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Authors
Christiansen, Blaine A
Emami, Armaun J
Fyhrie, David P
et al.

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Blaine A. Christiansen[1]
Department of Orthopaedic Surgery,
University of California-Davis Medical Center,
4635 2nd Avenue, Suite 2000,
Sacramento, CA 95817;
Biomedical Engineering Graduate Group,
University of California-Davis,
Davis, CA 95616
e-mail: bchristiansen@ucdavis.edu

Armaun J. Emami
Department of Orthopaedic Surgery,
University of California-Davis Medical Center,
4635 2nd Avenue, Suite 2000,
Sacramento, CA 95817;
Biomedical Engineering Graduate Group,
University of California-Davis,
Davis, CA 95616

David P. Fyhrie
Department of Orthopaedic Surgery,
University of California-Davis Medical Center,
4635 2nd Avenue, Suite 2000,
Sacramento, CA 95817;
Biomedical Engineering Graduate Group,
University of California-Davis,
Davis, CA 95616

Patrick B. Satkunananthan
Department of Orthopaedic Surgery,
University of California-Davis Medical Center,
4635 2nd Avenue, Suite 2000,
Sacramento, CA 95817;
Biomedical Engineering Graduate Group,
University of California-Davis,
Davis, CA 95616

Michael R. Hardisty
Department of Orthopaedic Surgery,
University of California-Davis Medical Center,
4635 2nd Avenue, Suite 2000,
Sacramento, CA 95817;
Biomedical Engineering Graduate Group,
University of California-Davis,
Davis, CA 95616

Trabecular Bone Loss at a Distant Skeletal Site Following Noninvasive Knee Injury in Mice

Traumatic injuries can have systemic consequences, as the early inflammatory response after trauma can lead to tissue destruction at sites not affected by the initial injury. This systemic catabolism may occur in the skeleton following traumatic injuries such as anterior cruciate ligament (ACL) rupture. However, bone loss following injury at distant, unrelated skeletal sites has not yet been established. In the current study, we utilized a mouse knee injury model to determine whether acute knee injury causes a mechanically significant trabecular bone loss at a distant, unrelated skeletal site (L5 vertebral body). Knee injury was noninvasively induced using either high-speed (HS; 500 mm/s) or low-speed (LS; 1 mm/s) tibial compression overload. HS injury creates an ACL rupture by midsubstance tear, while LS injury creates an ACL rupture with an associated avulsion bone fracture. At 10 days post-injury, vertebral trabecular bone structure was quantified using high-resolution microcomputed tomography (μCT), and differences in mechanical properties were determined using finite element modeling (FEM) and compressive mechanical testing. We hypothesized that knee injury would initiate a loss of trabecular bone structure and strength at the L5 vertebral body. Consistent with our hypothesis, we found significant decreases in trabecular bone volume fraction (BV/TV) and trabecular number at the L5 vertebral body in LS injured mice compared to sham (−8.8% and −5.0%, respectively), while HS injured mice exhibited a similar, but lower magnitude response (−5.1% and −2.5%, respectively). Contrary to our hypothesis, this decrease in trabecular bone structure did not translate to a significant deficit in compressive stiffness or ultimate load of the full trabecular body assessed by mechanical testing or FEM. However, we were able to detect significant decreases in compressive stiffness in both HS and LS injured specimens when FE models were loaded directly through the trabecular bone region (−9.9% and −8.1%, and 3, respectively). This finding may be particularly important for osteoporotic fracture risk, as damage within vertebral bodies has been shown to initiate within the trabecular bone compartment. Altogether, these data point to a systemic trabecular bone loss as a consequence of fracture or traumatic musculoskeletal injury, which may be an underlying mechanism contributing to increased risk of refracture following an initial injury. This finding may have consequences for treatment of acute musculoskeletal injuries and the prevention of future bone fragility.
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Keywords: finite element modeling, vertebral body, trabecular bone, ACL rupture, mechanical testing, osteoporosis

