR, Reinwald S, Burr DB. Alendronate reduces bone toughness of ribs without significantly increasing microdamage accumulation in dogs following 3 years of daily treatment. Calcif Tissue Int. 2008; 82:354–360. [PubMed: 18463913]

6. Diez-Perez A, Guerri R, Nogues X, Caceres E, Pena MJ, Mellibovsky L, Randall C, Bridges D, Weaver JC, Proctor A, Brimer D, Koester KJ, Ritchie RO, Hansma PK. Microindentation for in vivo measurement of bone tissue mechanical properties in humans. J Bone Miner Res. 2010; 25:1877–1885. [PubMed: 20200991]

7. Reinwald S, Peterson RG, Allen MR, Burr DB. Skeletal changes associated with the onset of type-2, diabetes in the ZDF and ZDSD rodent models. Am J Physiol Endocrinol Metab. 2009; 296:E765–E774. [PubMed: 19158319]

8. Tang SY, Allen MR, Phipps R, Burr DB, Vashishth D. Changes in non-enzymatic glycation and its association with altered mechanical properties following 1-year treatment with risedronate or alendronate. Osteoporos Int. 2009; 20:887–894. [PubMed: 18850239]

9. Mashiba T, Turner CH, Hirano T, Forwood MR, Johnston CC, Burr DB. Effects of suppressed bone turnover by bisphosphonates on microdamage accumulation and biomechanical properties in clinically relevant skeletal sites in beagles. Bone. 2001; 28:524–31. [PubMed: 11344052]

