Citation: BoneKEy Reports **4**, Article number: 712 (2015) | doi:10.1038/bonekey.2015.81

www.nature.com/bonekey

ORIGINAL ARTICLE

In vivo reference point indentation measurement variability in skeletally mature inbred mice

Andrew Srisuwananukorn[1], Matthew R Allen[1], Drew M Brown[1], Joseph M Wallace[2,3] and Jason M Organ[1]

[1]Department of Anatomy and Cell Biology, Indiana University School of Medicine, Indianapolis, IN, USA. [2]Department of Biomedical Engineering, Indiana University-Purdue University at Indianapolis, Indianapolis, IN, USA. [3]Department of Orthopaedic Surgery, Indiana University School of Medicine, Indianapolis, IN, USA.

Reference point indentation (RPI) was developed to measure material-level mechanical properties of bone *in vivo*. Studies using RPI *in vivo* have discriminated between human subjects with previous skeletal fractures and those without and among dogs given different anti-remodeling drugs. Recently, this technology was extended to rats, providing the first *in vivo* data for rodents. The goal of the present study was to perform *in vivo* RPI measurements in mice, the most common animal model used to study bone. Twelve 16-week-old female C57BL/6 mice were subjected to RPI (three tests) on the anterior tibia, followed by a repeat test session on the contralateral limb 28 days later. A custom MATLAB program was used to derive several outcome parameters from RPI force-displacement curves: first cycle indentation distance (ID-1st), ID increase (IDI), total ID (TID), first cycle unloading slope (US-1st) and first cycle energy dissipation (ED-1st). Data within an individual were averaged across the three tests for each time point. Within-animal variation of all RPI parameters on day 1 ranged from 12.8 to 33.4% and from 14.1 to 22.4% on day 28. Between-animal variation on day 1 ranged from 11.4% to 22.8% and from 7.5% to 24.7% on day 28. At both time points, within- and between-animals, US-1st was the least variable parameter and IDI was most variable. All parameters were nonsignificantly lower at day 28 compared with day 1. These data are important to demonstrate the feasibility of collecting bone material property data longitudinally in mice and will inform the design of future studies in terms of statistical power and appropriate sample size considerations.

BoneKEy Reports **4**, Article number: 712 (2015) | doi:10.1038/bonekey.2015.81

Introduction

Ex vivo mechanical testing has long been the gold standard for assessing bone mechanical properties at the structural and material levels. However, measuring mechanical properties *ex vivo* precludes longitudinal investigations of the efficacy of different interventions in enhancing these properties. Recently, reference point indentation (RPI) was developed to enable assessment of bone mechanical properties *in vivo*.[1,2] This technique utilizes one of the two instruments to assess bone material-level properties: BioDent Hfc and OsteoProbe (ActiveLife Scientific, Santa Barbara, CA, USA). These instruments both indent the surface of bone in order to assess material properties, but they differ in a number of important details. Biodent uses a reference probe to stabilize the bone with a reference force prior to indenting the cortical bone surface with a separate test probe in a cyclic manner (typically 10–20 cycles at 2 Hz) at a variable force (typical studies use between 2 and 10 N). OsteoProbe uses only a single probe, with the reference force provided by the test probe itself prior to triggering a single indentation cycle at ~45 N of force. According to the manufacturer, BioDent was designed for use in the laboratory setting for *ex vivo* assessment of bone material properties, whereas Osteoprobe was designed for use in the clinical setting for *in vivo* use.

Early reports using RPI *in vivo* have been promising, in that the outcome data distinguish patients with previous skeletal fractures from those who have not fractured previously[3] and patients administered bisphosphonate treatment from those who were treatment naive.[4] A study in dogs demonstrated differences in RPI parameters measured *in vivo* between controls and animals treated for 6 months with raloxifene at clinically relevant doses.[5] These studies show the real potential for this tool in a clinical setting, even while we are only beginning to understand how RPI outcomes compare with properties measured using traditional mechanical testing modalities.[6–9]

The present study is targeted at a more fundamental level than the clinical and preclinical assessments of RPI described

Correspondence: Dr JM Organ, Department of Anatomy and Cell Biology, MS 5035, Indiana University School of Medicine, 635 Barnhill Dr., Indianapolis, IN 46202, USA. E-mail: jorgan@iupui.edu

Received 29 September 2014; accepted 8 May 2015; published online 17 June 2015

above. Rodents are the most common animals used in skeletal research, owing in large part to the biological tools (for example, genetic manipulation) available for these taxa.[10,11] Using rodent models allows for controlled experimental studies, which provide the foundation for preclinical studies in larger animal models with bone that better approximates human morphology (that is, dog, pig and/or monkey).

