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Deferoxamine reverses radiation induced hypovascularity during bone regeneration & repair in the murine mandible

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Abstract

Background—Deferoxamine (DFO) is an iron-chelating agent that has also been shown to increase angiogenesis. We hypothesize that the angiogenic properties of DFO will improve bone regeneration in distraction osteogenesis (DO) after x-ray radiation therapy (XRT) by restoring the vascularity around the distraction site.

Material & Methods—Three groups of Sprague-Dawley rats underwent distraction of the left mandible. Two groups received pre-operative fractionated XRT, and one of these groups was treated with DFO during distraction. After consolidation, the animals were perfused and imaged with microCT to calculate vascular radiomorphometrics.

Results—Radiation inflicted a severe diminution in the vascular metrics of the distracted regenerate and consequently led to poor clinical outcome. The DFO treated group revealed improved DO bone regeneration with a substantial restoration and proliferation of vascularity.

Conclusions—This set of experiments quantitatively demonstrates the ability of DFO to temper the anti-angiogenic effect of XRT in mandibular DO. These exciting results suggest that DFO may be a viable treatment option aimed at mitigating the damaging effects of XRT on new bone formation.

Keywords

distraction osteogenesis; angiogenesis; angiogenesis inducing agents; head and neck cancer; postirradiation

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Background

In the US, cancer affects over 11 million people, about half of whom receive radiation therapy (XRT) as part of treatment [1]. Although XRT increases survival rates, it can be exceedingly detrimental to bone resulting in an unacceptably high incidence of devastating complications such as osteoradionecrosis and the debilitating problem of late pathologic fractures [2–5]. XRT significantly changes the biologic environment of bone resulting in a severe attenuation of cellularity, fibrosis, as well as obliteration of small blood vessels [6–12]. Bone subjected to XRT demonstrates reduced mechanical strength and bone atrophy as a consequence of increased bone resorption and decreased osteogenesis [13,14]. The recovery of irradiated bone is usually poor and the structural and functional degradation often leads to significant morbidity. The corrosive impact of these XRT induced side effects can be unrelenting and their complex management is rarely remedial.

For patients with head and neck cancer (HNC) requiring mandibular reconstruction, the pernicious effects of XRT on bone dictates utilizing free tissue transfer [15,16]. Distraction Osteogenesis (DO), the creation of new bone by the gradual separation of two osteogenic fronts, offers a less invasive reconstruction method for HNC. Uniquely, DO generates an anatomical and functional replacement of deficient tissue from local substrate while avoiding donor site morbidity; a form of endogenous tissue engineering [17,18]. Unfortunately, the drastic impairment of new bone formation and attenuation of vascularity after XRT precludes extending this innovative reconstructive strategy, in its present form, to the setting of cancer and irradiation.

Deferoxamine (DFO), a chelating agent used in the treatment of iron toxicity and hemochromatosis, functions to sequester iron which is a cofactor required for the degradation of hypoxia-inducible factor-1 α (HIF), thereby stimulating vascular endothelial growth factor (VEGF) production and other downstream angiogenic factors [19]. DFO has been demonstrated to bolster fracture repair and bone regeneration during DO in long bones by mounting an augmented angiogenic and osteogenic response during bone healing [20]. The purpose of this study was to document the degree by which Human Equivalent XRT decreased vascularity during bone regeneration in the murine mandible and to then determine the efficacy of DFO to mitigate or reverse that process. We used an irradiated murine mandibular DO model to gauge the extent by which DFO injections into the distraction gap during the DO procedure can restore vascularity to the distraction site and overcome the deleterious effects of XRT in order to promote bone regeneration.

Material & Methods

Adult male Sprague-Dawley rats (n=24, 375–400 grams) were randomly assigned to 3 groups (control, xDO, and xDFO) and treated as follows: all groups underwent DO, the xDO group received XRT, and the xDFO group received XRT and DFO treatment. The XRT protocol utilizes 5 fractional doses of 7 Gray, which produces the normalized equivalent dose a human mandible experiences for HNC [21].

The DO protocol has been previously described, but briefly, a two week recovery period was allowed between the completion of XRT and operative distractor placement [22]. All groups underwent placement of a custom-built bilateral external fixator with unilateral distraction device and a left mandibular osteotomy posterior to the molars. Following four days of latency, distraction began at a rate of 0.3mm every 12 hours to a total of a 5.1 mm DO gap. In previous studies, 5.1mm was shown to be a critical-size defect using this model [22]. During active distraction, the DFO treatment group was injected with 200 μ M DFO (Desferal; Hospira, Lake Forest, USA) (300 μ L) into the regenerate (RG) every other day for

a total of five doses. After an additional 28 days of consolidation, vascularity and bone healing were assessed.

