Temporal modifications in bone following spinal cord injury in rats

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Abstract

Introduction: The aim of this study was to investigate the temporal modifications in bone mass, bone biomechanical properties and bone morphology in spinal cord injured rats 2, 4 and 6 weeks after a transection.
Material and methods: Control animals were randomly distributed into four groups (n = 10 each group): control group (CG) – control animals sacrificed immediately after surgery; spinal cord-injured 2 weeks (2W) – spinal cord-injured animals sacrificed 2 weeks after surgery; spinal cord-injured 4 weeks (4W) – spinal cord-injured animals sacrificed 4 weeks after surgery; spinal cord-injured 6 weeks (6W) – spinal cord-injured animals sacrificed 6 weeks after surgery.
Results: Biomechanical properties of the right tibia were determined by a three-point bending test and injured animals showed a statistically significant decrease in maximal load compared to control animals. The right femur was used for densitometric analysis and bone mineral content of the animals sacrificed 4 and 6 weeks after surgery was significantly higher compared to the control animals and animals sacrificed 2 weeks after surgery. Histopathological and morphological analysis of tibiae revealed intense resorptive areas in the group 2 weeks after injury only.
Conclusions: The results of this study show that this rat model is a valuable tool to investigate bone remodeling processes specifically associated with SCI. Taken together, our results suggest that spinal cord injury induced bone loss within 2 weeks after injury in rats.

Key words: rat, spinal cord injury, bone loss.

Introduction

One of the main consequences of spinal cord injury (SCI) is the significant bone tissue loss within a few months to a few years after trauma, leading to a decline in bone mineral density (BMD) and increased risk of fractures [1, 2]. In neurologically complete motor SCI, bone loss proceeds at a rate of 1% per week for the first 6-12 months, inducing rapid osteoporosis [3]. Also, Rittweger *et al.* [4] observed a decrease of 33% in the BMD at the distal femur metaphysic and a decrease of 43% at the proximal tibia metaphysic in SCI patients paralyzed for 6 years on average. The most commonly affected sites are long bones of the lower limbs and the trabecular metaphysical-epiphyseal areas of the distal femora and proximal tibiae.

Many factors contribute to the decrease of bone mass and its pathophysiology is complex. Certainly, disuse may play an important role [5]. The loss of mechanical stimuli to the bone is considered one of the main influences in maintaining bone integrity. Immobility leads to a changing pattern of loading in the paralyzed areas, which respond by alteration in skeletal structure. Also, the osteoporosis induced by SCI is related to the impairment of calcium and phosphate metabolism and the parathyroid hormone (PTH)-vitamin D axis, poor nutritional status, venous and capillary vascular stasis, gonadal function and neural factors [6]. Moreover, many authors have demonstrated that sympathetic nervous tone controls both bone formation and bone resorption [7]. In SCI patients this control is affected, aggravating bone loss [5].

Considering the high prevalence of SCI in society and that osteoporosis is one of its most serious complications, SCI experimental animal models have an important role in the understanding of the physiological mechanisms involved in bone loss after SCI [2]. Some studies have already led to meaningful insights into bone turnover processes and plasticity after immobilization [8]. However, there is still a critical need to characterize the temporal structural changes occurring especially in short periods after the injury in rats. This knowledge may allow us to develop more efficient treatments which could be applied in earlier stages, helping to prevent the development of osteoporosis. In this context, the aim of this work was to investigate the temporal changes in biomechanical, densitometric and morphological properties in bone after 2, 4 and 6 weeks of a spinal cord transection in rats.

Material and methods

Animals and experimental design

Forty male Wistar rats (aged 8 weeks and weighing 290 ±6.8 g) were used in this study. They were maintained under controlled temperature (22 ±2°C), light-dark periods of 12 h and with free access to water and commercial diet. All animal handling and surgical procedures were strictly conducted according to the Guiding Principles for the Care and Use of Laboratory Animals. This study was approved by the São Paulo Federal University Animal Care and Use Committee guidelines (1617/08).

Rats were randomly distributed into four groups (n = 10 each group): control group (CG) – control animals sacrificed immediately after surgery; spinal cord-injured 2 weeks (2W) – spinal cord-injured animals sacrificed 2 weeks after surgery; spinal cord-injured 4 weeks (4W) – spinal cord-injured animals sacrificed 4 weeks after surgery; spinal cord-injured 6 weeks (6W) – spinal cord-injured animals sacrificed 6 weeks after surgery.

