ARTICLE

A comprehensive study of long-term skeletal changes after spinal cord injury in adult rats

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Spinal cord injury (SCI)-induced bone loss represents the most severe osteoporosis with no effective treatment. Past animal studies have focused primarily on long bones at the acute stage using adolescent rodents. To mimic chronic SCI in human patients, we performed a comprehensive analysis of long-term structural and mechanical changes in axial and appendicular bones in adult rats after SCI. In this experiment, 4-month-old Fischer 344 male rats received a clinically relevant T13 contusion injury. Sixteen weeks later, sublesional femurs, tibiae, and L4 vertebrae, supralesional humeri, and blood were collected from these rats and additional non-surgery rats for micro-computed tomography (μCT), micro-finite element, histology, and serum biochemical analyses. At trabecular sites, extreme losses of bone structure and mechanical competence were detected in the metaphysis of sublesional long bones after SCI, while the subchondral part of the same bones showed much milder damage. Marked reductions in bone mass and strength were also observed in sublesional L4 vertebrae but not in supralesional humeri. At cortical sites, SCI induced structural and strength damage in both sub- and supralesional long bones. These changes were accompanied by diminished osteoblast number and activity and increased osteoclast number and activity. Taken together, our study revealed site-specific effects of SCI on bone and demonstrated sustained inhibition of bone formation and elevation of bone resorption at the chronic stage of SCI.

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INTRODUCTION

Osteoporosis is a well-known secondary complication of spinal cord injury (SCI).1–2 Shortly after the injury, sublesional bone density and mass decline rapidly and linearly. This is particularly deleterious to the cancellous bone located in the metaphyseal-epiphyseal area of the distal femora and proximal tibiae, which experiences a 1%–4% per month bone loss in the first 6–12 months after SCI.3–5 This rate is 4-, 10-, and 30-fold greater than those observed during microgravity, prolonged bed rest, and early menopause, respectively. Hence, severe osteoporosis, with at least a 40% reduction in bone mineral content, is common in SCI patients.6 Studies in patients demonstrate that serum or urine levels of bone resorption markers, such as type I collagen C-telopeptide and N-telopeptide, increase within 2 weeks post-SCI and reach extremely high levels within 2–4 months.7 On the other hand, bone formation markers are at normal or slightly above normal levels during this acute phase.7–8 These data suggest that excessive bone resorption is the major contributor for the rapid bone loss in the acute phase.

During the chronic phase that begins 1–2 years after injury, bone mass reaches a nadir at a very low level9–10 but bone loss continues.11 As a result, 50% of SCI patients will sustain a low-impact or osteoporotic fracture at some...
Spinal cord injury-induced bone damage

T Lin et al

To delineate the damaging effect of SCI on the entire skeleton in a clinically relevant setting, we performed a comprehensive analysis of long-term structural and mechanical changes in axial and appendicular bones in adult male rats after SCI. Our study revealed site-specific effects of SCI on bone and demonstrated sustained inhibition of bone formation and elevation of bone resorption at the chronic stage of SCI. These results provide mechanistic insight for developing new effective treatments for SCI-induced severe osteoporosis.

METHODS

SCI surgery and tissue harvest

All animal studies were reviewed and approved by the Institutional Animal Care and Use Committees (IACUC) at Rutgers University and the University of Pennsylvania. Four-month-old male Fischer 344 rats (Taconic, Hudson, NY, USA, n = 5) were anesthetized by isoflurane and their back skin was incised at the mid-line. Muscle was sharply dissected from the spinal column to expose the T9–10 dorsal processes. After cutting the dorsal intravertebral ligament, a T9–10 laminectomy was used to expose the T13 cord. Rats were then moved to a New York University Impactor where their T13 spinal cord received modest injury delivered by a 10-g rod dropped from a 25 mm height. Muscle and skin were then closed with stitches and stainless steel clips, respectively. Post-surgery, manual bladder expression was performed 1–2 times per day throughout the entire experiment. The control group consisted of age- and gender-matched Fischer 344 rats (n = 6). Sixteen weeks later, rats were weighed, subjected to cardiac puncture for blood collection, and perfused with 4% paraformaldehyde (PFA). Sublesional bones, including femurs and tibiae, and supralesional bones, including fourth lumbar vertebrae (L4) and humeri, were collected and fixed further in 4% PFA for subsequent measurements. Before fixation, the full lengths of long bones were measured by a Vernier caliper.