Micro-computed tomography (MicroCT) imaging in combination with vessel perfusion was used to quantify angiogenesis. MicroFil (FloTech) perfused, harvested, and decalcified bones were scanned at an 18- μ m isotropic voxel size. A 5.1mm volume of interest encompassing the RG was selected. Radiomorphometrics for vascularity were performed using a threshold of 1000 for three-dimensional angiogram reconstruction with MicroView software (GE Healthcare). All data is presented as mean $\pm$ SE. Statistical analysis was performed using SPSS Statistics software (SPSS, Chicago, IL). The data were compared using ANOVA with Tukey's post hoc method and two-tailed Students t-test. Levene's test was used to determine distribution of the data. Significance was assigned as $p=0.05$. One animal in the xDFO group was excluded from analysis due to failure to thrive following XRT.

Results

At harvest, the control group demonstrated complete bony regeneration. The xDO group demonstrated gaps in the RG, incomplete bridging, and fibrous unions; three samples were classified as non-union and five samples had a fibrous union (Fig. 1). In contrast, the xDFO group revealed improved bony bridging of the distraction gap in six samples with one sample exhibiting a fibrous union.

Three-dimensional reconstructions of the microCT angiography showed decreased vascularity in the xDO group compared to controls (Fig. 2). Quantitative analysis revealed a profound diminution in the metrics of microvasculature in response to XRT. There was a statistically significant increase in vessel separation (VS) (1.99 v 1.18; $P<0.005$) and decrease in vessel volume fraction (VVF) (0.029 v 0.046; $P=0.005$) indicating an overall decrease in vascular density. Similar measurable reductions in vessel number (VN) (0.53 v 0.82; $P<0.001$) and vessel volume (VV) (5.53 v 9.63; $P<0.001$) demonstrated a severe attenuation or obliteration of vascularity within the regenerate (Fig. 3).

We found a substantial proliferation of vascularity in the xDFO group when compared to the xDO group (Fig. 2). DFO effected a significant increase in VN (1.02 v 0.53; $p<0.001$) and an expected concomitant decrease in VS (0.94 v 1.99; $p<0.001$). Our data revealed a massive increase in the number of vessels which were now packed closer together in the experimental group. This strong increase of vascularity with DFO in the radiated RG could be appreciated grossly with microCT image reconstruction (Fig. 2) as well as objectively measured by the increased VVF (0.05 v 0.029; $p<0.001$).

Our results show that the xDFO group reversed the radiation induced hypovascularity resulting from XRT as demonstrated by a substantial restoration of all vascular metrics. Furthermore, there were no statistically significant differences between VN, VS, and VVF when comparing the xDFO group to the controls: VN (1.02 v 0.82; $P=0.38$), VS (0.94 v 1.18; $P=0.71$), and VVF (0.050 v 0.046; $P=0.97$).

Discussion

Pathologic effects of radiation are mediated in part through destruction and suppression of microvasculature [23]. Mandibular bone is particularly prone to XRT damage due to its limited vascular supply and compact bony structure [24]. In the mandible, XRT results in progressive obliterative endarteritis, leading to decreased blood flow and fibrosis [25]. Radiation's disruption of bone microvasculature progressively worsens over time, causing decreased vascular density and obliteration of small blood vessels.

In the present study we document and quantify the pathologic effect of XRT on microvasculature in DO of the murine mandible. XRT damages the angiogenic capacity of the DO RG to such an extent that the vascular response is considerably less than that observed in controls. Adequate angiogenesis is known to be required for bone regeneration, as indicated by animal models showing defective or delayed angiogenesis exhibiting impaired healing of DO [26]. Our data support the hypothesis that the pathologic effect of XRT on the microvasculature impedes new bone formation during DO.

Wang et al has demonstrated that the activation of HIF-1 α leads to increased bone deposition proportional to an increase in vascularity [27]. Therefore, they posit that the osteogenic process appears to be driven by angiogenesis, which is stimulated through HIF activation and VEGF production. The potential to exploit the capability of angiogenic factors to promote bone healing led us to explore the usefulness of small molecules, such as DFO, to target the HIF pathway to activate angiogenesis to combat the hypovascular effect of XRT.