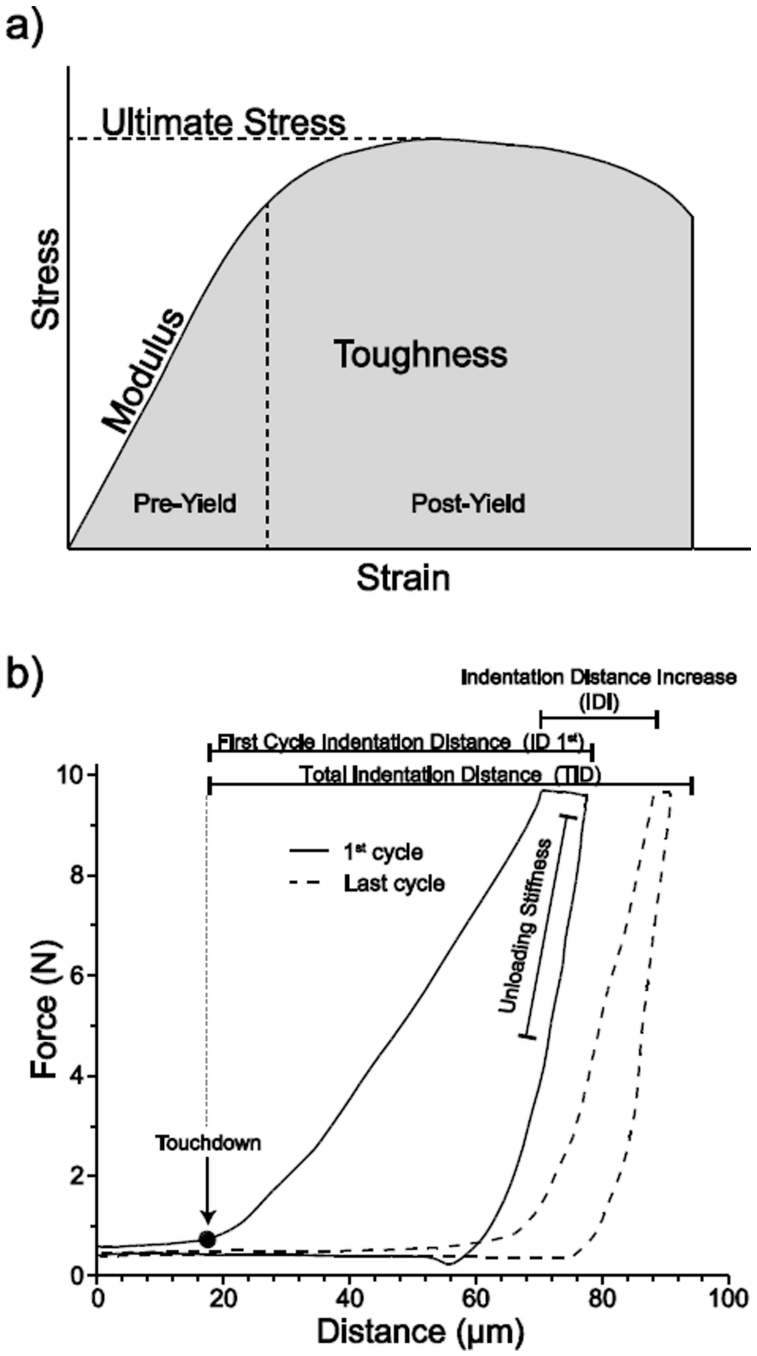

Figure 1. Mechanical testing curves

a) Generic stress-strain curve of a long bone obtained from a 3- point bending test. **b)** Example of an RPI testing curve from the ZDSD rat femur group. To simplify the design, only the first and last cycle (20^{th}) of the testing.