Introduction

Traumatic injuries can have systemic consequences, as activation of the immune system and the early inflammatory response after trauma can lead to tissue destruction at sites not affected by the initial injury [1,2]. ACL rupture in humans causes an immediate flare of inflammatory cytokines [3,4] and biomarkers of cartilage damage [5–8] in the affected joint. Importantly, the increased matrix turnover observed after ACL injury may not be limited to the injured knee, as concentrations of aggrecan, cartilage oligomeric matrix protein, and matrix metalloproteinase-3 are also elevated in the uninjured knee of ACL rupture patients [9]. Similarly, surgically creating a tibial bone defect in rats increases the bone formation rate at distant, unrelated skeletal sites [10]. Altogether, these results suggest a possible systemic effect of musculoskeletal injuries that may be catabolic to the entire system.

We have developed a noninvasive knee injury model in mice, which uses tibial compression overload to induce ACL injury [11]. The loading rate of this model can be controlled to create ACL injuries via either midsubstance tear or ligament rupture with an associated avulsion bone fracture [12]. We observed a 20–44% loss of trabecular bone mass in the femoral and tibial epiphysis of the affected limb by 7–14 days post-injury using this model. Significantly, we also observed a 3–12% decrease in BV/TV in the contralateral knee at 7 days post-injury relative to 1 day post-injury. This suggests that the injury in our mouse model could induce a systemic catabolic effect resulting in a loss of bone volume (and potentially bone strength) throughout the body. However, we have yet to investigate bone loss following acute knee injury at a distant, unrelated (non-contralateral) skeletal site.

In the current study we utilized our noninvasive mouse knee injury model to determine whether acute knee injury causes a mechanically significant trabecular bone loss at a distant,

[1]Corresponding author.
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unrelated skeletal site (L5 vertebral body). We quantified vertebral trabecular bone structure using high-resolution μCT, then determined differences in mechanical properties using FEM and compressive mechanical testing. We hypothesized that knee injury would initiate a loss of trabecular bone structure and strength at the L5 vertebral body. Results from this study reveal a novel and potentially important mechanism of systemic loss of bone structure and strength after musculoskeletal injury. Ultimately these data may affect the treatment of acute musculoskeletal injuries and the prevention of future bone fragility.

Methods

Animals. A total of 23 male C57BL/6 mice (10 weeks old at time of injury) were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN). Mice underwent a two-week acclimation period in a housing facility before injury. Mice were maintained and used in accordance with National Institutes of Health guidelines on the care and use of laboratory animals. All procedures were approved by our Institutional Animal Care and Use Committee.

Knee Injury Via Tibial Compression Overload. Noninvasive knee injury of mice was performed as previously described [11,12]. Briefly, mice were anesthetized with isoflurane inhalation, and were placed in materials testing system (ELF 3200, Bose, Eden Prairie, MN) with platens designed for tibial compression of mice. The right lower leg of each mouse was preloaded with 1–2 N compressive force, then dynamically compressed at either 1 mm/s (low-speed (LS) injury; $n = 8$) or 500 mm/s (high-speed (HS) injury; $n = 8$) to a target displacement of -1.7 mm. Our previous study showed that LS injury creates ACL rupture with an associated avulsion fracture from the distal femur, while HS injury creates ACL rupture without avulsion (midsubstance tear). ACL rupture was noted by a release of compressive force during the dynamic displacement cycle, and with an auditory "click." Sham mice ($n = 7$) were anesthetized and subjected to the 1–2 N preload only. After injury or sham loading, each mouse was returned to normal cage activity for the remainder of the study.

