Title: Spatial Relationships between Bone Formation and Mechanical Stress within Cancellous Bone

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ABSTRACT

Bone adapts to mechanical stimuli. While in vivo mechanical loading has been shown to increase the density of cancellous bone, theory suggests that the relationship between tissue stress/strain and subsequent bone formation occurs at the scale of individual trabeculae. Here we examine bone formation one week following mechanical stimulus. Three bouts of cyclic loading (300 cycles/day on 3 consecutive days) were applied to caudal vertebrae of female rats (n=7). Bone formation was determined using three-dimensional images of fluorescent markers of bone formation (0.7x0. x5.0µm) and local tissue stress/strain was determined using high-resolution finite element models. Three days of mechanical stimuli resulted in an increase in mineralizing surface (loaded: 17.68±2.17%; control: 9.05±3.20%; mean ± SD) and an increase in the volume of bone formed (loaded: 7.09±1.97%; control: 1.44±0.50%). The number of bone formation sites was greater in loaded animals (650.71±118.54) than pinned not loaded controls (310.71±91.55), a difference that was explained by the number of formation sites at regions with large local tissue strain energy density (SED). In addition, the probability of observing bone formation was greater at locations of the microstructure experiencing greater SED, but did not exceed 32%, consistent with prior work. Our findings demonstrate that bone formation in the week following a short term mechanical stimulus occurs near regions of bone tissue experiencing high tissue SED, although the ability of finite element models to predict the locations of bone formation remains modest and further improvements may require accounting for additional factors such as osteocyte distribution or fluid flow.

1.0 INTRODUCTION

The idea that bone adapts to habitual mechanical stimulation originated over 150 years ago and was based on observations that the alignment of cancellous bone microarchitecture matches the orientation of principal stresses (see (Cowin, 2001) for a thorough review). The degree to which bone density and internal and external morphology is altered in response to mechanical stimuli is key to understanding the potential of biophysical stimuli to improve bone microstructure and is also useful for the interpretation of trabecular bone alignment in rare specimens from the fossil record (Ryan and Ketcham, 2002).

Experimental application of controlled mechanical loads to the bones of live animals has provided a wealth of information on bone functional adaptation (van der Meulen and Hernandez, 2013). In cortical bone, in vivo mechanical loading leads to increases in periosteal bone formation at the locations of greatest tensile and compressive strains (Torrance et al., 1994). In cancellous bone, in vivo loading has been shown to result in increases in bone density and bone volume fraction (Fritton et al., 2005; Lambers et al., 2011; Morgan et al., 2015; van der Meulen et al., 2006; Webster et al., 2010). The vast majority of studies examining in vivo mechanical loading of cancellous bone have addressed changes at the regional level (regions of cancellous bone 1-5 mm in size), yet theoretical analysis suggests that functional adaptation in bone occurs at the local level (as close as 10-100 micrometers)(Carter, 1982). In cortical bone, new bone formation after an acute mechanical stimulus has been correlated with local periosteal stresses and strains (Kotha et al., 2004). In cancellous bone, local mechanical stress and strain associated with long-term loading (stimulus 5 days per week for 4 or more weeks) has been shown to be moderately predictive of locations of new bone formation (conditional probability no better than 45%) (Schulte et al., 2013).

While the relationship between local tissue stress and strain and new bone formation has been established in cancellous bone for long-term loading (4 weeks or more of regular loading) (Schulte et al., 2013; Webster et al., 2012) it is not known how well local mechanical stimulus is associated with new bone formation over shorter time periods. Determining the spatial associations between mechanical stress/strain and new bone formation at the level of individual trabeculae immediately following an acute stimulus has the potential to provide a more direct evaluation of mechanosensory mechanisms. While stress and strain in mineralized tissue are believed to be the primary stimulus, there is evidence that fluid flow within the lacunar canalicular network (Jacobs et al., 2010) and

fluid flow and pressure in the marrow space have been associated with bone formation (Hu et al., 2012; Kwon et al., 2010; Metzger et al., 2015; Qin et al., 2003; Webster et al., 2015). The ability to assess the spatial association between bone formation and mechanical stress and strain is necessary to differentiate among mechanosensory mechanisms, particularly if such measures are made soon after loading.

The long term goal of this line of investigation is to determine the mechanotransduction mechanisms in cancellous bone. Here we examine the spatial relationship between local mechanical stress/strain and bone formation in the first week after a mechanical loading stimulus. Specifically we determine the relationship between tissue stress and strain within cancellous bone and individual locations of bone formation stimulated by mechanical loading.