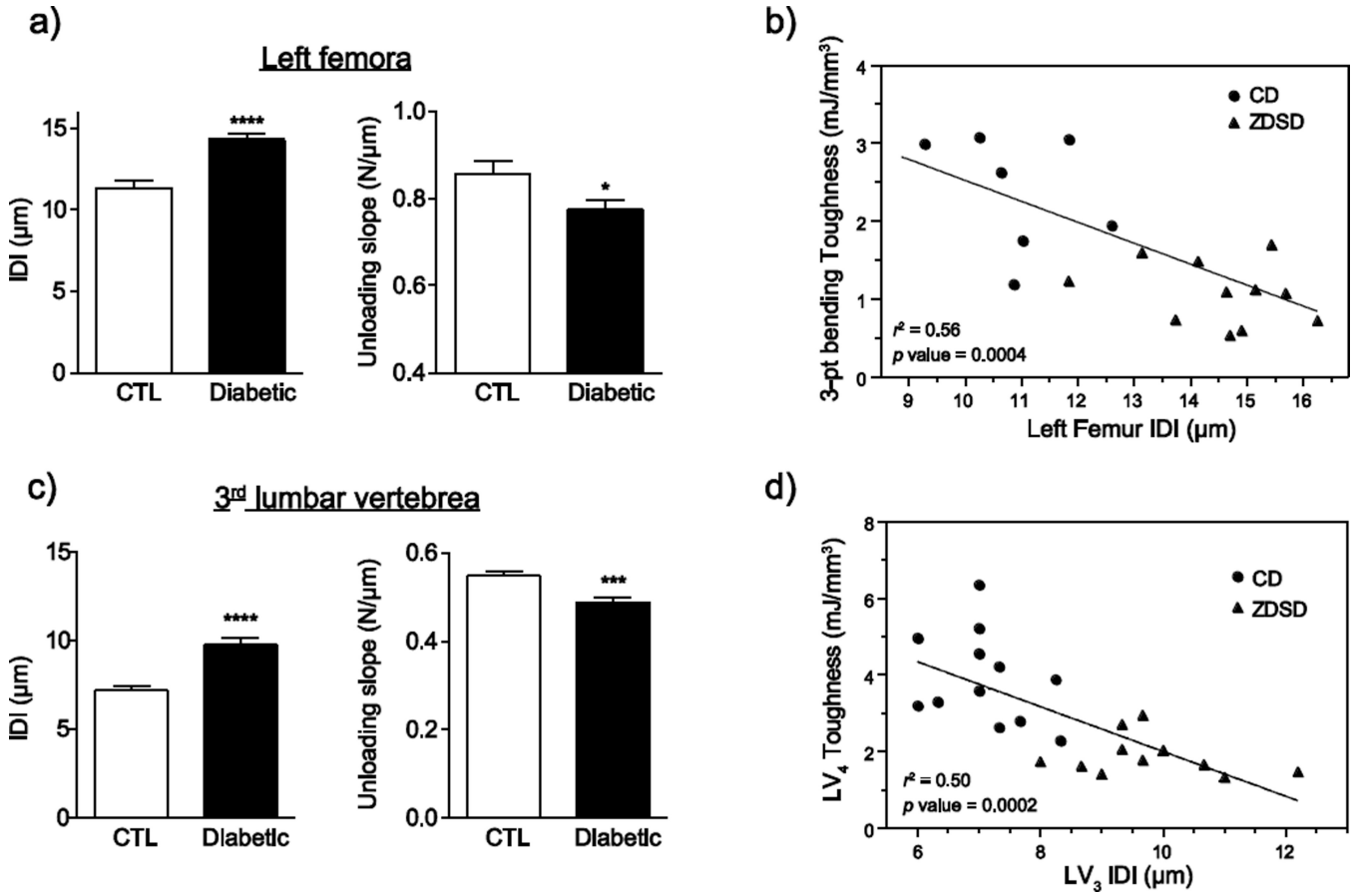

Figure 2. Reference-point indentation of rat bones

Diabetic rat femurs (**a**) and vertebral cortical shell (**c**) showed higher IDI and lower unloading stiffness than control (CD) animal. IDI from both the femurs (**b**) and vertebrae (**d**) correlated with toughness obtained from traditional mechanical testing. *: $p<0.05$, ***: $p<0.001$, ****: $p<0.0001$.

