Nutritionally Induced Delayed Union Model of Femur Fractures in Mice

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Disclosures:

Introduction: Fracture healing, a complex biologic process requires the synchronization of several cell types to regain mechanical competence and allow weight bearing. Failure in coordinating, or completing, this biologic process results in a non-union of the fractured bone. Inorganic phosphate is an essential mineral for maintaining healthy bones and has been shown to be a critical component for bone healing [1]. The goal of this study was to generate a nutritionally induced murine non-union model of fracture healing. It was hypothesized that progressively longer periods of phosphate deficiency would lead to development of nonunion. This hypothesis was tested by comparing the time course of fracture healing of control mice fed a diet containing normal levels of phosphate and calcium to mice placed on a high calcium phosphate deficient diet for varying periods of 5 to 20 days. Radiological, structural, mechanical, histological and mRNA expression assessments were made to examine the effects of varying lengths of dietary phosphate deficiency on fracture healing.

Methods: Animal model. All protocols were approved by BUSM Animal Care and Use Committee. Standard chow (0.65% phosphorus) or a phosphate-restricted diet (PRD; 0.06% Pi) were from Teklad 2018, Madison, WI, USA. Eight to 10 week old male C57Bl/6J (B6) mice were purchased from Jackson Laboratories Bar Harbor, ME, USA. Closed, stabilized fractures [2] were created in the right femora of male mice. Dietary phosphate restriction was initiated two days prior to producing the closed fracture of the femur with mice being maintained on the phosphate deficient diet for 5, 10, 15, and 20 (post-operative days(POD)) prior to being returned to the normal diet. Groups sizes were N= 8 for Micro-Computed Tomography (μCT), N=12 for mechanical testing, N=5 for histology, and N=3 for mRNA Cartilage Analysis by Contrast-Enhanced (μCT). Calluses were scanned at a resolution of 12 μm/voxel (μCT40, Scanco Medical, Bruttisellen, Switzerland) before and after eight hours of incubation in a cationic contrast agent, CA4+ [3]. As validated previously [4], registration and then subtraction and thresholding of the pre- and post-incubation images was performed to quantify: the volumes of cartilage, mineralized cartilage as well mineralized tissue, and total callus; the mean intensity of the contrast-labeled cartilage; and the mean and standard deviation of the tissue mineral density (considering only the well mineralized tissue). The preexisting cortex was excluded from these calculations. For these measurements μCT analysis of various treatment groups and controls were assayed at POD 10,14,18 and 21. Mechanical Testing. For these measurements μCT analysis followed by torsion testing were carried out on callus tissues for various treatment groups and controls at POD 14, 21, 28 and 35 [5]. Histology and qPCR. Calluses in the second phase of enrollment were processed for histological evaluation (n=3/group) or PCR (n=3/group) [2]. Statistical Analysis: Measures were compared among time-points using analyses of variance (ANOVA) with Tukey post hoc tests (JMP 10, SAS, Inc., Cary, NC).

Results: Delayed bone healing was induced by the phosphate restricted diet. The phosphate restricted diet led to a time dependent impairment of healing where mice maintained on longer periods of phosphate restriction (i.e. 15 & 20 days) showed a more prolonged period of impaired healing. Plain x-ray analysis presented in Figure 1 shows these findings in which the gap remains open and the tissue is undermineralized in the two longer periods of Pi restriction. Molecular analysis of two representative mRNAs for bone or cartilage development showed that phosphate restriction did not inhibit chondrogenesis, but rather led to a delayed and enhanced expression of chondrogenic markers, indicating that the callus remained primarily cartilage. On the other hand, compared to controls, osteogenic gene expression was blunted and showed a progressive delay in initiation of osteogenic gene expression with progressively longer periods of dietary phosphate restriction. When phosphate was re-introduced to the diet, the process of fracture healing reinitiated; however, progressively longer periods of time were required to achieve union based on the initial period of phosphate restriction.

Discussion: Phosphate restriction demonstrated an inhibitory effect on bone healing by impairing osteogenesis, but not necessarily chondrogenesis. The data suggest that dietary restriction of phosphate can be used as a model of nutritionally induced delayed union of femur fracture, while allowing identification of, the molecular events that promote cartilage mineralization and initiation of osteogenesis.

Significance: These studies suggest that phosphate restriction provides a non-surgical model of generating delayed fracture healing and will be useful to assess therapies that will promote healing after return to the normal diet. Comparative analysis of these tissues will identify those molecular regulators that are related to phosphate metabolism that control mineral deposition which will have importance in developing approaches to increase bone mineral density. Finally this animal model can be used to compare human fracture healing in patients that are vitamin D deficient and whom experience deficient bone healing after fracture.

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Figure 2. Gene expression data of a second gene (Cover: CO) in osteogenic and chondrogenic (CO). Solid line represents the direct drive group and dotted line is the control group.