Surgical procedure

The animals were anesthetized by an intraperitoneal injection of ketamine (90 mg/kg) and xylazine (10 mg/kg) and a laminectomy was executed at Th9-10. In injured rats, the dura mater was exposed and the spinal cord was completely transected with microscissors. During the surgical procedure, body temperature was kept at 37-38°C using a heat pad. Bladders were manually emptied three times daily until sufficient bladder-emptying function returned (about 7 to 10 days after surgery). All rats received preoperative care involving administration of 1 ml of lactate-Ringer's solution, 5 mg/kg Baytril (Bayer, Toronto, ON), and 0.1 mg/kg buprenorphine (Schering-Plough, Pointe-Claire, QC). Postoperative care consisted of lactate-Ringer's solution (2 ml/day, *s.c.*), buprenorphine (0.2 mg/kg/day, *s.c.*), and Baytril (5 mg/kg/day, *s.c.*) administration for 4 days.

Locomotor function

To reveal temporal changes to the locomotor function after SCI, an evaluation was carried out by using the Basso, Beattie and Bresnahan (BBB) Locomotor Rating Scale [9]. The BBB Assessment via the BBB scale started 24 h after surgery and it was performed once a week until the end of the experiment. Each rat was placed on a dimpled plastic floor where its behavioral recovery was observed and recorded for 4 min. The observation period was monitored and recorded by two observers working simultaneously but independently. The BBB rating scale is a 21-point system based on operationally defined behavioral features to follow up recovery progression from complete paralysis to normal locomotion. This scale is able to predict anatomical and behavioral outcomes and provides a view of the recovery process after SCI [9]. Only animals with no locomotor recovery were kept in the study.

Tissue preparation

At week 0, 2, 4 or 6 after surgery, animals were deeply anesthetized with urethane (1.25 mg/kg) and sacrificed via transcardiac perfusion with 100 ml of isotonic saline at room temperature, followed by 500 ml of fixation fluid (4°C) over a period of 6 min. The fixative consisted of 4% paraformaldehyde (Merck, Darmstadt, Germany) in 0.1 M phosphate buffer, pH 7.4. Tibiae and femora were removed for analysis.

Mechanical test

Biomechanical properties of the right tibia were determined by a three-point bending test in an Instron® Universal Testing Machine (USA, 4444

model, 1 KN load cell). Tibiae were placed on a 3.8 cm-long metal device, which provided a 1.8-cm-distant double support on the bone diaphysis. The load cell was perpendicularly positioned at the middle point of the bone. A 5 N pre-load was applied in order to avoid specimen sliding. Finally, the bending force was applied at a constant deformation rate of 0.5 cm/min until fracture occurred. From the load-deformation curve, the maximum load at failure (N), structural stiffness (N/mm) and energy absorption (J) were obtained.

Densitometry

To measure bone mineral content (BMC g/cm^2) and bone mineral density (BMD g/cm^2) of the right femur densitometry (DEXA Hologic Inc Discovery model-Belford, MA, USA) analysis was carried out by using specific software for small animals.

Histopathological analysis

For histopathological analysis, the left tibia was removed upon sacrifice, and then fixed in 10% buffer formalin (Merck, Darmstadt, Germany) for 48 h, decalcified in 4% EDTA (Merck) and embedded in paraffin blocks. Five-micrometer-thick/long/wide slices were obtained in a serially sectioned pattern and stained with hematoxylin and eosin (H.E. stain, Merck).

Morphometric analysis

To confirm the interpretation of the microscopic analysis in tibia of rats, we used a 64-square reticule to perform morphometric assessment of the photomicrographs, the details of which have been described previously [9]. A total of 10 representative areas from each specimen were analyzed by systematic sampling at nominal 400× magnification. Point counting was performed on the bone tissue or medullar area. When all fields were analyzed, the volume density was calculated, based on the principle that each fraction is equal to the mean volume density occupied by its related component [10]. Results were presented as the mean volume density for calcified tissue or medullar tissue in each examined group.