2.0 METHODS

2.1 In vivo Loading and Micro-Computed Tomography

Three month old, female Sprague Dawley rats were used in this study (n=14). Animals were housed individually in a 12 hour light 12 hour dark cycle and fed standard rat chow ad libitum. Animal use was approved by the local IACUC.

Mechanical stimulus was applied to regions of cancellous bone using the rat tail loading model (Chow et al., 1993; Guo et al., 2002). The animals were anesthetized using isoflurane inhalation and threaded Kirschner wires (K-wire, dia. = 1.6 mm, Zimmer, Warsaw, IN) were inserted into the seventh (Cd7) and ninth caudal vertebra (Cd9). K-wire positioning was ensured using digital radiographs (Preva, Progeny, Lincolnshire, IL). Following surgical implantation, K-wires were secured in a single piece aluminum external fixator to prevent displacement and rotation of the K-wires relative to one another, thereby unloading the eighth caudal vertebrae (Fig 1A,B). Animals were returned to normal cage activity for 3 weeks to allow healing around the implants.

Three weeks after surgery, animals were anesthetized, K-wires were removed from the external fixator, and micro-computed tomography images (CT-120, GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) of the eighth caudal vertebrae were acquired (25µm voxel size, 80kVp, 32mA, 1200 projections, 100ms integration time). Immediately following image acquisition, the K-wires were secured into custom fixtures within a materials testing device (Bose EnduraTech LM1 test bench, Bose Corporation, Minnetonka, MN). Animals were divided into two groups, a loaded and pinned not loaded control (n=7 per group). Animals in the loaded group were anesthetized

through isoflurane inhalation and compressive loading was applied through the two K-wires onto the eighth caudal vertebra. Loading was applied first in a ramp from 0N to 25N at 1N/sec, followed by cyclic sinusoidal compressive loading from 25N to 100N at 0.5Hz for 300 cycles. Preliminary assessment with strain gages indicated that the maximum applied load of 100N was associated with surface strains of $2141 \pm 644 \mu\epsilon$ on the dorsal and $771 \pm 534 \mu\epsilon$ (mean $\pm$ SD, n=5) on the ventral surfaces (Goff et al., 2014). Animals in the pinned not loaded control group were anesthetized and had K-wires secured into the custom loading fixtures for the same length of time but no load was applied. The protocol was repeated once per day for 3 consecutive days and K-wires were secured in the external fixators between loading sessions. Bone formation markers were administered via intraperitoneal injection at 2 days (90mg xylene orange/ kg body weight) and 7 days (10mg calcein/ kg body weight) after loading. Animals were euthanized 11 days post loading and bones were collected for analysis.

2.2 Finite Element Modeling

The distribution of tissue stress/strain within the eighth caudal vertebrae during loading was determined using high resolution finite element models generated from micro-computed tomography images (micro-CT) collected prior to loading. Following the application of a Gaussian filter (radius = 1 voxel) the bone was segmented using a locally adaptive threshold based on edge detection (Waarsing et al., 2004). Finite element (FE) models were created by converting each voxel into 8 node linear brick elements using custom software (Lynch et al., 2010) (element size 25 μ m which has been shown to be sufficient for modeling cancellous bone microstructure (Guldberg et al., 1998; Niebur et al., 1999)). Bone tissue was assigned a Young's modulus consistent with surface strains achieved in preliminary experiments ($E = 9.4$ GPa) (Goff et al., 2014) and Poisson's ratio $\nu = 0.3$. The intervertebral discs were included in the model as cylindrical discs attached at the ends of the bones with a diameter of 3 mm and height of 0.5 mm. Discs were modeled with $E = 4.0$ MPa and $\nu = 0.3$ (Elliott and Sarver, 2004). The peak load applied to the bone in vivo was simulated by applying a compressive load of 100N across the caudal discs and constraining axial displacement at the distal disc (Fig 1C). The finite element models were implemented with ABAQUS (Version 6, Waltham, MA) on the Gordon supercomputer (XSEDE, vSMP node, for ~140 total computing hours) (Ray et al., 2014). Models consisted of 3.85 ± 0.25 million elements (mean $\pm$ SD). Strain energy density (SED), maximum principal strain, and von Mises stress within each element was used to evaluate local mechanical stimuli. Similar trends were found with all 3 assays of local mechanical stimuli so only SED is reported

for consistency with other work (Huiskes, 2000; Lambers et al., 2011; Schulte et al., 2013). Finite element models were performed for the pinned not loaded controls to provide a null control for SED distributions.