Evaluation of bone microarchitecture by micro-computed tomography

All bones were scanned by a compact fan-beam-type vivaCT40 (Scanco Medical AG, Bassersdorf, Switzerland) at a 15 μm nominal voxel size. The scanned and analyzed areas of each type of bone are summarized in Table 1. All images were first smoothed by a Gaussian filter (sigma = 1.2, support = 2.0) and then thresholded corresponding to 392.1 (trabecular bone) and 582 mgHA-cm⁻³ (cortical bone). Three-dimensional analyses were performed to calculate trabecular parameters, such as trabecular volumetric bone mineral density (vBMD), bone volume fraction (BV-TV⁻¹), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.Sp), and structure model index (SMI), and cortical parameters, such as cortical...
BMD, cortical area (Ct.Ar), cortical thickness (Ct.Th), periosteal perimeter (Ps.Pm), endocortical perimeter (Ec.Pm), porosity, and polar moment of inertia (pMOI) as described by Bouxsein et al.23 Trabecular bone stiffness was calculated for humeri, femora, and L4 vertebrae based on thresholded microcomputed tomography (µCT) images as previously described.24 Briefly, microstructural finite element (µFE) models were generated by converting each bone voxel to an 8-node brick element. Bone tissue was modeled as an isotropic, linear elastic material with a Young’s modulus of 15 GPa and a Poisson’s ratio of 0.3. A uniaxial compression was applied along the axial direction of the model and the model was subjected to a linear elastic analysis to determine the bone stiffness.

Bone histology analysis
After µCT scans, right tibiae were decalcified and processed for paraffin sections. Five-µm longitudinal sections were stained either by hematoxylin and eosin (H&E) for counting the number of cuboidal and plump bone lining osteoblasts, or by tartrate-resistant acid phosphatase (TRAP) assay kit (Sigma-Aldrich, St. Louis, MO, USA) for counting the number of TRAP-positive multinucleated osteoclasts within the secondary spongiosa. All images were captured by Nikon Eclipse 90i and quantified using Bioquant Osteo Software (Bioquant Image Analysis, Nashville, TN, USA).

Serum chemistry
Blood was collected via cardiac puncture at euthanasia and left at room temperature for at least 30 min before centrifuging at 200 × g for 10 min to separate serum. Serum calcium levels were measured by Calcium Colorimetric Assay (Sigma-Aldrich). Osteocalcin and TRACP 5b level were determined by Rat Osteocalcin EIA Kit (Biomedical Technologies, Stoughton, MA, USA) and RatTRAPTM Assay (Immunodiagnostic Systems, Scottsdale, AZ, USA), respectively.

Statistics
Data are expressed as means ± standard error (SEM) and analyzed by unpaired, two-tailed Student’s t-test for comparison between control and SCI groups using Prism 5 software (GraphPad Software, San Diego, CA, USA). Values of P < 0.05 were considered statistically significant.

RESULTS
General observations
Initial body weight was the same among control (308 ± 15 g) and SCI (305 ± 14 g) groups. Sixteen weeks after surgery, the weight of SCI rats (289 ± 15 g) was 30% (P < 0.001) lower than that of controls (417 ± 8 g). Both sublesional and supraspinal long bone lengths were significantly affected in SCI rats compared to controls (femur: control 41.5 ± 0.2 mm, SCI 37.8 ± 0.9 mm, P < 0.01; humerus: control 31.6 ± 0.2 mm, SCI 29.1 ± 0.5 mm, P < 0.01). These results suggest that SCI impairs normal weight gain and longitudinal bone growth.