A number of studies have evaluated RPI measurements in rodents *ex vivo*,[6,12–14] but there are very few *in vivo* data reported.[15] Recently, we assessed the variation of *in vivo* RPI parameters in the skeletally mature rat, within and between animals.[15] The present study aims to examine *in vivo* RPI parameters measured with BioDent Hfc in the skeletally mature mouse. In particular, we assess variation in RPI measures within and between individuals, as well as variation over time, in order to provide data essential to design adequately powered mouse experiments. These data allow us to appreciate the variability of RPI parameters measured in treatment naive individuals so that we may better understand and control for variation in future interventional studies. We expect that variability of *in vivo* RPI measurements within- and between-animals to be similar to what has been previously reported for other animal models.

Results

A total of 103 indentation tests were performed on 12 animals over the 2 time points. Of these tests, 31 were unsuccessful based on operator observation of various problems during testing, including the measurement unit shifting during the test, or parameters measured outside the range of realistic values (for example, negative indentation distances, or decreasing displacement in the first few cycles resulting in a negative unloading slope). After removal of these erroneous tests, analyses were performed on a total of 72 tests (12 animals $\times$ 3 measurements $\times$ 2 time points).

All animals survived *in vivo* RPI testing without incident. Within-animal variation on the first testing date ranged from 12.8 to 33.4% (**Table 1**, **Figure 1a**). On the second testing date, within-animal variation ranged from 14.1 to 22.4%. At both time points, the least variable parameter was first cycle unloading slope (US-1st), whereas the most variable was indentation distance increase (IDI). For all parameters except US-1st, the within-animal variation was lower for the second test session compared with the first.

Between-animal variation on the first testing date ranged from 11.4 to 22.8%; on the second testing date between-animal variation ranged from 7.5 to 24.7% (**Table 2** and **Figure 1b**). At both time points, the least variable parameter was US-1st. At the first time point, IDI was the most variable along with first

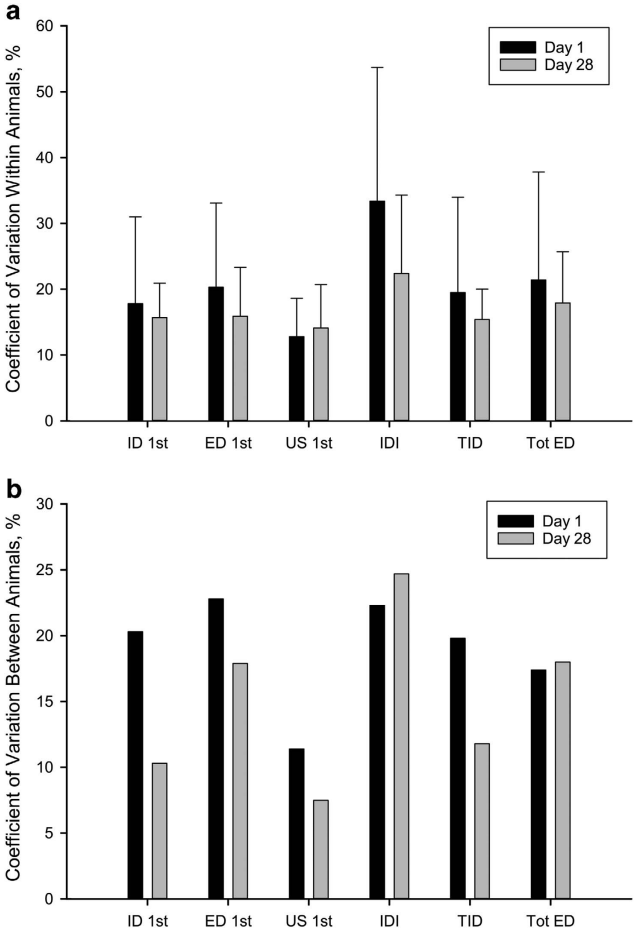

Figure 1 Measurement variation in RPI parameters. (**a**) Within-animal variation is presented as the mean and s.d. of the coefficient of variation (%) within a given animal. (**b**) Between-animal variation is presented as the coefficient of variation (%) for each variable measured across all animals. ID 1st, first cycle indentation distance; ED 1st, first cycle energy dissipated; US 1st, first cycle unloading slope; IDI, indentation distance increase; TID, total indentation distance; Tot ED, total energy dissipated.

