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Spatial relationship between bone formation and mechanical stimulus within cortical bone: Combining 3D fluorochrome mapping and poroelastic finite element modelling

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ABSTRACT

Bone is a dynamic tissue and adapts its architecture in response to biological and mechanical factors. Here we investigate how cortical bone formation is spatially controlled by the local mechanical environment in the murine tibia axial loading model (C57BL/6). We obtained 3D locations of new bone formation by performing 'slice and view' 3D fluorochrome mapping of the entire bone and compared these sites with the regions of high fluid velocity or strain energy density estimated using a finite element model, validated with ex-vivo bone surface strain map acquired ex-vivo using digital image correlation. For the comparison, 2D maps of the average bone formation and peak mechanical stimulus on the tibial endosteal and periosteal surface across the entire cortical surface were created. Results showed that bone formed on the periosteal and endosteal surface in regions of high fluid flow. Peak strain energy density predicted only the formation of bone periosteally. Understanding how the mechanical stimuli spatially relates with regions of cortical bone formation in response to loading will eventually guide loading regime therapies to maintain or restore bone mass in specific sites in skeletal pathologies.

1. Introduction

Though it has been recognized for centuries that bone adapts its architecture in response to applied loads (Wolff, 1892), we still do not understand how the process is spatially regulated. In particular, it is not well known how the local mechanical environment correlates with regions of bone apposition. Does bone adaptation occur where a particular mechanical stimulus is high? What is that mechanical stimulus?

We currently have limited understanding of the spatial regulation of mechanoadaptation because: 1) it is challenging to accurately investigate a spatially and temporally varying mechanical field in the bone during loading; and 2) it is difficult to identify regions of adaptation throughout the bone, when the amount of local bone formation can be on the order of 10 μm or less. Previous studies have used a combination of finite element modelling, in vivo microCT imaging, and bone histomorphometry to correlate the mechanical environment and regions of cortical bone formation (Webster et al., 2012; Razi et al., 2015a; Moustafa et al., 2012; Birkhold et al., 2017; Gross et al., 1997; Judex et al., 1997). In a mouse vertebral loading model, it was found that strain energy density averaged within a cross-sectional regions of cortical bone predicted regions of bone formation along the length of the vertebrate (Webster et al., 2012). However, these studies did not identify how bone adaptation and mechanical stimuli within a cross-section related to each other in specific locations. In a turkey radius loading model (Gross et al., 1997), peak circumferential strain gradients (which closely relates to fluid flow), calculated with a finite element modelling in 24 sectors of a single mid-diaphyseal section, strongly correlated with the specific regions of periosteal bone formation while strain energy density did not. Similarly, peak strain gradients correlated with sites of periosteal bone formation in an exercise-

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induced rooster model (Judex et al., 1997). More recently, in the mouse tibial loading model, "4D" in-vivo microCT was used to identify regions of bone formation and resorption from sequential in-vivo scans in a region of interest spanning 5% of the tibial length (Birkhold et al., 2015; Birkhold et al., 2016). For each modelling event, the principal strains in that region (~10 μm) were determined from a finite element model (Birkhold et al., 2016). Regions of bone formation correlated with high principle strain at the periosteal surface, but not at the endosteal surface (Razi et al., 2015a; Birkhold et al., 2016). Other studies using the mouse tibial loading model concluded that there is no correlation between regions of high peak longitudinal strain and bone formation in tibial cortical bone when the stimuli was compared to regions of bone formation in two specific histomorphometric sections (37% and 75% of the bone length) (Moustafa et al., 2012).

These previous studies indicate a possible spatial relationship between local mechanical stimuli and cortical bone adaptation. However, there are limitations in relying on in-vivo microCT with nominal isotropic resolution of 10 μm to detect events occurring on the length scale of 10 μm. Also, examining only a small portion of the bone or a few cross-sectional areas, and only the periosteal surface limits the ability to draw conclusions about the relationship between the mechanical environment and bone formation throughout the entire cortical bone. In addition, previous studies only examined strain-dependent stimuli (strain energy density, longitudinal strain, and principal strain), which capture bone's sensitivity to load magnitude but not load rate, and do not predict endocortical formation events, as the stimulus is maximum on the outer surface. Time dependent stimuli (such as fluid velocity) capture the rate-dependent effects, periosteal and endocortical bone formation (Pereira et al., 2015) and are related to shear stresses at the osteocytic level (Weinbaum et al., 1994; You et al., 2001). Thus, in this study we use the murine tibial loading model to determine spatial relationships between the mechanical environment and bone formation in the tibial cortical bone. We examine both strain and fluid flow dependent stimuli in a finite element model. Regions of bone formation are detected with three-dimensional imaging of fluorochrome labels in a novel slice-and-view technique for cortical bone histomorphometry. The mechanical stimuli (strain energy density or fluid velocity) are compared visually and quantitatively to regions of bone formation.