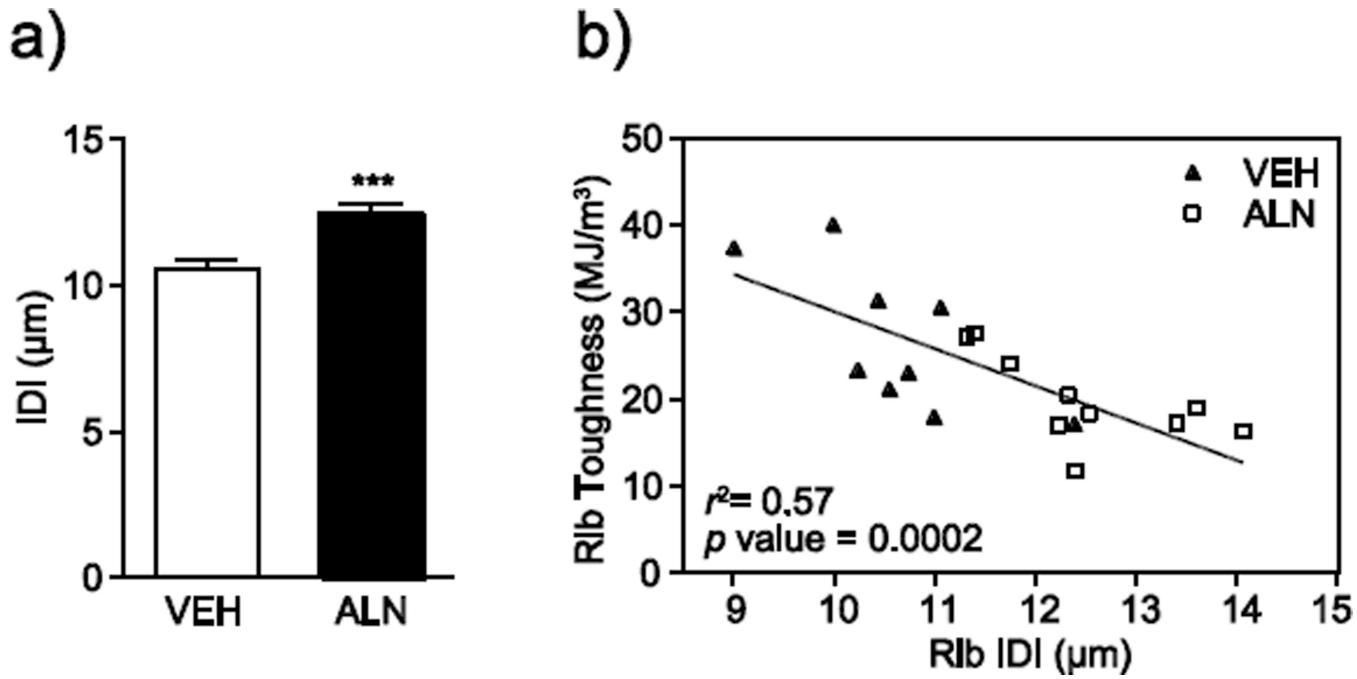

Figure 3. Reference-point indentation of bisphosphonate-treated dig ribs

a) IDI of the ribs was greater in alendronate-treated dogs compared to vehicle-treated animals. **b)** Rib material toughness from 3-point bending correlates significantly with IDI obtained from RPI. ***: $p<0.001$

