Bone 48 (2011) 433–442

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

In vivo micro-computed tomography allows direct three-dimensional quantification of both bone formation and bone resorption parameters using time-lapsed imaging

Friederike A. Schulte, Floor M. Lambers, Gisela Kuhn, Ralph Müller *

Institute for Biomechanics, ETH Zurich, Wolfgang-Pauli-Strasse 10, 8093 Zurich, Switzerland

ARTICLE INFO

Article history:
Received 31 May 2010
Revised 30 September 2010
Accepted 6 October 2010
Available online 13 October 2010

Edited by: R. Baron

Keywords:
Bone formation
Bone resorption
Dynamic morphometry
Mechanical loading
In vivo micro-computed tomography

ABSTRACT

Bone is a living tissue able to adapt its structure to external influences such as altered mechanical loading. This adaptation process is governed by two distinct cell types: bone-forming cells called osteoblasts and bone-resorbing cells called osteoclasts. It is therefore of particular interest to have quantitative access to the outcomes of bone formation and resorption separately. This article presents a non-invasive three-dimensional technique to directly extract bone formation and resorption parameters from time-lapsed *in vivo* micro-computed tomography scans. This includes parameters such as Mineralizing Surface (MS), Mineral Apposition Rate (MAR), and Bone Formation Rate (BFR), which were defined in accordance to the current nomenclature of dynamic histomorphometry. Due to the time-lapsed and non-destructive nature of *in vivo* micro-computed tomography, not only formation but also resorption can now be assessed quantitatively and time-dependent parameters Eroded Surface (ES) as well as newly defined indices Mineral Resorption Rate (MRR) and Bone Resorption Rate (BRR) are introduced. For validation purposes, dynamic formation parameters were compared to the traditional quantitative measures of dynamic histomorphometry, where MAR correlated with $R=0.68$ and MS with $R=0.78$ ($p<0.05$). Reproducibility was assessed in 8 samples that were scanned 5 times and errors ranged from 0.9% (MRR) to 6.6% (BRR). Furthermore, the new parameters were applied to a murine *in vivo* loading model. A comparison of directly extracted parameters between formation and resorption within each animal revealed that in the control group, i.e., during normal remodeling, MAR was significantly lower than MRR ($p<0.01$), whereas MS compared to ES was significantly higher ($p<0.0001$). This implies that normal remodeling seems to take place by many small formation packets and few but large resorption volumes. After 4 weeks of mechanical loading, newly extracted trabecular BFR and MS were significantly higher ($p<0.01$) in the loading compared to the control group. At the same time, ES was significantly decreased ($p<0.01$). This indicates that modeling induced by mechanical loading takes place primarily by increased area, not width of formation packets. With these results, we conclude that the non-invasive direct technique is well suited to extract dynamic bone morphometry parameters and eventually gain more insight into the processes of bone adaptation not only for formation but also resorption.

Introduction

Bone is able to change its microstructure by mechanisms called modeling and remodeling. Modeling is needed to adapt the micro-architecture to external influences such as changes in mechanical loading [1]. Remodeling keeps the structure capable of coping with daily physical requirements [2]. This adaptation process is governed by bone-forming cells called osteoblasts and bone-resorbing cells called osteoclasts. As a result, formation and resorption are two separate processes whose coordinated execution eventually results in a global net gain or loss of bone tissue. In order to investigate this cellular interplay on an experimental basis, it is of particular interest to have quantitative access to the outcomes of bone formation and bone resorption separately.

Traditionally, the outcomes of dynamic bone cell activity are measured by two-dimensional (2D) histomorphometry. In order to quantify rates of bone formation, the bone is stained *in vivo* with a substance able to incorporate into actively mineralizing osteoid, e.g., calcein or tetracyclin. A short time period between a first and a second labeling injection allows a time-dependent assessment of the mineralization fronts, visible as epifluorescent lines. From these lines, a number of bone formation parameters including Bone Formation Rate (BFR), Mineral Apposition Rate (MAR), and Mineralizing Surface (MS) are determined [3].

Conversely, histomorphometric assessment of bone resorption suffers from the lack of an equivalent dynamic marker [4]. Alternatively, people study resorption cavities in terms of their relative and absolute extent [4]. Eroded Surface (ES) is measured as the percentage of crenated surfaces per total surface; furthermore,

* Corresponding author. Fax: +41 44 632 1214.
E-mail address: ram@ethz.ch (R. Müller).

8756-3282/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bone.2010.10.007

markers exist that are able to visualize the osteoclast number and activity (Oc.N) [5]. Nevertheless, these bone resorption parameters are, in contrast to the available bone formation parameters, only snapshots of a single moment and do not allow an assessment of temporal rates of bone resorption. Besides the difficulty in assessing rates of bone resorption, quantitative histomorphometry suffers from its destructive, laborious, and operator-dependent nature [6,7].

Thus, there is a need for an alternative assessment of bone formation and bone resorption rates. This should be non-destructive and allow time-lapsed *in vivo* measurements of these rates. Waarsing et al. [8] proposed a three-dimensional (3D) method to analyze *in vivo* scans from a non-invasive micro-computed tomography (micro-CT) system. This technique was based on image registration of two subsequent scans of the same animal, where voxels only present in the earlier measurement were considered resorbed bone volumes and voxels only present in the latter measurement were considered formed bone. Qualitative comparison of endosteal formation areas indicated by *in vivo* micro-CT and calcein labeling resulted in very good agreement. Here, we go one step further and show that it is not only possible to detect and track local changes but also to quantify the formation and resorption rates.

