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# Region-Dependent Bone Loss in the Lumbar Spine Following Femoral Fracture in Mice

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## Abstract

We previously showed that after femur fracture, mice lose bone at distant skeletal sites, including the lumbar vertebrae. This bone loss may increase the risk of subsequent vertebral fractures, particularly if bone is lost from high-strain bone regions, which are most commonly found adjacent to the superior and inferior endplates of the vertebral body. To determine regional bone loss from the lumbar spine following femur fracture, we evaluated the cranial, center, and caudal portions of the L5 vertebral bodies of Young (3 month-old) and Middle-Aged (12 month-old) female C57BL/6 mice two weeks after a transverse femur fractures compared to Young and Middle-Aged uninjured control mice. We hypothesized that greater bone loss would be observed in the cranial and caudal regions than in the center region in both Young and Middle-Aged mice. Trabecular and cortical bone microstructure were evaluated using micro-computed tomography, and osteoclast number and eroded surface were evaluated histologically. In Young Mice, fracture led to decreased trabecular and cortical bone microstructure primarily in the cranial and caudal regions, but not the center region, while Middle-Aged mice demonstrated decreases in trabecular bone in all regions, but did not exhibit any changes in cortical bone microstructure after fracture. No significant differences in osteoclast number or eroded surface were observed at this time point. These data suggest that bone loss following fracture in Young Mice is concentrated in areas that contain a large amount of high-strain tissue, whereas bone loss in Middle-Aged mice is less region-dependent and is restricted to the trabecular bone compartment. These results illustrate how systemic bone loss after fracture could lead to decreases in vertebral strength, and how distinct regional patterns and age-dependent differences in bone loss may differentially affect vertebral fracture risk.

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Credit Author Statement:

**Erica V. Ely:** Conceptualization, Investigation, Writing – Original Draft, Visualization. **Benjamin Osipov:** Validation, Formal Analysis, Investigation, Writing – Review & Editing. **Armaun J. Emami:** Conceptualization, Methodology, Validation, Investigation, Writing – Review & Editing. **Blaine A. Christiansen:** Conceptualization, Methodology, Resources, Writing – Review & Editing, Visualization, Supervision, Funding Acquisition.



Conflict of Interest Statement:

The authors have no conflicts of interest to disclose.

**Keywords**

fracture; osteoclasts; bone resorption; systemic bone loss

## Introduction:

Osteoporosis is associated with decreased bone mass and increased risk of fracture, and vertebral fractures are the most common type of osteoporotic fracture [1, 2]. Our previous study in mice investigated systemic skeletal effects of femur fracture and found that mice lost bone at distant skeletal sites (e.g., the lumber spine) within two weeks following a femur fracture [3]. Specifically, we observed 11% and 18% lower trabecular bone volume fraction (BV/TV) at the L5 vertebral body of young (3 month-old) and middle-aged (12 month-old) fractured mice, respectively, compared to age-matched controls. We also observed similar bone loss in the cortical shell of the lumbar vertebral bodies, with 10% and 13% lower cortical thickness in young and middle-aged fractured mice, respectively, compared to age-matched controls. These microstructural changes resulted in significantly decreased compressive stiffness of the lumbar vertebral bodies predicted by finite element analysis in fractured mice compared to uninjured control mice.

The specific location of resorbed bone tissue is a crucially important factor affecting mechanical properties and fracture risk for whole bones. Loss of bone tissue in areas of high strain results in considerable changes in strain distribution and deterioration of whole-bone mechanical properties, while removal of bone from low-strain areas results in almost no changes in mechanical properties [4, 5]. Another study in human lumbar vertebrae found that the amount of “high-risk tissue” (bone tissue at the highest risk of initial failure within the vertebral body when subjected to compressive loading) was significantly greater adjacent to the superior and inferior growth plates of a vertebral body in comparison to the center region [6]. Therefore, loss of bone volume from the superior and inferior regions of the vertebral body may have a considerable effect on whole-bone mechanical properties and vertebral fracture risk. However, the region-dependent loss of bone from the lumbar spine following femur fracture has not been investigated.