Microcomputed Tomography of L5 Vertebral Body Trabecular Bone. All mice were sacrificed 10 days after injury, and L5 vertebrae were removed for analysis. Bones were scanned using microcomputed tomography (SCANCO, Model μCT 35, Brüttisellen, Switzerland) with 6 μm nominal voxel size, 55 kVp energy, 114 mA intensity, and 900 ms integration time. A global threshold of 543.9 mg HA/cm^3 was used to segment bone from non-bone voxels. Trabecular bone was analyzed at the L5 vertebral body, including the full trabecular region enclosed by the superior and inferior growth plates, excluding the cortical shell and posterior elements (Fig. 1). BV/TV, trabecular number, trabecular thickness, trabecular separation, and bone mineral density were measured using the manufacturer's 3D analysis tools.

FEM of L5 Vertebral Bodies. Finite element (FE) models were constructed based on μCT scans of the L5 vertebral body of each mouse ($n = 7$–8 per group), and were used to estimate compressive mechanical properties. FE analysis of vertebrae followed our published methods [13–15]. Using custom software, a morphologically accurate three-dimensional FE model was created from μCT images for each bone. All models had a vertical dimension of 2.1 mm (350 slices), and excluded the posterior elements, superior and inferior growth plates. The hard tissue in the models was assumed isotropic and uniform with a Young's modulus of 10 GPa and a Poisson's ratio of 0.3. The top of each model was compressed uniformly to an apparent strain of 100% and the resulting load and stiffness were calculated. The top and bottom of the model were constrained using fixed boundary conditions to simulate mechanical testing between glue-bonded platens. Tests were performed with the entire ends of the bone being compressed

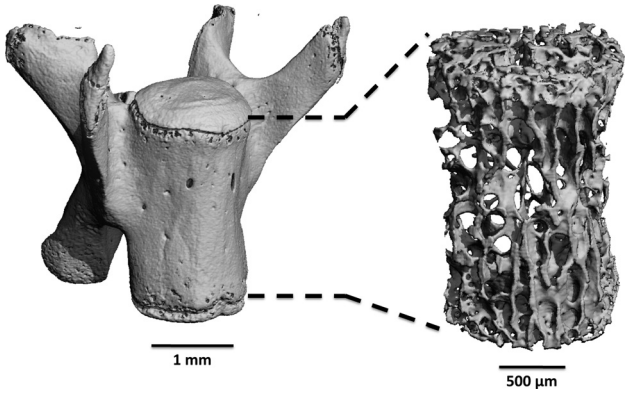

Fig. 1 Mouse L5 vertebra orthogonal view (left). Trabecular bone structure of the L5 vertebral body was analyzed with microcomputed tomography in a volume excluding the endplates and posterior elements (right).

(including both the trabecular region and cortical shell), and also with compression of the trabecular region only (Fig. 2). Trabecular bone compression was simulated by loading 1.002 mm (167 pixel) diameter circular areas axially aligned on the top and bottom of the models. Analysis was performed on a DELL 64 bit PC using custom linear FEM software. All mechanical simulations were linear elastic, therefore both the magnitude of the compressive displacement and the uniform Young's modulus are irrelevant to the between-group comparisons.

Compression Testing of L5 Vertebral Bodies. Following scanning with μCT, L5 vertebral bodies were mechanically tested in compression in order to determine mechanical properties as previously described [16–18]. Posterior elements were trimmed from each vertebral body at the pedicle, and endplates were cut parallel using a precision saw (Isomet 1000, Buehler, Lake Bluff, IL), such that the samples had a vertical length of approximately 1.5 mm. Samples were affixed to loading platens with cyanoacrylate, then compressed to failure at 0.05 mm/s using a electromagnetic materials testing system (Bose ELF 3200). Specimens were rehydrated with phosphate-buffered saline prior to testing, and were kept hydrated during testing. Compressive stiffness and ultimate load were calculated from the force/displacement curve for each specimen.

Statistics. All data were compared using one-way analysis of variance with posthoc analysis by Fisher's protected least significant difference. Significant differences between groups were designated as $p < 0.05$.

