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Effects of spinal cord injury and hindlimb immobilization on sublesional and supralesional bones in young growing rats

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ABSTRACT

Both spinal cord injury (SCI) and hindlimb cast immobilization (HCI) cause reduction in maturation-related bone gain in young rats, but the effects of the two interventions on bone pathophysiology may be different. The objective of this study was to compare the effects of SCI and HCI on the sublesional/supralesional bones and bone turnover indicators in young rats. Forty male Sprague–Dawley rats (six-week-old) were randomized into four groups, with ten rats in each group. The groups were classified as follows: base-line control, age-matched intact control, HCI, and SCI groups. Bone tissues, blood, and urine samples were studied at 4 weeks after treatments. The tibial dry weights and ash weights in SCI were remarkably reduced by 7.5% (dry weights) and 8.2% (ash weights) compared with HCI. SCI rats showed lower areal bone mineral density in the proximal tibiae compared with HCI rats (− 14%). Cortical thickness and cortical area of the tibial midshaft in SCI were lower than HCI (− 23%, − 33% respectively). The bone surface/bone volume, trabecular separation, trabecular number, connectivity of the trabecular network, and structure model index of the proximal tibiae were remarkably different between SCI and HCI groups. In SCI tibiae, the mineralizing surface, mineral apposition rate, and surface-based bone formation rate were significantly higher than HCI groups (12%, 47%, and 29% respectively). In the compression test, the ultimate load, the energy of ultimate load, and Young's modulus of the proximal tibiae in SCI rats were significantly lower than HCI rats. The serum levels of osteocalcin and the urinary levels of deoxypyridinoline in SCI were higher than those in HCI. There were no significant changes in supralesional bones between SCI and HCI rats. SCI results in a rapid bone loss with more deterioration of trabecular microstructure and cortical bone geometric structure in sublesional bones. High bone turnover rate and low biomechanics strength were found in tibiae in SCI rats. This might be the result of the imbalance of bone resorption and bone formation induced by the impaired neuronal function.

Introduction

Bone loss is one of the most common and important complications of spinal cord injury (SCI), characterized with low bone mass [8,16,50], bone ultrastructure deterioration [34], and high fracture risk [20,40]. Disuse is generally thought to be one of the major causes for bone loss in SCI [27,44,49], but it is currently unclear whether it is solely responsible for the severity of bone loss. The pathogenesis of bone loss in SCI is very complicated and a number of factors have been implicated in this process [22,24].

SCI causes more damage to bone mass, bone structure, biomechanical properties and bone metabolism than sciatic neurectomy (NX) in animal models [25], and such bone loss is associated with increased bone resorption and decreased bone formation [23,36]. Clinically, the bone loss following SCI is more severe than in bed-rest, so SCI patients show different clinical features when compared with disuse individuals [38,49]. Furthermore, SCI results in significant bone loss that is hardly reversed completely by functional exercise [2,26,29] and drug therapy [35]. Therefore, understanding the mechanisms of bone loss induced by SCI is necessary for therapy and prophylaxis of bone loss after SCI.

To our best knowledge, there are no comparative studies of the reduction in maturation-related bone gain between SCI and hindlimb cast immobilization (HCI) rats. This study was undertaken based upon the hypothesis that the changes of bone microstructure and bone pathophysiology in young rats after SCI would be different from those after HCI and the fact that the neural lesion itself is involved in the bone loss [9,25]. The objective of the study was to compare the effects of SCI and HCI on the sublesional/supralesional bone and biochemical indicators involved in bone metabolism at an early stage in young rats.

Materials and methods

Animals

All experimental procedures were performed in accordance with the guidelines about the care and use of animals defined by the University Animal Welfare and Ethical Review Committees. Rats were purchased and raised at the centralab of our institution. Forty male Sprague–Dawley rats, six-week-old and weighing 140 to 161g, were randomly assigned into 4 groups, with ten rats each group. The groups were categorized as follows: base-line control (BLC), age-matched intact control (INC), HCI, and SCI

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groups. Rats in SCI were anaesthetized by intraperitoneal injection of ketamine (75 mg/kg) and xylazine (10 mg/kg). The spinal cord was exposed by laminectomy at the T10–12 level, and then was completely transected with a sharp scalpel. The urinary bladders of SCI rats were emptied manually at least three times a day for the first 2 weeks until spontaneous micturition recovered. Rats in HCI were performed according to the previously described procedures [4]. Rats were lightly anaesthetized in order to attach the plaster of a Paris cast. Briefly, bilateral hindlimbs were fixed from the waist down with the hips at extension, the knees at 110° flexion and the ankles at 150° plantarflexion. The casts were extended to 1cm below the feet, making weight bearing impossible [48]. All rats were housed in a controlled environment at 22 °C with a twelve-hour light/dark cycle.