2.3 Image Acquisition and Processing

The eighth caudal vertebrae were collected and soft tissue was removed. The endplates were cut away with a low speed diamond saw (Isomet, Beuhler, Lake Bluff, IL) and marrow was removed using a low pressure water jet and short periods of ultrasonication. Specimens were fixed in 70% ethanol for 48 hours, dehydrated in increasing concentrations of ethanol and embedded undecalcified in methyl methacrylate made opaque by the addition of sudan black dye in preparation for image acquisition with serial milling (Slyfield et al., 2012). Serial milling is a destructive imaging technique that results in a three-dimensional image of the bone and two different fluorescent markers with a voxel size of $0.7 \times 0.7 \times 5.0 \mu\text{m}$ (Slyfield et al., 2009) (please see Supplementary Materials for a more detailed description of the technique). Following image reconstruction, three-dimensional dynamic bone histomorphometry was performed to measure three-dimensional mineralizing surface per bone surface (3D MS/BS), the number of bone formation sites, and the volume of bone formed during the experiment (FV/BV, formation volume fraction).

2.4 Spatial Associations between Bone Formation and Mechanical Strain

Images collected using serial milling were registered to finite element models using commercial software (AffineRegistration, AMIRA, v5.3.3, Visage Imaging, San Diego, CA) which includes some downsampling of serial milling images and upsampling of FE models for registration (Fig. 2). Regions of cancellous bone were segmented manually by a trained observer. The central region of each specimen contained negligible amounts of trabecular bone and was removed from analysis (Lambers et al., 2011). The region of each bone analyzed therefore comprised of two regions of cancellous bone, each 1.4 mm in height located 1 mm away from each growth plate.

Four different techniques were used to test the idea that bone formation was preferentially located at regions of increased tissue stress/strain: an object number-based spatial correlation approach, a volume-based spatial correlation approach (Goff et al., 2014), measuring SED at locations of bone formation vs. SED at all bone surfaces, and assessing the probability of observing bone formation as a function of local tissue stress/strain (Schulte et al., 2013).

The object number-based spatial correlation tests the idea that two types of objects are preferentially near one another. We tested the idea that bone formation events were near locations of high SED and also considered the converse questions that locations of high SED were near bone formation events. High SED was defined as the 80th percentile of SED within each specimen (a preliminary parametric analysis revealed that our conclusions were not altered by the numerical value of this choice for a range of 70th to 95th percentile).

The volume-based spatial correlation tests the idea that the volumes occupied by two different types of objects in a three-dimensional image are preferentially near one another (Goff et al., 2014). In this case we test the idea that the formation volumes were preferentially near locations of high SED and the converse question that high SED volume was near bone formation. The volume-based spatial correlation is determined as the volume of newly formed bone near tissue experiencing high SED divided by the volume of bone tissue selected at random that is near tissue experiencing high SED. A value greater than one indicates a positive correlation (new bone formation and tissue with high SED are near one another) while a value equal to one indicates no spatial correlation (new bone formation is not preferentially near or far from tissue experiencing high SED). The volume based spatial correlation was determined at distances ranging from 25µm to 100µm (the average trabecular thickness).

The average SED (calculated as the geometric mean) was determined for all bone surface voxels and bone surfaces within 25 microns of bone formation. A ratio of SED near bone formation to the SED at all bone surfaces was determined per specimen and compared to 1.0. A ratio greater than 1.0 indicates that SED is higher than average surface SED near locations of bone formation.

The probability of observing bone formation was determined as the percentage of newly formed bone tissue at each value of SED (Schulte et al., 2013). Locations of SED in the probability analysis were confined to the surface voxels of the bone.

2.5 Statistics

Measures of bone formation were compared between loaded and control groups using a student's t-test. A t-test was used to determine if the ratios describing the volume-based spatial correlation and differences in SED differed from 1.0 (no difference).

3.0 RESULTS

The three-dimensional mineralizing surface in loaded animals ($17.68 \pm 2.17\%$, mean $\pm$ SD) was greater than that observed in pinned not loaded controls ($9.05 \pm 3.20\%$) (Fig. 3A). The volume of bone formed between the formation labels in loaded animals was more than four times greater than that in pinned not loaded controls (loaded: $7.09 \pm 1.97\%$; control: $1.44 \pm 0.50\%$; Fig. 3B). Although only 3 days of load had been applied, BV/TV was slightly greater in loaded animals ($p = 0.09$, Fig. 3C). The increase in bone formation in loaded specimens was a result of an increase in the number of bone formation sites as well as larger formation sites (Fig. 3D, 3E). Regions of new bone formation in the cancellous envelope were lamellar, but each of the loaded specimens showed regions of woven bone on the periosteal surfaces (no periosteal woven bone was observed in pinned not loaded control animals).