The utilization of DFO to maintain HIF activity for the purpose of bone repair has previously shown success in improving vascularity and bone regeneration in both fracture repair and DO of the long bone [19,27]. DO uniquely demonstrates an intense angiogenic response during the early days of distraction that also corresponds with a peak in blood flow followed by a decrease in the number of vessels [28,29]. We hypothesize that the angiogenic properties of DFO would facilitate bone regeneration in the radiated mandible during DO by increasing vascularity around the distraction site. In the present study, we quantitatively demonstrate the ability of DFO therapy to temper the anti-angiogenic effect of XRT.

Our results from this study suggest that DFO may be a viable treatment option aimed at mitigating the damaging effects of XRT on bone formation. Furthermore, this study establishes DFO's ability to propagate angiogenesis in a pathologic model where healing is not routinely observed. No study to date has observed this effect in a pathologic model. The DFO induced increase in vascularity of the irradiated mandible was accompanied by a tremendous increase in the rate of bony union, establishing an important clinical link between pharmacologically triggered angiogenesis and bone healing.

Although the results of this report are quite striking the experiments outlined did have some limitations in both the study design and the animal model. Our use of microCT angiography, though important, limits our evaluation to only the vascular response of DFO in mandibular DO. In an attempt to go beyond the vascular response we also reported on the ability to achieve union, however, the quality of such a union would require a more thorough biomechanical evaluation of the RG. Furthermore, only one therapy protocol which utilized DFO during active distraction was employed based on data gleaned from the literature. Dose response studies as well as attempts at differing delivery protocols would be required for full optimization of DFO therapy.

Additional research will be needed to further validate and characterize the clinical utility of DFO therapy for mandibular DO in humans. In particular, future studies should first assess the quality of the DO RG with different methods of analysis including histology, microCT of the bone, and biomechanical testing. Furthermore, modifications of our DFO therapy protocol will need to be optimized in order to establish the relationship between clinical DO parameters and angiogenesis. Finally, since XRT is usually utilized as an adjuvant treatment for cancer, studies will need to be undertaken to demonstrate that DFO treatment is not associated with tumor recurrence.

Our results would suggest the potential to extend DFO's utility for a broader range of skeletal repair, including osteoradionecrosis, and pathologic fracture. The low cost and

relative stability of DFO make it advantageous and suggest promise in its ability to improve blood supply to skeletal and other tissues that require angiogenesis to regenerate. The overall efficacy and lack of overt toxicity of DFO are also encouraging. Since DFO is a drug that is already on formulary at many hospitals and is FDA approved for other medical applications, the potential for its immediate use and the translation of our experimental results from the bench to the bedside are all the more promising.

Highlights

We model the equivalent radiation dose for head and neck cancer in a murine model of mandibular distraction osteogenesis. > We examine the effect of radiation and deferoxamine therapy on vascularity and bone regenerate. > Radiation induces hypovascularity to the distraction gap which is reversed by deferoxamine therapy. > Deferoxamine improves angiogenesis and bone regeneration in distraction osteogenesis.

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Figure 1. Micro-CT Reconstruction. Representative 3-dimensional micro-CT isosurface reconstructions illustrating non-union XRT DO samples (A) and bony union in XRT DO with DFO samples (B).

Figure 2. Micro-CT angiography. After 28 days of consolidation, rats were sacrificed and perfused with contrast agent. The bones were decalcified and scanned. Representative 3-dimensional micro-CT vascular reconstructions of control, xDO, and xDFO groups are shown.

Figure 3.

Quantitative Radiomorphometrics. Quantitative analysis of the micro-CT data showed decreased vessel number (VN) and vessel volume fraction (VVF), and subsequent increased vessel separation (VS) with radiation. DFO treated groups revealed and increased VN and VVF with expected decreased VS. (Results compared by ANOVA and post hoc Tukey's * $p < 0.05$)

Table 1

MicroCT Results

Analysis	DO	xDO	xDFO
VN	0.82 [*] ± 0.05	0.53 [*] ± 0.05	1.02 [*] ± 0.05
VV	9.63 [*] ± 0.81	5.53 [*] ± 0.61	6.12 ± 0.98
VS	1.18 [*] ± 0.07	1.99 [*] ± 0.21	0.94 [*] ± 0.05
VVF	0.046 [*] ± 0.004	0.029 [*] ± 0.003	0.05 [*] ± 0.003

VN, vessel number; VV, vessel volume; VS, vessel separation; VVF, vessel volume fraction.

* Outcomes with provided standard error values are significant ($p \leq 0.05$) between DO-xDO and xDO-xDFO groups.