Results

Microcomputed Tomography of L5 Vertebral Body Trabecular Bone. Both low-speed (LS; 1 mm/s loading rate) and high-speed (HS; 500 mm/s loading rate) knee injury initiated bone loss at the L5 vertebral body, but the magnitude of this bone loss was greater for LS mice (Fig. 3). For example, BV/TV was 5.1% lower in HS mice than Sham mice ($p = 0.110$), and 8.8% lower in LS mice than Sham mice ($p = 0.010$). Similarly, trabecular number was decreased 2.5% in HS mice ($p = 0.053$) and 5.0% in LS mice ($p < 0.001$) compared to Sham, while trabecular separation was increased 3.4% ($p = 0.022$) and 5.3% ($p = 0.001$) in HS and LS mice, respectively. No significant differences in trabecular thickness were observed between groups.

FEM of L5 Vertebral Bodies. Simulation of vertebral body compression using FE models revealed no significant differences in compressive stiffness when full specimen compression was

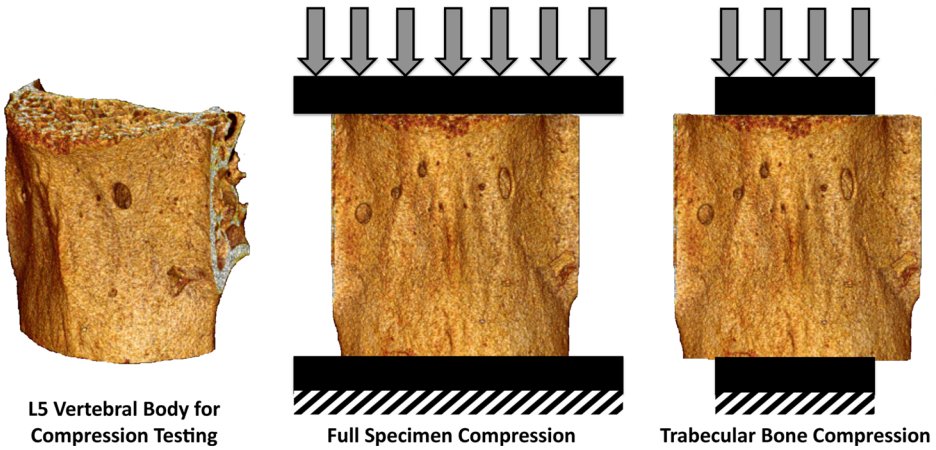

Fig. 2 Representative L5 vertebral body (reconstructed from μCT scan) used for finite element analysis with parallel-cut ends and posterior elements trimmed at the pedicle (left). FE models were compressed with two different boundary conditions (center/right). Full specimen compression was simulated by loading to the entire cross section at the top and bottom, including both trabecular bone and the cortical shell. Trabecular bone compression was simulated by loading 1.002 mm (167 pixel) diameter circular areas axially aligned on the top and bottom of the models.

