

Original article

# The effects of spinal cord injury on bone loss and dysregulation of the calcium/parathyroid hormone loop in mice<sup>☆</sup>

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## Abstract

**Objective:** To map the progression of osteoporosis following spinal cord injury in mice in specific areas and analyze changes in parathyroid hormone (PTH) and ion levels which could be responsible for overall bone loss.

**Summary of background data:** Spinal cord injury rapidly induces severe bone loss compared to other conditions, yet the cause of this bone loss has not been identified. Studies suggest the bone loss after injury is not solely due to disuse.

**Methods:** To quantify bone loss we weighed individual bones and measured bone mineral density using dual energy X-ray absorptiometry at acute (1 week) and chronic (4 week) time points following a T9 contusion. An ELISA was used to measure blood PTH levels at 1 and 4 weeks after injury. Calcium and phosphate levels were also analyzed at 4 weeks following injury at the University of Miami pathology core.

**Results:** We observed a significant decrease in bone mineral density in hind limbs after an acute injury, and found this bone loss to progress over time. Furthermore, following chronic injury a decrease in bone mineral density is also observed in bones above the level of injury and in the total bone mineral density. We observed a significant decrease in parathyroid hormone levels in injured mice at the chronic time point, but not at the acute time point which suggests this could be involved in the global bone loss following injury. We also observed a significant increase in serum calcium levels following injury which could account for the imbalance of PTH levels.

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**Keywords:** Bone; Osteoporosis; Spinal cord injury; Parathyroid hormone; Calcium

## 1. Introduction

Spinal cord injury (SCI) induces a severe bone loss, putting patients at high risk for osteoporosis and bone fracture [1–4]. The severe bone loss following SCI differs from osteoporosis seen in other conditions since it occurs at a much faster rate [1,4,5]. Following SCI, bone loss has been found to occur at a

rate of 1.25% per week and patients can lose up to 41% of sublesional bone mineral density within the first year [6,7]. The fragility of their skeletons puts SCI patients at a higher risk for bone fractures despite their lack of physical activity [1–3]. The incidence of fracture risk increases with time following injury and can reach up to 39% at 15 years post-injury [8].

Although it has been previously thought that this bone loss is mostly due to lack of weight bearing below the neurological level, recent evidence suggests other factors may be playing a role. First and foremost, weight bearing exercises seem to have little to no effect in preventing bone mineral density (BMD) loss following SCI [9,10]. Secondly, the bone loss rate following SCI is much higher than that seen in patients who have undergone microgravity or prolonged bedrest [6,11,12].

<sup>☆</sup> Significance: Here we map out the progression of bone loss throughout the organism following spinal cord injury and measure concurrent changes in parathyroid hormone, calcium, and phosphate in the blood.

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Finally, paraplegic patients also exhibit bone loss in upper extremities which are still functional [7]. This data underlines the importance of investigating factors other than lack of weight bearing which may induce osteoporosis in SCI.

Various ions and hormones in the blood are responsible for maintaining bone homeostasis. Parathyroid hormone is a key modulator of bone mass since it is capable of inducing both bone degradation and bone formation [13,14]. When calcium levels are low, PTH is synthesized by chief cells in the parathyroid gland and activates target receptors on kidneys, gut, and bones. Upon binding to receptors on bone cells, PTH releases calcium stored within the bone matrix by initiating bone resorption or bone degradation [15,16]. Osteoclasts are multinucleated cells which break down the bone during bone resorption and PTH has been found to increase both osteoclast activity and levels of bone resorption markers, such as RANKL, which promote osteoclastogenesis [17,18].

Although parathyroid hormone increases bone resorption, it simultaneously induces bone formation by activating signaling pathways in osteoblasts (bone forming cells) which promote synthesis of the bone matrix. While most therapies for osteoporosis are antiresorptive and prevent osteoclast degradation of the bone, intermittent PTH is the only anabolic therapy which actually increases bone formation, making it one of the most effective therapies [13,14,16]. PTH is capable of changing bone composition leading to bone accrual by developing both trabecular and cortical bone [19]. PTH therapy has also been found to reduce the risk of fracture in postmenopausal women with osteoporosis [20,21]. Therefore, any significant change in PTH levels, whether it be an increase or decrease, could be causing bone loss following injury. Previous research has shown aberrant levels of PTH following spinal cord injury with both increases and decreases depending on the time after injury [22,23].

