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Original Article

Evaluating effects of deferoxamine in a rat tibia critical bone defect model

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ABSTRACT

Objectives: Evaluate the use of deferoxamine in a calcium sulfate carrier to promote fracture healing in a critical bone defect model.

Methods: 43 female retired breeders were divided randomly into Control, Carrier, DFO and BMP groups and appropriate agents placed at the osteotomy site.

Results: There was a significant difference in the mean gap between groups Control vs DFO and Control vs BMP. A higher mean number of cortices were bridged in the DFO group as compared to the Control group.

Conclusions: Our study demonstrated that DFO helped reduce the gap in this critical tibia defect.

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1. Introduction

A critical bone defect is defined as a bony defect that will result in a non-union unless an intervention is undertaken. A variety of recent advances have been made in the treatment of critical bony defects and/or non-unions including technical aspects of surgery, hardware modifications and orthobiologics such as bone allograft and/or Bone Morphogenetic Protein (BMP). Bone autografting remains a gold standard for atrophic non-union but it causes long term donor site morbidity in a high percentage of patients.¹ Allograft has its own shortcomings including infection and lower rates of union.² BMP is expensive and has limited Food and Drug Administration

(FDA) approval.³ An ideal surgery or bone substitute, that would cause 100% union rate in critical bone defects, is yet to be found. A compromised vascular supply is a factor in the approximately 10% of fractures that show a delay in healing.

Over the past few years, a variety of studies have been conducted on deferoxamine (DFO) demonstrating some positive effects on fracture healing.^{4,5} DFO is currently an FDA approved medication for the chelation of iron in the treatment of iron toxicity and has been used for many decades for this indication. It has been observed to induce upregulation of HIF (Hypoxia Inducible Factor) which in its turn upregulates VEGF (vascular endothelial growth factor).⁶ VEGF has been found to play an important role in the fracture healing response as well as in recruiting new vasculature. Recently, it has been

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demonstrated in an unimpaired, closed fracture healing model that injection of DFO at the injury can increase vascularity and bone volume at the fracture site.⁷ As repeated injection of DFO may not be practical or effective clinically, recent *in vitro* work was undertaken and it was found that DFO can be incorporated into a calcium sulfate (CS) carrier and still maintain its angiogenic potential.⁵ In tissue culture, DFO induces marked increases in vascularity of fetal mouse metatarsals and, in studies performed in our laboratory (Hertzberg et al), it is readily incorporated in calcium sulfate pellets and easily released from them.⁶

So far, there have been no *in vivo* studies using DFO in a carrier in an impaired fracture healing model. We hypothesized that DFO may have the potential to heal a critical bone defect and we wished to evaluate this potential.

The goal of this study was to evaluate the use of DFO in a CS carrier as an easily implemented and cost-effective means to promote fracture healing in a critical bone defect model and compare the results with BMP as a positive Control.

2. Material and methods

We used the rat tibial non-union model developed by Miles et al.⁸ In this study, a non-union was reproducibly induced by creating a simple transverse osteotomy in the rat tibia and placing a 3 mm Poly Ether Ether Ketone (PEEK) spacer between the bone ends. The spacer was secured on the intra-medullary K-wire and maintained the gap/defect. This 3 mm gap results in 100% non-union ("critical defect"). After obtaining approval from the Institutional Animal Care and Use Committee, we obtained a total of 43 female Sprague-Dawley (SD) retired breeders (Charles River Labs) for this study. The rats were weighed and divided randomly into 4 groups.

Group Control: Negative Control group: They received no treatment other than insertion of the 3 mm PEEK spacer and reduction and stabilization with a K-wire ($N = 11$).

Group Carrier: Calcium sulfate carrier group: These rats received morselized fragments of CS placed around the PEEK spacer intra-operatively after placement of a K-wire ($N = 11$).

Group DFO: Experimental group: These rats received morselized beads of CS loaded with deferoxamine (1 mg/kg body weight) placed around the PEEK spacer intra-operatively after placement of a K-wire ($N = 11$).

Group BMP: Positive Control group: These rats received placement of a collagen sponge (BIOCOL) loaded with 10 μ g of rhBMP-2 around the PEEK spacer intra-operatively after placement of a K-wire ($N = 10$).

2.1. Technical aspects

Each rat was identified with its specific group and anesthetized with isoflurane vapor anesthesia. The right leg was used in all the animals. The fur on the right leg was clipped and the operative area was prepped and draped in a sterile fashion with betadine. A 2 cm longitudinal incision was made along the anteromedial border of the proximal tibia. The patellar tendon insertion was identified. The osteotomy site was 13 mm distal to the patellar tendon insertion (close to the metaphyseal-diaphyseal junction). Soft tissue dissection was

conducted with a hemostat passed underneath the tibia to protect the soft tissues as well as expose the tibial shaft area. Care was taken not to strip excessive periosteum during dissection. The osteotomy was performed with a small rotary saw using copious saline irrigation. One saw blade was used to perform no more than 2 osteotomies in order to prevent heat necrosis at the osteotomy site secondary to blade wear. A 0.9 mm inch diameter stainless steel wire with a partially threaded distal tip was then used to cannulate the proximal tibia in a retrograde fashion. A PEEK spacer of 3 mm length was threaded over the distal end of the steel wire at the osteotomy site and the wire was inserted and screwed in an antegrade fashion into the distal tibia to create a stable construct. The proximal end of the wire was bent 90° to resist wire back-out.