Bone microarchitectural impairment in sublesional extremities after SCI
In SCI patients, the most severe bone loss occurs at the knee joint, including both distal femur and proximal tibia. There are three types of trabecular bone within this region: primary and secondary spongiosa in the metaphyseal area and subchondral trabecular bone in the epiphyseal area. All three types of bone are formed through endochondral ossification. During development, the two metaphyseal sites are formed within the primary ossification center and the epiphyseal site is formed within the secondary ossification center. While most previous studies focused on analyses of trabecular bone in the secondary spongiosa, there are very few reports describing the changes in trabecular bone at the other two sites under physiological and pathological conditions. To gain detailed knowledge about SCI damage on bone in the knee joint, all three sites were scanned by µCT at a high resolution at 16 weeks post-surgery. At the distal femoral site, we observed that the most drastic bone loss occurred in the secondary spongiosa immediately followed by the primary spongiosa, while, surprisingly, there was only modest bone loss in the subchondral region (Figure 1a). Specifically, 3D analysis of µCT data revealed striking 54% and 65% reductions in vBMD and Bv-Tv−1, respectively, in the secondary spongiosa from the SCI group compared to those from controls (Figure 1b). This was mainly due to significant decreases in Tb.N (36%) and Tb.Th (26%), and an

| Bone          | Scanned area                      | Analyzed area                                                                 |
|---------------|-----------------------------------|-------------------------------------------------------------------------------|
| Femur         | Subchondral bone                  | 2–3 mm below the distal growth plate within the secondary ossification center |
| Femur         | Primary spongiosa                 | 1–2.5 mm above the distal growth plate within the primary ossification center  |
| Femur         | Secondary spongiosa               | 2.5–4 mm above the distal growth plate within the primary ossification center  |
| Femur         | Mid-shaft cortical bone           | 0.5 mm above to 0.5 mm below the midline of a femur                           |
| Humerus       | Secondary spongiosa               | 0.75–2.25 mm below the proximal growth plate within the primary ossification center |
| Humerus       | Mid-shaft cortical bone           | 4.5–5.25 mm below the proximal growth plate within the primary ossification center |
| L4 vertebra   | Trabecular bone                   | 1.5 mm below the top growth plate to 1.5 mm above the bottom growth plate      |

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Bone Research (2015) 15028
Spinal cord injury-induced bone damage

T Lin et al

Figure 1. SCI causes severe trabecular bone loss and structural deterioration in the metaphyseal area but relatively moderate damage in the subchondral bone area in distal femur. (a) Representative longitudinal μCT images of distal femurs in control and SCI rats at 16 weeks after injury. Brackets define the regions of three types of trabecular bone: subchondral trabecular bone (STB), primary spongiosa (PS), and secondary spongiosa (SS) in the metaphysis. (b) μCT measurement of trabecular structural parameters in the secondary spongiosa area. (c) μCT measurement of trabecular structural parameters in the primary spongiosa area. (d) μCT measurement of trabecular structural parameters in the subchondral trabecular area. *P < 0.05; **P < 0.01; ***P < 0.001 vs control (con).

increase in Tb.Sp (73%). Furthermore, a 2.9-fold increase in SMI suggests that SCI impairs the structural integrity of trabecular bone. In the primary spongiosa, similar but relatively smaller reductions in vBMD (47%), BV·TV⁻¹ (56%), Tb.N (34%), Tb.Th (30%), and Tb.Sp (70%), and an increase in SMI were observed in SCI rats compared to controls (Figure 1c). By contrast, much milder trabecular bone damage (−16% in vBMD, −19% in BV·TV⁻¹, −6% in Tb.N, −13% in Tb.Th, +7% in Tb.Sp, and +69% in SMI) was detected in the subchondral area (Figure 1d), implying that the extent of trabecular bone loss induced by SCI is site-specific even within the same bone. Similar patterns of trabecular bone damage were also observed in the proximal tibial region (data not shown).

