Title page

Does subchondral bone of the equine proximal phalanx adapt to race training?

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Summary

Sagittal fractures of the first phalanx are a common, potentially catastrophic injury in racehorses. These fractures are often linked to an acute, one time, biomechanical event; however, recent evidence implies that chronic exposure to stress can lead to accumulative bony changes that affect the structural integrity of the bone and increase the likelihood of fracture. The aim of the study was to compare variations of two common metrics of bone adaptation - subchondral bone density and thickness across the proximal articular surface of the first phalanx in Thoroughbred horses that 1) raced but never experienced a first phalanx fracture (Raced Control); 2) horses that raced and had experienced fracture of the contralateral first phalanx (Contralateral to Fracture); 3) horses that had never raced nor experienced a first phalanx fracture (Unraced Control). A total of 22 first phalangeal bones were sampled post-mortem and imaged using micro-computed tomography calibrated for mineral density measures. Measurements of volumetric subchondral bone mineral density and thickness were taken from images at five sites from medial to lateral, in three coronal planes (25%, 50% and 75% dorsal-palmar). At each of the 15 sites, measurements were repeated and averaged across ten adjacent micro-computed tomography slices of bone, spanning 0.75mm. The magnitude and variance of these measurements were compared between sites and between cohorts with non-parametric statistical tests.

Across the proximal osteochondral surface of the first phalanx, the pattern of subchondral bone volumetric bone mineral density and thickness varied with each coronal section studied. The subchondral bone thickness was greater for the central and dorsal coronal sections, compared to the palmar section. For the race-fit groups (Raced Control and Contralateral to Fracture), the highest volumetric bone mineral density was in the central sagittal groove. The volumetric bone mineral density was significantly greater in the sagittal groove in the central coronal section in the raced compared to the unraced group. The Contralateral to Fracture group demonstrated significantly greater variance of volumetric bone mineral density compared to the Raced Control and Unraced Control ($P<0.0001$), with no difference in variance noted between the Raced Control and Unraced Control groups. There was a small (R rank = 0.3) but significant correlation between subchondral bone volumetric bone mineral density and thickness in the Contralateral to Fracture group ($P=0.005$). The findings demonstrate that differences exist in subchondral

bone volumetric bone mineral density and thickness across the proximal osteochondral surface of the equine first phalanx in horses with different training histories. The findings demonstrate that the subchondral bone of the sagittal groove of the equine first phalanx adapts to race-training in the race-fit groups (Raced Control and Contralateral to Fracture) with an increase in volumetric bone mineral density relative to un-raced controls. Within the race-trained groups, the Contralateral to Fracture bones had a greater variance of volumetric bone mineral density suggesting that stress induced bone adaptation had become more erratic, potentially contributing to the aetiology of sagittal fractures of the first phalanx in the Thoroughbred racehorse.

Keywords: Equine, subchondral bone density, bone adaptation

Introduction

Sagittal fractures of the equine proximal phalanx (P1) are relatively common in Thoroughbred racehorses during training (Ely et al., 2009, Ramzan and Palmer, 2011), constituting 40% of fatal distal limb fractures during flat racing on turf (Parkin et al., 2004). Most P1 fractures in the Thoroughbred racehorse occur in a predictable configuration, along a sagittal plane through the proximal sagittal groove (Ellis et al., 1987, Stover, 2003, Murray et al., 2006). One theory of aetiology for sagittal fractures of P1 is that the rotary movement between the sagittal groove of the proximal P1 and the distal third metacarpal bone (McIII) sagittal ridge, induces compressive and torsional forces that ultimately lead to fracture as an acute biomechanical event or monotonic overload (Markel and Richardson, 1985, Ellis et al., 1987, Holcombe et al., 1995).

