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Metaphyseal and Diaphyseal Bone Loss in the Tibia Following Transient Muscle Paralysis are Spatiotemporally Distinct Resorption Events

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Abstract

When the skeleton is catabolically challenged, there is great variability in the timing and extent of bone resorption observed at cancellous and cortical bone sites. It remains unclear whether this resorptive heterogeneity, which is often evident within a single bone, arises from increased permissiveness of specific sites to bone resorption or localized resorptive events of varied robustness. To explore this question, we used the mouse model of calf paralysis induced bone loss, which results in metaphyseal and diaphyseal bone resorption of different timing and magnitude. Given this phenotypic pattern of resorption, we hypothesized that bone loss in the proximal tibia metaphysis and diaphysis occurs through resorption events that are spatially and temporally distinct. To test this hypothesis, we undertook three complimentary *in vivo*/μCT imaging studies. Specifically, we defined spatiotemporal variations in endocortical bone resorption during the 3 weeks following calf paralysis, applied a novel image registration approach to determine the location where bone resorption initiates within the proximal tibia metaphysis, and explored the role of varied basal osteoclast activity on the magnitude of bone loss initiation in the metaphysis using μCT based bone resorption parameters. A differential response of metaphyseal and diaphyseal bone resorption was observed throughout each study. Acute endocortical bone loss following muscle paralysis occurred almost exclusively within the metaphyseal compartment (96.5% of total endocortical bone loss within 6 days). Using our trabecular image registration approach, we further resolved the initiation of metaphyseal bone loss to a focused region of significant basal osteoclast function (0.03 mm³) adjacent to the growth plate. This correlative observation of paralysis induced bone loss mediated by basal growth plate cell dynamics was supported by the acute metaphyseal osteoclastic response of 5-wk vs. 13- month-old mice. Specifically, μCT based bone resorption rates normalized to initial trabecular surface (BRR_{BS}) was 3.7-fold greater in Young vs. Aged mice (2.27 ± 0.27 μm³/μm²/day vs. 0.60 ± 0.44 μm³/μm²/day). In contrast with the focused bone loss initiation in the metaphysis, diaphyseal bone loss initiated homogeneously throughout the long axis of the tibia predominantly in the second week following paralysis (81.3% of diaphyseal endocortical expansion between days 6 and 13). The timing and homogenous nature is consistent with *de novo* osteoclastogenesis mediating the diaphyseal resorption. Taken together, our data suggests that tibial metaphyseal and diaphyseal bone loss induced by transient calf paralysis are spatially and temporally discrete events. In a broader context, these findings are an essential first step toward clarifying the timing and origins

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of multiple resorptive events that would require targeting to fully inhibit bone loss following neuromuscular trauma.

Keywords

Bone; Bone Resorption; MicroCT; Muscle Paralysis; Osteoclast; Image Registration

1.0 INTRODUCTION

Profound catabolic alterations in skeletal morphology are induced by numerous exogenous and endogenous stimuli [1-6]. Induced bone loss ranges in extent from focal bone resorption caused by localized deficits in the skeletal loading environment [7, 8] to broad or systemic osteopenia, such as occurs following ovariectomy [9, 10], best-rest [11, 12], or as a complication of chronic inflammatory diseases [13, 14]. Given this variability and the differing permissiveness to resorption of trabecular and cortical bone, it has proven difficult to identify common cellular mechanisms that account for such a wide range of resorptive outcomes.

In this context, heterogeneous bone resorption (both between and within individual bones) has been identified in a variety of induced bone loss models [15, 16]. In these models, trabecular bone has been observed to be more susceptible to osteoclastic resorption than cortical bone [16-19]. However, both genetic (e.g., gender and genetic background) and non-genetic (age and skeletal location/time period investigated) factors can alter, and in some cases transpose, this generality [20-23]. Cancellous and cortical bone alterations have, as a result, predominantly been studied as independent outcomes. A more complete understanding of how bone loss initiates, propagates and interacts among and between distinct skeletal structures would be an important first step towards understanding the variability of cellular dynamics that may underlie the range of tissue-level resorption in these models.

We have previously demonstrated that transient muscle paralysis of the murine calf muscles induces rapid and profound osteoclastic bone resorption in both trabecular and cortical compartments within the tibia adjacent to the paralyzed muscles [24]. Further, we have previously characterized the temporal nature of this bone loss through acute time course μ CT imaging of trabecular bone (proximal tibia metaphysis) and cortical bone (tibia diaphysis) resorption and have found that the timing and magnitude of bone resorption differs greatly in these compartmental tissues [25]. Specifically, significant trabecular resorption in the metaphysis is first observed 3 days following muscle paralysis [25]. Cortical resorption within the tibia diaphysis was slower to occur, with endocortical expansion (as a surrogate for endocortical resorption) first identified 9 days later [25]. While these μ CT measures may suggest temporally distinct bone loss occurred between trabecular and cortical compartments in the model, the experimental design does not allow determination if this arose through distinct cellular events of varying robustness in the spatially disparate metaphyseal and diaphyseal compartments or due to a single continuum of osteoclast resorption initiating in the metaphysis and migrating between compartments. The distinction between a single prolonged or multiple temporally and spatially separate resorptive responses following acute muscle paralysis would be an essential first step toward clarifying the signaling pathway(s) underlying such rapid and profound osteoclastogenesis. Specifically, multiple resorptive responses would be indicative of multiple signaling pathways that would each require individual targeting to completely inhibit bone loss.