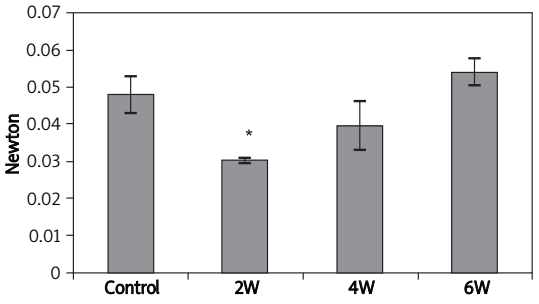

Figure 1. Maximal load
*Control group, 2W – animals sacrificed 2 weeks after surgery, 4W – animals sacrificed 4 weeks after surgery, 6W – animals sacrificed 6 weeks after surgery; *vs. Control*

Statistical analysis

The distribution of all variables was tested for normality by using Shapiro-Wilk's W test. Data were analyzed by Kruskal-Wallis one-way analysis of variance by ranks followed by the Student-Newman-Keuls post-hoc test. Statistica version 7.0 (data analysis software system – StatSoft Inc.) was used to carry out statistical analysis. Values of $p < 0.05$ were considered statistically significant.

Results

General findings

The lesion procedure caused severe degradation in behavioral performance, as measured by the BBB score. SCI animals did not present any recovery in their general motor behavior and none of them presented plantar placement of the paw with weight support during the experimental period.

Biomechanical analysis

Figure 1 shows the values obtained for the maximal load evaluation of all experimental groups ($p < 0.05$). Two weeks after surgery the injured animals showed a statistically significant decrease in maximal load compared to control animals ($p < 0.05$). Interestingly, the animals sacrificed 4 and 6 weeks after surgery did not demonstrate any difference in the biomechanical evaluation when compared to controls ($p < 0.05$).

Densitometry

Bone mineral density and bone mineral content data are shown in Figures 2 and 3. Body mass density showed a significant decrease 2 and 4 weeks after SCI ($p < 0.05$). Interestingly, BMD of the animals sacrificed 6 weeks after surgery did not show a statistical difference when compared to the control group ($p < 0.05$). Bone mineral content of the animals sacrificed 4 and 6 weeks after surgery was significantly higher compared to the control animals and animals sacrificed 2 weeks after surgery ($p < 0.05$) (Figures 2 and 3).

Histopathological analysis

The subjective morphological analysis revealed cortical bone containing osteocytes, the medullary area and periosteum in the control group. Intense resorptive areas were observed in the experimental group 2 weeks after injury (Figure 4 B) when compared to the control group (Figure 4 A). This was represented by lower cortical areas when compared to the control group. The experimental groups

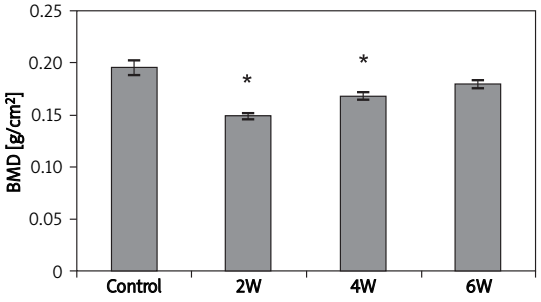

Figure 2. Bone mineral density
*Control group, 2W – animals sacrificed 2 weeks after surgery, 4W – animals sacrificed 4 weeks after surgery, 6W – animals sacrificed 6 weeks after surgery; *vs. control*

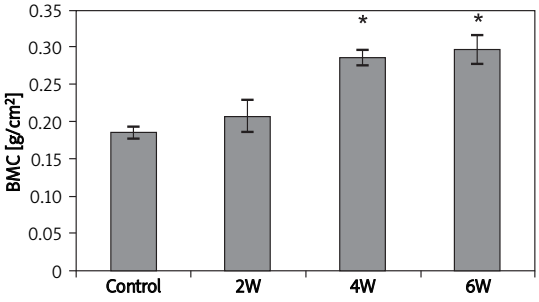

Figure 3. Bone mineral content
*Control group, 2W – animals sacrificed 2 weeks after surgery, 4W – animals sacrificed 4 weeks after surgery, 6W – animals sacrificed 6 weeks after surgery; *vs. control*

sacrificed after 4 and 6 weeks did not show remarkable changes when compared to the control group (Figures 4 C and D, respectively).