A slightly more nuanced, but complementary, interpretation is that an inadequate adaptive response of the P1 subchondral bone to exercise, diminishes the ability of bone to withstand the compressive and torsional forces placed upon it (Riggs and Boyde, 1999, Firth and Rogers, 2005a, Murray et al., 2006). This view suggests that the acute event is predicated on longer term repetitive loading that promotes bone remodelling that can weaken the bone as it attempts to respond to the low level but frequent stimulus (Riggs and Boyde, 1999, Firth and Rogers, 2005b, Ramzan and Powell, 2010, Whitton et al., 2010, van Oers et al., 2011, Vickerton et al., 2014).

The ontogenic template, as well as the molecular pathways and cellular responses of bone to mechanical stimuli, are coded for genetically; but structural “normality” can only be achieved by adaptive responses to load bearing (Lanyon, 1984). Loads are not distributed evenly across the articular surface of joints and vary according to the surface topography of the articulation, as well as the level of physical activity and of the animal’s overall limb conformation (Brama et al., 2001, Holopainen et al., 2008, Brama et al., 2009, Beccati et al., 2011). Subchondral bone (SCB) mineral density and thickness also vary across the articulating surface of a joint and may be influenced by the dominant loading pattern, as well as by the shape of the joint (Eckstein et al., 1997). SCB volumetric bone mineral density (vBMD) is affected by anatomical site, SCB thickness, physical activity and maturation. Exercise induces changes of the SCB and these are preserved for up to six months after the

82 exercise regime has changed, highlighting the importance of defining optimum training for
83 osseous health (Firth and Rogers, 2005b, Brama et al., 2009).

84 Since biomechanical properties of the SCB are important to susceptibility of bone to fracture
85 (Riggs and Boyde, 1999, Riggs et al., 1999, Rubio-Martínez et al., 2008a, Barr et al., 2009,
86 Anthenill et al., 2010), a better understanding of the SCB of the equine proximal phalanx is
87 necessary. Mechanical properties of bone such as: elastic modulus, yield stress and strain
88 and energy to failure are highly related to the physical properties of bone, such as SCB
89 mineral density (Rubio-Martínez et al., 2008a, Madry et al., 2010). Knowledge of
90 subchondral bone mineral density and thickness of the proximal articular surface of P1
91 would allow inferences regarding the variation in mechanical properties of bone across the
92 articular surface.

93 Previously, studies have investigated the biomechanical properties of commonly fractured
94 bones in the equine distal forelimb. The distal condyles of equine McIII have been analysed
95 to document the effect of alteration in subchondral bone density and mechanical
96 properties (yield stress and strain, trabecular thickness and separation, bone volume
97 fraction and connectivity) on fracture occurrence (Young et al., 1991, Riggs and Boyde,
98 1999, Rubio-Martínez et al., 2008a). Hypertrophy within the subchondral cancellous
99 architecture of the third carpal bone has been documented and compared between horses
100 undergoing different exercise regimes and these alterations appear more prominent in high
101 intensity exercise groups (Young et al., 1991, Firth et al., 1999, Murray et al., 2006). As yet,
102 the only study on SCB mineral density and thickness of P1 has been conducted on growing
103 horses at two specific sites in the proximal articular surface: the medial facet under constant
104 weight-bearing and the dorsomedial margin that is loaded intermittently at a high rate
105 during exercise (Holopainen et al., 2008, Brama et al., 2009).

106 The aim of this study was to characterise and compare variations in SCB mineral density and
107 thickness across the proximal articulating surface of P1 from Thoroughbred racehorses with
108 and without P1 fractures, and with P1 bones from non-racing horses. By comparing bones
109 from horses with a catastrophically fractured P1 to both raced and unraced control groups,
110 we aimed to determine if the fractured bones failed to adapt to exercise completely or
111 simply deviated from the normal pattern of adaption. Our hypotheses were that the

subchondral bone thickness and density would vary significantly between the three groups and across the proximal articular surface of P1.