In part due to our limited understanding of resorption kinetics in our model, we recently developed a novel μ CT image registration approach capable of identifying focal osteoclastic resorption in the tibia diaphysis following muscle paralysis [26]. This goal was achieved by identifying a temporally unaltered image registration landmark in the tibia that enabled accurate superpositioning of serial μ CT diaphyseal volumes obtained up to 21 days apart. Utilizing this approach, we were able to quantify, with high precision, the complex spatial dynamics of endocortical osteoclast activity within the diaphyseal region. As this technique is amenable to use in any bone compartment in which a consistent image registration volume can be identified, we anticipated that with modifications to facilitate trabecular alignment this approach could be used to detect the initiating site(s) and progression of focal bone loss (if any) between the metaphysis and diaphysis of the tibia following muscle paralysis.

Given the differences in timing and magnitude of acute bone resorption in the tibia metaphysis and diaphysis following muscle paralysis and the spatial distance between these compartments, we hypothesized that bone loss in the proximal tibia metaphysis and diaphysis occur through spatially and temporally distinct resorption events. To test this hypothesis, we first implemented an imaging strategy that allowed for spatiotemporal assessment of bone loss following muscle paralysis throughout the metaphyseal and diaphyseal compartments. These data indicated that focal endocortical bone loss initiation occurred immediately adjacent to the proximal growth plate and that metaphyseal and diaphyseal bone loss were likely to occur through distinct osteoclastic events. Two follow-up *in vivo* studies were used to clarify these observations. In the first, we were able to co-localize metaphyseal bone loss initiation to the location of high basal osteoclast activity within the trabecular bone of the proximal metaphysis. The final study then examined whether the level of basal osteoclastic activity influenced the magnitude of acute bone resorption following muscle paralysis.

2.0 METHODS

2.1 Transient Muscle Paralysis Model

Studies were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee, University of Washington. All mice were female C57Bl/6 obtained from Jackson Laboratories. Transient muscle paralysis was induced in the right calf muscle group of mice via a single injection of botulinum neurotoxin A (BTxA; 2U/100 g body weight) [25]. This intervention induces temporally distinct degradation of trabecular bone tissue in the metaphyseal and cortical bone tissue in the diaphyseal compartments [24, 25]. Mice received BTxA treatment on day 0 and were allowed free cage activity for the remainder of each experiment. Calf paralysis was confirmed 24 hours post-injection by visual examination of reduced toe extension and ankle plantarflexion in the affected limb [24].

2.2 Study Design

2.2.1 Study 1: Spatiotemporal Tracking of Bone Loss—The purpose of this study was two-fold. The first was to determine if the acute bone loss initiation previously identified in the trabecular bone of the proximal tibia metaphysis [24, 25] was due to trabecular permissiveness to osteoclast resorption or due to a more acute osteoclastic response in the metaphysis compared to the diaphysis. The second was to determine whether a continuum of osteoclastic resorption temporally and spatially migrates from the metaphysis to the diaphysis or occurs in two distinct events as hypothesized. For this experiment, a group of 16 week old mice (n=6) received BTxA injection in the right calf immediately following a single μ CT scan that encompassed both the proximal tibia

metaphyseal and diaphyseal envelopes (day 0). Mice in this group were subsequently imaged on days 6, 13 and 20, and euthanized immediately following the last scan.

2.2.2 Study 2: Spatial Initiation of Trabecular Bone Loss—The second study investigated the timing and location of bone loss initiation identified within the metaphyseal compartment in the previous experiment. Based on preliminary data we specifically sought to define the spatial location of bone resorption initiation in the highly metabolically active trabecular tissue within the proximal tibia metaphysis. Prior to the experiment, we first assessed the accuracy and precision of our image registration approach. For this, untreated mice (Registration Control, n=6, 16wk) received a set of non-contiguous μ CT scans of the proximal tibia metaphysis and the tibia mid-diaphysis. Mice were then removed and re-inserted into the μ CT scanner and a second identical set of scans was obtained. This group of mice was used to determine the resolution at which focal bone loss initiation can be identified in the trabecular compartment of the metaphysis. Next, two groups of mice (n=6/gp, 16wk) received intramuscular injections of either BTxA (BTxA Registration group) or an equal volume of saline (Saline Registration group) in the right calf on day 0 followed directly by metaphyseal and diaphyseal μ CT scans. As we have previously shown that a saline injection in the calf does not alter muscle, trabecular or cortical bone morphology as assessed by μ CT [24], saline injected mice were used as controls to maintain consistency vs. BTxA groups (e.g., stress of injections and mouse handling). Mice were subsequently scanned on day 2 and day 3.

2.2.3 Study 3: Basal Cell Activity Modulates Bone Loss Initiation—The final study sought to determine if baseline growth plate cellular dynamics alters the magnitude of acute bone loss initiation following muscle paralysis. A group of 5-week old (Young, n=6) and 13-month old (Aged, n=6) mice received a BTxA injection in their right calf followed immediately by a μ CT scan of their right proximal tibia metaphysis on day 0 and a follow-up scan on day 3. The end point of this experiment was chosen based on previous studies with this model [25].