Experimental design

Body weights were measured from the initial study. The rats were injected with tetracycline hydrochloride (30 mg/kg; Sigma, St. Louis, MO, USA) at day four and day fourteen before being euthanized. Firstly, at 4 weeks, urine samples were collected in the morning. Then all animals were anesthetized, and blood samples were taken by exsanguinations. Secondly, the left gastrocnemius muscles, tibiae, and humeri were removed. The gastrocnemius muscles were immediately weighted. The tibiae and humeri were utilized for measurements of bone length, wet weight, and bone volume. Then, they were measured for bone mineral content (BMC) and areal bone mineral density (aBMD) by dual-energy X-ray absorptiometry (DXA) and were subsequently used for biomechanical tests. After that, the fractured bone specimens were processed for measurements of dry weight and ash weight. Thirdly, the right tibiae and humeri were scanned by micro-computed tomography (micro-CT) for cortical geometric structure and trabecular microstructure. Finally, the right samples continued to be processed for bone dynamic histomorphometric analysis.

Muscle weight, wet weight, bone length, bone volume, bone density, dry weight, and ash weight

The gastrocnemius muscles removed from the left tibiae were immediately weighted using a 1:10 000 balance (Sartorius BS2105, Germany). Bone length of tibiae and humeri was measured using a dial caliper. For wet weight measurement, the sample was placed in a volumetric flask filled with deionized water. The flask was placed in a desiccator under a vacuum for 2 h. After trapped air had diffused out of the bone, wet weight of the bone was obtained, using a Sartorius balance with a thin wire to which the blotted bone was attached. The bone was weighed again after submersion in deionized water. The difference between the weight of the bone in air and that in water is the volume of the bone. Wet weight and volume were used for calculation of bone density. After finishing biomechanical tests, the fractured bones were dehydrated twice with 70% ethanol and chloroform (1:1) solution for 48 h each time. The dehydrated and defatted bones were then dried in an oven at 80 °C for 48 h, and weighted for dry weight [22]. Then, these samples were dried in an oven at 100 °C for 24 h and ashed in a muffled furnace at 400 °C for 48 h to determine the ash weight [21].

BMC and aBMD measurement

BMC and aBMD of the left tibiae and humeri were measured by DXA on a Hologic Discovery-A QDR Bone Densitometer (Bedford, MA, USA) with software modified for small animals (Regional High Resolution version 4.76; Hologic). The bones were set in a petri dish. To simulate soft-tissue density surrounding the bones, water was poured around the bones to achieve a depth of 1cm. The BMC of the whole tibiae and humeri was obtained. The regions of interest for aBMD included two segments: the proximal quarter and the diaphyseal portion. The aBMD was calculated as BMC divided by bone area. The percentage coefficients of variations (%CVs) for these parameters in our laboratory were less than 2.0%.

Micro-CT measurement

Fixed tibiae and humeri were analyzed using a micro-CT (μCT40, Scanco Medical, Bassersdorf, Switzerland). Approximately two hundred and fifty 1024 × 1024 two-dimensional (2-D) axial slices were yielded, covering the entire width of the bone. These 2-D images had an element size of 12.5 μm in all three spatial dimensions and were segmented into bone and marrow regions by applyin1g a visually fixed threshold for all samples after smoothing the image with a 3-D Gaussian low-pass filter. Trabecular microstructural analysis was performed on the cancellous bone area 1.0 to 4.0 mm distal to the growth plate and cortical geometric structure analysis performed at midshaft. Three-dimensional analysis was performed to calculate morphometric indices including bone surface (BS), bone volume (BV), total volume (TV), cortical thickness (Ct.Th), marrow area (Ma.Ar), cortical area (Ct.Ar), volumetric cortical bone mineral density (vCtBMD), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp) and various ratios including density bone volume fraction (BV/TV), BS/BV, BS/TV [41]. In addition, trabecular architecture was quantified by calculating the structure model index (SMI) and the connectivity of the trabecular network (Conn.D) [14,37]. SMI indicates whether the trabeculae are more rod-like (SMI=3) or more plate-like (SMI=0). Conn.D is a topological measure that represents the number of closed loops in a trabecular bone network. All the parameters above were used to confirm the effects of SCI and HCI on cortical bone and cancellous bone.