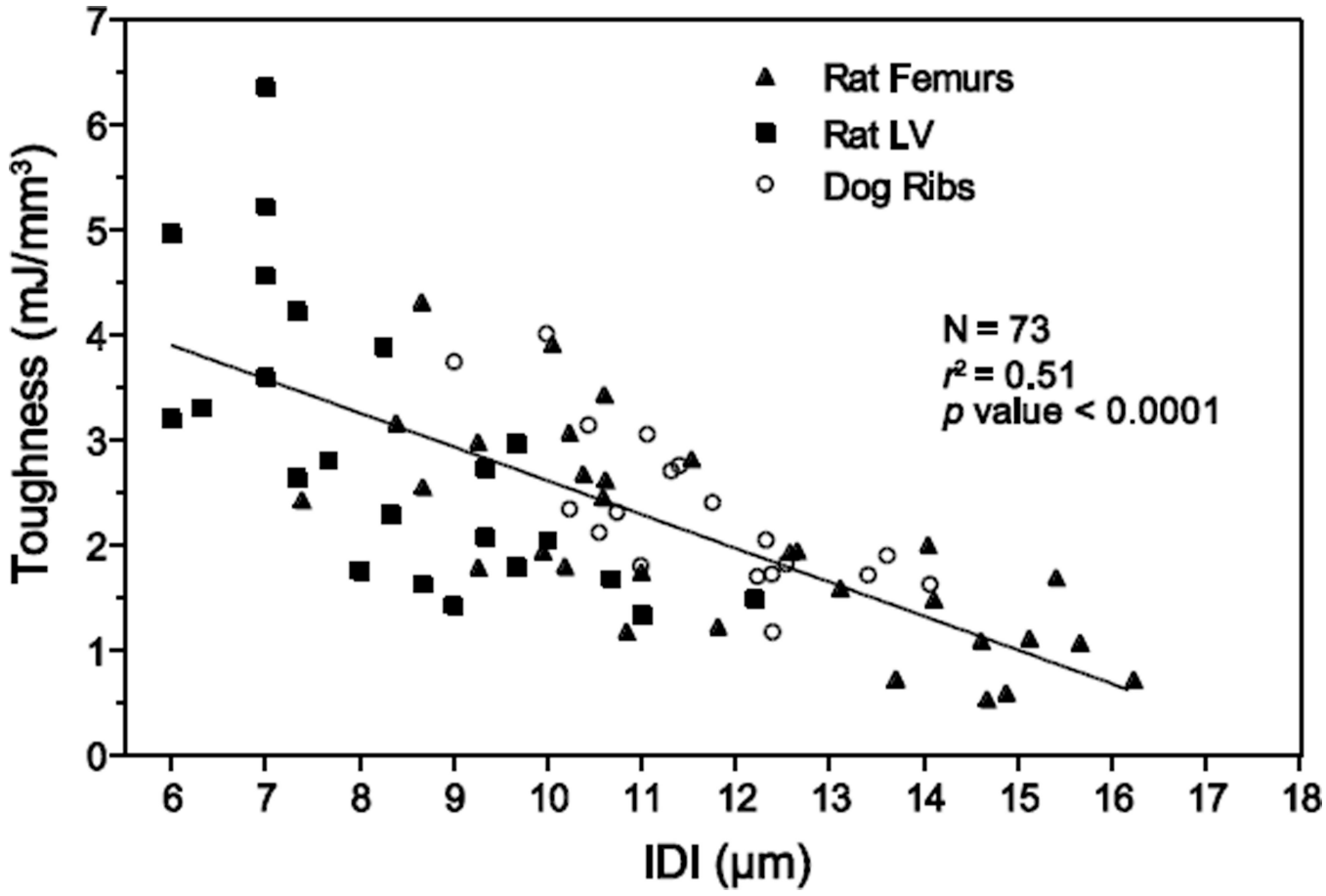

Figure 4. Global correlation of toughness and IDI

Results from different experiments were pooled and analyzed for correlation between toughness from traditional mechanical testing and IDI. The data shows that a significant correlation exists between toughness and IDI from different sites and species.

Table 1

Material properties of rat femurs and 4th lumbar vertebrae.

	Material Properties	**Control Mean ± SD**	**Diabetic Mean ± SD**	**T-test**
Right Femoral Midshaft: 3-pt Bending	Ultimate Stress (MPa)	52.7 ± 6.63	44.8 ± 7.65	0.021
	Modulus (MPa)	1,667 ± 345	1,493 ± 224	0.184
	Toughness (mJ/mm^3)	2.18 ± 0.7	1.09 ± 0.4	< 0.001
	Post-Yield Toughness (mJ/mm^3)	1.58 ± 0.72	0.52 ± 0.35	< 0.001
LV_4 Body: Axial Compression	Ultimate Stress (MPa)	101.2 ± 16.8	72.8 ± 11.4	< 0.001
	Toughness (mJ/mm^3)	3.93 ± 1.15	1.58 ± 0.53	< 0.001

Table 2

Correlations between BioDent outcomes and traditional mechanical properties.

		Mechanical Properties					*Material Properties*			
	BioDent Outcomes	**Ultimate Load**	**Stiffness**	**Post-yield displ.**	**Energy to failure**	**Post-yield E to fail.**	**Ultimate Stress**	**Modulus**	**Toughness**	**Post-yield Toughness**
Femur	IDI	−0.70 **	ns	−0.70 **	−0.75 ***	−0.72 ***	−0.57 *	ns	−0.75 ***	−0.72 ***
	1st cycle distance	ns	ns	0.51 *	0.48 *	0.50 *	ns	ns	0.47 *	0.48 *
	Total distance	ns	ns	ns	ns	ns	ns	ns	ns	ns
	Unloading slope	ns	ns	ns	ns	ns	ns	ns	ns	ns
Lumbar vertebrae	IDI	−0.77 ***	ns	–	−0.70 ***	–	−0.61 **	ns	−0.71 ***	–
	1st cycle distance	−0.37	ns	–	−0.36	–	−0.36	ns	−0.35	–
	Total distance	−0.53 **	ns	–	−0.50 *	–	−0.48 *	ns	−0.49 *	–
	Unloading slope	0.62 **	0.60 **	–	0.46 *	–	ns	0.44 *	0.40	–
Dog rib	IDI	ns	ns	−0.42	−0.51 *	−0.53 *	−0.51 *	ns	−0.75 ***	−0.74 ***
	1st cycle distance	ns	ns	ns	ns	ns	ns	ns	ns	ns
	Total distance	ns	ns	ns	ns	ns	ns	ns	ns	ns
	Unloading slope	ns	ns	ns	ns	ns	ns	ns	ns	ns

* p<0.05,

** p<0.01,

*** p<0.001, ns: not significant

– not calculated, correlations close to significance (0.10>p>0.05) are included.