The effect of local delivery of Iron Chelators on Bone Regeneration and Osteoclast Mediated Bioceramic Bone Graft Resorption.

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Introduction: The clinical success of any bioresorbable bone graft substitute for the treatment of bone defects relies on rapid vascularization to provide nutrients and progenitor cells for graft remodeling and new bone growth. However, temporizing graft resorption to maintain the scaffold’s structure and strength during bone ingrowth is also necessary. Iron chelators such as the widely available desferoxamine (DFO), have recently been investigated as angiogenic and anabolic agents for bone regeneration. Local injection of DFO within fracture sites and distraction gaps of rodent models has shown both augmented vascular growth and bone formation. This response is likely mediated through activation of the Hypoxia Inducible Factor-1a (HIF-1a) signaling pathway. Ferric iron is critical to the function of propyl hydroxlase proteins involved in the oxygen dependent regulation of HIF-1a. Chelation results in the unrestricted activation of HIF-1a, upregulating a cascade of hypoxia response genes of which VEGF is highly induced. Hypoxic conditions are also known to induce osteoclast differentiation and resorptive function through HIF-1a activation. However, mimicking this effect with iron chelators has interestingly shown contradicting in vitro results. This study investigates the in vivo use of iron chelators on monetite (CaHPO4) bone graft remodeling in a mid-sized animal model. Specifically we aimed to determine the effect of the local delivery of DFO on new bone growth and graft resorption in a rabbit ulnar defect bridged by anatomical 3D-printed monetite grafts. Secondly we aimed to accurately quantify the effect of iron chelator delivery on osteoclast mediated resorption using a monetite graft cranial onlay model.

Methods: Graft design: A 3D calcium phosphate powder printer was used to fabricate 1cm microporous brushite grafts that matched the anatomy of the rabbit ulnar shaft. Two small pegs were added on either end of the graft to stabilize the grafts within the intermedullary canal. The grafts were subsequently thermally converted to monetite using a standard autoclave. For the onley model, cylindrical monetite grafts (9mm diameter/ 4mm thick) were prepared by thermal conversion of preset microporous brushite cements. Implantation: Sixteen 5-month-old New Zealand rabbits were used in this study in compliance with the ethical committee of the study institution. For six rabbits, bilateral 10mm mid-diaphyseal segmental defects of the ulnae were created and two 3D printed grafts were press fit within the defects. Starting on postoperative day 4, 600ul of a 200uM solution of DFO was injected into one of the grafts of each rabbit every 48 hours for a total of 6 doses. The contralateral limb received saline injections. For 10 rabbits, two cylindrical grafts were implanted subperiosteally onto exposed cranial surface and held in place with a 5mm screw. Starting on post-operative day 4, four rabbits received injections of DFO (600ul of 200uM solution) into each graft every 48hr for total of 6 doses (n=8 grafts). Four rabbits were given control saline injections (n=8). To verify if the results could
be replicated using another iron chelator, two rabbits were injected with 1,10 phenantroline (600ul of 200uM solution) in an identical fashion (n=4). Analysis: After 8 weeks all grafts were explanted. Explants were scanned using a bench top micro-CT (Skyscan 1172). For the ulnae model a set cuboid volume of interest was used to encompass the full osteotomy site of each sample excluding the radial cortex. New bone was easily distinguishable from graft material using various thresholding operations. New bone volume/tissue volume and remaining graft volume were compared between the DFO and saline treated limbs. For the cranial model a set VOI was used to encompass the graft starting at the first axial level at which cranial endplate was no longer visible. As new bone and graft were not reliably distinguishable in this model, total mineralized volume/tissue volume of each sample was used as a proxy for graft resorption. All explants were subsequently embedded in poly-methyl-methacrylate. 15 um sections were stained with methylene blue and basic fuchsine to qualitatively assess new bone growth and graft resorption. 5um sections of the onlay grafts were stained with tartrate resistant acid phosphatase. The total area of TRAP positive cells/area of bone graft interface was calculated for each sample. ANOVA with tukey post-hoc analysis was used for all 3-group analysis and student T-test for all 2 group analysis.

**Results:**

**Ulnar bone defect:** At 8 weeks post implantation, Micro-CT bone metric analysis demonstrated an increase in new bone growth in the DFO group compared to the saline group (new bone volume / tissue volume 19.5% vs 13.65% (p=0.042) (figure 1). Graft resorption was similar between the two groups (77.8 vs 75.3 mm3). Histological analysis of coronal sections showed increased bone within the osteotomy gap, more bone integrated with the graft’s surface as well a more mature soft tissue callus in the DFO group. Cranial onlay model: Micro-CT demonstrated decreased graft resorption in the DFO and PHT group as compared to saline controls (figure 2). Histological analysis of coronal 15um cut through the center of each graft showed marked decreased resorption in the DFO and PHT groups compared to saline controls (figure 2). TRAP stain quantification showed a 3 fold significant increase of TRAP positive stain/interface area in the saline controls as compared to chelation groups (figure 2).

**Discussion:** This study demonstrates that the local delivery of DFO substantially increased new bone volume in a long bone defect bridged by a bioresorbable bone graft substitute compared to saline controls. Graft resorption was similar between the 2 groups. In vivo bioceramic graft resorption occurs by a combination of passive dissolution and cell-mediated resorption. Weight bearing and muscle contractions expose the graft to fragmentation forces and elevated fluid circulation. Passive dissolution likely largely influences graft resorption in the long bone model thereby explaining the relatively similar graft volumes observed post explantation between the two groups. The cranial onlay model exposes the grafts to a more static environment whereby cell mediated resorption can be tracked from a single front. We have shown that local delivery of DFO as well as PHT, reduced graft resorption and osteoclast numbers at the bone graft interface. During osteoclast differentiation iron uptake is known to be highly unregulated and acts as an essential factor for mitochondrial biogenesis. This study proposes a possible second mechanism by which iron chelators display bone anabolic properties- in addition to acting as HIF-1a activators they may also reduce osteoclast mediated resorption by an independent mechanism of mitochondrial biogenesis inhibition.

**Significance:** The clinical application of iron chelators for bone regeneration is of interest to the orthopaedic community given the safety, cost and stability of these drugs. In this study we show the bone regenerative properties of DFO in a mid sized animal bone defect model. To our knowledge this is
the first study that has demonstrated an inhibitory effect of locally delivered iron chelators on osteoclast-mediated resorption in vivo.
Figure 1: Representative 3D coronal cuts of the osteotomy site. Grey represents new bone and pink represents the remaining graft material.

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