2.3 μ CT Imaging and Segmentation

High-resolution μ CT images of the right tibia were obtained from all mice on day 0 (Scanco μ CT 40; 10.5 μ m voxel size, 55 kVp, 145 μ A). While anesthetized with isoflurane, the right hindlimb of each mouse was secured in a custom apparatus in order to provide reproducible leg orientation and stability during the scan process. The imaged region(s) varied for each experiment. In Study 1, a single scan of 6.3 mm beginning at the distal end of the proximal growth plate and continuing through the midshaft of the tibia was obtained. The scanning location and time points were designed to detect the occurrence, or lack thereof, of osteoclast migration between compartments. In Study 2, a set of scans encompassed 0.85 mm of the proximal tibia metaphysis immediately distal to the growth plate (Region of Interest, ROI; Figure 1A) and 4.45 mm of the tibia midshaft (starting 0.65 mm proximal from the tib-fib junction; Image Registration Volume, IRV; Figure 1A). The midshaft scan region was identical to that used previously for image registration in this model [26]. In Study 3, a metaphyseal scan region was obtained identically to that described in Study 2.

All raw μ CT image data were preprocessed using a Gaussian Filter algorithm to remove image noise (Sigma=1.2, Support=2.0) followed by bone segmentation within the scan volume using standard image thresholding techniques [27]. Two different μ CT thresholds were used for bone tissue identification. In scan regions in which trabecular bone tissue was analyzed and/or segmented from the cortical shell, a threshold of 441.23 mg HA/cm³ was used as to provide the best comparisons between 2-D binarized images and the original gray-scale images [28]. When cortical bone was exclusively quantified, a manufacturer

recommended threshold for cortical bone identification was used (705.97 mg HA/cm³). All thresholds were verified through manual investigation.

2.4 Image Registration

In Study 2, image registration of serial scans was performed to spatially define bone loss within metabolically active trabecular tissue. Image registration was achieved by leveraging phenotypic patterns of bone loss induced by transient muscle paralysis. Specifically, within the timespan investigated (up to 3 days following muscle paralysis) cortical morphology remains unaltered [25]. As such, all IRV scans obtained over this time period were presumed volumetrically identical. Multiple μ CT scans obtained within a single scanning session (e.g. paired metaphyseal and diaphyseal scans) were mapped onto a global coordinate system within the Scanco μ CT 40 scanner. This allowed for the relative position of the non-contiguous scans to be identified and thereby rotated and translated as a single rigid body. For all mice in Study 2, spatial superposition of the ROI scans (i.e., trabecular scan volume) was achieved by first registering the volumetrically identical IRV in the primary (Day 0) and secondary scans (all subsequent scans) as previously described (Scanco IPL Registration Command: Figure 1B) [26]. Given the acute timespan of this study, we improved the accuracy of our image registration technique by using both the periosteal and endocortical surfaces as image registration landmarks (previous long term studies used only the unchanged periosteum). The rotational and translational parameters determined during IRV image registration were then applied to the ROI scan volumes producing spatially aligned trabecular volumes (Figure 1B).

2.5 Study Specific Image Analysis

2.5.1 Study 1: Spatiotemporal Tracking of Bone Loss—We assessed endocortical bone loss across 6.3 mm of the tibia distal to the growth plate, as to avoid the confounding influence of the enhanced resorbable surface to volume ratio of trabecular versus cortical tissue. Though initial endocortical surface varied along the scan volume, our previous data showed that generally less than 50% of the available surface (i.e., endocortical surface) undergoes significant resorption in the 3 weeks following muscle paralysis [26]. As the resorptive ‘carrying capacity’ of the endocortical surface is not approached, we chose raw endocortical expansion data and not percent change in endocortical surface as the least biased approach available to examine spatiotemporal bone alterations. We therefore assessed endocortical volume (E.Vol) expansion as the primary outcome measure to simultaneously define the onset of osteoclast activity in both metaphyseal and diaphyseal compartments. To accomplish this, all trabecular bone was digitally segmented from the surrounding cortical shell using automated custom software (MATLAB). The scan volume (minus trabecular architecture) was discretized into six bins (each 1.05 mm) beginning at the growth plate. Endocortical volume (E.Vol) within bins was individually determined, and alterations occurring between each μ CT scan were integrated to assess the spatiotemporal nature of endocortical resorption over the length of the tibia from just below the growth plate to the mid-diaphysis.

2.5.2 Study 2: Spatial Initiation of Trabecular Bone Loss—As the translational and rotational parameters used to superimpose the trabecular ROI are based on cortical bone registration (i.e., IRV registration), the accuracy of the IRV registration was first determined in all groups. This was achieved by calculating the percent misalignment (i.e., the percent of non-overlapping scan volume to the average scan volume of the IRV scans) of the center 3.4 mm of the IRV scan. Given that trabecular registration (i.e., ROI registration) occurs through rigid body rotations and translations, accuracy of IRV registration was assumed to be proportional to accuracy of ROI registration allowing for quantification of alterations of

trabecular bone architecture within the highly metabolically active metaphyseal compartment.

Prior to determining the location(s) where bone loss initiates in the trabecular compartment following muscle paralysis, we examined whole tissue changes in the experimental animal groups using standard trabecular μ CT analysis measures [28]. This analysis included quantification of endocortical volume (E.Vol or TV), trabecular fraction (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th) and trabecular spacing (Tb.Sp).