Just as PTH regulates serum calcium levels, calcium regulates the production of PTH through a negative feedback loop [24,16]. The parathyroid gland contains G-protein coupled calcium-sensing receptors which regulate PTH synthesis. Calcium acts as a ligand for these receptors which inhibit the secretion of PTH upon binding via second messengers such as calcium and inositol phosphates [16,25]. Therefore, when serum calcium levels drop, more PTH is produced in order to restore normal calcium levels and vice versa. Phosphate has also been found to regulate PTH levels. Hyperphosphatemia directly stimulates PTH synthesis and phosphate restriction also prevents growth of the parathyroid gland [26]. Phosphate and calcium have both been found to bind to PTH RNA elements and affect its half-life and stability [27].

In this study we characterized the degree of bone loss in acute and chronic spinal cord injury by measuring BMD at different time points in naïve and injured mice. After observing a decrease in BMD, we then quantified blood plasma PTH levels and serum calcium and phosphate levels at these time points following injury. Our aim was to characterize bone loss at different time points following injury and identify changes in hormone and ion levels which could be responsible for the corresponding bone loss.

## 2. Materials and methods

### 2.1. Animals

Adult female C57BL/6 mice were purchased from Jackson Laboratories (USA) Mice were housed in virus/antigen-free environments under diurnal lighting conditions and allowed free access to food and water. Surgeries were performed at approximately 4 months of age. All experiments were approved by the Institutional Animal Care and Use Committee (IACUC Protocol number 11-062).

### 2.2. Surgery

Prior to surgery animals were anesthetized with an IP injection of .02 ml ketamine and .01 ml xylazine. A laminectomy was performed to expose the spinal cord in the T9 vertebrae. Animals were injured by severe contusion with a predetermined force of 70 k Dynes at T9 delivered by an IH impactor (Infinite Horizon Impactor IH 0400 by Precision Systems and Instrumentation), and the exposed area was then sutured. Post-operative care included daily treatments with lactated Ringer's solution and gentamycin. Manual bladder expression was performed daily until needed.

### 2.3. Bone mass

Bones were collected and the muscle was cleaned off before weighing the bones on a high precision electronic scale (American Scientific S/P 182).

### 2.4. Dual energy X-ray absorptiometry

Animals were anesthetized with a ketamine and xylazine cocktail (30 and 10 mg/kg of total body weight) and were laid down flat, facedown with limbs spread out. Bone mineral density (BMD) was measured using the pDXA Sabre Bone Densitometer and the pDXA Sabre Software version 3.9.4 (Norland Medical Systems, Fort Atkinson, WI), both of which were especially designed for small animals. The research mode scan option was used for the measurements. Pixel spacing for the scan was set to  $0.5 \times 0.5$  mm and the scan speed to 4 mm/s. We analyzed total BMD, an area defined as the entire skeleton except for the skull, as well as BMD in certain regions of interest such as the femur, tibia, and humerus. These areas were defined as the total area from one end of the bone to the other end of the bone. For each animal both right and left bones were analyzed and the average of the two was used for analysis.

### 2.5. PTH ELISA

Blood was collected in EDTA coated tubes and samples were centrifuged for 15 min at  $1000 \times g$  within 30 min of collection. Plasma was removed and an ELISA (Immunotopics) kit was used to measure intact parathyroid hormone (PTH 1–84) levels. Results were normalized to total protein in the blood which was measured by Lowry assay.

## 2.6. Calcium and phosphate analysis

Blood was collected and allowed to clot for two hours at room temperature before centrifugation. Serum was removed and analyzed by the University of Miami Comparative Pathology Department.

## 2.7. Statistical analysis

Student's T-test was used to analyze significant differences between naïve and SCI groups. Paired T-test was used to analyze significant differences in 1 week post injury and 4 week post injury groups for DEXA imaging data. One way ANOVA was used to analyze parathyroid hormone levels.

## 3. Results

### 3.1. Progression of bone loss following injury

We determined at what time points bone loss can be observed following thoracic (T9) contusion injury and mapped the progression of osteoporosis by measuring the extent of bone loss over time. Four weeks following injury we found there to be a significant decrease in bone mass below the level of injury in the tibia (Fig. 1). A significant reduction in BMD is observed as soon as 1 week following injury, but this is restricted to areas below the level of injury (Fig. 2). In chronic injuries (4 weeks), BMD continues to decrease in areas below injury, and a significant decrease in BMD is also seen in bones above the level of injury (humeri). A significant decrease is also seen in the total bone mineral density at this time point.