Dynamic histomorphometry analysis

Tetracycline hydrochloride, a fluorochrome that is incorporated into the ossifying bone, was used to mark osteoid deposition and mineralization in vivo. At 4 weeks, the right tibiae and humeri were dehydrated in graded alcohols, embedded in methylmethacrylate without decalcification and sectioned longitudinally in 8 μm thick with a Leica/Jung 2065 microtome. Histomorphometry was performed in the regions between 1.0 mm and 4.0 mm distal to the growth plate in the proximal tibiae and humeri in order to exclude the primary trabecular spongiosa. Dynamic histomorphometry analysis was performed using a morphometric program (OsteoMeasure, OsteoMetrics, Atlanta, GA, USA) at a magnification of 100×. Bone formation was expressed by the mineral apposition rate (MAR), mineralizing surface (MS/BS) and surface-based bone formation rate (BFR/BS). Nomenclature and units were used according to the recommendation of the Nomenclature Committee of the American Society for Bone and Mineral Research [39].

Biomechanical testing

The left tibiae and humeri were tested by a three-point bending method using a mechanical testing machine (MZ-500D, Maluto, Tokyo, Japan). The specimens were placed on two support bars (20 mm apart) with a convex side facing toward a loading bar. The load was applied at a strain rate of 1 mm/min until breakage. The ultimate load and energy were automatically determined from a load–displacement curve by a connected computer. Then the load–displacement data were normalized to obtain intrinsic material properties, such as ultimate stress and elastic modulus, which are independent of cross-sectional size and shape [46]. The mechanical strength of the proximal tibiae was evaluated by another compression test using the same instrument. The tibiae were cut 10 mm distal to the proximal end using an electric saw and then were placed on the test apparatus with the anterior side up. A compression force was applied to the proximal tibiae at a speed of 1 mm/min until a fracture occurred. Similar parameters were obtained.

Biochemical assay

After an overnight fast, urine samples were collected throughout 12 h from 6:00P.M. to 6:00A.M. and blood samples (3 ml) were carried out between 6:00A.M. and 8:00A.M. The samples were immediately centrifuged at 1600g 4 °C and the supernatant was stored at − 80 °C until further assay, not longer than two months. Serum levels of osteocalcin (OC) were measured using a rat sandwich enzyme-linked immunosorbent assay (ELISA) kit (BPB Biomedical, Stroughton, MA, USA). Urinary levels of deoxypyridinoline (DPD) were also measured by a rat sandwich ELISA kit (ADL, Fremont, CA, USA). All the assay procedures were followed by the manufacturer's protocols and all samples were assayed in duplicate.

Statistics analysis

All data were presented as means ± SD. Comparisons of data among the groups were performed by analysis of variance (ANOVA) with Student–Newman–Keuls (SNK) test for multiple comparisons. All statistical analysis was performed using SPSS 11.5 software (SPSS Inc., Chicago, IL, USA). A significance level of $P < 0.05$ was used for all comparisons.

Results

General observations

All rats in INC and HCI groups survived until the end of the experiment. However, one rat in SCI group died and the general mortality rate was 2.5%. Dead rats were excluded in data analysis. The animals in SCI group showed paraplegia, muscle spasticity and atrophy.

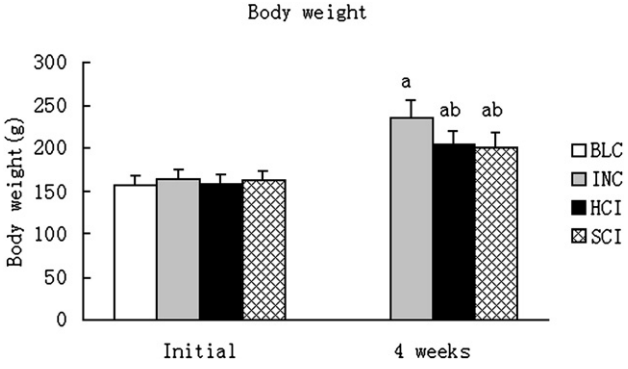

Fig. 1. Body weights (g) in all groups at initial time and 4 weeks after treatments. [a]$P < 0.05$ vs. Initial; [b]$P < 0.05$ vs. INC. Data are expressed as mean ± SD. Multiple comparisons of data were performed by two-factor ANOVA BLC, base-line control; INC, age-matched intact control; HCI, hindlimbs cast immobilization; SCI, spinal cord injury.