After fixation of the osteotomy site, the remainder of the surgery was conducted according to group. The plaster beads containing deferoxamine, the plaster only beads and the collagen sponge containing BMP were placed around the PEEK spacer. The wound was then closed with absorbable 4-0 vicryl sutures subcutaneously. Each animal was given 6 ml of warmed Ringer's lactate solution subcutaneously with a 22 gauge needle. Oral analgesia was provided with acetaminophen 250 mg/kg (5 ml elixir per 100 ml of drinking water) for 21 days postoperatively. Injectable analgesia was provided with buprenorphine 0.03 mg/kg daily for three days postoperatively. The rats were returned to individual cages after adequate recovery from anesthesia and monitored closely.

The rats were euthanized eight weeks postoperatively and weighed. Tibias from both the operated legs and contralateral non-operated legs had the surrounding soft tissues removed before potting the specimens for radiographic examination and biomechanical testing.

2.1.1. Radiographic evaluation

This was carried out using orthogonal views using a cabinet radiographic unit (Faxitron Series by Hewlett Packard; HP 43804 X-Ray System). The total numbers of completely bridged cortices at the osteotomy site were counted. Then the total length of the gap was measured at all 4 cortices (2 on AP view and 2 on lateral view). These measurements were done by 2 independent examiners blinded to the treatment groups. The "total number of cortices" was given a score of '0' for no bridging, '1' if one cortex is bridged through '4' if both cortices bridged on both views. A cortex was defined as bridged where there was bone to bone connection from both ends. Total length of gap was defined as sum of the gap distance at each of the four cortices on the two radiographic views. Mean gap was defined as the mean of the total gap recorded by the two examiners.

2.1.2. Biomechanical testing

To assess the mechanical competence of the healed fracture, the stiffness, ultimate torque to failure, and energy to ultimate load of the fracture callus was determined by torsional testing in external rotation on a uniaxial servohydraulic material testing machine fitted with fixtures to convert the axial motion to rotary motion. Specimens were torqued at a constant rate of six degrees/s until failure or 60 degrees of rotation. The torque and deflection angle were recorded and torsional

Table 1 – Radiographic and μ CT measures.

Treatment groups	Number of cortices bridged	Total gap at fracture- 4 cortices (mm)	Mineralized callus volume (mm^3)
Control	0.33 ± 0.67	7.2 ± 3.4	5.00 ± 3.13
Carrier	0 ± 0	6.6 ± 2.7	NA
DFO	0.56 ± 0.97	4.5 ± 2.1^a	7.27 ± 2.20
BMP	4 ± 0^a	0 ± 0^a	NA

^a Statistical difference from Control group.

properties computed. The ultimate torque was recorded as the maximum torque prior to 35 degrees of angulation being achieved. Displacement greater than 35° without reaching a peak torque was felt to be indicative of tensioning of fibrous callus rather than bony union.

Specimens from only the DFO group and the Control group were then scanned on a μ CT (μ CT-40 Scanco Medical system) for evaluation of the mineralized tissue volume. This was measured only over the 3 mm defect site as defined by the PEEK spacer and did not include bone to either side of the defect. μ CT evaluation was not performed for Plaster and BMP groups (for cost savings).

2.1.3. Statistical analysis

A one-way ANOVA or Kruskal Wallis ANOVA on ranks followed by multiple comparison testing was used to determine statistical differences between the groups. Significance was set at $P < 0.05$.

3. Results

Three rats each from the Control and Carrier groups and 2 rats from the DFO group were lost secondary to self mutilation and infection. One rat from the BMP group was removed from testing secondary to placement of an incorrect BMP dose for that specimen. 1 rat from DFO group was lost during the process of removal of the potting material and performing micro-CT.

3.1. Radiographic evaluation

A mean gap (SD) of 7.2 mm (3.5), 6.6 mm (2.7), 4.5 mm (2.1) and 0 mm (0) was noted in Control, Carrier, DFO and BMP groups respectively as shown in Table 1. There was a significant difference in the mean gap between groups Control vs DFO

Table 2 – Mean weights-preoperatively and postoperatively.

Treatment groups	Pre-op mean weight (g)	Post-op mean weight (g)
Control	312.182 ± 53.01	301.50 ± 56.40
Carrier	309.55 ± 46.44	312.25 ± 43.19
DFO	319.45 ± 53.67	315.89 ± 56.31
BMP	304.20 ± 52.09	304.00 ± 55.24

Table 3 – Ultimate torque at 35° in non-operative limb.