Materials and Methods

The P1 bones were collected from three groups of horses: contra-lateral to a fractured P1 (CF), raced controls (RC) and unraced controls (UC). The raced control bones (n=10, 5 pairs) were obtained from Thoroughbred racehorses euthanized for reasons other than limb fractures. The CF group bones (n=6; 2 left, 4 right) were obtained from Thoroughbred racehorses euthanized for catastrophic P1 fracture of the opposite, contralateral, limb. The unraced control group bones (n=6; 3 left, 3 right) were obtained from unraced Warmblood/Thoroughbred crosses euthanized for conditions unrelated to the musculoskeletal system. All bones were from mature horses. Racing and training of Thoroughbred horses in the United Kingdom is varied as regards direction of travel and surface. Bones were collected as part of a previous studies [(Parkin et al., 2004, Tranquille et al., 2012) both Horserace Betting Levy Board funded)] for which ethical approval had been obtained (RC and CF bones) or after informed consent from the owner (UC bones).

The bones were wrapped in moist paper towels and were stored frozen at -20^{o}C until imaging with microcomputed tomography (µCT) (Metris X-tek custom 320 kV bay system, Henry Moseley Imaging Facility, University of Manchester). The bones were defrosted, wrapped in three layers of plastic and then scanned in pairs. The µCT images were acquired at 75-µm isotropic voxel resolution with exposure factors of 90kV and 85µA (Figure 1). A calibration phantom (M32-HA-30, QRM, Germany) with densities of 0, 200, 800 and 1200 mgHA/cm^3 was scanned with the same exposure factors as the bone samples. Image data were subsequently imported into image analysis software Image J (Schneider et al., 2012). A calibration curve was plotted (Microsoft Excel) based on the hydroxyapatite (HA) concentrations of the phantom and image voxel values. The calibration equation was subsequently used to convert voxel values into the volumetric mineral density (mgHA/cm^3) of the subchondral bone at the test sites (vBMD, mgHA/cm^3).

An image standardised protocol was established to ensure that the measurement sites were consistent among the bones. The isometric bone image data were all aligned in the same orientation with the longitudinal axis of the bone parallel to the z axis and with the mid-sagittal plane parallel to the x axis. The isometric image stack was then resliced to generate coronal (x-y) images running from the dorsal to palmar surface. Across the proximal articular surface, ten consecutive µCT image slices were analysed at 25% (dorsal), 50% (middle) and 75% (palmar) of the distance from dorsal to palmar (Figure 1). Within each of the three regions, five measurement sites were selected from lateral to medial (Figure 2) encompassing the sagittal groove and the SCB either side. These sites are illustrated in Figure 2 and are referred to in a lateral to medial direction as: lateral fovea, lateral ridge, sagittal groove, medial ridge and medial fovea. Overall, 15 sample sites were selected across the proximal articulating surface of P1 for the measurements.

For each site, both subchondral bone (SCB) vBMD and SCB thickness were measured in ten adjacent µCT slices of bone, with the average of these ten values used for the analysis. Subchondral bone thickness was measured in pixel co-ordinates along a line segment running perpendicular to the articular surface and then multiplied by the pixel resolution to convert the value to millimetres. Average values for the ten slices were recorded to the nearest mg/cm3 for vBMD and the nearest 10 microns for thickness. One person (the primary author) selected all the sites and took all the measurements to ensure consistency of site selection across bones. The values were imported into Microsoft Excel 2010 and Past version 3.09 (Hammer, 2001) for further analysis.

Statistical Analysis

Measurement reliability was assessed by completing a repeatability investigation. One bone was chosen at random from each group and one of the fifteen sites was chosen at random. The chosen site on each bone was measured for SCB vBMD and thickness five times in total on different days. Repeatability tests were conducted on these measurements using an analysis of variance, after the normality of the data was assessed. These tests indicated that the between group variation was statistically greater than the within group variation, meaning the measurements taken can be deemed reliable ($P = <0.001$). Preliminary Mann-

Whitney tests did not demonstrated a significant difference between left and right RC P1 bones for SCB vBMD and thickness overall or at each site, so the data were pooled for subsequent analysis.