In this study, we analyzed trabecular and cortical bone at the L5 vertebral body of Young (3 month-old) and Middle-Aged (12 month-old) mice 2 weeks after transverse femoral fracture [3]. The cranial (superior), center, and caudal (inferior) regions of the vertebral body were individually analyzed in order to determine region-dependent bone changes relative to vertebrae from Young and Middle-Aged uninjured mice. Micro-computed tomography (μCT) was used to quantify trabecular and cortical microstructure, and tartrate-resistant acid phosphatase (TRAP) staining of histological sections was used to measure osteoclast number and the percentage of eroded bone surface. We hypothesized that greater bone loss would be observed in the cranial and caudal regions than in the center region in both Young and Middle-Aged mice, which could lead to diminished mechanical properties of the whole bone and increase risk of subsequent fracture.

## Methods

### Animals:

Thirty-six female C57BL/6 mice, 3 months old (Young) or 12 months old (Middle-Aged) at the start of the study, were subjected to transverse femur fracture (Fractured) or no surgical procedure (Control). Transverse femur fractures were stabilized by a 0.01 in. stainless steel intramedullary pin inserted in the right femur via a method developed by Bonnarens and Einhorn [7]. Fractures were created with a controlled impact using a modified fracture apparatus [8, 9]. Uninjured Control mice were subjected to anesthesia and analgesia only. Full weightbearing and unrestricted activity were permitted postoperatively. Buprenorphine (0.05 mg/kg) was administered every 12 h for 48 h post-fracture. Mice were euthanized on day 14 after fracture (n =7-12 mice/age/experimental group) and L5 vertebrae were removed for analysis. Mice had ad libitum access to food (Harlan irradiated 2918 chow) and autoclaved water and were monitored by husbandry staff at least once a day, 7 days a week, with monthly health care checks by a veterinarian. All procedures were approved by the UC Davis Institutional Animal Care and Use Committee.

### Micro-Computed Tomography Analysis of Trabecular and Cortical Bone Microstructure:

L5 vertebrae were scanned using micro-computed tomography (SCANCO Medical AG, μCT 35, Brüttisellen, Switzerland), with 6 μm nominal voxel size (X-ray tube potential = 55 kVp, current = 114 mA, integration time = 900 ms, number of projections = 1000/180°) according to the JBMR guidelines for μCT analysis of rodent bone microstructure [10]. Trabecular and cortical bone were analyzed at the L5 vertebral body in three regions: cranial, center, and caudal (Figs. 1–2). These regions were designated by equally dividing the L5 vertebral body into thirds along the cranial-caudal axis from the cranial growth plate to the caudal growth plate, excluding the transverse processes. Trabecular volumes of interest were manually drawn on transverse images excluding the cortical shell. Cortical shell volumes of interest were contoured from the cranial growth plate to caudal growth plate excluding the trabecular compartment and the transverse processes. A global threshold of 471.3 mg HA/cm$^3$ was used to segment bone from non-bone voxels. Microstructural outcomes for trabecular bone and the cortical shell were determined using the manufacturer's 3-D analysis software.

### TRAP Staining Analysis of Osteoclast Number and Activity:

Vertebrae were fixed in 4% paraformaldehyde for 24-48 hours and were subsequently preserved in 70% ethanol. Following μCT imaging, bones were decalcified in 0.34 M EDTA for 30 days with solution changes every 2–3 days. Vertebrae were embedded in paraffin, frontal sections were taken through the center of the vertebral body at a thickness of 5 μm, and sections were stained using the tartrate-resistant acid phosphatase (TRAP) staining system (Sigma-Aldrich, St. Louis, MO). The trabecular bone region of the vertebral body was divided into cranial, center, and caudal regions as described for μCT volumes of interest (Fig. 3). TRAP-stained osteoclasts near eroded bone surfaces and total eroded surface length were measured using ImageJ. Total bone surface length was also measured within each region of interest. Number of osteoclasts per bone surface (N.Oc/BS) and eroded surface per bone surface (E.Pm/B.Pm) were calculated. All samples had the same scale of 5.88 microns/pixels.

### Statistical Analysis:

For all outcomes, data from each age group of mice were analyzed using 2-way ANOVA stratified by injury status and region of interest. Post-hoc analyses were performed using t-tests. Significant differences were designated at $p < 0.05$.