There were, on average, more than twice as many sites of bone formation in the loaded animals than in the pinned not loaded controls (loaded: 650.71 ± 118.54 ; control: 310.71 ± 91.55 ; Fig. 4). However, in the loaded animals, half of the bone formation sites were near regions of high SED (within $25\mu\text{m}$). Hence in loaded animals, the increase in the number of bone formation sites was caused by formation associated with high SED. As many as $46.74 \pm 7.37\%$ of the locations of high SED were near regions of bone formation in loaded animals (as compared to $16.80 \pm 3.58\%$ in controls).

The ratio of SED near bone formation to SED at all bone surfaces was greater in loaded specimen (Fig 5). Average SED was found to be 7.96 ± 2.49 kPa (geometric mean $\pm$ SD) with a 95% confidence interval of 5.66 to 10.27 kPa. (see also supplementary material).

In loaded specimens, the probability of observing bone formation at a location in the microstructure was greater at regions experiencing greater SED (Fig 6). In controls there was no relationship between the probability of observing bone formation and tissue SED and the probability of observing bone formation was lower than that in loaded specimens. Volume based spatial correlations did not indicate a correlation between the volume of tissue experiencing high SED and the volume of bone formation (Fig. 7).

4.0 DISCUSSION

The objective of this study was to determine the spatial relationship between local mechanical stress/strain and bone formation in the week after a short-term mechanical loading stimulus. We found a spatial correlation between bone formation stimulated by mechanical loading and the tissue stress/strain environment at the scale of 25

micrometers. Hence, even early functional adaptation responses in bone within a week of a short-term stimulus are associated with local tissue stress/strain.

The use of four different assays of the spatial association between bone formation and high SED provides a full picture of the association between bone formation and tissue stress and strain. The increase in number of bone formation sites caused by loading was due to formation sites near tissue experiencing high SED. Strain energy density was greater near bone formation than the average on all bone surfaces. The probability of observing bone formation increased at greater SED magnitudes. Therefore three measures indicated a spatial association between bone formation and tissue stress and strain. However, the volume-based spatial correlation did not observe an association between newly formed bone and bone volume experiencing high SED. We interpret these results as indicating that many new formation sites appeared near regions of the tissue experiencing high SED, but a large proportion of the bone volume formed was due to expansion of bone remodeling events that were ongoing prior to mechanical stimulus.

The probability of observing new bone formation at the regions of greatest SED may seem small (32%) but is similar in magnitude to the largest probability (45%) reported using the mouse tail loading model that involved 4 weeks of daily loading and higher resolution finite element models (10.5 μm elements) (Schulte et al., 2013). As we expect larger amounts of bone formed over 4 weeks to be associated with mechanical loading, we consider our findings after 3 days of loading to be consistent with that of Schulte and colleagues. Given the consistency in our measure, it is unlikely that improvements in finite element resolution would improve our predictive capabilities. We feel that it is more likely that variation in material properties and the osteocyte network could improve prediction.

There are several strengths to the current work. First, we have shown that small misalignments during experimental loading will not alter the locations of high SED identified by finite element models (Goff et al., 2014), providing confidence that the localization of mechanical stress and strain was accurate. Second, the use of serial milling makes it possible to directly measure small amounts of bone formation in three-dimensions. The serial milling approach has some advantages over the more commonly used sequential in vivo micro-computed tomography technique for assessing bone formation (Kohler et al., 2005; Liu et al., 2012; Schulte et al., 2012). Serial milling, as we have performed, has a higher resolution (1-5 μm voxels) than existing in vivo micro-computed tomography systems (10.5 μm voxels), making it possible to measure bone formation over periods as short as a

week. Additionally, measures of bone formation using serial milling are based on fluorescent markers of bone formation, which are the gold standard for assessment of bone formation and, due to the increased resolution have smaller image registration/segmentation errors. For example, when using sequential micro-computed tomography imaging, small errors in image registration and partial volume effects on bone surfaces can influence measures of bone formation and resorption (Lan et al., 2013). To avoid registration error some practitioners disregard the outer layer of voxels on bone formation and resorption surfaces (de Bakker et al., 2015), limiting the accuracy of measures of the number and size of formation and resorption events. An important limitation of serial milling is that it is a destructive imaging approach and cannot be performed in vivo. Sequential micro-CT imaging provides accurate measures of net changes in bone volume and microarchitecture between scans and is therefore a useful method of measuring changes in microarchitecture over longer time periods.