Next, we analyzed cortical bone structural parameters at the femoral mid-shaft. As shown in Figure 2, cortical BMD remained the same after SCI. However, compared to those from the control group, bones from the SCI group were slimmer with 14% and 8% reductions in Ps.Pm and Ec.Pm, respectively, resulting in a 16% decrease in Ct.Th and a 30% decrease in Ct.Ar. These structural changes led to a drastic 45% reduction in pMOI, a parameter reflecting the bending strength of cortical bone.

The effects of SCI on bone microarchitecture in forelimbs. In addition to the paralysis of trunk and lower extremities below the injury site, SCI causes remarkable neuronal and hormonal changes throughout the entire body, which might affect the bones in the upper extremities. To explore this in our SCI rat model, the supralesional proximal humeri were scanned and analyzed by μCT. We did not detect any differences in trabecular bone within the subchondral site and the primary spongiosa after SCI (Figure 3a). In the secondary spongiosa, a trend of structural impairment was observed, with 15%, 20%, 21%, and 4% decreases in vBMD, BV·TV⁻¹, Tb.N, and Tb.Th, respectively, and a 29% increase in Tb.Sp (Figure 3b) in the SCI group compared to controls, but none of the changes reached statistical significance. By contrast, we observed significant changes in the mid-shaft cortical bone of humeri in SCI rats (Figure 3c and 3d). Bone parameters, Ps.Pm and Ec.Pm, decreased by 9% and 12%, respectively, in the SCI group compared to controls, but
bone thickness (Ct.Th) increased by 9%. A small but significant increase in cortical BMD (3%) was also detected after SCI. Overall, those changes led to a trend of decrease in bending strength after SCI (a 17% decrease in pMOI, \( P = 0.056 \)). Taken together, our results indicate that SCI also has harmful effects on the mobilized forelimbs, albeit to a much lesser extent compared to affected immobilized hindlimbs.

SCI is detrimental to vertebral trabecular bone

Clinical studies describe conflicting conclusions about whether sublesional axial bones are as severely affected by SCI as appendicular bones.25–26 In our study, SCI rats lost significant amounts of trabecular bone in the sublesional L4 vertebral body, particularly within the central region (Figure 4a). Trabecular vBMD and \( BV·TV−1 \) in L4 from SCI rats were 31% and 37%, respectively, less than those from controls, and were accompanied by remarkable decreases in Tb.N (16%) and Tb.Th (27%) as well as increases in Tb.Sp (32%) and SMI (Figure 4b).

The site-specific effect of SCI on trabecular bone strength

\( \text{FE analysis was performed to measure the integrated mechanical competence of trabecular compartments in femur, humerus, and vertebra (Figure 5). Strikingly, the} \)

Figure 2. SCI alters cortical bone structure and impairs cortical bone strength in femur. (a) Representative cross-sectional \( \mu \)CT images of femoral mid-shaft in control and SCI rats at 16 weeks after injury. (b) \( \mu \)CT measurement of cortical structural parameters and bending strength. \( *P < 0.05; **P < 0.01; ***P < 0.001 \) vs con.

Figure 3. SCI results in a trend of trabecular bone loss and significant cortical bone damage in supralesional humerus. (a) Representative longitudinal \( \mu \)CT images of proximal humeri in control and SCI rats at 16 weeks after injury. Brackets define the regions of the secondary spongiosa (SS). (b) \( \mu \)CT measurement of trabecular structural parameters in the secondary spongiosa area. (c) Representative cross-sectional \( \mu \)CT images of humeral mid-shaft in control and SCI rats at 16 weeks after injury. (d) \( \mu \)CT measurement of cortical structural parameters and bending strength. \( *P < 0.05; **P < 0.01; ***P < 0.001 \) vs con.
Figure 5. SCI has site-specific effects on trabecular bone stiffness. Based on a μFE model, bone stiffness was calculated from the secondary spongiosa area of femur and humerus and from the L4 vertebral trabecular bone. **P < 0.01; ***P < 0.001 vs con.