## Results

### Micro-Computed Tomography Analysis of Trabecular Bone

Changes in trabecular bone microstructure as a result of fracture were significantly greater in the cranial and caudal regions of the vertebral body than in the center region in Young mice. Specifically, trabecular bone volume fraction (BV/TV) was 11% lower in the cranial region ($p = 0.004$) and 11% lower in the caudal region ($p = 0.003$) of Young Fractured (YF) mice compared to Young Control (YC) mice, while no significant differences between YF and YC mice were observed at the center region (Fig. 1). In contrast, changes in trabecular bone microstructure as a result of fracture were not dependent on regions of the vertebral body in Middle-Aged mice. Middle-Aged Fractured (MAF) mice had significantly lower BV/TV in the cranial (−17%) and caudal (−15%) regions compared to Middle-Aged Control (MAC) mice, and 23% lower BV/TV in the center region, though this difference was not statistically significance ($p = 0.139$). Trabecular thickness (Tb.Th) was decreased in YF mice compared to YC mice at all regions (Fig. 1). YF mice had 8%, 9%, and 6% lower Tb.Th in the cranial, center, and caudal regions, respectively. Similarly, MAF mice had 11%, 12%, and 11% lower Tb.Th in the cranial, center, and caudal regions, respectively, compared to MAC mice. No significant differences were observed between Fractured and Control mice for trabecular number (Tb.N). Bone Tissue Mineral Density (TMD) was significantly reduced in YF mice compared to YC mice, but the same effect was not seen in Middle-Aged mice (data not shown).

### Micro-Computed Tomography Analysis of the Cortical Shell

Microstructure of the cortical shell of the lumbar vertebral body was also altered in YF mice compared to YC mice, and these changes differed by region (Fig. 2). YF mice had significantly lower cortical bone area (B.Ar) in the cranial region (−12%) and caudal region (−17%) compared to YC mice, while the center region was not significantly different between YF and YC mice (−9%, $p = 0.11$). Similarly, YF mice had significantly lower cortical thickness in the cranial region (−9%) and caudal region (−16%) compared to YC mice, while the center region was nearly statistically different between YF and YC mice (−5%, $p = 0.054$). In contrast, there were no significant differences in cortical shell microstructure outcomes in Middle-Aged mice at any region (Fig. 2).

### Region-Dependent Differences in Osteoclast Number and Eroded Surface

Analysis of TRAP-stained slides from the L5 vertebral body revealed no significant differences between Fracture and Control mice for osteoclast number and eroded surface (Fig. 3).

## Discussion

In this study we quantified bone loss from specific regions of the L5 vertebral body following femoral fracture in mice. We found that fracture caused greater trabecular and cortical bone loss in the cranial and caudal regions of the L5 vertebral body in Young mice, while regional differences in post-fracture bone loss were not observed in Middle-Aged mice. These findings may partially explain how the healing response to a bone fracture at any skeletal site can initiate changes in bone microarchitecture in the vertebral bodies, which could compromise mechanical strength and increase fracture risk in the spine.

Age-related differences in bone loss from cortical and trabecular bone are potentially important from the perspective of load sharing in the cortical and trabecular compartments in the vertebral body [11–13]. In human vertebrae, the fraction of compressive force supported by trabecular bone varies from 66-89% near the endplates to 37-62% at the midtransverse plane [11, 13]. With age, trabecular bone loss leads to a significantly increased load-carrying role of the cortical shell [12]. Similarly, a study in rats found that trabecular bone accounted for 11-57% of the whole-bone strength of the L5 vertebral body, and this percentage was decreased by ovariectomy-induced bone loss [14]. Therefore, the lack of changes in cortical bone morphology in older mice following fracture may be due to the greater mechanical role of the cortical shell in these mice. In support of this hypothesis, a previous study that examined trabecular bone loss in rats during pregnancy and lactation found that trabecular bone volume was lost to a greater degree in the proximal tibia, where it had less of a mechanical role, than in the lumbar spine, where the trabecular bone provided a much larger mechanical role [15].

In addition to changes in bone volume, changes in bone quality following fracture may also have an effect on bone strength and fracture risk. For example, previous studies in humans found that women who had low-trauma fractures in the absence of low BMD had significantly different lamellar width and osteonal structure than age-matched women without prevalent fractures [16, 17]. In the current study we observed a small but significant decrease (~1%) in TMD in vertebral trabecular bone of Young mice following fracture. This difference may reflect an increased microporosity volume or a change in bone tissue composition, however this remains to be investigated.