To determine focal alterations in trabecular morphology, trabecular bone in the registered proximal tibia metaphysis (i.e., ROI) was segmented from the surrounding cortical shell using custom software (MATLAB). Unlike the analysis of whole tissue changes in which separate user-defined ROI's were determined for primary and secondary scan volumes of varied spatial orientation, this technique allowed for a single automated ROI to be defined for both primary and registered secondary scans reducing the amount of variation in the data acquisition. The trabecular compartment of the ROI was then discretized into five equally spaced volumetric elements in each anatomical dimension based on the anatomical extremes of the primary volume (125 total elements). Element locations were identified numerically from medial to lateral, posterior to anterior, and proximal to distal, in that order (e.g., Element^{1,1,1} representing the most medial, posterior and proximal element). To quantify bone loss as a function of the proximity to the growth plate, elements within the same transverse plane were grouped (proximally to distally) into Stacks (e.g., Elements^{1-5,1-5,1-5} = Stack¹).

The precision of the trabecular image registration was determined in the Registration Control group in order to quantify the resolution at which focal bone resorption could be identified. Specifically, the average difference and standard deviation of bone volume between corresponding non-zero Elements in the primary and registered secondary images were calculated. The average difference and standard deviation of the Registration Control group were then used to identify significant alterations in the experimental groups (i.e., BTxA and Saline Registration groups).

2.5.3 Study 3: Basal Cell Activity Modulates Bone Loss Initiation—During the course of Study 3 we attempted to implement trabecular image registration in Young and Aged animals using simultaneously obtained IRV scans. However, the rapid bone alterations in the diaphysis of Young animals made accurate image registration impossible and we therefore did not include image registration data in this study. Thus we first performed standard trabecular μ CT based analysis on all metaphyseal scans [24]. Given baseline morphologic differences in the trabecular bone of Young and Aged animals and its potentially confounding effect on interpreting standard bone loss metrics, μ CT based dynamic bone morphometry parameters normalized to initial trabecular structure were also determined. Bone resorption rates (BRR) were quantified and then normalized either to day 0 trabecular bone volume (BRR_{BV}) or day 0 trabecular bone surface (BRR_{BS}), as previously described [29]. Prior to data analysis in this study, one Young mouse was removed due to a failed physical examination of a positive BTxA injection.

2.6 Statistics

In all studies, $p < 0.05$ was considered to be statistically significant unless otherwise stated.

2.6.1 Study 1: Spatiotemporal Tracking of Bone Loss—For Study 1, we first determined the longitudinal location of acute endocortical bone loss initiation by performing a one-way ANOVA with Tukey post-hoc analysis with bin location as the independent factor and day 0 to day 6 endocortical expansion as the dependent variable. Once

endocortical bone loss initiation was identified in the metaphysis, we next analyzed if osteoclast activity migrated to the diaphysis. For this, we performed a univariate analysis of variance with diaphyseal bin location (Bins C-F) and scanning time periods as independent factors and endocortical expansion as the dependent variable. TUKEY post-hoc analysis was performed where main effects achieved significance without significant main effect interactions.

2.6.2 Study 2: Spatial Initiation of Trabecular Bone Loss—For whole bone changes, a multivariate analysis of variance was performed with scanning time point (day following treatment) and treatment (BTxA versus Saline) as independent factors and standard trabecular analysis metrics (e.g. BV, BV/TV, etc.) as the dependent variables. For tibial long-axis variations in bone loss in BTxA and Saline treated mice, univariate analysis of variance was performed with location (Stack) and treatment (BTxA versus Saline) as main effects and loss of trabecular bone volume the dependent variable. To analyze focal changes in trabecular architecture, the calculated accuracy and precision of our image registration technique was used. Significant differences in percent misalignment within the IRV were determined using unpaired T-tests. Significant alterations in Element bone volume in the experimental groups were required to be greater than 3 standard deviations from the mean difference calculated in the Registration Control animals ($p < 0.003$). This stringent threshold was used to minimize the potential for Type 1 error within the large number of comparisons between Elements (125 total Elements $\times$ 2 time points = 250 total comparisons).

2.6.3 Study 3: Basal Cell Activity Modulates Bone Loss Initiation—To compare the baseline trabecular structure (day 0) in Young and Aged animals, a multivariate analysis of variance was performed with mouse age (Young vs. Aged) as the independent factor and standard trabecular analysis metrics (e.g. BV, BV/TV, etc.) as the dependent variables. To compare the osteoclast dynamics induced with the first 3 days following muscle paralysis in Young and Aged animals, a multivariate analysis of variance was performed with mouse age (Young vs. Aged) as the independent factor and dynamic bone morphometry measures (BRR_{BV} and BRR_{BS}) the dependent variables.

3.0 RESULTS

3.1 Study 1: Spatiotemporal Tracking of Bone Loss

Following transient muscle paralysis, endocortical bone resorption in the proximal tibia metaphysis was distinct in both spatial distribution and timing compared to endocortical resorption in the diaphysis. During the first 6 days, 96.5% of observed endocortical bone loss occurred in the metaphyseal region of the scan volume (Figure 2B). Specifically, post-hoc analysis determined that endocortical bone resorption focally initiated in the metaphysis nearest the growth plate, as endocortical expansion was significantly elevated in Bin A versus all other bins during the day 0 to day 6 time period (Figure 2B, $p < 0.001$).

Migration of the initial endocortical osteoclast response in the metaphysis was not supported by spatiotemporal analysis of endocortical expansion in the diaphysis. In contrast to the endocortical resorption in the metaphyseal region, diaphyseal bone loss was homogenously distributed across bins and occurred almost entirely between days 6 and 13 (81.3% of diaphyseal bone loss occurred within day 6 to day 13; Figure 2C). Analysis of variance revealed a significant timepoint main effect on diaphyseal endocortical expansion following transient muscle paralysis, while location and location:timepoint interaction were not significant (Figure 2C). Post-hoc analysis confirmed that endocortical bone loss in the diaphyseal bins (Bin C-F) was significantly elevated during the day 6 to day 13 period versus day 0 to day 6 and day 13 to day 20 periods (Figure 2C, $p < 0.01$).