Figure 4. The vertebral trabecular bone is impaired by SCI. (a) Representative longitudinal μCT images of L4 vertebrae in control and SCI rats at 16 weeks after injury. Brackets define the central trabecular region of vertebra (CR). (b) μCT measurement of trabecular structural parameters inside the vertebra. ***P < 0.001 vs con.

Between bone-forming and bone-resorbing cells was further attested by a decreased serum level of bone-formation marker (osteocalcin) and a trend of increased amount of resorption marker (TRAP; Figure 6b). Serum calcium level is usually elevated at the acute SCI stage due to the hyperactivation of osteoclasts (14). However, it returned to normal in SCI rats at this chronic stage (Figure 6b).

DISCUSSION
Bone is a dynamic tissue that undergoes constant remodeling, and coordination between osteoblastic and osteoclastic activities is required for optimal bone homeostasis. After SCI, the interaction between osteoblasts and osteoclasts favors bone resorption, leading to severe bone loss particularly in the sublesional appendicular bones and increased risk of low impact fractures in these bones. In this study, we used an adult rat T13 contusion model to reproduce the effects of SCI in mature patients and performed a comprehensive analysis of the long-term effects of SCI on bone structure and mechanics at multiple clinically relevant skeletal sites. Our high resolution μCT scans revealed that the most severe trabecular bone loss and structural deterioration occurred in the metaphyseal area ofibia and femur. By contrast, more modest but significant damage occurred in vertebral trabecular bone, and only a trend of trabecular bone loss was noted in humerus. Further μFE analysis confirmed this sequence in terms of the degree of mechanical property changes. At cortical sites, SCI does not induce bone demineralization but reduces bone amount and strength in both sub- and supraprofessional bones. Many factors, including local ones (immobilization and bone denervation) and systemic ones (hormonal and metabolic changes), contribute to
SCI-induced bone damage. Our data indicate that trabecular bone is primarily sensitive to local factors while cortical bone is also sensitive to systemic factors.

Remarkably, we found that SCI has distinct site-specific effects on trabecular bone even within the same appendicular bone. In mammals, long bone is developed through endochondral ossification and eventually has two ossification centers. The primary ossification center is formed in the center of diaphysis at the embryonic stage by converting the hypertrophic cartilage in the growth plate into trabecular bone and marrow space. It consists of primary spongiosa, which is directly connected with the growth plate and is mainly shaped by bone modeling during longitudinal growth, and secondary spongiosa, which is derived from the primary spongiosa by a bone remodeling process. Shortly after birth, the epiphyseal cartilage is excreted by canals invaginated from the perichondrium to form the secondary ossification center. The trabecular bone within this center is anatomically located below the articular cartilage and is termed subchondral bone. Previous studies have principally examined pathological effects of SCI on trabecular bone in the metaphyseal region, whereas no studies have attempted to characterize the response of subchondral bone. Surprisingly, we found that subchondral bone is much more resistant to SCI damage than trabecular bone in the metaphyseal area. Moreover, unlike the other trabecular sites, where the bone loss is caused by both trabecular thinning and loss of trabecular bone, the SCI-induced subchondral bone loss occurs mainly through decreasing Tb.Th with little loss of Tb.N. To our knowledge, this is the first report delineating selective responses of trabecular bone within different anatomical regions of the same bone to the same injury. Further investigation will be required to identify whether this is specific for SCI only or represents a more general response to skeletal perturbations and to define the functional mechanisms.