This study is the first to report region-dependent bone changes in the vertebral bodies following femur fracture in mice, including changes in trabecular bone and the cortical shell of the vertebral body. We found important region-dependent differences in bone adaptation in Young mice, and reported notable age-related differences in trabecular and cortical bone loss patterns. However, there are several limitations of this study that must be acknowledged. First, only one time point was investigated (2 weeks post-fracture). This is notable because our previous study [3] showed possible age-related differences in recovery from post-fracture bone loss, and greater (statistically significant) differences in osteoclast activity were observed at an earlier time point (3 days post-fracture). Additionally, the current study was cross-sectional, and did not directly measure bone loss in the same mice over time. This study investigated only female C57BL/6 mice; the systemic bone loss response following fracture may be considerably different for male mice or other genetic mouse strains. Also, it

is currently unclear if the post-traumatic systemic bone loss observed in mice would also occur in large animal models or humans, therefore the translatability of these findings is uncertain. It is also possible that our study did not include enough mice to detect significant differences between groups. Considering trabecular bone volume fraction (BV/TV) as the primary outcome, nine mice per group provided at least 80% power to detect differences between YF and YC mice at the cranial and caudal regions, but only ~60% power to detect differences between MAF and MAC mice at these regions. Additionally, our statistical analysis did not include any corrections for data dependency within a given animal.

Despite these limitations, this study reports significant region-dependent and age-dependent differences in bone loss from lumbar vertebral bodies following a femur fracture. Bone loss that preferentially affects the regions adjacent to the vertebral end plates may have considerable consequences for the mechanical properties and fracture resistance of the whole bone, and bone loss in these regions could be an underlying mechanism contributing to increased fracture risk following an initial fracture.

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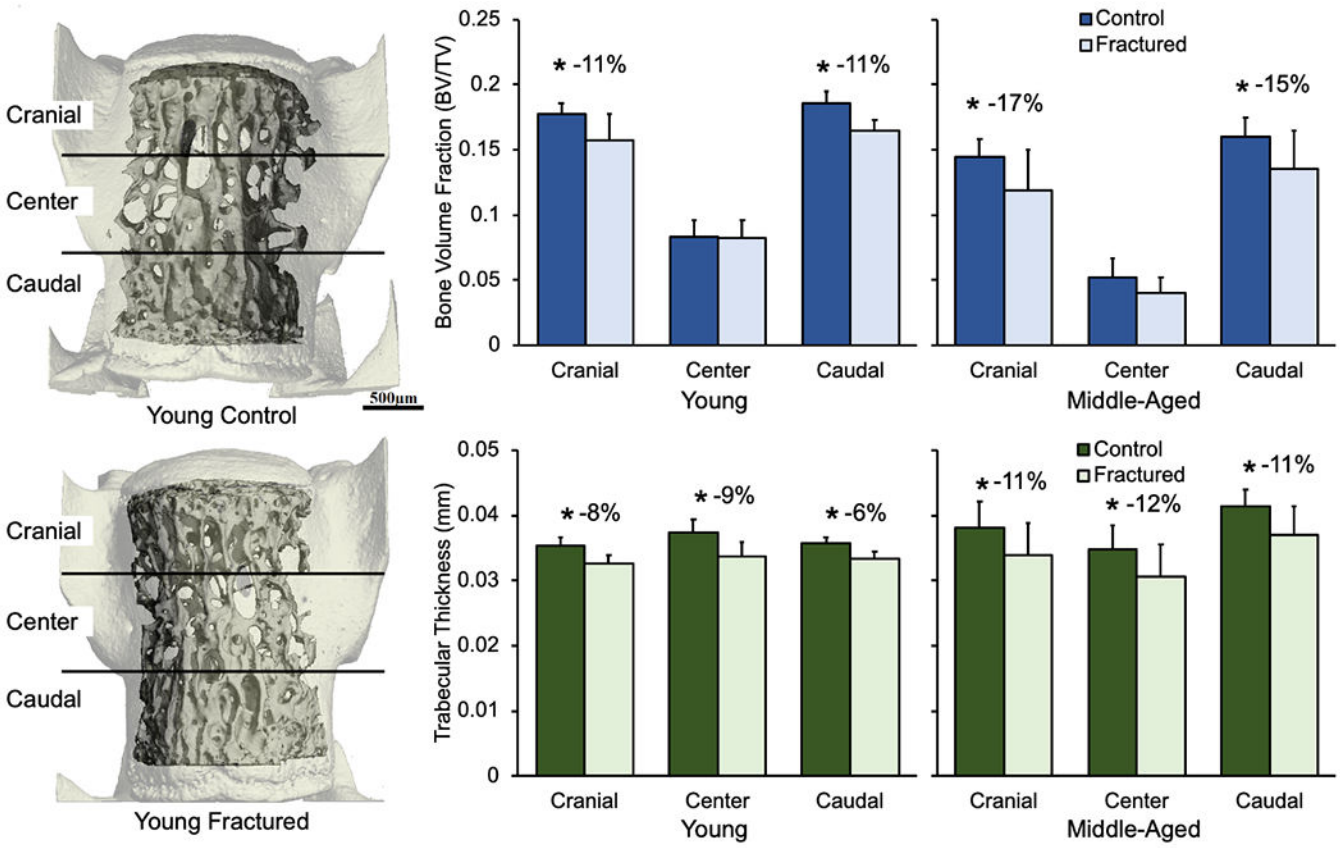