3.2 Study 2: Spatial Initiation of Trabecular Bone Loss

Image registration was highly accurate within both the cortical IRV and the trabecular ROI. The mean ($\pm$ s.d.) percent misalignment within the IRV of Registration Controls was $2.0 \pm 0.3\%$. Within the ROI, the average volumetric difference between Elements in the primary and registered secondary scan volumes of the Registration Controls was $2.3e3 \pm 1.4e5 \mu\text{m}^3$ ($0.01 \pm 0.44\%$ when normalized to total Element volume). In the experimental groups, percent misalignment of the IRV was not significantly different in BTxA treated animals on day 2 or day 3 compared to Registration Controls ($1.8 \pm 0.1\%$ and $2.1 \pm 0.2\%$, respectively; $p > 0.23$).

In BTxA treated animals, image registration allowed for detection of focal bone loss initiation at time points where bone resorption was undetectable using standard μCT analysis. Standard μCT analysis of un-registered whole trabecular morphology indicated that muscle paralysis did not alter any bone measurement within the analyzed trabecular bone volume of BTxA treated mice compared to Saline controls within 3 days of treatment (Table 1). When registered serial trabecular images were analyzed in longitudinal stacks (Figure 3A), no significant bone volume differences could be identified on day 2 ($p > 0.053$; Figure 3B) or on day 3 ($p > 0.132$; Figure 3C). However, bone loss nearest the growth plate (Stack¹) in BTxA treated mice was 8.8-fold greater than in any other Stack analyzed (Figure 3C-D).

Further increasing analysis resolution to the level of an individual Element allowed for statistically significant bone alterations to be identified in both BTxA and Saline mice. When quantified in three-dimensions (Figure 4), observed patterns of bone loss in BTxA treated mice reflected an amplification of baseline resorptive events observed in saline treated mice. In saline treated animals, a single discretized element (from 125 total) demonstrated a significant decrease in bone volume at both day 2 and day 3 when compared to day 0 bone volumes (Figure 4A&C). Specifically, calculated bone volume decreased within Element^{3,4,1} from -8.0% on day 2 to -15.7% on day 3 (mean difference of intra-animals day 0 comparisons). In the BTxA treated group, Element^{3,4,1} was also the only element to demonstrate a significant decrease in bone volume at day 2 (-7.9% mean difference; Figure 4B). Unlike the Saline treated group, the number of elements in which significant bone loss occurred expanded in BTxA treated mice by day 3. Seven discretized elements demonstrated significant bone loss (ranging from -7.2 to -31.4% , Figure 4D) at day 3, of which five elements either included or were adjacent to Element^{3,4,1} (Figure 4D).

3.3 Study 3: Basal Cell Activity Modulates Bone Loss Initiation

Osteoclastic resorption was more profound in the metaphysis of Young versus Aged mice following muscle paralysis. Baseline volumetric measures of metaphyseal trabecular bone did not differ between Young and Aged mice (BV: $p > 0.98$, BV/TV: $p > 0.45$; Table 2). However, the structural morphology of the trabecular bone clearly differed between Young and Aged animals. The trabeculae of Young animals were more numerous (TB.N: $p < 0.001$; Table 2) and densely packed (TB.Sp: $p < 0.001$; Table 2), but were thinner than the trabeculae of Aged mice (TB.Th: $p < 0.001$; Table 2). As a result, Young mice demonstrated a nearly two-fold greater bone surface than Aged animals (BS: $p < 0.03$; Table 2).

μCT based dynamic bone resorption parameters, referent to trabecular morphology, enabled normalized comparison of the effect of acute osteoclastic activity induced following muscle paralysis in Young and Aged mice (Figure 5A). BRR_{BV} in the 3 days following muscle paralysis was greater in Young versus Aged groups ($13.05 \pm 1.53 \text{ \%/day}$ vs. $2.15 \pm 1.56 \text{ \%/day}$, $P < 0.001$; Figure 5B). Bone resorption rate normalized to bone surface (BRR_{BS}) was also significantly increased in Young versus Aged mice ($2.27 \pm 0.27 \mu\text{m}^3/\mu\text{m}^2/\text{day}$ vs. $0.60 \pm 0.44 \mu\text{m}^3/\mu\text{m}^2/\text{day}$, $P = 0.005$; Figure 5C).

4.0 DISCUSSION

Combining a μ CT image registration strategy with an *in vivo* model of acutely induced osteoclastic resorption allowed us to explore, for the first time, the temporal and spatial relation of bone resorption in adjacent metaphyseal and diaphyseal compartments of the tibia. Consistent with our hypothesis, spatiotemporal tracking of compartmental bone loss identified conspicuous differences in both the timing and spatial distribution of osteoclastic resorption between the compartments. Endocortical bone loss was most rapidly initiated in the metaphyseal region following induction of muscle paralysis and, given its location, appeared to occur via amplification of baseline osteoclastic activity nearest to the growth plate. This conclusion was clarified by demonstrating that elevated basal osteoclastic resorption was associated with an enhanced magnitude of initial trabecular resorption. The absence of evidence suggesting osteoclast migration between the metaphysis and diaphysis, when combined with the temporal separation in the onset of resorption, suggests that the initiations of osteoclastic resorption in the two compartments are distinct cellular events.