A limitation of the current work is the presence of woven bone on the periosteum of loaded vertebra. The presence of woven bone on the surfaces has the potential to influence physiological processes in the endosteal and cancellous bone envelopes. However, bone formation on the endosteal surface and in cancellous regions remained lamellar and the probability of observing bone formation at regions of high tissue SED (largest value 32%) was similar to that reported by others following 4 weeks of loading (45%, see below (Schulte et al., 2013)), hence it is unlikely that the presence of woven bone on the periosteal surfaces influenced our conclusions regarding the spatial association of bone formation and tissue stress and strain.

It is useful to consider the current work in the context of prior investigations. Chow et al. showed that as little as one loading session of 300 cycles is capable of increasing bone formation rate through increases in both double labeled surface and mineral apposition rate (Chow et al., 1993). A series of recent investigations using tail loading in the mouse and sequential micro-CT imaging, demonstrated that long-term mechanical loading (4 weeks or more, 5 days/week) resulted in noticeable alterations in cancellous bone microarchitecture that could be explained, to some degree by tissue strain energy density (Schulte et al., 2013). Another difference between our work and prior investigations is related to the external fixation and disuse present prior to applied loading bouts. In the current study we used a one piece external fixator resulting in unloading of the eighth caudal vertebra in the three weeks between pin placement and mechanical stimulus. Recent work using the mouse tail loading system did not utilize external fixation, presumably maintaining habitual activity (Lambers et al., 2011; Schulte et al., 2013;

Webster et al., 2008) and the resulting probability of observing bone formation in the microstructure showed some relationship to SED even in the pinned not loaded control animals (Schulte et al., 2013).

While increases in mechanical stimuli lead to new bone formation, the mechanosensory mechanisms are complicated. Determining the spatial correlation between bone formation and tissue stress/strain immediately following an acute stimulus has the potential to allow more direct identification of mechanosensory mechanisms. For example, although tissue stress/strain is believed to be the primary mechanical stimulus, one group has reported that fluid pressure in the marrow space was more strongly associated with the locations of bone formation ($R^2 = 0.83$) than strain energy density within mineralized tissues ($R^2 = 0.55$) (Webster et al., 2015). Gene expression in the marrow differs from that in cancellous bone as well as that in cortical bone (Kelly et al., 2014). Hence, identifying the most compelling the spatial correlations between formation and mechanical stress/strain will identify which material to isolate to identify signaling processes.

In conclusion, we demonstrated that bone formation within the first week after a mechanical stimulus is spatially associated with regions of increased tissue stress/strain within cancellous bone.

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Figure 1. (A) K-wires were inserted into the Cd7 and Cd9. (B) K-wires were secured into an external fixator between loading sessions. (C) Micro-CT images were converted to finite element models with intervertebral discs and boundary conditions as shown. The strain energy density (SED) distribution is shown for a cross section of the whole vertebra.

Figure 2. (A) A region of cancellous bone with locations of high SED indicated in purple is shown. (B) Three-dimensional images of fluorescent markers of bone formation (green) were used to identify regions of bone formation that occurred in the week after loading. (C) The spatial relationship between bone formation and regions of high SED was determined after image registration of the finite element model and serial mill images.

Figure 3. (A) Mineralizing surface was twice as large in the loaded group as compared to controls. (B) In loaded animals the volume of bone formed in the week after loading was greater than in pinned not loaded controls. (C) Bone volume fraction was not significantly increased. (D) The number of bone formation sites increased due to loading. (E) The volume of each bone formation site was larger in loaded specimens as compared to unloaded controls. (F) Three-dimensional images of bone (tan) and bone formation (green) are shown for a control and loaded specimen.

Figure 4. The number of bone formation sites in the control group is shown for each of the specimens. The number of bone formation sites in loaded specimens near locations of high SED (within 25 μm) and not near high SED is indicated. The number of bone formation sites near locations of high SED in loaded specimens is similar to the number of formation sites observed in pinned not loaded control animals.

Figure 5. The ratio of mean SED near bone formation (within 25 μm) to SED at all bone surfaces is greater than 1.0 ($p=0.02$) in loaded specimens, therefore SED is greater at locations of bone formation than elsewhere. The ratio in control specimens is not greater than 1.0.

Figure 6. The probability of observing bone formation within 25 μm of a given SED range increases in loaded specimen and decreases in controls. Locations of SED values were constrained to the surface of bone. Error bars are standard error.

Figure 7. The volume based spatial correlation of loaded specimens is shown. (A) The likelihood of bone formation near regions of high SED is shown and (B) the likelihood of high SED near regions of bone formation is shown. A ratio greater than 1.0 indicates bone formation is preferentially near high SED. When the ratio is less than 1.0 bone formation is preferentially distant from high SED.

Figure1

Figure2

Figure3

Figure4

Figure5

Figure6

Figure 7