Because of complicated post-surgery care, the majority of previous rodent studies examined bone phenotypes shortly (i.e., usually within 3 weeks) after SCI. The longest follow-up of bone phenotypes was 6 months after SCI in young (6-week-old) male Sprague–Dawley rats by Jiang et al. Similarly to our results, they reported a striking 76% reduction in BV/TV in tibial metaphysis and a relatively milder 34% reduction in L4 vertebra at this chronic stage. However, their densitometric analysis and mechanical testing did not detect any changes in distal radius and humerus, respectively, leading to their conclusion that SCI has a negligible effect on supralesional bones. The discrepancy between our data and theirs on supralesional appendicular bone can be attributed to differences in rat strains (F344 vs Sprague–Dawley), type of SCI model (contusion vs transection), outcome measurement (areal BMD vs volumetric BMD), and animal age (4-month-old vs 6-week-old). While we found that adult SCI rats barely gained weight or even lost weight after paralysis, adolescent SCI rats almost doubled their body weight after 6 months. These data imply that SCI might have greater systemic effects on adult rats than on young rats.

Currently, there is no conventional treatment for SCI-induced osteoporosis at the chronic phase. Given the extreme degree of bone loss following SCI, which far exceeds that induced by other insults, such as estrogen deficiency, neurological deficit, and mechanical unloading, and the notable loss of osteoblasts at later stages, antiresorptive agents such as bisphosphonates would seem to be ineffective choices for treating bone loss at chronic SCI. The great reduction (−71%) in osteoblast number in tibial metaphysis revealed in our study suggests that, in contrast to acute SCI when the osteoblast number and activity are unaffected or even elevated, chronic SCI almost completely eliminates the osteoblasts in the metaphyseal region of tibia and femur. Therefore, in order to restore bone mass, to repair micro-architectural damage, and to reduce risk of fracture, anabolic treatments that greatly stimulate new bone formation via promoting osteoblast number and activity should be suitable therapies for SCI patients at the chronic stage. The treatment choices include teriparatide (recombinant human PTH1-34), the only FDA-approved anabolic treatment for severe postmenopausal osteoporosis, and monoclonal antibody against Sclerostin (Scl-Ab), which has shown potent efficacy in patients with postmenopausal osteoporosis in a phase 2 clinical trial. A recent patient study suggested a trend of bone improvement after 1 year of teriparatide injection together with gait training. However, it did not reach statistical significance, likely due to the limited number of subjects. In contrast, the preclinical data with Scl-Ab are very promising. Three weeks of Scl-Ab injections immediately after SCI completely restored the trabecular bone structure and cortical bone strength in rats.

Our study has several limitations. First, we used age- and gender-matched naive rats instead of sham-operated rats as controls. According to our experience, sham operation does not have detectable effects on rat behavior and neuron functions and should not significantly affect our conclusions. Second, unlike human subjects, rats never cease longitudinal bone growth. As shown in our recently published paper, at age of 8 months, rat proximal tibial growth rate is still at 3 μm/day. Therefore, we cannot completely exclude the confounding factor of growth on the metaphyseal region, especially the primary spongiosa. Indeed, we still observed reduced bone length in both femur and humerus. Applying a 3D image registration approach we developed recently on in vivo longitudinally scanned μCT images should further improve the accuracy and significance of our data.
in terms of analyzing bone phenotypes and studying the underlying mechanism, this adult rat model is much more clinically relevant than young adolescent rats.

In conclusion, our comprehensive study has demonstrated that chronic SCI has deleterious effects on the entire skeleton, with the most severe bone loss and structural deterioration in the lower extremities followed by sublesional vertebrae. The upper extremities also experience bone damage but to a much lesser extent. Furthermore, our findings of sustained inhibition of bone formation and continuous elevation of bone resorption at the chronic stage of SCI strongly suggest that anabolic treatments should be vigorously pursued for promoting bone health and preventing fractures in patients with chronic SCI.

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