As with any series of *in vivo* studies, our data should be considered in the context of the general limitations of the approaches we implemented. To determine whether bone loss in the metaphysis and diaphysis were distinct resorption events, we temporally tracked endocortical alterations in a scanned bone volume that encompassed both compartments. This approach differed from our previous studies (which investigated metaphyseal trabecular bone and a non-contiguous volume of cortical bone in the diaphysis) and therefore allowed simultaneous assessment of osteoclastic activity across both compartments. Due to the large scan lengths (6.3 mm) and scan duration (≈ 1 h) required for Study 1, we were limited to three total *in vivo* scanning sessions to minimize the possible effects of both anesthesia and radiation exposure on bone cell activity [30]. For that reason, we chose equally spaced scanning time points (≈ 1 /wk) which encapsulated the initiation and peak resorption events previously observed separately in each compartment [25]. If osteoclasts were to have migrated from the location of bone loss initiation in the metaphysis to areas of temporally distinct bone loss in the diaphysis, we would have anticipated a location main effect or a timepoint:location interaction in the spatiotemporal analysis of the diaphysis (Figure 2C). The lack of such findings suggests that metaphyseal and diaphyseal resorption are not connected through an expansive migratory response. As each scan is an individual ‘snapshot’ of bone resorption at a single moment in time, it is possible that our approach would be insensitive to extremely rapid or complex 3-dimensional osteoclast migration. However, the speed at which active osteoclasts have been reported to migrate *in vivo* (≈ 0.28 mm/wk; [31, 32]) would produce maximum weekly cell movements that were less than 50% of the bin lengths used in this experiment. Thus, individual osteoclasts would be unlikely to travel between non-adjacent bins within a single scanning time period.

Defining the focal initiation of trabecular resorption required development of a more spatially precise approach capable of separating induced osteoclast activity from baseline bone remodeling. This goal was accomplished through the application of serial μ CT image registration, which requires the identification of invariant landmarks capable of defining accurate image superposition. However, such invariant landmarks are commonly lacking in models of profound bone resorption [33]. For this reason, previous studies in models of bone tissue alteration have used operator-defined regions of interest and/or best-fit algorithms to enable alignment of μ CT images across time [34-39]. While these strategies have been successfully applied to qualitatively examine focal bone alterations in a variety of bone loss/bone formation models, quantitative assessment is difficult without a means to simultaneously quantify registration accuracy. By adapting our previous diaphyseal image registration paradigm [26], we created a novel strategy capable of tracking trabecular alterations using the morphologically quiescent (i.e., within 3 days after muscle paralysis)

mid-diaphysis as the registration landmark. This refinement allowed us to quantify the accuracy of our image registration even in the presence of profound trabecular alterations, and thereby enabled identification of focal trabecular alterations in a model of profound osteoclastogenesis. The flexibility of this technique makes it applicable for high-resolution tracking of osteoclast (i.e., resorption) dynamics in any model in which an unaltered registration landmark is identifiable.

To begin to clarify distinctions between metaphyseal and diaphyseal bone resorption following muscle paralysis required mitigation of one potentially confounding difference in the manner these compartments are traditionally analyzed. Previous studies, including ours, generally focus on trabecular alterations within the metaphysis while quantifying cortical alteration at the diaphysis [25, 40-42]. However, given the relative differences in the surface area available for resorption and mineral density in these distinct bone tissues [43, 44], relating bone resorption (and thus osteoclast activity) between these compartments has been previously interpreted in the context of a perceived permissiveness of trabecular bone to resorption. When using endocortical expansion as a common measure of bone resorption spanning the compartments, the data indicated that bone resorption was more acute and profound in the proximal metaphyseal compartment than in the adjacent diaphysis. While not contradicting the possibility of trabecular bone hyper-sensitivity to osteoclastic resorption, these data suggested that increased basal metabolic activity in the metaphyseal compartment might underlie why trabecular bone loss occurs more rapidly than cortical bone loss following muscle paralysis.

Subsequent studies identified a role of the basal cellular microenvironment in mediating the acute metaphyseal response following muscle paralysis. The resolution provided by three-dimensional examination of registered trabecular bone allowed for identification of focal bone alterations not identifiable by either traditional imaging based assessment (Table 1) or by dividing the metaphysis into five equal thickness stacks (Figure 3). Using our novel image registration approach, we found that trabecular bone loss following calf paralysis was anatomically discrete and first occurred in areas also observed to have high basal levels of osteoclast activity (anterior portion immediately adjacent to the growth plate). As well, given the compatibility in timing and location of metaphyseal endocortical resorption in Study 1 and metaphyseal trabecular resorption in Study 2, it is likely that endocortical and trabecular resorption were a result of a single metaphyseal resorptive event. This finding suggests that the bone microenvironment (e.g., metaphyseal compartment) and not the perceived permissiveness of trabecular bone to resorption, may control the acute response of trabecular bone in many models of bone resorption.

Directly testing the hypothesis that growth plate bone cell dynamics mediates acute trabecular bone loss following muscle paralysis is inherently challenging. Both genetic (e.g., mouse strain; [45]) and epi-genetic (e.g., mouse age, physical stimuli gain/loss; [46, 47]) factors alter bone cell activity in the growth plate/proximal metaphyseal region. However, these factors also inevitably alter the basal trabecular environment (as emphasized by our observation of a nearly two fold greater trabecular bone surface in Young vs. Aged animals despite nearly equivalent bone volume). As such, increased bone resorption following muscle paralysis in Young mice might occur either by increased osteoclastic activity or due to increased resorptive potential of the high surface area trabecular bone.