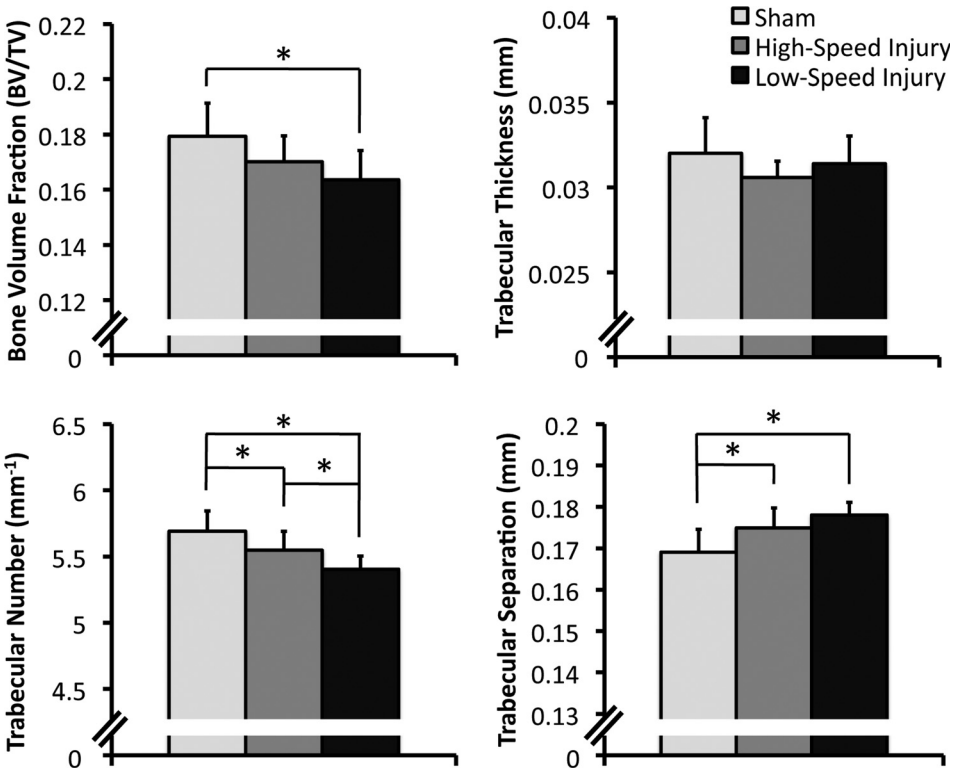

Fig. 3 L5 vertebral body trabecular bone structural parameters. BV/TV was 8.8% lower in LS injured mice than sham mice. Similarly, trabecular number was decreased 2.5% and 5.0% for HS and LS injured mice, respectively, while trabecular separation was increased 3.4% and 5.3% in HS and LS injured mice, respectively, compared to sham mice. No significant differences were observed for trabecular thickness. * $p < 0.05$

used, but significant decreases in stiffness of injured specimens compared to sham when compression was applied directly to the trabecular bone region (Fig. 4). Compression of FE models using the full specimen boundary conditions revealed no decreases in stiffness of HS or LS injured vertebral bodies compared to sham; in fact there was a trend toward increased compressive stiffness in injured specimens, although this was not statistically significant. However, when compression was applied to the trabecular region only, we observed a 9.9% decrease in stiffness in HS injured vertebral bodies ($p = 0.007$), and an 8.1% decrease in stiffness in LS injured vertebral bodies ($p = 0.022$) compared to sham. Compression of vertebral bodies using full specimen boundary conditions resulted in compressive loads primarily being borne by the cortical shell of the vertebral body. In contrast, compression of the trabecular regions at the top and bottom of the model resulted in a relatively greater distribution of loads being borne by the trabeculae, although the cortical shell was still engaged, especially near the midtransverse plane.