Table 1 Within-animal variation of reference point indentation parameters in skeletally mature mice

	First cycle indentation distance, ID-1st (μm)	*First cycle energy dissipated, ED-1st (μJ)*	*First cycle unloading slope, US-1st (N/μm)*	*Indentation distance increase, IDI (μm)*	*Total indentation distance, TID (μm)*	*Total energy dissipated, Tot-ED (μJ)*
Day 1						
Mean coefficient of variation within animal (%)	17.8	20.3	12.8	33.4	19.5	21.4
Standard deviation (%)	13.2	12.8	5.8	20.3	14.5	16.4
Day 28						
Mean coefficient of variation within animal (%)	15.7	15.9	14.1	22.4	15.4	17.9
Standard deviation (%)	5.2	7.4	6.6	11.9	4.6	7.8

Table 2 Between-animal variation of reference point indentation parameters in skeletally mature mice

	First cycle indentation distance, ID-1st (μm)	*First cycle energy dissipated, ED-1st (μJ)*	*First cycle unloading slope, US-1st (N/μm)*	*Indentation distance increase, IDI (μm)*	*Total indentation distance, TID (μm)*	*Total energy dissipated, Tot-Ed (μJ)*
Day 1						
Mean	31.6	28.3	0.2	7.9	36.0	86.9
Standard deviation	6.4	6.4	0.0	1.8	7.1	15.2
Coefficient of variation (%)	20.3	22.8	11.4	22.3	19.8	17.4
Animals needed in each of two groups to detect a 25% treatment effect	12	14	7	15	11	9
DAY 28						
Mean	28.0	23.6	0.2	6.9	32.2	82.7
Standard deviation	2.9	4.2	0.0	1.7	3.8	14.9
Coefficient of variation (%)	10.3	17.9	7.5	24.7	11.8	18.0
Animals needed in each of two groups to detect a 25% treatment effect	4	10	4	17	5	10
Paired *t*-test of parameter means, *P*-value (day 1 vs day 28)	0.124	0.069	0.374	0.105	0.304	0.154

cycle energy dissipated (ED-1st), but at the second time point IDI showed more variation compared with ED-1st. For all parameters, the coefficient of variation either remained stable (total energy dissipation, Tot ED) or was lower (all other parameters) at the second testing session compared with the first.

Percent change in all RPI parameters over the 28 days was calculated for each animal and then averaged; all parameter were, on average, lower at day 28 compared with day 1 (**Figure 2**), although paired *t*-tests on the parameter means were not significant (**Table 2**).

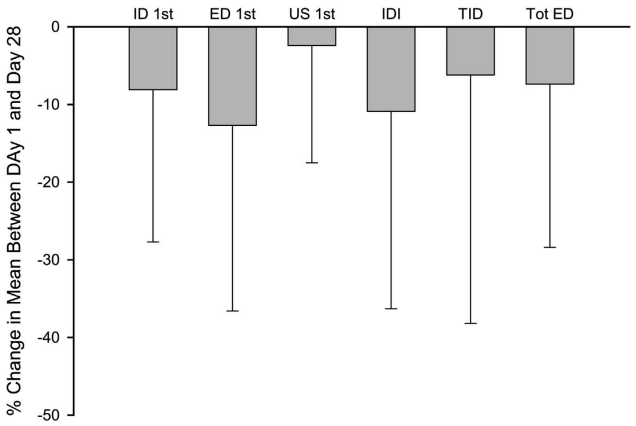

Figure 2 Average percent difference in measurement variation of *in vivo* RPI parameters measured 28 days apart, on contralateral limbs of treatment naive, skeletally mature female mice. ID 1st, first cycle indentation distance; ED 1st, first cycle energy dissipated; US 1st, first cycle unloading slope; IDI, indentation distance increase; TID, total indentation distance; Tot ED, total energy dissipated.

