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μCT-based, in vivo dynamic bone histomorphometry allows 3D evaluation of the early responses of bone resorption and formation to PTH and alendronate combination therapy

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ABSTRACT

Current osteoporosis treatments improve bone mass by increasing net bone formation: anti-resorptive drugs such as bisphosphonates block osteoclast activity, while anabolic agents such as parathyroid hormone (PTH) increase bone remodeling, with a greater effect on formation. Although these drugs are widely used, their role in modulating formation and resorption is not fully understood, due in part to technical limitations in the ability to longitudinally assess bone remodeling. Importantly, it is not known whether or not PTH-induced bone formation is independent of resorption, resulting in controversy over the effectiveness of combination therapies that use both PTH and an anti-resorptive. In this study, we developed a μCT-based, in vivo dynamic bone histomorphometry technique for rat tibiae, and applied this method to longitudinally track changes in bone resorption and formation as a result of treatment with alendronate (ALN), PTH, or combination therapy of both PTH and ALN (PTH+ALN). Correlations between our μCT-based measures of bone formation and measures of bone formation based on calcein-labeled histology ($r = 0.72–0.83$) confirm the accuracy of this method. Bone remodeling parameters measured through μCT-based in vivo dynamic bone histomorphometry indicate an increased rate of bone formation in rats treated with PTH and PTH+ALN, together with a decrease in bone resorption measures in rats treated with ALN and PTH+ALN. These results were further supported by traditional histology-based measurements, suggesting that PTH was able to induce bone formation while bone resorption was suppressed.

Introduction

The healthy human skeleton is continuously renewed via coupled and balanced bone remodeling. After menopause, the bone turnover rate accelerates, and bone remodeling is shifted toward a negative balance between bone formation and resorption, resulting in bone loss [1]. Current osteoporosis treatments include anti-catabolic agents, which reduce bone resorption [2,3], and anabolic agents, which increase bone formation [4]. However, due to coupling of bone resorption and formation, drugs that inhibit resorption often also inhibit formation, and those that increase formation also increase resorption, thereby limiting their potential benefits [2–4]. Recent data suggest that intermittent parathyroid hormone (PTH) treatment, currently the only FDA-approved anabolic agent, may increase bone formation partly through a modeling-based mechanism (bone formation without prior resorption) [5–9]. Therefore, it has been hypothesized that combination therapy of PTH with an anti-catabolic agent could use this modeling-based pathway to concurrently activate new bone formation and block resorption, leading to improved bone quality. In support of this hypothesis, our recent study found that treatment with both PTH and alendronate (ALN), a bisphosphonate, resulted in an improved trabecular structure at the rat proximal tibia beyond what occurred with either treatment alone [10].

However, results from clinical and preclinical studies of combination therapy have been highly variable [11–21], and the ability of PTH treatment to stimulate bone formation while osteoclast resorption is inhibited, remains controversial. This is partly due to technical limitations in the ability to directly assess bone resorption and formation in a simultaneous and longitudinal manner. Although bone formation can be assessed using dynamic histomorphometry with double fluorochrome labeling, this technique is limited by its two-dimensional, destructive nature and its inability to quantify bone resorption. To improve the quantification of bone remodeling, a three-dimensional

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(3D) dynamic histomorphometry method has been developed that can simultaneously identify bone formation and resorption sites [22–26]. This method gives highly precise morphological measurements of bone formation and can indirectly measure resorption cavities; however, its destructive nature makes it difficult to longitudinally monitor disease progression or drug effects over multiple time points.

The increased availability of in vivo micro-computed tomography (μCT) scanners allows the possibility of quantifying bone formation and resorption in a non-invasive, longitudinal manner. The ability to identify bone remodeling sites using in vivo μCT was first demonstrated by Waarsing et al., who used registered in vivo μCT images to investigate changes in trabecular microarchitecture and qualitatively identify regions of bone remodeling in the rat tibia [27]. More recently, Schulte et al. developed an innovative method to longitudinally assess trabecular bone formation and resorption in mouse caudal vertebrae based on in vivo μCT images [28]. After registering longitudinal μCT scans from the same animal at two different time points, the two images are superimposed, allowing for measurement of bone remodeling. Resorption is identified as the bone areas only present in the earlier images, while bone formation is identified as the bone areas only present in the later scans. This technique provides accurate quantification of bone remodeling in mouse caudal vertebrae in models of postmenopausal osteoporosis [29] and mechanical loading [28,30,31].

Despite the utility of this technique in measuring bone remodeling in the caudal vertebra, the trabecular regions of the tibia, femur, and lumbar spine are more functionally relevant skeletal sites in rodent models of metabolic diseases and drug treatments, and are therefore more commonly used to study bone quality. However, the unclosed growth plate and continuous skeletal growth in the long bones of adult mice and rats significantly affect bone geometry and size over time [27,32–34]. Although trabecular bone undergoes minimal changes due to longitudinal growth, the dramatic changes that occur in the surrounding cortical bone morphology make it difficult to identify and precisely align the same trabecular bone regions in sequential μCT scans. For this reason, there is a great need for a robust imaging technique that is able to quantitatively track long-bone trabecular remodeling in rodents.