Morphometric results

As seen in the morphological description, the histomorphometric results showed statistically significant differences ($p < 0.05$) in the experimental group sacrificed 2 weeks after injury when compared to the control group. No significant differences ($p > 0.05$) were noted for other experimental groups. All the results are summarized in Figure 5.

Discussion

The goal of this study was to evaluate the early temporal changes in bone tissue after spinal cord transection in rats. In addition, these findings will allow better interpretation of osteoporosis pathogenesis following SCI in order to help to optimize management and prevention.

Bone mineral loss following SCI is well documented in clinical studies [11, 12], but the time course changes in experimental approaches are still rare and controversial. We observed that at 2 and 4 weeks after a total SCI, femur presents bone min-

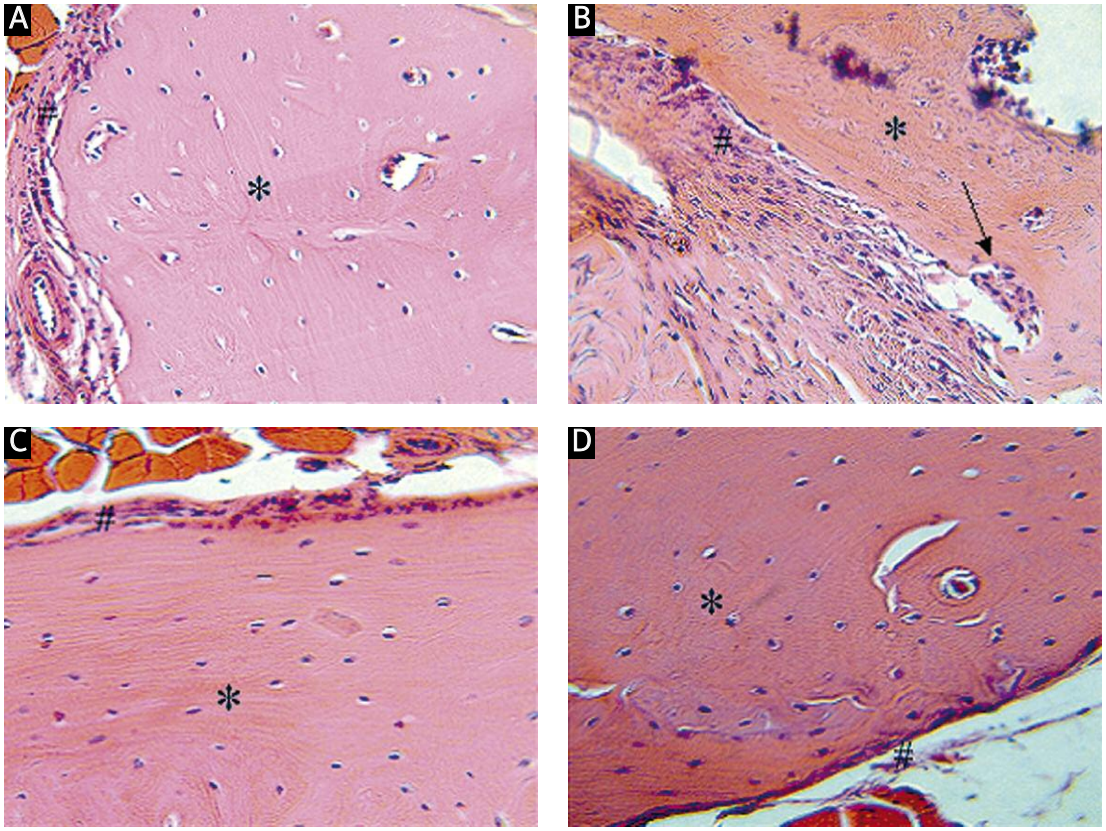

Figure 4. Histological analysis of hematoxylin-eosin staining. Control group (**A**), 2W – animals sacrificed 2 weeks after surgery (**B**), 4W – animals sacrificed 4 weeks after surgery (**C**), 6W – animals sacrificed 6 weeks after surgery (**D**)
**Indicates cortical bone; #indicates periosteum; arrow indicates a resorptive bone area containing osteoclast (multinucleated cell)*