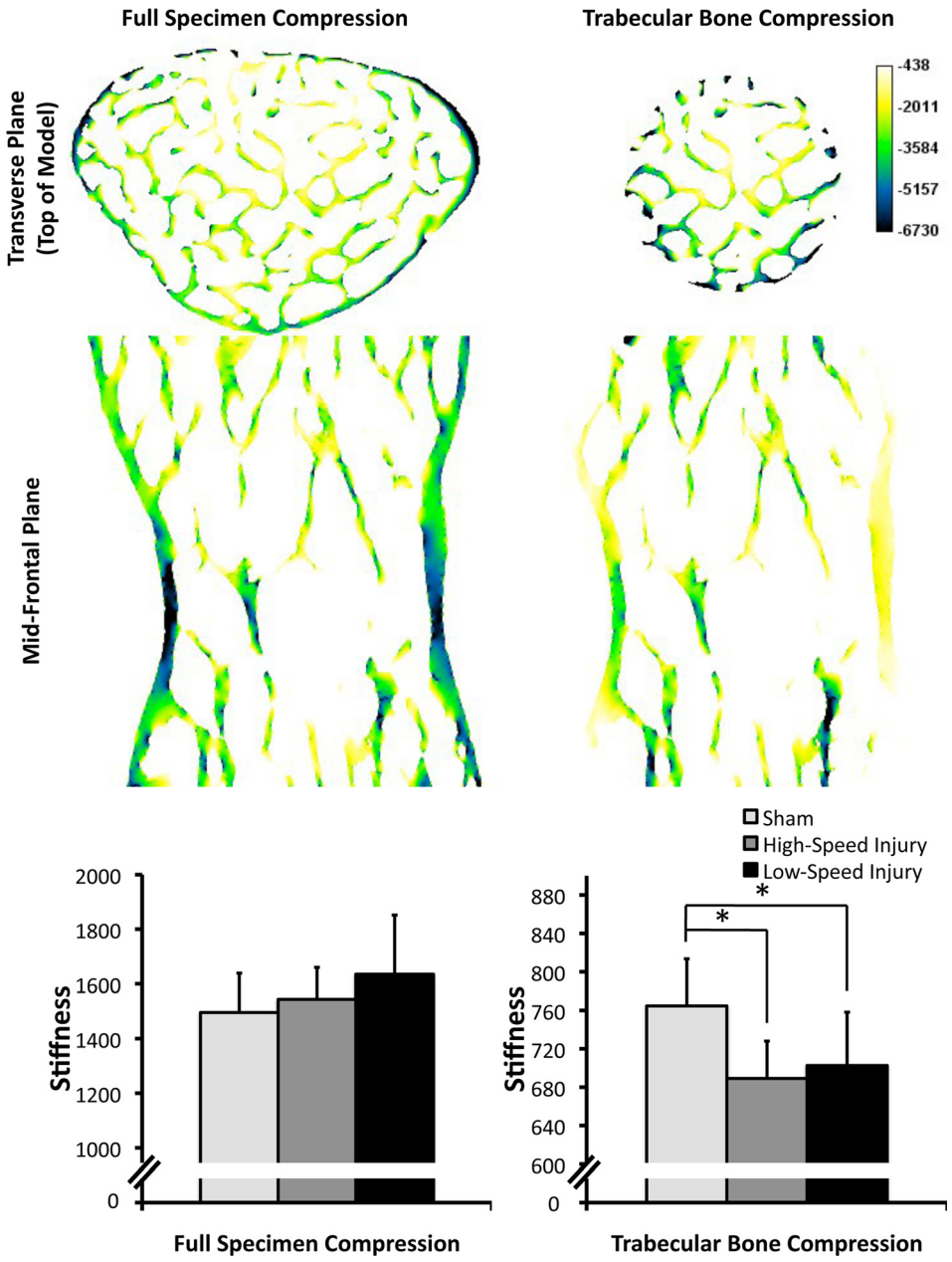

Fig. 4 Representative stress distribution in the direction of the principal loading axis at the top boundary of the model (top row) and midfrontal section (middle row). Full specimen compression (left column) did not predict any significant differences between HS injured, LS injured, or sham specimens (bottom left). However, trabecular bone loading (right column) predicted significantly lower compressive stiffness for HS injured (−9.9%) and LS injured (−8.1%) specimens compared to sham (bottom right). * $p < 0.05$

Compression Testing of L5 Vertebral Bodies. Mechanical testing of isolated vertebral bodies revealed no significant differences in compressive stiffness or ultimate load between experimental groups (Fig. 5). Compressive stiffness exhibited a trend toward decreased values in HS and LS injured mice compared to sham mice (−10.0% and −20.9%, respectively); however, this decrease was not statistically significant. No significant differences were observed for ultimate load.