**Figure 1:**

Trabecular bone microstructure of the L5 vertebral bone in Young and Middle-Aged mice following femur fracture. (Left) Representative images of trabecular bone in the L5 vertebral body of Young Fractured and Control mice. (Right) Trabecular bone volume fraction (BV/TV) and Trabecular Thickness (Tb.Th) of the cranial, center, and caudal regions of the vertebral bodies. Trabecular bone volume was primarily decreased in the cranial and caudal regions, while trabecular thickness was reduced in all regions for both age groups. No significant differences were observed for trabecular number (Tb.N).

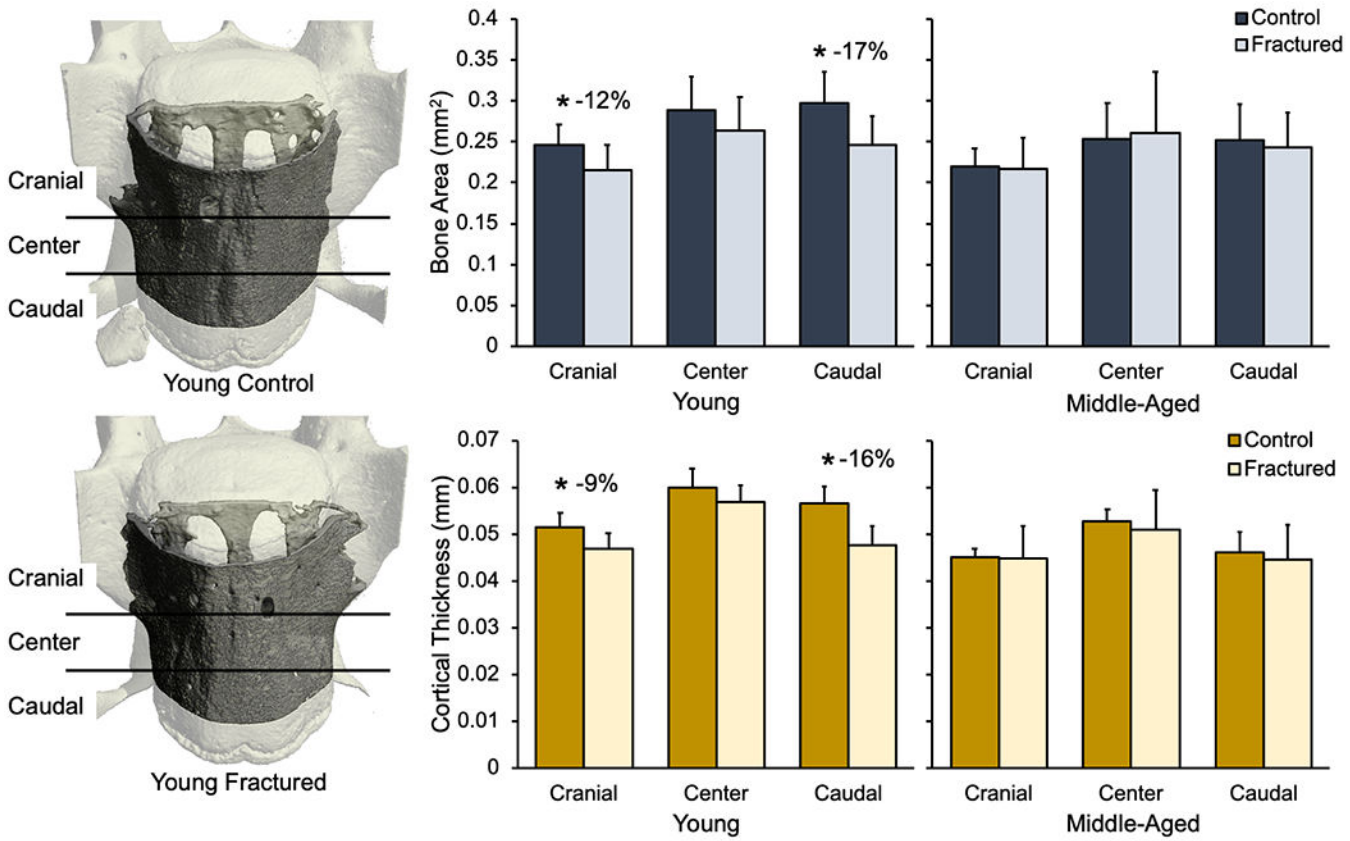


**Figure 2:**

Microstructure of the cortical shell of L5 vertebral bodies from Young and Middle-Aged mice following femur