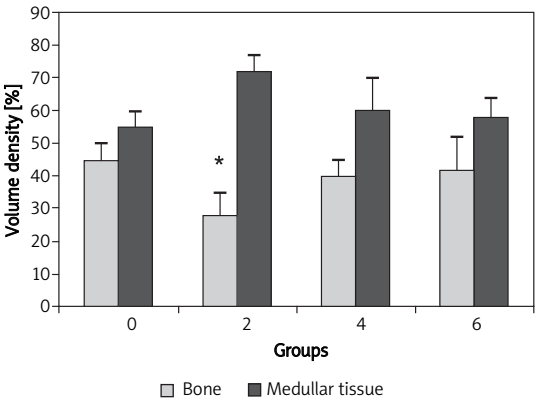

Figure 5. Morphometric data as volume density (%) to bone and medullary tissue for groups at 0, 2, 4 and 6 weeks after spinal transection. Value of $p < 0.05$ when compared to negative control (zero)

eral density loss, and these findings are not maintained at 6 weeks after injury. Biomechanical analysis supported these findings since a decrease of biomechanical strength was noted at 2 weeks after injury, and no significant difference was found at 4 or 6 weeks after injury. The decrease of biomechanical strength might be the result of the low BMD and deteriorated bone structure at 2 weeks after injury, as these findings suggest that SCI leads to a reduction in both bone quantity and quality 2 weeks after the lesion. Similar results were found by Jiang *et al.* [2] when the energy to the ultimate load of the femora, proximal tibiae, and lumbar vertebrae increased significantly from 3 to 6 weeks in injured animals. However, these parameters were not obviously changed from 6 weeks to 6 months in control rats, while there was a significant decrease in SCI rats. In a study conducted by Sugawara *et al.* [13] significant differences between SCI and control rats were found in maximum torque needed to produce failure in the femoral shaft and in compressive load to produce failure in cross-sectional specimens of the distal femur and proximal tibia only at 24 weeks after injury. It is important to stress that site-specific changes have also been found from different strains, suggesting that genetics can influence bone morphology and define bone response to mechanical unloading [14].

The proposed mechanisms of bone loss following SCI involve disrupted vasoregulation, immobilization and several hormonal changes [12]. The altered balance between osteoblast and osteoclast activity is reflected in the change in bone turnover markers after SCI [15]. Bone resorption markers, urinary hydroxyproline, pyridinoline and deoxypyridinoline, C-telopeptide (CTX-1) and N-telopeptide (NTX-1) tend to rise by 2 weeks after injury, generally peaking at 2 and 3 months, whereas bone formation markers, procollagen type I propeptide, osteocalcin, and bone-specific alkaline phosphatase may or may not rise and if so, rise to a much lesser extent than resorption markers [16]. Our histopathological results demonstrated intense resorptive areas in the cortical bone of animals submitted to surgery 2 weeks after injury only. This finding is new, and, therefore, difficult to discuss properly. We support the notion that the period of 2 weeks probably plays a critical role in inducing bone loss following spinal cord injury in rats. This requires further study.

It is important to clarify that some differences in bone loss progression can also be detected between most models of disuse and age/hormone-related models, suggesting that differences in bone remodeling mechanisms may exist in elderly vs. young immobilized rats [17]. Results from SCI patients suggest that both a decrease of osteoblastic activity and an increase of osteoclastic activity contribute to bone loss after trauma [18]. This finding is in close agreement with some results obtained in a rat model of disuse showing a rapid decrease of osteocalcin and a sharp increase of acid phosphatase (i.e., markers of osteoblastic and osteoclastic activities respectively) within a few days to a few weeks after immobilization [19]. Moreover, the BMD and BMC measurements were conducted on the whole femora in this setting, which makes it difficult to directly compare histopathological changes with densitometric ones. Also, note that although bone remodeling is mainly discussed here, we cannot exclude the possibility that reduced bone growth was a critical factor that contributed to some of these adaptive post-injury bone changes in rats. Therefore, it will be of interest in future experiments to determine whether comparable changes in biomarker levels can be found in SCI rats. Anyway, the results of this study show that this rat model is a valuable tool to investigate bone remodeling processes specifically associated with SCI.

In conclusion, our results suggest that spinal cord injury induced bone loss within 2 weeks after injury in rats, and that these changes have an important relationship with bone modifications observed in long periods after SCI. However, this suggestion should be verified by further investigation using chronically injured animals.

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