Discussion

In this study we investigated decreased bone structure and mechanical strength at a distant, unrelated skeletal site (L5 vertebral body) as a consequence of noninvasive knee injury in mice. Consistent with our initial hypothesis, we found significant decreases in trabecular structure 10 days following injury, with mice subjected to low-speed injury (ACL rupture with avulsion fracture) being affected to a greater degree than mice subjected to high-speed injury (midsubstance tear). Contrary to our hypothesis, this decrease in trabecular bone structure did not translate to a significant deficit in mechanical properties assessed by mechanical testing or full compression of FE models. However, we were able to detect a significant decrease in compressive stiffness in injured specimens when FE models were loaded directly through the trabecular bone region only. These data may point to a systemic bone loss as a consequence of musculoskeletal injury, which may be an underlying mechanism contributing to increased risk of refracture following an initial bone fracture.

Noninvasive ACL injury via high-speed and low-speed tibial compression allowed us to create comparable joint injuries that

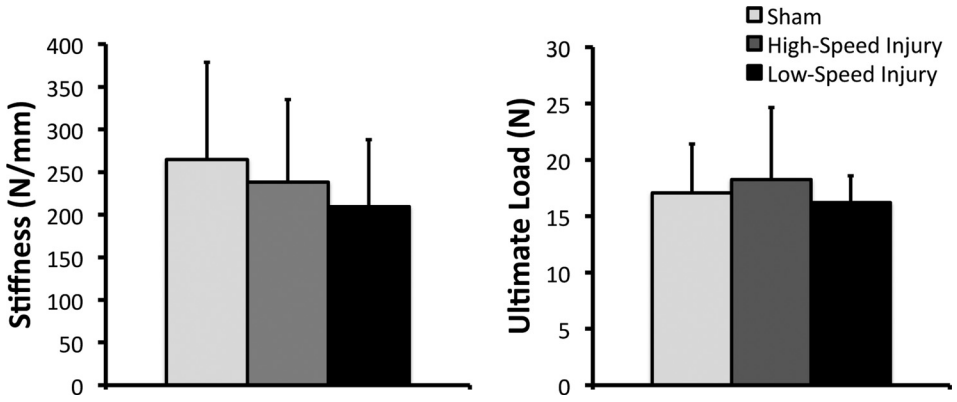

Fig. 5 Results for compressive mechanical testing of isolated mouse L5 vertebral bodies. No significant differences were observed between HS injured, LS injured, or sham specimens for stiffness or ultimate load.

involved either soft tissue damage only (HS injury) or soft tissue damage combined with direct bone damage via avulsion fracture (LS injury). Our previous study using these two injury modes showed that trabecular bone loss in the injured knee at 10 days post-injury is greater for LS injured mice than HS injured mice [12]. The current study showed a similar pattern of trabecular bone loss at a distant, unrelated skeletal site (L5 vertebral body), supporting the conclusion that musculoskeletal injuries that involve bone damage may result in a greater systemic bone loss than injuries involving soft tissue only. This finding points to a potential mechanism underlying the well-established epidemiological observation of increased osteoporotic fracture risk for patients with a non-osteoporotic (index) bone fracture. Subjects with a previous bone fracture are approximately 2–5 times more likely to sustain a future fracture [19–22], even after controlling for bone mineral density [19,23,24]. The risk of future fractures increases with the number of prior fractures [19], and is maintained even when the previous fracture occurs early in life [24–27] or at an unrelated skeletal site [19,20,28]. This increased risk of subsequent fracture is not constant following an initial fracture, but is highest in the first 1–2 yr following an initial fracture, then decreases over subsequent years [28–30], but remains higher than that of the general population with no pre-existing fracture. The specific mechanisms and risk factors associated with this immediate and time-dependent high fracture risk are not known. With our current results, we hypothesize that the healing response to a fracture or significant musculoskeletal injury causes systemic bone loss. Specifically for the mouse, ACL rupture caused mechanically significant decreases in vertebral (L5) trabecular bone volume and stiffness.