fracture. (Left) Representative images of the cortical shell of the L5 vertebral body of Young Fractured and Control mice. (Right) Cortical bone area (B.Ar) and Cortical Thickness (Ct.Th) of the cranial, center, and caudal regions of the vertebral bodies. B.Ar and Ct.Th were significantly decreased in the cranial and caudal regions, but not the center region, of Young Fractured mice. No significant differences in cortical shell microstructure were observed in Middle-Aged mice following fracture.

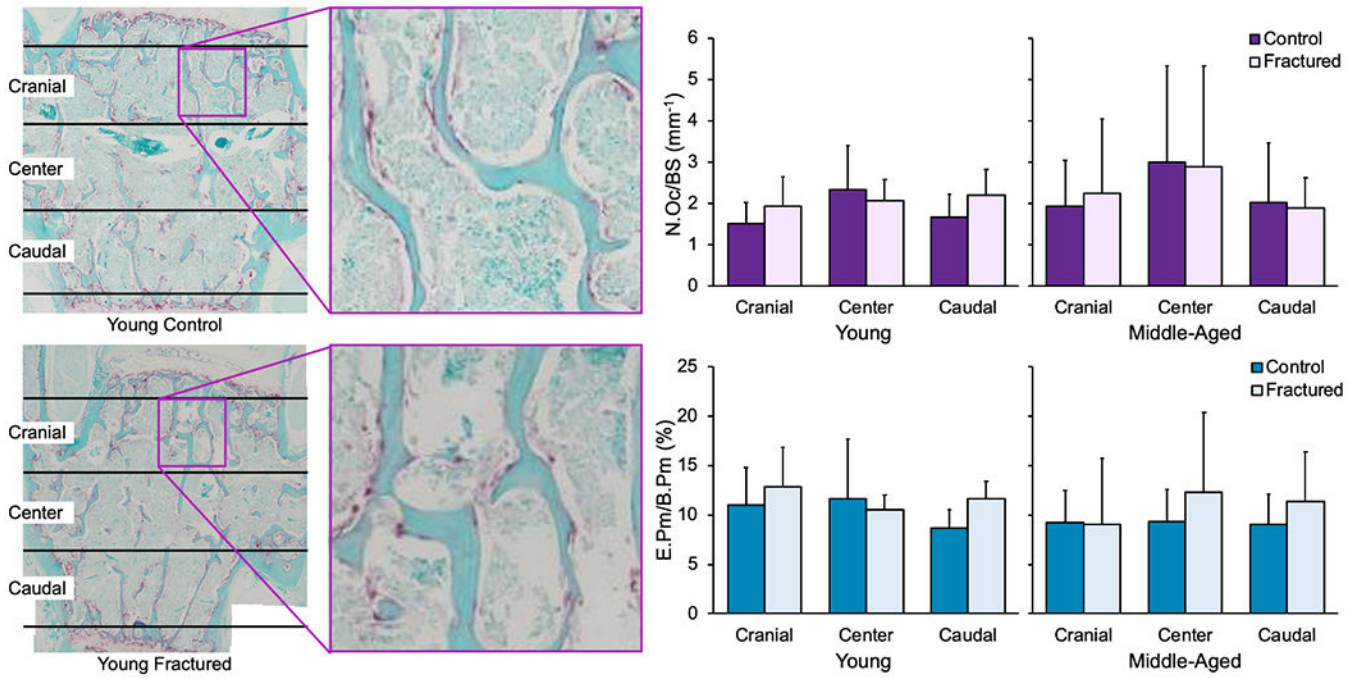


**Figure 3:**

Quantification of osteoclast number and resorbing surface in L5 vertebral body trabecular bone using tartrate-resistant acid phosphatase (TRAP) staining. No significant differences were observed between Fractured and Control mice from either age group.