Treatment groups	Mean (N*mm)	Standard deviation
Control	193.27	58.15
Carrier	239.37	77.85
DFO	211.13	39.86
BMP	196.60	51.15

($P = 0.005$) and Control vs BMP ($P < 0.001$). The number of bridged cortices for each group is shown in Table 1. All cortices were bridged in the BMP group as compared to a mean number of 0.33 cortices bridged in the Control group. A higher mean number of cortices were bridged in the DFO group (0.56) as compared to the Control group (0.33) but this difference was not statistically significant.

3.2. Biomechanical testing

No significant difference between the experimental groups in body weight were found preoperatively or postoperatively ($P = 0.3936$ and 0.922 respectively; Table 2).

No significant difference in the torque at 35° was found between the groups for the non-operated limb ($P = 0.390$; Table 3).

A significant difference was noted in the mean ultimate torque and mean energy between the Control and BMP groups. No statistically significant differences were detected between the Control vs DFO and Control vs Carrier groups (Fig. 1).

Specimens from the DFO group and the Control group were then scanned on a μ CT system for evaluation of the volume of mineralized tissue at the 3 mm defect site. No significant difference was found between the DFO group and the Control group although DFO did have a trend for higher bone volume than Control group ($P = 0.116$) (Table 1, Figs. 2 and 3).

* Statistically significantly different from Control ($P < 0.05$)

Fig. 1 – Ultimate torque of the experimental groups.

Fig. 2 – Mineralized callus volume.

4. Discussion

A number of studies have documented the critical role of angiogenesis and increased blood flow for successful bone repair in the surgical patient. Over the past few years, deferoxamine (DFO) has been shown to improve vascularity and healing at the fracture site via the Hypoxia Inducible Factor (HIF)⁹ and VEGF pathways.⁶ DFO is an FDA approved iron chelating agent that has been found incidentally to stimulate several angiogenic programs by altering the metabolism of Hypoxia Inducible Factor-1 α (1–3).⁶ At the molecular level, the initial signals for blood vessel invasion into bone are unknown, but tissue hypoxia is believed to be critical for commencement of the angiogenic cascade.¹⁰ Hypoxia triggers a chain of events leading to stabilization of HIF α (Hypoxia Inducible Factor-alpha) which ultimately leads to HIF-1-regulated gene expression. The HIF (Hypoxia Inducible Factor) complex consists of 1 of 3 α subunits (HIF-1 α , HIF-2 α , or HIF-3 α) bound to the aryl hydrocarbon receptor nuclear translocator (ARNT), also known as HIF β . The level of HIF-1 α and HIF-2 α proteins is regulated by ongoing ubiquitination and proteasomal degradation following enzymatic prolyl hydroxylation on an oxygen-dependent degradation domain.¹¹ Vascular endothelial growth factor (VEGF) is a potent endothelial cell-specific cytokine that has

been shown to have mitogenic and chemotactic effects on endothelial cells *in vitro*.¹² Wang et al showed that HIF α promotes angiogenesis and osteogenesis by elevating VEGF levels in osteoblasts.¹³

In addition, VEGF has been shown to induce angiogenesis in many *in vivo* models. Increased VEGF mRNA expression has been shown in membranous bone fracture healing *in vivo* and in isolated osteoblasts stimulated by indirect and direct angiogenic growth factors *in vitro*.¹⁴ *In vivo* induction of VEGF mRNA expression during fracture healing is likely secondary to alterations in the fracture microenvironment.

Our study demonstrated that DFO helped reduce the gap in this critical tibia defect. However, it was not able to produce bridging of this critical defect and thus, the torsional properties of the callus were not significantly improved. μ CT testing showed a nonsignificant increase in the volume of mineralized tissue in the DFO group as compared to the Control group. We believe that further testing needs to be conducted in order to optimize the DFO dosage, release time, and delivery method. In the current study, the DFO was delivered in morselized calcium sulfate pellets which may have delivered the DFO over a suboptimum time frame. In addition, combining DFO with an osteoconductive bone allograft may be a more effective approach to achieve union in this critical defect model. Our study has provided additional data demonstrating the ability of rhBMP-2 to promote union across this PEEK spacer critical bone defect model.

Stewart et al conducted similar testing in a 5 mm rat femur defect model.⁴ They used a 5 mm bone defect bridged by a scaffold. They showed similar results in respect to complete healing of the defect in the BMP group. Bone volume measured via μ CT showed increased bone volume in the DFO group vs Control but it did not reach statistical significance.

There are limitations in this study. First, the number of rats in each group is small. Second, the evaluations were performed at a single time point of eight weeks. Third, we did not perform μ CT evaluations for groups Carrier and BMP. We elected not to expend resources in evaluation of these two groups since rats in the BMP group had completely bridged the defect on the radiographs whereas the Carrier group was only a secondary Control. Fourth, these results are applicable only to a rat tibia critical defect model and we are unable to predict their exact implications in humans.

Specimen- DFO

Specimen- Control

Fig. 3 – Representative images from micro-CT.

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Conflicts of interest

All authors have none declare.

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