Despite these limitations, our final experiment provides *in vivo* support for the conclusions drawn from the initial experiments. The age of mice in the final experiment were chosen based on their clear differences in growth plate cellular activity [46]. To control for varied surface areas between groups, we utilized direct 3-D quantification of dynamic osteoclast activity via time-lapse μ CT imaging [29]. We found that paralyzing the calf muscle of very

young mice with high growth plate cellular activity induced a more profound acute trabecular bone loss response (both morphometrically and when osteoclast induced bone resorption was normalized to surface area) than in 13-month old animals in which longitudinal tibial growth had ceased [46]. While a number of additional factors could also contribute to this observation (e.g., osteoclast potential for resorption could differ with age, a role of altered osteoblastic activity), we believe these data clearly extend the data of the initial correlative experiments and represents an initial confirmation of our developing thesis that basal osteoclast activity, in part, mediates the magnitude and rate of acute trabecular bone resorption following transient muscle paralysis.

One explanation for the enhancement of bone resorption nearest the growth plate within just 3 days of muscle paralysis may be enhancement of RANKL signaling. In a previous study, we identified upregulation of RANKL positive cells throughout the growth plate in the week following muscle paralysis [48]. Given the ability of RANKL signaling to both amplify and initiate osteoclastic activity, focal amplification (or initiation) of osteoclastic activity in these areas would not be surprising [49]. However, determining if this spatially discrete pattern of bone loss in the metaphysis arises solely from the amplification of normal growth plate osteoclast function or is further potentiated by activation of functionally distinct marrow populations within the trabecular microenvironment remains to be explored.

Following calf paralysis, diaphyseal bone resorption differed from metaphyseal resorption in both timing (predominantly between day 6 and day 13) and spatial homogeneity (i.e., does not initiate from a secondary foci and spread from that point). Specifically, the timing of bone loss initiation in this diaphyseal compartment (day 6 at the earliest) coincides with the amount of time required to form osteoclasts from monocyte/macrophage precursors *in vitro* and *in vivo* (which is too slow to account for the onset of metaphyseal resorption [50, 51]). As such, osteoclastogenesis within the marrow cavity may drive a secondary wave of bone resorption in the diaphysis in a manner distinct from the enhanced basal osteoclast activity observed in the metaphysis.

Though we believe our data suggests that functionally distinct upregulation of osteoclast activity in the metaphysis and the diaphysis are the likely causes of bone resorption following muscle paralysis, the role of altered osteoblast activity in this process was not specifically addressed. Given the role of osteoblast activity in models of disuse [52, 53], it is possible that decreased bone formation has an effect on the magnitude of bone loss in the metaphyseal or diaphyseal compartments. This is especially pertinent when considering the origins of the paralyzed soleus muscle in our model of calf paralysis spatially correspond to the metaphyseal region in which bone resorption was most profound. However, our own recent studies have demonstrated that direct inhibition of osteoclastogenesis via induced NFATc deletion completely inhibits bone loss following muscle paralysis [48]. As well, rather than acute loss of osteoblast function in the actively remodeling growth plate region, our registered imaging approach in Study 2 actually suggested a non-significant increase in osteoblast function in the 3 days immediately following paralysis. This coupled with the unchanged diaphyseal bone volume needed to accurately register the bone suggests that within the first 3 days following muscle paralysis osteoblast function is not materially altered in the metaphyseal or diaphyseal compartments. That said, the long term effects of muscle paralysis on osteoblast function, if any, remain to be resolved [25, 54, 55].

Taken together, our data suggests that tibial metaphyseal and diaphyseal bone loss induced by transient calf paralysis are spatially and temporally discrete events whose magnitude and location are influenced by basal bone cell activity. Initial bone resorption following paralysis was likely achieved through an increase in basal osteoclast activity within the proximal tibia metaphysis, while profound *de novo* osteoclastogenesis within the marrow may be the

predominant mechanism driving resorption in the diaphysis. In addition, we found that mouse age can affect the dynamics of this induced bone resorption by reducing the magnitude of bone loss initiation in the metaphysis. These results highlight the importance of understanding the basal bone resorption dynamics when the skeleton is catabolically challenged, as the relative susceptibility of a given compartment or bone type (cortical versus trabecular) may be dependent on a variety of external factors. In conclusion, we believe that extension of the imaging approach we have developed and implemented holds potential to clarify the differing signaling mechanisms that may underlie osteoclastogenesis in different bone compartments induced by a variety of neuromuscular trauma.

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Highlights

We examined metaphyseal and diaphyseal bone resorption in the tibia following transient muscle paralysis.

Identified focal trabecular bone loss initiation using an automated μ CT image registration strategy.

Metaphyseal bone resorption following muscle paralysis initiated in locations of high basal osteoclast activity adjacent to the growth plate.

Diaphyseal bone resorption was homogenously distributed and occurred secondary to metaphyseal resorption.

Fig. 1.

Trabecular image registration within the metabolically active metaphysis was achieved through coupling with image registration in the unchanged diaphysis. After obtaining two non-contiguous scan volumes within a single scanning session (1A), rotational and translational parameters were defined by spatially registering the volumetrically identical IRVs and then applied to the ROI volumes. This approach enabled unbiased superimposing of the trabecular compartment despite the potential for morphologic alteration on any bone surfaces (1B).

Fig. 2.