Discussion

Longitudinal experimental designs allow researchers to examine the sequences of change in a given outcome and require fewer animals to achieve adequate statistical power versus studies with cross-sectional experimental designs. Currently, there are a number of tools that allow researchers to study bone structure and cellular processes *in vivo* (serum/urine biomarkers of bone turnover or high-resolution structural imaging). Studies of bone mechanical properties, however, by nature of their destructive outcomes (bone breaking) have required cross-sectional experimental designs, which limits the inferences that can be made. The recent development of RPI as a method for assessing material-level mechanical properties *in vivo* has the potential to revolutionize the field.[3–5] Whereas a number of studies have measureed RPI outcomes in rodents *ex vivo*,[6,12–14] only a single study has examined these measures *in vivo*.[15] The objective of this study was to assess the variability of RPI parameters measured *in vivo* within and among treatment naive, skeletally mature mice, because mice are among the most common first-line model in skeletal research. These data are needed to adequately power future interventional studies with *in vivo* RPI parameters as outcomes.

The main RPI parameter is IDI, which is the increase in penetration depth from the first to the last cycle of each test procedure. Other outcomes include first cycle ID (ID-1st), ED-1st, US-1st, total indentation distance (TID) and Tot ED. Among previously published studies of *in vivo* RPI measures, considerable variation has been reported within individual RPI parameters. In human patients, within-individual variation of IDI ranged between 15% and 24%, and within-individual variation of TID ranged from 10 to 17%.[3,4] In dogs, within-animal variation ranged from 5% in US-1st to 27% in ID-1st and ED-1st.[5] In rats, within-animal variation ranged from 13% in Tot ED and US-1st to 21% in IDI.[15] The data presented here for mice exceed the upper limit of these ranges at the first test date, as the variation in IDI within-animal exceeds 30%. However, by the second test date, variation in all parameters decreases to within the ranges noted above, with IDI reducing to 22% and the other variables generally reducing in variation as well. We attribute the lower variation at the second test session to a learning curve, as the primary individual running the tests was new to the system. In our animal studies, RPI measurements were taken along a length of tibia ~6–8 mm long, but the total length of the tibia varies significantly among these taxa (~18 mm in mice, ~40 mm in rats and ~100 mm in dogs). Given the size differences among these species, it is remarkable that the

variation seen across taxa is similar, because a larger proportion of the bone was sampled in the smaller species than in the larger ones. This suggests that the inherent variation along the length of the bones in these taxa is similar.

Our data also show an interesting pattern of variation *among* the RPI parameters. At both testing dates, the parameter with the least within-animal variation is US-1st, which is also observed in dogs[5] and rats.[15] The parameter with the most variation in the current study is IDI, which is also seen in rats. Although IDI is not the most variable parameter in dogs, it is toward the upper end of the range of variation. The US-1st is an indicator of material stiffness, whereas IDI is the best RPI predictor of material toughness.[6] IDI is a small linear measurement ($\sim 7\,\mu m$), and any small deviation (of even a single micrometer) around the mean for an individual has a disproportionately larger influence on the variation around that mean than it would for other linear measures at the same scale, such as ID-1st or TID (both are ~ 30–$35\,\mu m$). It is not surprising, then, that the CVs for IDI are high. Yet, it is also quite remarkable that IDI has the strongest correlation to traditional mechanical testing outcomes.

Even with the variation in RPI parameter measurements described here, the technique is still the only one capable of measuring bone mechanical properties longitudinally *in vivo*. To that end, this study provides the between-animal variation data necessary to design mouse experiments with sufficient statistical power. For example, a study designed to detect a 25% difference between two groups with 80% power would require anywhere from 4 to 17 animals per group at any single time point (**Table 2**).

The variation in RPI measures comes from three general sources: methodological error, measurement error and biological variation. Methodological error is the error associated with violating assumptions of contact mechanics analysis, which leads to error in the data structure. Contact mechanics requires that the indent is applied normal to the surface, that the surface is an infinite flat elastic half space, that the indenter is spherical and that the indent depth is relatively shallow compared with the radius of the indenter. For the most part, these assumptions are not grossly violated, with the possible exception of the bone being approximated as an infinite elastic half space. We assume, therefore, that the variation in RPI parameters is driven instead by probe placement, heterogeneities in the sample, presence of soft tissue, etc. These are all sources of variation that can be attributed to measurement error or biological noise. The purpose of this paper was to describe the variation associated with measurement error precisely so that properly powered experiments can be designed to investigate the variation associated with biological response.