### 3.2. Decrease in blood PTH levels

In our mouse model we observed no significant change in parathyroid hormone levels in blood plasma at one week following injury, but we did observe a decrease in PTH levels

after four weeks following injury (Fig. 3). Although the PTH levels appeared to be dropping over time following injury, there was no significant difference between one week and four week injured animals.

### 3.3. Increase in blood calcium levels

We then chose to analyze both calcium and phosphate levels after injury since these could account for changes in parathyroid hormone levels. In our animals we found there was a significant increase in serum calcium levels at 4 weeks following injury while phosphate levels remained within the same range (Fig. 4).

## 4. Discussion

Although osteoporosis is very prevalent within the spinal cord injury community, the cause of this bone loss has yet to be identified. In a group of men with SCI, 61% met the World Health Organization criteria for osteoporosis, and 19.5% were osteopenic [28]. Patients with spinal cord injury often show the greatest reduction in bone below the level of injury, but have also shown bone loss throughout the body [29,30]. Here we have shown the progression of bone loss following a T9 contusion in mice, which can begin as early as one week following injury in hind limbs. The total BMD and the BMD in areas above the level of injury is not affected until chronic stages of the injury. If bone loss is occurring above the level of injury where there is still locomotor function, we can deduct that not all bone loss following injury is caused by disuse or immobilization. Instead, this data suggests chronic spinal cord injury evokes a global dysregulation of bone homeostasis not limited to areas below injury, which directed our focus to injury induced hormone level changes.

We observed a drop in parathyroid hormone levels only at the chronic time point after injury which paralleled the progression of bone loss in areas above the level of injury, but no significant difference was observed at the acute stage. This data demonstrates that spinal cord injury has no immediate effect on PTH levels, but exerts an effect at a chronic time point. This suggests that the decrease in PTH levels could be responsible for the total bone loss, but not the local bone loss, following injury.

After observing a decrease in parathyroid hormone following injury we decided to investigate different factors which could be affecting parathyroid hormone levels. Parathyroid hormone production is mainly dependent on calcium levels in the blood. High levels of calcium inhibit PTH synthesis via calcium sensing receptors in the parathyroid gland, while hyperphosphatemia induces PTH production. We found that calcium levels were increased after chronic spinal cord injury which would induce the decrease in PTH levels we previously observed. Although there was only a 0.48 mg/dL (0.12 mmol/L) difference in serum calcium levels between naïve and injured mice, only a very slight shift in calcium levels is required to induce a massive change in PTH levels. A study in post menopausal women showed a 1–2% rise in

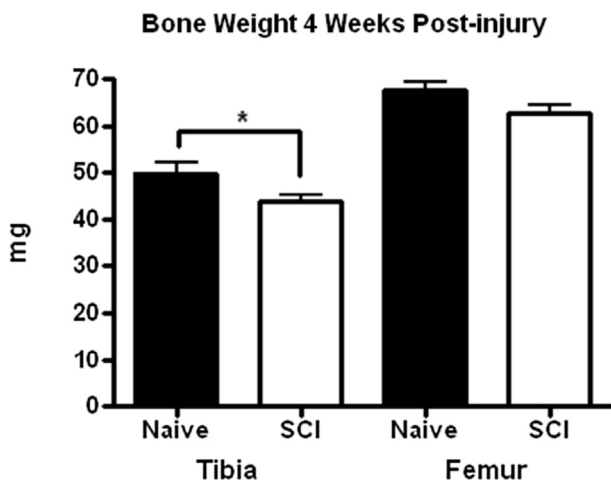


Fig. 1. Bone Mass of hind limbs. Chronically injured mice exhibit a decrease in bone mass in both the femur and tibia, with a significant decrease in bone mass the tibia (injured  $n = 4$ , naïve  $n = 4$ ) ( $p < .05$ , student's t-test).