The following three steps are necessary to propose a new technique for the calculation of established parameters. First, the results from the new technique need to be compared to the results from the established method. Second, the reproducibility of the new technique needs to be shown. Third, the new method has to be applied to experimental data in order to verify its sensitivity. For this reason, we validated the new 3D technique against traditional quantitative histomorphometry, investigated its reproducibility, and applied it to an experimental *in vivo* animal loading model. The focus of this article was on the validation of the new algorithm. Nevertheless, the evaluation of the *in vivo* model also provided new insights into trabecular bone formation and resorption during normal remodeling and during mechanical loading.

Materials and methods

Materials

All experimental data used in this study were taken from an animal *in vivo* loading model following the approach of Webster et al. [9]. Briefly, *in vivo* bone adaptation was induced in 15-week-old female C57BL/6 mice (RCC, Füllinsdorf, Switzerland) by subjecting their sixth caudal vertebra to mechanical loading at 0 or 8 N for 3000 cycles at 10 Hz, 3 times per week for 4 weeks. Loading was applied through pins inserted in the adjacent vertebrae with a previously developed device [10]. Contrary to the original cross-sectional experiment [9,10], the loaded vertebra was scanned weekly with *in vivo* micro-CT (vivaCT 40, Scanco Medical, Brüttisellen, Switzerland) at an isotropic voxel resolution of 10.5 μm. In order to prevent motion artifacts, the tails were tightly clamped. Previous radiation control studies showed that no radiation effects could be observed in mice scanned 5 times as compared to animals where no *in vivo* imaging was performed (data not shown). During all treatments, the animals underwent isoflurane anesthesia. All animal procedures were approved by the local animal care and use committee (Kantonales Veterinäramt Zürich, Zürich, Switzerland).

For comparison between micro-CT and histomorphometry, data from an experiment with 6 loaded and 6 control mice were used. For quantitative comparison, data from a larger experiment with 9 loaded and 8 control mice were taken. In this experiment, *in vivo* loading resulted in a 22% net gain of trabecular bone volume fraction (BV/TV) and a 13% increase in trabecular thickness (Tb.Th) for the loaded group, and a 7% net gain of trabecular BV/TV for the control group. These results confirmed adequate trabecular bone remodeling and were thus considered appropriate for this article.

For histomorphometry, calcein (Sigma-Aldrich, Buchs, Switzerland) was injected on the days of the 4th and the 5th micro-CT measurement (21 and 28 days after start of loading). Mice were sacrificed 24 h after the second injection. The dissected vertebrae were fixed in phosphate-buffered formaldehyde and then submitted to a dehydration process in an ascending series of ethanol solutions (70%, 80%, 90%, 96%, 100%), before degreasing in xylene. Samples were infiltrated for 7 days at 4 °C in MMA (methyl methacrylate) solution. This solution consisted of methacrylic acidmethylester (Fluka, Basel, Switzerland), dibuthylphthalate (Merck, Darmstadt, Germany), and perkadox 16 (Dr. Grogg Chemie AG, Stettlen-Deisswil, Switzerland) in a proportion 89.5:10:0.5. Thereafter, the samples polymerized at room temperature. Sagittal sections of 6-μm thickness were prepared using a microtome (Leica SP1400, Wetzlar, Germany). Afterwards, fluorescent images were taken from these sections using a fluorescence microscope (Zeiss, Aalen, Germany) and stitched together with Photoshop CS3 (Adobe, San Jose, USA). Histomorphometric analysis was executed using Image Pro Analyzer 6.3 (Media Cybernetics, Silver Spring, USA). For statistical analysis, the software package R (R, Auckland, New Zealand) was used.

Image processing

In order to assess bone formation and resorption sites separately, we followed the approach of Waarsing et al. [8], where a latter measurement of an *in vivo* measured animal was superimposed onto an earlier measurement of the same animal. Bone areas only present in the earlier measurement were considered resorbed bone areas, while areas only present in the latter measurement corresponded to formed bone areas [8,11,12].

Superimposing measurements taken from one animal at different points in time requires a procedure called image registration because the mouse tail cannot be fixed twice in the exact same position. Therefore, data sets of different measurements have to be matched by rotating and translating one record with respect to the other. Several registration algorithms have been proposed in the field of micro-computed tomography [8,11,13]. The approach applied here consists of an intensity-based least-squares algorithm proposed by Thevenaz et al. [14]. B-splines were chosen as the interpolation method [15].

In the image processing chain, the unfiltered initial grayscale scan (week 0) was aligned according to its principal axis, and the unfiltered second grayscale scan (week 4) was registered to it. Both transformed measurements were Gaussian filtered (support 1, sigma 1.2) and thresholded at a global level (threshold 220). Afterwards, the binary registered data set of week 4 was added onto the binary data set of week 0. This procedure resulted in a three-colored image containing voxels present in both volumes, voxels only present in the former volume, and voxels only present in the latter volume (see Fig. 1a). These three-colored data sets served as input for the direct extraction of dynamic bone formation and bone resorption rates.

Dynamic bone morphometry

Histomorphometry defines the Mineral Apposition Rate (MAR) as the distance between the midpoints or the corresponding edges of two consecutive labels, divided by the time between the label injections, given in micrometer per day (μm/d) [3]. In histomorphometry, there is no corresponding parameter such as Mineral Resorption Rate (MRR), given in μm/d. Mineralizing Surface (MS) is defined by the total extent of labeled surface, usually normalized by total bone surface (BS) and given in percentage. The extent of labeled surface can be specified as the mean between separately measured first and second label length, the second label length alone, or the total label length, where the specification and validation of the chosen method are, according to Parfitt et al. [3], the responsibility of the investigator. Eroded Surface (ES) is usually determined by the length of crenated surfaces per total bone surface, also given in percentage.