Although we observed significant decreases in trabecular bone structure in L5 vertebral bodies of HS and LS injured mice, this did not translate to a detectable deficit in mechanical properties assessed by FEM when displacement was applied to the full cross section of the vertebral body. This is not unexpected, as the cortical bone compartment is known to bear much of the vertebral body compressive loads, particularly near the midtransverse plane [31–33]. Compression of full specimen FE models supported these previous findings, as the cortical shell bore much of the compressive load, particularly near the midtransverse plane of the model (Fig. 4). The lack of a significant difference in compressive mechanical testing of vertebral bodies is also not surprising, as these techniques are strongly dependent on consistent cutting and potting of samples, and often produces a high degree of variability in the resulting data. For example, although the measured compressive stiffness values follow a similar trend to the data collected with μCT, power analysis reveals that at least 50 mice per group would be required to show significant differences between Sham and LS groups given the current means and standard deviations.

Although we were unable to show a mechanical deficit in the vertebral body using full specimen boundary conditions, we were able to detect a significant decrease in compressive stiffness in both injury modes when FE models were directly loaded through the trabecular bone region only. This finding may be particularly important for future fracture risk, as damage within vertebral bodies has been shown to initiate within the trabecular bone compartment [34,35]. For example, Eswaran et al. showed that during compressive loading of human vertebral bodies, high-risk tissue was first observed in the trabecular bone region, and the largest proportion of high-risk bone tissue was in the trabecular bone [34]. They also found that the least amount of high-risk trabecular tissue was near the midtransverse plane, where the majority of the compressive load is borne by the cortical shell. Altogether, these results support our hypothesis of systemic trabecular bone loss following an index fracture contributing to future risk of osteoporotic refracture.

This study is somewhat limited because it did not investigate specific mechanisms contributing to the observed trabecular bone loss at distant skeletal sites following knee injury. These mechanisms may include disuse or reduced mechanical loading following joint injury [36], systemic inflammation [1], or increased systemic bone turnover [10]. Decreased or altered mechanical loading in particular may contribute to structural bone changes at distant skeletal sites following injury, since decreased voluntary movement or unloading of one limb would likely cause changes in the mechanical loading of other skeletal sites. However, changes in gait or voluntary movement in injured mice are yet to be quantified, and the translation of these loading changes to forces seen at the L5 vertebral body may be difficult, although computational methods exist to estimate loading history from trabecular microstructure [37]. Future studies will investigate these potential mechanisms, and will begin to determine their individual contributions to systemic bone loss. However, despite this limitation, we were able to show for the first time significant reductions in trabecular bone structure and compressive stiffness of the trabecular bone compartment at a distant, unrelated skeletal site following noninvasive joint injury. This finding may have important ramifications for treatment of fractures or other significant musculoskeletal injuries in human patients, and for the assessment and prevention of future fracture risk.

This study is also limited because we only analyzed trabecular bone structure at one skeletal site, and did not analyze cortical bone structure at any site. Therefore, it is still unknown whether the observed bone loss phenomenon can be considered a "systemic" response. Additionally, the current study only investigated one time point post-injury. In our previous study we observed differences in bone structure in the affected leg at different time points post-injury [11]. It is therefore possible that bone changes at distant skeletal sites will also be dependent on the time

post-injury. For example, in the affected leg we observed an initial loss of trabecular bone by 7–14 days, followed by a partial recovery of bone structure by 4 weeks post-injury. Since the current study only quantified L5 trabecular bone at one time point (day 10), it is unclear whether there is a recovery of trabecular bone at this skeletal site by 4–8 weeks post-injury. Additionally, the current study did not include a baseline group, which would have been useful to confirm that our sham group did not exhibit any bone changes as a result of anesthesia, analgesia, or tibial compression preload. Our future investigations of this phenomenon will quantify both cortical and trabecular bone at multiple skeletal sites and at multiple time points post-injury.

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