To provide an overview, standard metrics of mean and standard deviation were calculated for each cohort at each site. Ranges across all sites were also calculated. These are presented in Tables 1 & 2. Statistical tests to investigate how the three groups differed from each other, and how the parameters varied between locations across the proximal articulating surface of P1 were conducted in Past v3.09 (Hammer, 2001). Differences between sites within cohorts and differences between cohorts for individual sites were evaluated using the non-parametric two-tailed (Wilcoxon) Mann-Whitney U tests for equal medians. Differences of the coefficient of variation between cohorts were evaluated with a Fligner-Kileen test (Donnelly and Kramer, 1999). An association between subchondral bone vBMD and thickness for each of the fifteen sample sites was investigated with the Spearman's non-parametric rank order correlation coefficient. For all of the analysis, the limit of statistical significance was set at ≤ 0.05.

Results

SCB vBMD

The SCB vBMD (mgHA/cm^3) varied across the proximal osteochondral unit of the P1 bones in each experimental group, with ranges of: 581-807 for CF; 622-780 for RC; and 636-759 for UC (Table 1 and Figure 3). Comparing all sites between cohorts (Table S1) revealed highly significant differences in the coefficients of variation between CF and RC ($p < 0.0001$) as well as between CF and UC ($p < 0.0001$) but not between RC & UC ($p = 0.074$). These differences of variability were also significant at specific sites, including 2, 5, 7-8, & 10-12 (see Table S1; Figure 3). There were slightly significant differences of the median densities (Table S1) between RC and UC cohorts (RC vs UC: site 6 $p = 0.044$; site 8 $p = 0.011$; site 9 $p = 0.045$). These findings indicate that the magnitude of the density value may distinguish raced from unraced with the raced cohort having denser bone, whilst perhaps most significantly, increased variability in the density of bone appears to differentiate raced horses that have experienced fracture. Pairwise tests showed that there were significant differences of

density between sites within each cohort, particularly within the raced group in which the central most part of the sagittal groove (site 8) tended to be significantly denser than all other sites apart from site 14 (Table S2).

SCB thickness

SCB thickness varies across the proximal osteochondral surface of P1, with the pattern differing for each of the coronal sections studied (Table 2 and Figure 4). In general, when comparing sites within each cohort the median SCB thickness tended to be slightly thinner in the palmar section apart from the palmar medial fovea (site 15). The palmar medial fovea was the thickest site within RC and UC. Also within the RC cohort there was a significant increase of median thickness in the central most region of the sagittal groove (site 8) compared to other sites. Taking all the sites into account there was a small significant difference of median thickness between UC and CF ($p=0.003$) but not with regard to the RC cohort (Table S3). Small but significant cohort differences of the median values were observed (Table S3) at a few specific sites as well, including site 2 (UC vs CF, $p=0.045$), site 3 (RC vs CF, $p=0.015$), site 9 (RC vs CF, $p=0.034$), site 10 (RC vs UC, $p=0.011$ & UC vs CF, $p=0.005$) and site 11 (UC vs CF, $p=0.031$), as well as site 14 (RC vs CF, $p=0.034$) and site 15 (RC vs UC, $p = 0.003$ & UC vs CF, $p=0.005$). Significant differences in the coefficients of variation were observed at site 1 (RC vs UC, $p=0.030$), site 7 (RC vs UC, $p = 0.028$) and site 15 (RC vs CF, $p=0.008$ & UC vs CF, $p=0.018$). These findings are not as clear as those for density in differentiating the three cohorts but highlight sites that may be biomechanically important. The correlation analysis showed no significant relationship between thickness and density in the raced and unraced cohorts ($p=0.084$ & 0.143, respectively). There was, however, a small (R rank = 0.3) significant positive correlation in the contralateral fracture cohort ($p=0.005$).