2. Materials and methods

2.1. In-vivo tibial loading model

The right leg of five female C57BL/6 mice (22 week old, Charles River Company, UK) was loaded with a custom tibial loading rig (De Souza et al., 2005). The left leg was not loaded and used as control. In this study, trapezoidal load cycles were applied for 0.1 s, with a peak load of 12 N and a rest period of 10 s, using a regime of 40 cycles a day, 3 times a week for 2 weeks (De Souza et al., 2005). Calcein was administered intraperitoneally once (on day 5), which was about one-third of the way through the experimental period (14 days). Mice were sacrificed on day 15. Mice were maintained under standard laboratory conditions and experiments were conducted in compliance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines for reporting. Briefly, mice were housed up to 4 per cage in polypropylene cages with wood chip and paper bedding and provided standard rodent maintenance diet (Special Diet Services, South Witham, UK) and water ad libitum throughout the study. All procedures complied with the UK Animals (Scientific Procedures) Act 1986 and were reviewed and approved by UK Home Office and local ethics committee of the Royal Veterinary College (London, UK).

2.2. Strain map on the bone surface using digital image correlation

Right and left tibiae of the five mice subjected to the mechanical loading protocol were considered. Tibiae were exposed by removing soft tissues, and a thin layer of matt, water-based, white paint with subsequent matt, acrylic, black ink speckles was applied using a high precision air brush (Carriero et al., 2014). Speckles were randomly distributed with a 45% black/white density and dots of about 8 pixels in diameter (Carriero et al., 2014). Legs were inserted in the loading cups and loaded to 12 N to replicate the load in vivo (Instron 5800, High Wycombe, UK) while two CCD cameras (100 mm lenses, GOM GmbH, Germany) mounted on a tripod recorded images of the medial side of the tibiae surface with a resolution of 7.5 × 10.9 μm at 1 N interval (GOM GmbH, Germany) (Carriero et al., 2014). Square facets (19 × 19 pixels) with 15 pixels step facet were used for the post-processing of the images using ARAMIS 5 M System (GOM GmbH, Germany) (Carriero et al., 2014). Paired images were taken in the undeformed state to determine the amount of experimental error (noise), and the surface of each tibia were imaged at least two times to demonstrate repeatable strain fields. These ex-vivo strain maps determined strain magnitudes and distribution patterns in mature tibiae with and without prior adaptation to applied load (right and left tibia, respectively). Peak and average strain at 12 N on the medial surface of the control non-adapted (left) leg and the load adapted (right) leg were calculated, averaged across the specimens of each group, and statistically compared. Homogeneity of variance and normality of the variables were assessed using the Levene's test and the Shapiro-Wilk test, respectively (SPSS, IBM, Somers, NY, USA). Difference between the two groups was assessed using paired *t*-test. Statistical significance was set at $p < 0.05$.

2.3. Bone architecture using 3D Micro-Computed Tomography

After determination of surface strains across the entire tibia by DIC, bones were dissected and scanned by microCT at 10 μm resolution. Images were acquired using a Skysan 1172 micro-CT system (Skyscan, Kontich, Belgium) with the x-ray tube operated at 50 kV, 200 μA, 1600 ms exposure time with a 0.5 mm aluminium filter and a focal spot size of 5 μm. The microCT images were imported into image-processing software (Mimics V15, Materialise, Leuven, Belgium). Tibiae were segmented, (with fibula removed from the image) and aligned along their longitudinal axis. Minimum moment of area (Imin,) along the tibial diaphysis was determined using ImageJ (Schneider et al., 2012; Pereira, 2014). We previously determined Imin was more sensitive to regions of adaptation than Imax because of the location of regions of adaptation and in relation to the principle axes (Pereira, 2014). The longitudinal distance between the proximal and distal tibia-fibula junctions were normalised for all bones.

Cross-sectional morphology from microCT of the loaded and unloaded tibiae identified general regions along the length of the bone where adaptation occurred. Box plots were used to represent the minimum moment of area along the entire length of the tibia. Statistical significant difference between load adapted and control leg was considered when no overlap exists between the confidence interval (CI) of the blocks of the two groups.

2.4. Location of bone formation using 3D fluorochrome mapping

Loaded and control tibiae of one mouse were then fixed and embedded in an opaque methyl-methacrylate (PMMA) containing Sudan Black dye (2%) to preserve calcein labels and provide an opaque embedding material to block out fluorescence behind the plane of imaging. Each embedded bone block was then mounted in the "histocutter" (Fig. 1) that allows serial cutting (Leica RM-2265 Microtome) and imaging (Nikon AZ-100 Fluorescence Microscope) of the 'block face' in order to create a 3D histological reconstruction (3.3× projection lens, zoom 2, 4.585 mm field of view), similar to what 3D techniques used for trabecular bone in other groups (Kazakia et al., 2007; Bigley et al., 2008; Goff et al., 2012; Goff et al., 2014; Matheny et al., 2013; Slyfield et al., 2009; Slyfield et al., 2012a; Slyfield et al., 2012b; Tkachenko et al., 2009). The microscope was equipped with a wheel with multiple emission filters