The overall goal of this study was to develop a μCT-based, 3D in vivo dynamic bone histomorphometry technique that would allow us to longitudinally and simultaneously quantify changes in bone formation and resorption in rat tibiae as a result of treatment with ALN, PTH, and combination therapy of both PTH and ALN (PTH+ALN). We validated this novel approach through comparison with standard 2D dynamic bone histomorphometry and a serum resorption marker, and then applied this method to longitudinally track the short-term responses of bone resorption and formation activities to treatment with ALN, PTH, or combination therapy. We hypothesized that adding ALN to the PTH treatment would inhibit bone resorption while maintaining the elevated bone formation activities induced by the PTH treatment, thus resulting in an additive, beneficial effect on trabecular bone.

Materials and methods

Animals

A total of 37 three-month-old, female, Sprague–Dawley rats were used in this study. Eight rats were excluded due to inadequate image quality caused by motion artifacts during in vivo μCT scans. The remaining 29 rats belonged to 4 treatment groups: PTH (PTH, n = 10), alendronate (ALN, n = 6), combined PTH and ALN (PTH+ALN, n = 6) or vehicle (Veh, n = 7). As described in [10], the rats in the PTH and PTH+ALN groups were given daily subcutaneous injections of PTH (PTH (1–34), 60 μg/kg/day dissolved in saline, Bachem, Bubendorf, Switzerland) starting on day 0 for a total of 12 days; the rats in the ALN and PTH+ALN groups were given subcutaneous injections of alendronate (alendronate sodium trihydrate, 50 μg/kg, Sigma-Aldrich, St. Louis, MO) every three days starting three days prior to day 0 (day −3) until day 12; and the rats in the Veh group were given daily subcutaneous saline injections for 12 days starting from day 0. All rats were sacrificed on day 12. All experiments were approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

In vivo μCT scans

The right proximal tibia of each rat was scanned by μCT (vivaCT 40, Scanco Medical AG, Brüttisellen, Switzerland) on days 0 and 12 at 10.5 μm resolution. Rats treated with PTH and PTH+ALN received additional scans on days 4 and 8. A recent study has demonstrated protective effects of PTH treatment against radiation damage [35], and our previous study showed high rates of bone formation in rats treated with PTH despite having received additional scans [10]; thus it is expected that scans on days 4 and 8 had no detrimental effects. As described in [10] and [34], rats were anesthetized (4.0/1.75% isoflurane), and the right leg of each rat was inserted into a custom holder to ensure minimal movement during the scan. A 4 mm region of the tibia distal to the proximal growth plate was scanned at 10.5 μm resolution, 55 keV energy, 145 μA intensity, 200 ms integration time, and 1000 projections, using a 0.5 mm Al filter and a standard, manufacturer-provided beam-hardening correction algorithm, resulting in a total scan time of about 20 min and an approximate radiation dose of 0.307 Gy per scan. A subset of rats (n = 18: PTH: 6, PTH+ALN: 5; ALN: 4, Veh: 3) was also scanned eight days prior to treatment (day -8) to allow for pretreatment measures.

Registration between baseline and follow-up scans

For each rat, the day 0 and day 12 scans were aligned using linear, mutual-information-based registration software (ITK, NLM) [33,36]. For the subset of rats also scanned on day -8, the same trabecular volume was aligned in the scans made at day -8, day 0 and day 12 (Fig. 1). This image registration scheme allowed the same volume of trabecular bone to be followed over time so that local instances of bone formation and resorption could be identified within the subvolume.

In each set of sequential images, trabeculae were aligned precisely using a three-step registration procedure: first, baseline (**b**, day 0) and follow-up (**f**, day 12) scans were registered to derive a transformation matrix $\mathbf{T_1}$ by aligning the cortical bone in each image. After this step, the two images (**b** and $\mathbf{f^1}$; $\mathbf{f^1} = \mathbf{T_1 f}$) were oriented approximately the same way, but, due to linear growth, the trabecular compartments were offset from each other [33] (Fig. 2A). To eliminate this offset, a landmark-initialized registration of the trabecular compartment (excluding the cortical bone) was performed to more precisely align the trabecular structures (Fig. 2B) and to derive a second transformation matrix $\mathbf{T_2}$. Finally, a 146 × 146 × 96 voxel trabecular volume of interest (VOI) in the secondary spongiosa starting 2.5 mm distal to the proximal growth plate and spanning to 3.5 mm distal to the growth plate was identified in the baseline scan and extracted from the two images (**b** and $\mathbf{f^2}$, $\mathbf{f^2} = \mathbf{T_2 f^1}$), and was registered to align individual trabecular structures within the VOI. Therefore, a third transformation matrix $\mathbf{T_3}$ was derived. At each step, a visual inspection of the registration result was performed to prevent misalignment caused by local optima of the registration algorithm. Transformation matrices of the three, sequential registration steps were then combined to derive the overall transformation matrix **T** ($\mathbf{T} = \mathbf{T_3 T_2 T_1}$.).

In the traditional image registration scheme, a transformation is applied to the follow-up image (moving image) to match with the baseline image (fixed image). However, transformation of an image inherently causes a certain amount of resampling error, resulting in a difference in image quality between the fixed image and the moving image. In order to eliminate such a difference, we developed a new image registration scheme, where **T** was divided equally so that one half of the rotation, $\mathbf{T}_a$ was applied to the follow-up image (**f**) while