Discussion

Overall summary

This study documents the variation in SCB vBMD and thickness in multiple sites across the proximal osteochondral surface of the equine proximal phalanx. The present study

demonstrates that P1 bones from the race-fit groups (RC and CF) have different patterns of SCB vBMD and thickness compared to the unraced controls. More importantly, there are differences between the two race-fit groups, in particular, the variance in the SCB vBMD which is greatest in the CF group. The CF group represents a group of horses that have failed to withstand the rigors of racing, with a fracture in the opposite limb. Assuming that right and left bones from the same horse undergo the same load during training and racing, and adapt in a similar manner to that load (Rubio-Martínez et al., 2008b, Rubio-Martínez et al., 2008a, Rubio-Martínez et al., 2010), this study indicates that adaptation of the subchondral bone to race training was different, less constrained and seemingly less robust, in the CF group compared with the RC group.

The normal functional adaptation to exercise involves SCB modelling, which initially results in an increase in the SCB density (Rubin, 1984, Riggs and Boyde, 1999, Firth et al., 1999, Easton and Kawcak, 2007, Tidswell et al., 2008, Brama et al., 2009, Beccati et al., 2011), while a high intensity exercise regime that causes bone microcracks, leads to excessive or inappropriate remodelling which can be harmful, predisposing horses to catastrophic injury (Riggs et al., 1999, Stover and Murray, 2008, Anthenill et al., 2010, van Oers et al., 2011, Valence et al., 2011). Initially, areas with microcracks will undergo bone resorption to remove damaged tissue, with subsequent osteoid and then new bone deposition to repair the defect. In the early phase, bone resorption will predominate, creating areas of bone porosity and low density due to a temporal delay in bone deposition relative to removal. Therefore, the end result of the modelling in response to exercise and remodelling in response to focal microcracks is areas with variable SCB bone density (Martin et al., 1997, Riggs and Boyde, 1999, Stover and Murray, 2008, Ramzan and Powell, 2010). Whilst the present study cannot differentiate modelling and remodelling events, the changes of density seen in the CF group are consistent with bone turnover linked to increased fracture risk (Melton et al., 1997, De Laet et al., 1997). As such we propose that the CF group bones were undergoing remodelling at the time of bone failure in the opposite limb, accounting for significant variance in SCB density in this group, which would lead to a decrease in biomechanical integrity and ultimately, increased propensity to fracture (Riggs and Boyde, 1999, Firth and Rogers, 2005b, Ramzan and Powell, 2010, Whitton et al., 2010, van Oers et al., 2011, Vickerton et al., 2014). Evidence that bone remodelling occurs in the P1 sagittal groove region has been shown radiologically and with magnetic resonance imaging (MRI)

(Ramzan and Powell, 2010, Beccati et al., 2011, Dyson et al., 2011, Smith and Wright, 2014). Therefore, the maladaptation of P1 SCB to exercise with a failure to develop the biomechanical properties required for racing may provide an alternate explanation to the one time biomechanical event theory for sagittal fractures of P1. In reality, the sagittal fracture of P1 likely results from a combination of the rotary movement of P1 relative to MC3 (biomechanical theory) superimposed on an area of stress remodelling that decreases the overall biomechanical integrity of the bone.