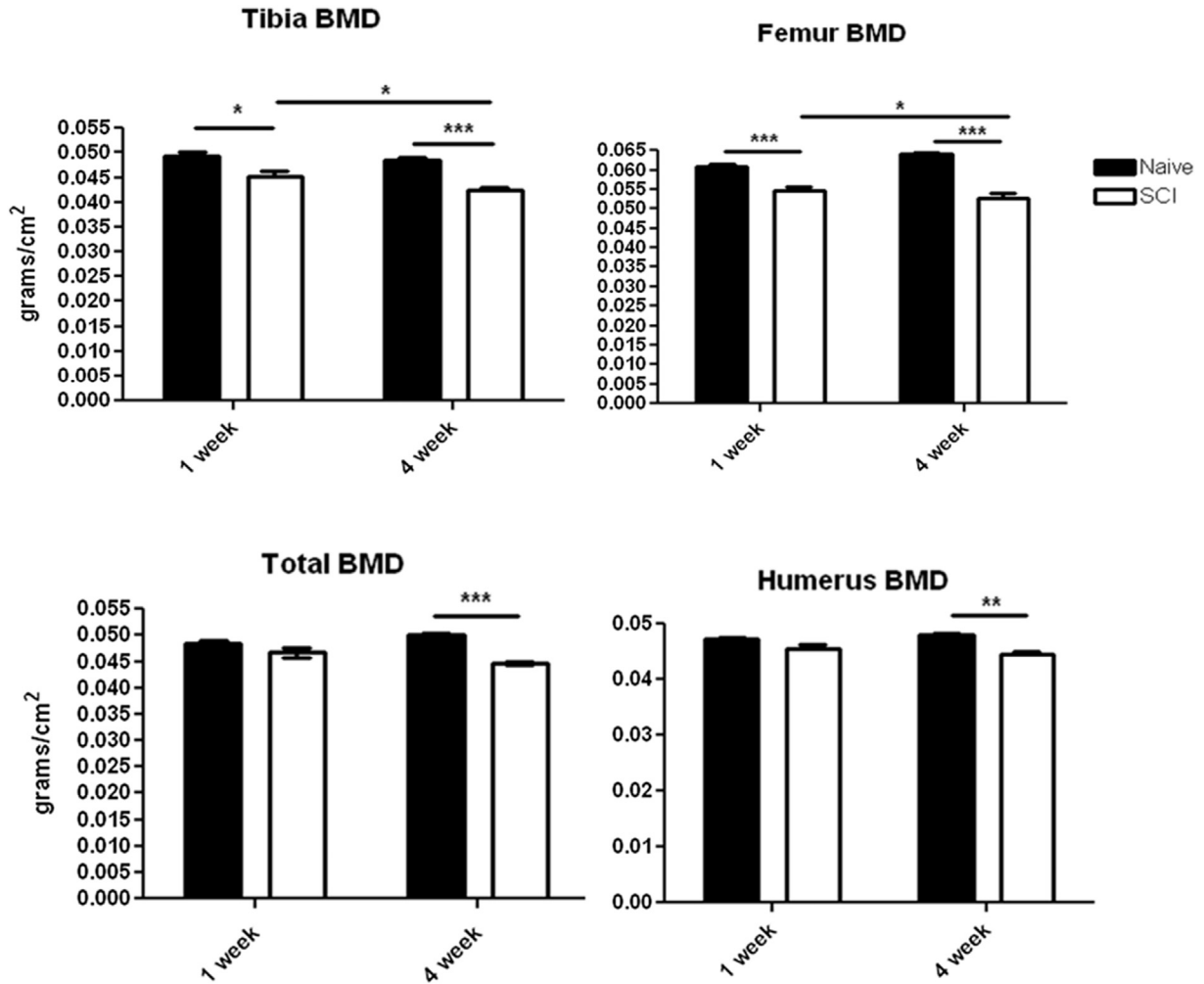


Fig. 2. Bone Mineral Density in hind limbs, humerus, and throughout the skeleton. A significant decrease in BMD is seen as soon as 1 week after injury in both tibia ( $p < .05$ , Student's t-test) and femur ( $p < .001$ , Student's t-test). BMD loss continues to progress at 4 weeks after injury in both hind limbs ( $p < .05$ , Paired t-test). 1 week following injury we see no significant difference in neither total BMD nor BMD in upper limbs. At 4 weeks following injury we see a significant decrease in BMD in both total BMD ( $p < .001$ , Student's t-test) and BMD in upper limbs ( $p < .01$ , Student's t-test). (injured  $n = 5$ , naïve  $n = 4$ ).

