M043

BrdU Tracking of Preosteoblasts in Formalin Fixed, Decalcified Regenerating Bone. E. C. Wahl*[1], J. Aronson*[2], R. A. Skinner*[2], C. K. Lumpkin[2]. [1]Arkansas Children's Hospital Research Institute, Little Rock, AR, USA, [2]Departments of Orthopedics and Pediatrics, University of Arkansas for Medical Sciences, Little Rock, AR, USA.

Distraction osteogenesis (DO) is a limb lengthening procedure that induces rapid bone formation by slowly stretching a surgically introduced fracture at a prescribed rate and rhythm. In order to track the pre-osteoblasts in the distraction gaps of rats, we utilized two types of immunohistochemical markers. The first was an injection of bromodeoxyuridine (BrdU), a thymidine analog, followed by immunohistochemical staining for BrdU positive nuclei. The second was the antibody against proliferating cell nuclear antigen (PCNA). Four male Sprague Dawley rats (350g) underwent standard DO surgery. External fixators were placed on the left tibiae and mid-diaphyseal tibial osteotomies were performed immediately following fixator placement. Distraction began one day after surgery at 0.2mm b.i.d. (0.4mm/day) for 12 days. On the twelfth day of distraction the rats were given an intraperitoneal injection of BrdU labeling reagent (1ml/100g). A rat was sacrificed 2, 24, 48, and 72 hours after the injection. At the time of sacrifice, the distracted tibiae were harvested and stored in neutral buffered formalin (NBF) until processed for histology. A small section of intestine from each rat was also collected as a positive BrdU control. After fixation in NBF, the tibiae were decalcified in 5% formic acid, dehydrated, and embedded in paraffin. Serial sections were then stained with either a mouse monoclonal antibody against PCNA (PC-10), a monoclonal antibody against BrdU, or a normal mouse IgG for negative control. A comparison of PCNA+ cells and H&E stained cells in serial sections at each time point demonstrated that the PCNA+ cells were located in the zone outlining the new osteoid columns and in the sinusoids between the columns. No PCNA+ cells were noted in the new microcolumns. A comparison of BrdU+ cells and H&E stained cells in serial sections at the 2 and 24 hour time points demonstrated that BrdU+ cells were also located outside the microcolumns. At the 48 hour time point, BrdU+ cells first appeared scattered throughout periosteal new bone, microcolumns, and cartilage. This same pattern was observed at the 72 hour time point, except that a greater majority of cells in the new columns were labeled. The results demonstrate for the first time that during DO at least a subset of the proliferating cells, juxtaposed to the newly developing osteoid column tips, can be "tracked" over a 48 -72 hour period, into the osteoid columns, confirming their identification as preosteoblasts.

Disclosures: ***C.K. Lumpkin****, None.*

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M044

Comparative Analysis of Confocal and Light Microscopy for Detecting *In Vivo* Microdamage in Wild and Domesticated Animals. W. E. Anderson*[1], J. G. Skedros[2]. [1]Univ. Utah Dept. Orthop. Surg., Salt Lake, UT, USA, [2]UofU, SLC, UT, USA.

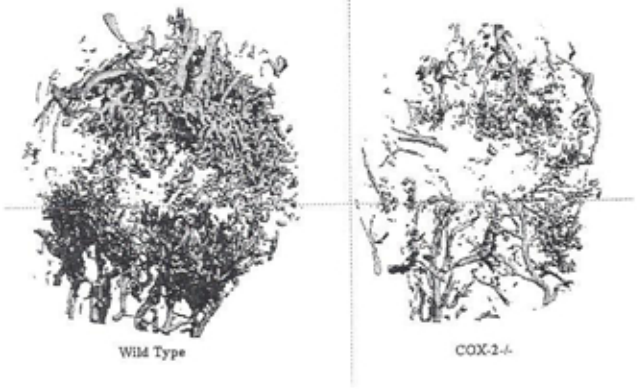
Microdamage (mdx) accumulation is believed to be important in the etiology of stress and fragility fractures. But detecting in vivo bone mdx is difficult. Confocal microscopy appears to be gaining popularity as the "gold standard" for detecting mdx because it is considered more sensitive than light microscopy. We tested this hypothesis using one calcaneus from each of 11 wild deer, 11 wild elk, 11 domesticated sheep, and 11 non-racing horses. In addition to examining entire bone sections, we also examined prevalent/predominant medial/lateral "shear" regions and caudal "tension" regions to see if these relatively more deleterious loading environments are associated with increased in vivo mdx when compared to cranial "compression" regions.14mm-thick mid-diaphyseal segments were bulk stained in 1% basic fuchsin [Burr & Stafford 1990 Clin. Orthop.]. Two investigators, blinded to the hypotheses, rigorously examined three 100micron-thick slices/bone at 10-40X using transmitted light and confocal microscopy for various forms of in vivo (fuchsin stained) mdx (linear microcracks, diffuse mdx, wispy mdx, and osteonal debonding). Confocal microscopy was conducted using a Nikon PCM-2000 with a green HeNe LASER (543nm wavelength excitation; 565 LP filter). Evaluation in light microscopy revealed 15 linear microcracks in 132 sections: nine in deer (4 bones), two in elk (1 bone), and four in horses (3 bones). In addition to confirming the presence of the 15 linear microcracks with light microscopy in the deer, elk, and horses, evaluation using confocal revealed 83 additional mdx entities (debonded, wispy, and diffuse), none of which could be detected with light microscopy. Frequencies of additional mdx entities included: deer (n=45; all bones), elk (n = 24; all bones), and horses (n=14; 9 bones). No mdx was detected in sheep calcanei using light or confocal microscopy. These results confirm the greater sensitivity of confocal microscopy. Higher levels of physical activity may explain why the wild animals exhibited significantly more mdx. Approximately 60% of mdx was concentrated in the caudal cortices where tensile strains are prevalent/predominant, possibly accounting for the high remodeling activity in this region. Confocal microscopy may help determine if bones subject to habitual bending incur similar regional variations in mdx prevalence and/or if these regional variations are minimized by regional adaptations (e.g., osteon densities, collagen orientation) that serve to "toughen" the bone. In turn, confocal microscopy may help determine how strain distributions might increase fracture propensity in some bones, especially with aging (e.g., proximal human femur).