The higher vBMD in the central and palmar sagittal groove regions of RC and CF groups compared to the UC group, suggests that the sagittal groove sustains an increased load compared with unraced horses (Firth and Rogers, 2005a). In focal samples from young warmblood foals (up to 11 months), habitual low intensity loading on the medial P1 fovea appears to elicit a greater response in SCB vBMD in quantitative terms compared to the high intensity, low frequency loading at the dorsoproximal aspect of P1 (Brama et al., 2009). Therefore, the results of the current study suggest that the central and palmar sagittal groove (site 8 and 13) is loaded more constantly in the racing horses due to the higher vBMD, compared to the unraced controls. The lower vBMD in the dorsal sagittal groove compared to the central groove, would be consistent with subchondral bone subjected to high intermittent peak loading (Easton and Kawcak, 2007, Brama et al., 2009). Both race-fit groups demonstrated higher vBMD in the central sagittal groove compared with much lower vBMD regions adjacent in the lateral and medial ridges of the sagittal groove. Substantial density gradients between adjacent areas of bone are hypothesised to cause stress concentration and shear forces that may predispose to fracture in McIII (Riggs et al., 1999, Riggs, 1999, den Hartog et al., 2009), so it is conceivable that shear stress across areas of different density also occur along the sagittal groove region of P1. A recent study of computed tomography images of P1 sagittal fractures in performance horses, demonstrated that the majority of P1 fractures originate within areas of SCB sclerosis within the mid-sagittal groove region (Brunishloz et al., 2014), which would correspond to the highest area of vBMD in racehorses. At the present time, there is no similar information regarding the site of fracture origination in the Thoroughbred racehorse.

The amount of pressure applied to the sagittal groove with loading has been investigated in a number of studies; with inconsistent conclusions. The sagittal groove was not an area that experienced particularly high loads or pressures in one *in vitro* loading system (Brama et al., 2001, Easton and Kawcak, 2007) based on pressure sensitive film and loading to 10,500N. However, converse results were found in a similar study using loads equivalent to walk, which demonstrated that in the majority of specimens, a linear increase in pressure was observed in the central sagittal groove through the stride, compared to a biphasic increase in the medial and lateral fovea of P1(den Hartog et al., 2009) . The steady increase in loading pressure may stimulate the high vBMD noted at this site in our study, compared to adjacent sites on the fovea that are not under constantly increasing loads . The differences among sites and groups may be related to functional adaption of the subchondral bone to topographically varying biomechanical demands (Brama et al., 2002) due either to joint geometry/morphometry or exercise regimes (Firth et al., 1999, Riggs and Boyde, 1999, Murray et al., 2001, Tidswell et al., 2008)**.**

There are a number of reasons that could explain the different conclusions in the aforementioned studies, complicating the ability to apply these findings to the current study. Firstly, in Brama et al. 2001, the assumption made was that the bones were loaded in the sagittal plane; however, kinematic measurements were not performed so movement outside of the sagittal plane may have occurred. There is clear evidence that out-of sagittal plane movement occurs between P1 and McIII (Denoix, 1999, Chateau et al., 2006, Clayton et al., 2007), which may have accounted for the different contact areas and pressure being reported. Metacarpophalangeal joint extension can induce collateromotion and axial rotation, which alters surface strains across the dorsoproximal surface of P1 (Singer et al., 2012) and the articulation between McIII and P1, altering the joint contact between these bones. It should also be noted that a study using an *in silico* finite element model of P1 concluded that whether the sagittal groove was loaded or not, higher von Mises stresses were experienced within the sagittal groove compared to other areas of the proximal surface of P1 (O'Hare et al., 2012). Therefore, there is evidence to indicate that the sagittal groove sustains significant stress during loading, which would be consistent with the high vBMD noted in this region in this study.

In conclusion, the SCB vBMD and thickness varies across the proximal articulating surface of P1 and differs between CF, RC and UC groups. The CF group represents a group in which the bones have not adapted as well as the RC group to the demands of training and racing, based on the large variance of SCB vBMD overall within this group of bones. The maladaptation to exercise and failure to produce bone with the biomechanical parameters required for racing is a possible explanation for the fracture of P1. Future studies should investigate the micromorphological features of the P1 SCB and trabecular bone, such as anisotropy and evidence of remodelling, to determine whether the structural alterations extend beneath the SCB and if these alterations could affect the bone biomechanical properties.

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Conflict of Interests

The authors do not have any conflicts of interest to declare.

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