Endocortical bone resorption in the metaphysis and diaphysis following transient muscle paralysis had distinct spatial and temporal patterns. Resorptive activity within the tibia following transient muscle paralysis was quantified in each of six discrete bins (2A) over a near three week time period. The initiation of endocortical expansion (mean $\pm$ s.e.) occurred exclusively within the metaphysis (2B). Within the first 6 days following muscle paralysis, endocortical expansion was focused nearest the growth plate (2B, * significantly different from Bins B-F at same time point, all $p < 0.001$). The resorptive initiation in the metaphysis did not migrate to the diaphysis. (2C). Instead, diaphyseal expansion occurred homogeneously in the day 6 to 13 time period (2C, ^ significantly greater than the day 0 to 6 and day 13 to 20 time periods at each bin location, all $p < 0.01$) and lacked a significant main effect of location or significant location:timepoint interaction.

Fig. 3.

Separating the metaphysis into equal-volume longitudinal stacks does not provide sufficient resolution to identify focal bone loss initiation in the proximal metaphysis. Bone volume alterations in registered and discretized trabecular bone volumes were first analyzed in layers of Elements (i.e., Stacks) perpendicular to the growth plate (3A, Stack¹ highlighted in blue). There was no significant difference in bone alterations at any location or across treatment groups within 2 (3B) or 3 days (3C), though in both cases the greatest bone loss was adjacent to the growth plate in BTxA treated animals. However, a representative coronal composite cross-section 3 days following BTxA treatment suggests that bone loss occurred most predominately nearest the growth plate (3D, Red=Bone Loss, Orange=Bone Formation).

Fig. 4. Metaphyseal trabecular bone resorption following transient muscle paralysis initiated in locations of high basal osteoclast activity nearest the growth plate. In both Saline and BTxA treated mice, significant bone loss occurred in Element^{3,4,1} by day 2 (4A-B, light red). Unlike the Saline group where the bone loss pattern was consistent across day 2 and day 3 (4C), significant bone loss expanded to adjacent bins in BTxA treated mice by day 3 (4D, dark red).

Fig. 5.

The rate of acute metaphyseal bone resorption following transient muscle paralysis varied between Young and Aged mice independent of trabecular morphology. Serial μ CT imaging of the proximal tibia metaphysis (5A) enabled quantification of dynamic bone morphology measures normalized to either bone volume or bone surface. Within the first 3 days following muscle paralysis, the Bone Resorption Rate per initial bone volume (BRR_{BV}) was significantly greater in Young versus Aged mice (5B, * $P < 0.001$). When normalized to initial bone surface, Bone Resorption Rate (BRR_{BS}) was also significantly elevated in Young versus Aged mice (5C, * $P < 0.005$).

Table 1 μ CT Outcome Measures for Study 2 [mean $\pm$ s.e.]

		Day 0	Day 2	Day 3
Endocortical volume, E.Vol or TV (mm ³)	Botox	1.991 $\pm$ 0.066	1.992 $\pm$ 0.075	2.017 $\pm$ 0.073
	Saline	1.999 $\pm$ 0.068	2.008 $\pm$ 0.075	1.995 $\pm$ 0.074
Trabecular fraction, BV/TV (%)	Botox	14.048 $\pm$ 0.632	14.045 $\pm$ 0.525	13.660 $\pm$ 0.536
	Saline	13.203 $\pm$ 0.957	13.337 $\pm$ 0.992	13.330 $\pm$ 0.998
Trabecular number, Tb.N (#/mm)	Botox	3.656 $\pm$ 0.122	3.673 $\pm$ 0.110	3.654 $\pm$ 0.117
	Saline	3.636 $\pm$ 0.236	3.527 $\pm$ 0.235	3.485 $\pm$ 0.237
Trabecular thickness, Tb.Th (mm)	Botox	0.059 $\pm$ 0.001	0.059 $\pm$ 0.001	0.058 $\pm$ 0.001
	Saline	0.059 $\pm$ 0.001	0.060 $\pm$ 0.001	0.060 $\pm$ 0.001
Trabecular spacing, Tb.Sp (mm)	Botox	0.268 $\pm$ 0.011	0.266 $\pm$ 0.009	0.269 $\pm$ 0.010
	Saline	0.274 $\pm$ 0.017	0.281 $\pm$ 0.017	0.288 $\pm$ 0.018

No significant differences in main effects of scanning time points ($p > 0.998$), treatment ($p > 0.053$) and time point:treatment ($p > 0.874$)

Table 2 μ CT Measures of Initial Trabecular Morphology for Study 3 [mean $\pm$ s.e]

Day 0		
Trabecular Bone Volume, BV (mm ³)	Young	0.163 $\pm$ 0.017
	Aged	0.161 $\pm$ 0.039
Trabecular fraction, BV/TV (%)	Young	10.650 $\pm$ 0.780
	Aged	9.297 $\pm$ 1.532
Trabecular number, TV.N (#/mm)	Young	4.350 $\pm$ 0.114 *
	Aged	2.341 $\pm$ 0.076
Trabecular thickness, Tb.Th (mm)	Young	0.043 $\pm$ 0.001 *
	Aged	0.075 $\pm$ 0.003
Trabecular spacing, TB.Sp (mm)	Young	0.230 $\pm$ 0.007 *
	Aged	0.443 $\pm$ 0.020
Trabecular bone surface, BS (mm ²)	Young	9.372 $\pm$ 0.898 *
	Aged	5.565 $\pm$ 5.371

*
(all p<0.03)