The data presented here should be considered within the context of the following limitations. (i) This experiment was our first attempt to perform *in vivo* RPI measurements in a mouse model; further refinement of our technique could result in lower variation in measures. (ii) Repeat tests were performed on contralateral limbs, likely increasing the variation across time points. We assumed that test sites would exhibit residual damage or healing in response to damage at the second test date. Therefore, we rationalized that testing the contralateral limb would result in less variation than the damaged/healing tissue on the same limb. However, when we examined the bones from the present experiment post mortem with scanning electron microscopy, we were unable to detect any healing response or residual damage; in fact, we were unable to locate the test sites at all. This suggests either that the low forces imparted into the bone during RPI-based indentation did not result in enough microdamage to promote a remodeling response or that the test sites healed rapidly. If the former is true, it might suggest that future studies can be conducted on the same limb, which may also lead to a further reduction in measurement variation.

In summary, the data we present here demonstrate the variability both within and between animals, and among variables, for RPI parameters measured *in vivo* in skeletally mature inbred mice. These data are important to demonstrate the feasibility of measuring bone material properties longitudinally in mice and will inform the design of future studies in terms of statistical power and appropriate sample size considerations.

Materials and Methods

Animals

Twelve 16-week-old female C57BL/6 mice were obtained and allowed to acclimate at the Indiana University School of Medicine (IUSM) housing facility for 7 days. Animals were subjected to *in vivo* RPI on day 1 and then again 28 days later. Between test dates, animals were allowed normal cage activity and had access to food and water *ad libitum*. The IUSM Animal Care and Use Committee approved all procedures prior to the start of the study.

RPI

In vivo RPI measurements were collected using the BioDent Hfc. We chose to use BioDent rather than OsteoProbe because the force of the OsteoProbe cannot be modulated, and a 45 N force would fracture a mouse tibia. Following the achievement of a stable anesthetic plane with inhalation Isoflurane, the left leg was shaved clean and prepared aseptically (washed with Betadine). Two local anesthetics—bupivacaine ($2.5\,mg\,kg^{-1}$) and lidocaine ($10\,mg\,kg^{-1}$)—were injected separately under the skin near the tibial test site. To increase the precision within and among animals a custom X–Y translational stage was designed that allowed movement of the animal in two directions (**Figure 3**). Using a BP3 probe (which has a flat, concentric reference probe and a spherical test probe) the skin was pierced, and the reference probe was placed on the anterior tibial cortex immediately proximal to the medial malleolus. As in our previous rat study, the periosteum was not scraped prior to indentation of the cortical bone due to the challenges of working in small areas and to prevent damage to the underlying cortical bone.[15] Indentation tests along the anterior tibial cortex were conducted for each animal: 4 preconditioning cycles (1 N force at 5 Hz frequency) followed by 10 testing cycles (2 N at 2 Hz), moving proximally from the distal tibia at 1 mm intervals for each successive test. Our goal was to obtain three successful measurements of each animal at both time points. If the first three tests were successful, no more tests were conducted on the individual. If a test was deemed unsuccessful by the RPI operator for any reason (that is, measurement unit slipped, or data were outside the range of possibility—for example, negative IDIs), it was noted and an additional test(s) was