Disclosures: ***W.E. Anderson****, None.*

M045

Assessment of Neovascularization in Fracture Healing of COX-2 -/- Mice Using Micro-Computed Tomography. C. Xie*[1], A. S. P. Lin*[2], E. M. Schwarz[1], R. E. Guldberg*[2], R. J. O'Keefe[1], X. Zhang[1]. [1]Orthopaedics, University of Rochester Medical Center, Rochester, NY, USA, [2]George W. Woodruff School of Mechanical Engineering, Georgia Institute of Technology, Atlanta, GA, USA.

Angiogenesis has been shown to play a key role in bone regeneration and fracture repair. We previously demonstrated that COX-2-/- mice exhibited impaired fracture healing, as evidenced by significant delay in mesenchyme differentiation and cartilage mineralization. To further determine whether delayed mineralization in COX-2-/- mice was associated with the deficiency in neovascularization, we used micro-computed tomography (Micro-CT) to examine the quantity and quality of vasculature in the fracture callus of COX-2-/- mice and their wild type control mice. A mid-diaphyseal fracture was created via 3 point bending in mouse femur and subsequently stabilized by a metal pin placed in the intramedulllary canal. MicroCT vascular imaging was obtained via perfusion of a lead chromate based contrast agent Microfil followed by complete decalcification. Segmentation was performed using software provided by Scanco to contour out the periosteal external callus and endosteal internal callus, excluding the cortex of the fractured femur. The three-dimensional reconstruction of microvasculature within fracture callus demonstrated a dramatic reduction of neovascularization in COX-2-/- mice compared to the wild type control at day 14 post-fracture (Figure). Clusters of highly connected, closely spaced, and isotropically oriented small vessels were observed in wild type mice, but were almost absent in COX-2 -/- mice. Quantitative analyses demonstrated that the volume fraction of mineralization within fracture callus was reduced by 2.5 fold, whereas the total vessel volume fraction was reduced by 3.75 folds in Cox-2-/- mice ($p<0.05$, n=3). Accordingly, vessel separation was increased by 1.5 fold and the average thickness of the vessels was decreased by 25% in the knockouts ($p<0.05$). The dramatic difference seen between COX-2 knockout and wild type mice was the connectivity of the vessels. Wild type mice demonstrated about 25 fold higher connectivity than COX-2-/- mice. Taken together, our data suggests that deficiency in angiogenesis may play a key role in delayed fracture healing in COX-2-/- mice.



Disclosures: ***X. Zhang****, None.*

M046

Automated Volumetric Imaging of *In Vivo* Bone Formation Labels. G. J. Kazakia*[1], M. Singh*[2], W. Yao[3], N. E. Lane[4], T. M. Keaveny[2]. [1]UC, San Francisco, CA, USA, [2]UC, Berkeley, CA, USA, [3]Stanford, Stanford, CA, USA, [4]UC Davis, Sacramento, CA, USA.

In vivo administered fluorescent labels are indispensable in the study of bone metabolism. Standard histomorphometry - particularly in mineralized tissues - is technically challenging and labor intensive. Moreover, histomorphometric measures based on thin sections allow only 2D measures of the 3D structures of interest. With renewed interest in the role of bone quality in osteoporosis, the distinction between 2D and 3D measures of histological structures such as remodeling spaces and microdamage may be important. The goal of this work was to apply a novel technique for visualizing 3D distribution of fluorochrome labeled components within large biological specimens to the reconstruction of an *in vivo* administered bone formation label. This method is based on computer controlled milling technology and combines an arrayed imaging technique with fluorescence capabilities, thus enabling 3D fluorescence imaging of bone specimens of any size at high resolution (on the order of a few microns). Biological specimens containing fluorescent labels are embedded in an opaque resin and mounted onto the system for serial milling and imaging. After each milling pass, an imaging station excites the exposed fluorochromes and captures the emitted fluorescence through a series of filters. As an example, an *in vivo* calcein-labeled femur from a six month old female Sprague-Dawley rat was harvested and imaged. The proximal femur was fixed in 10% NBF, dehydrated, and embedded in opaque MMA. Autofluorescence was exploited to capture bone morphology using a UV excitation filter and a DAPI emission filter. Calcein fluorescence was imaged using a FITC filter set. Two 2x3 image arrays (one per filter set) were captured for each exposed surface. A 5 mm length was imaged using 680 slices at an in-plane resolution of 3 microns/pixel and an out-of-plane resolution of 8 microns. Two superimposed volumetric data sets were rendered for visualization - one representing bone morphology and a second representing bone formation zones. Spatial distribution of bone forming zones and their relation to local bone architecture were clearly distinguished and substructures such as an entire osteon (length 1.36 mm; mean diameter 129 microns) were isolated. Imaging time