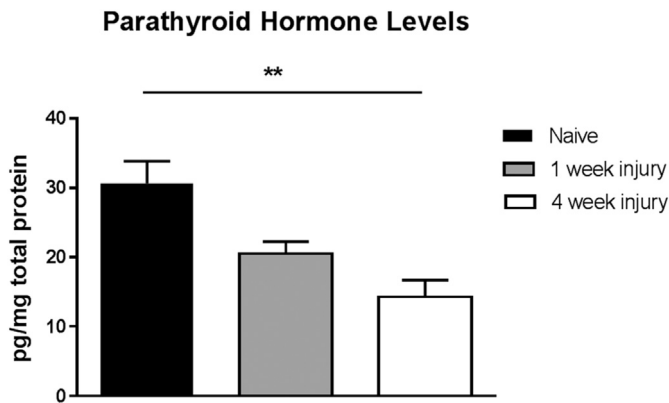


Fig. 3. Parathyroid hormone levels in the blood. There is a significant reduction in parathyroid hormone levels in the blood at 4 weeks after injury. Mean blood plasma PTH levels were 30.65 pg/mg for naïve mice, 20.5 pg/mg for 1 week injured mice, and 14.25 pg/mg for 4 week injured mice. ( $p < .01$ , One way ANOVA) (naïve  $n = 8$ , 1 week injured  $n = 4$ , 4 week injured  $n = 4$ ).

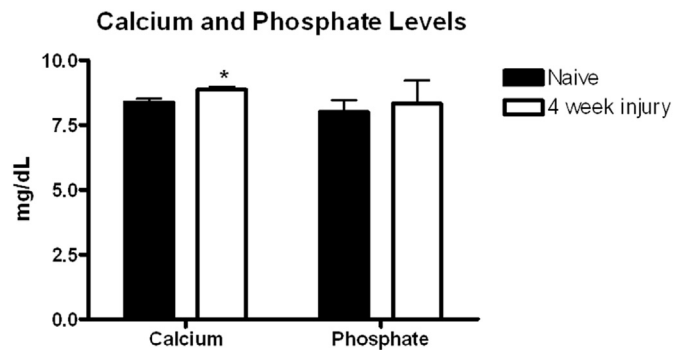


Fig. 4. Calcium and phosphate levels in the blood. There is a significant increase in calcium in the blood at 4 weeks after injury. There is no significant change in serum phosphate levels after injury. Mean serum calcium levels were 8.38 mg/dL (2.09 mmol/L) for naïve mice and 8.86 mg/dL (2.21 mmol/L) for 4 week injured mice. Mean serum phosphate levels were 8 mg/dL (2.58 mmol/L) for naïve mice and 8.32 mg/dL (2.69 mmol/L) for 4 week injured mice. ( $p < .05$ , Student's t-test) (naïve  $n = 4$ , 4 week injured  $n = 5$ ).

serum calcium resulted in 40% drop in serum PTH [31]. This data suggests total bone loss observed following chronic SCI could be caused by changes in calcium and PTH levels.

Currently bisphosphonates are the most common form of treatment for osteoporosis and function by inhibiting osteoclast activity and bone resorption, but these only prevent bone loss during a certain time frame following injury [32,33]. On the other hand, PTH is the only molecular compound known to stimulate bone formation [34]. Targeting PTH production following injury by manipulating calcium-sensing receptor activity could be beneficial. Previous studies in rats have shown that combined treatments for osteoporosis using a calcium sensing receptor antagonist along with bisphosphonates are more effective than bisphosphonates alone [35]. Although PTH treatment is currently being used to treat SCI induced osteoporosis, the underlying mechanism as to how it induces bone anabolism is not completely understood [34]. Although the findings in this study do not fully delineate a mechanism behind the bone loss following SCI, it is important to recognize that there is bone loss occurring above the level of injury and there is a correlation between this global bone loss and changes in blood PTH and calcium levels. Future studies should focus on identifying how a loss of PTH following injury is affecting bone accrual machinery in order to create effective therapies.

## 5. Conclusion

At earlier stages (1 week) following injury mice only exhibit bone loss on the hind limbs but not in the forelimbs. Chronically injured mice (4 weeks) have significant bone loss above the level of injury which may be induced by changes in calcium and parathyroid levels.

## Conflicts of interest

There are no conflicts of interest to declare with the present study.

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