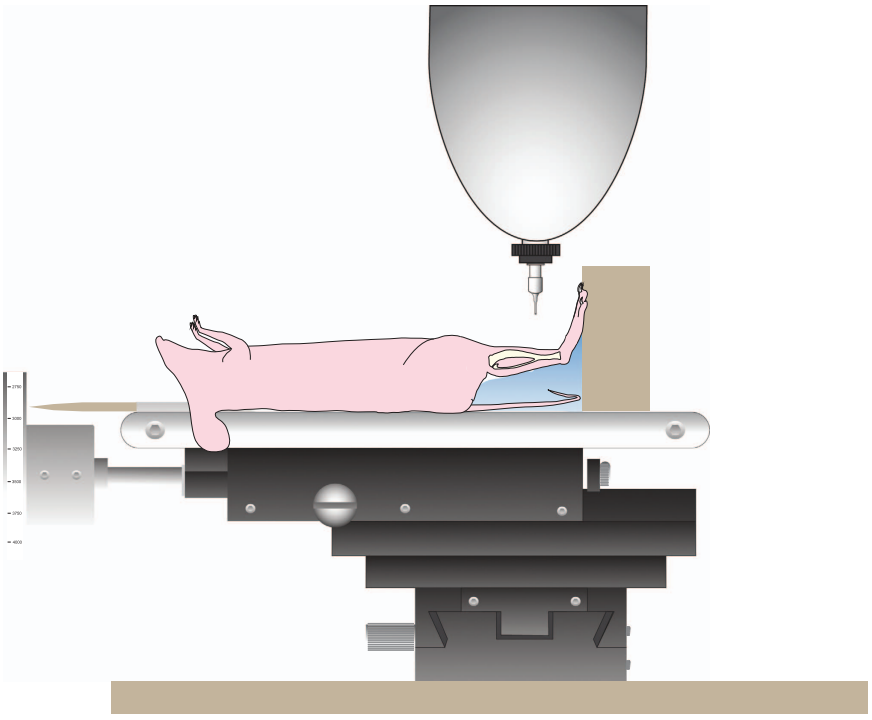

Figure 3 *In vivo* RPI testing setup for mice with custom X–Y translational stage. The animal's leg was flexed at the knee joint and placed on the translational stage so that the anterodistal tibia was perpendicular to the testing probe.

performed. Following the baseline tests, animals were allowed to recover in an empty cage and then returned to their normal cage upon full recovery. At the second test session, animals were euthanized by carbon dioxide inhalation, followed immediately by RPI indentation of the contralateral (right) leg. RPI data were analyzed using a custom MATLAB (MathWorks, Natick, MA, USA) program.[5] Key outcome parameters included ID-1st, ED-1st, US-1st, IDI, TID and Tot ED.

Statistics

Coefficients of variation (CVs) of each parameter were calculated for all tests within an animal to assess within-animal variation. CVs also were calculated for each parameter across all animals to assess between-animal variation at each time point. Paired *t*-tests (two-tailed; $\alpha = 0.05$) of the means within an animal at both time points were used to assess changes in each RPI parameter with time.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

Funding for this study was provided by the IUPUI Biomechanics & Biomaterials Research Center.

References

1. 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.
2. Bridges D, Randall C, Hansma PK. A new device for performing reference point indentation without a reference probe. *Rev Sci Instrum* 2012; **83**: 044301.
3. Diez Perez A, Güerri R, Nogues X, Cáceres E, Pena 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.
4. Güerri Fernández RC, Nogués X, Quesada Gómez JM, Torres del Pliego E, Puig L García Giralt N *et al.* Microindentation for *in vivo* measurement of bone tissue material properties in atypical femoral fracture patients and controls. *J Bone Miner Res* 2013; **28**: 162–168.
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.
6. 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.
7. Setters A, Jasiuk I. Towards a standardized reference point indentation testing procedure. *J Mech Behav Biomed Mater* 2014; **34**: 57–65.
8. 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. *J Mech Behav Biomed Mater* 2014; **37**: 174–185.
9. Rasoulian R, Najafi AR, Chittenden M, Jasiuk I. Reference point indentation study of age-related changes in porcine femoral cortical bone. *J Biomech* 2013; **46**: 1689–1696.
10. Thompson D, Simmons H, Pirie C, Ke H. FDA guidelines and animal models for osteoporosis. *Bone* 1995; **17**: S125–S133.
11. Gomes P, Fernandes M. Rodent models in bone-related research: the relevance of calvarial defects in the assessment of bone regeneration strategies. *Lab Anim* 2011; **45**: 14–24.
12. Newman CL, Moe SM, Chen NX, Hammond MA, Wallace JM, Nyman JS *et al.* Cortical bone mechanical properties are altered in an animal model of progressive chronic kidney disease. *PloS One* 2014; **9**: e99262.
13. Warden SJ, Galley MR, Hurd AL, Wallace JM, Gallant MA, Richard JS *et al.* Elevated mechanical loading when young provides lifelong benefits to cortical bone properties in female rats independent of a surgically induced menopause. *Endocrinology* 2013; **154**: 3178–3187.
14. Amugongo SK, Yao W, Jia J, Dai W, Lay Y-AE, Jiang L *et al.* Effect of sequential treatments with alendronate, parathyroid hormone (1–34) and raloxifene on cortical bone mass and strength in ovariectomized rats. *Bone* 2014; **67**: 257–268.
15. Allen MR, Newman CL, Smith E, Brown DM, Organ JM. Variability of *in vivo* reference point indentation in skeletally mature inbred rats. *J Biomech* 2014; **47**: 2504–2507.