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Localized Deferoxamine Injection Augments Vascularity and Improves Bony Union in Pathologic Fracture Healing after Radiotherapy

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Abstract

Background—Medically based efforts and alternative treatment strategies to prevent or remediate the corrosive effects of radiotherapy on pathologic fracture healing have failed to produce clear and convincing evidence of success. Establishing an effective pharmacologic option to prevent or treat the development of non-unions in this setting could have immense therapeutic potential. Experimental studies have shown that Deferoxamine (DFO), an iron- chelating agent, bolsters vascularity and subsequently enhances normal fracture healing when injected locally into a fracture callus in long bone animal models. Since radiotherapy is known to impede angiogenesis, we hypothesized that the pharmacologic addition of DFO would serve to mitigate the effects of radiotherapy on new vessel formation *in vitro* and *in vivo*.

Materials and Methods—*In vitro* investigation of angiogenesis was conducted utilizing HUVEC cells in Matrigel. Endothelial tubule formation assays were divided into four groups: Control, Radiated, Radiated + Low Dose DFO and Radiated + High Dose DFO. Tubule formation was quantified microscopically and video recorded for the four groups simultaneously during the experiment. *In vivo*, three groups of Sprague–Dawley rats underwent external fixator placement and fracture osteotomy of the left mandible. Two groups received pre-operative fractionated radiotherapy, and one of these groups was treated with DFO after fracture repair. After 40 days, the animals were perfused and imaged with micro-CT to calculate vascular radiomorphometrics.

Results—*In vitro*, endothelial tubule formation assays demonstrated that DFO mitigated the deleterious effects of radiation on angiogenesis. Further, high-dose DFO cultures appeared to organize within 2 hours of incubation and achieved a robust network that was visibly superior to all other experimental groups in an accelerated fashion. *In vivo*, animals subjected to a human equivalent dose of radiotherapy (HEDR) and left mandibular fracture demonstrated quantifiably

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Disclosure

All authors state that they have no conflicts of interest.

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diminished μ CT metrics of vascular density, as well as a 75% incidence of associated non-unions. The addition of DFO in this setting markedly improved vascularity as demonstrated with 3D angiographic modeling. In addition, we observed an increased incidence of bony unions in the DFO treated group when compared to radiated fractures without treatment (67% vs. 25% respectively).

Conclusion—Our data suggest that selectively targeting angiogenesis with localized DFO injections is sufficient to remediate the associated severe vascular diminution resulting from a HEDR. Perhaps the most consequential and clinically relevant finding was the ability to reduce the incidence of non-unions in a model where fracture healing was not routinely observed.

Keywords

Osteoradionecrosis; Deferoxamine; Pathologic Fracture; Angiogenesis; Non-union; Radiotherapy

(1.1) Background

The improved survival afforded by radiotherapy in the management of patients with Head and Neck Cancer (HNC) continues to be associated with the costly incidence of bone related complications [1,2]. While substantial undertakings have been employed to advance the delivery of ionizing radiotherapy, efforts to prevent the unavoidable damage to adjacent bone, or replenish and restore injured tissues remain constrained by comparison. The development of osteoradionecrosis (ORN), late pathologic fractures, and non-unions in the setting of radiation are particularly concerning as they subject cancer survivors to a life-long disability that is severely limiting and often profoundly painful. The management of these severe side effects is extremely challenging for the patient and the provider. In severe cases, non-unions develop and patients require surgical removal of necrotic bone and autologous tissue transfer [3,4]. Perhaps the most troubling clinical consequence is that when these measures fail, there are no other currently accepted management options available. Establishing a means to prevent the progression of pathologic fractures to subsequent non-unions in this setting would have immense therapeutic ramifications. Since radiotherapy is known to impede angiogenesis, we hypothesized that the pharmacologic addition of Deferoxamine (DFO), an iron chelator with demonstrated angiogenic properties, would serve to mitigate the effects of radiotherapy on new vessel formation *in vitro* and *in vivo*.

The intricate involvement of the vascular system during bone remodeling and fracture healing makes it a clear target for therapeutic optimization. Fracture healing is intimately dependent on an early activation of angiogenesis. This augmentation is crucial to the development of a vascular system capable of supplying the increased metabolic demands imparted by a fracture [5]. Radiotherapy impedes new vessel formation and diminishes vascularity, precluding the normal physiologic events of fracture healing [6–8]. Conceptually, therapeutic augmentation of the remaining vascular bed may provide a mechanism for the prevention of radiation-induced non-unions after pathologic fractures.

Investigators have recently demonstrated a novel use for Deferoxamine (DFO), a clinically accepted medication traditionally used in cases of transfusion related iron-overload [9]. Through a mechanism of iron chelation, DFO is able to remove excessive systemic iron stores from the blood. Similarly, in a localized and contained environment, DFO has experimentally demonstrated the capacity to up-regulate angiogenesis at exponentially smaller doses. DFO exerts this angiogenic function by triggering the hypoxia inducible factor (HIF 1- α) pathway both *in vitro* and *in vivo* when injected locally in small doses into a fracture callus [10]. Fracture calluses enclose and protect a fracture site from the surrounding environment, and exhibit a localized increase in blood circulation making this site an optimal vehicle for drug delivery. DFO triggers a transcriptional cascade of events by

favoring the accumulation of HIF 1- α . Iron is a co-factor required for the prolyl hydroxylation of HIF 1- α —a reaction that leads to its ultimate degradation. DFO inhibits prolyl hydroxylation by removing iron from the environment. This localized iron chelation leads to the constitutive and sustained presence of HIF 1- α that subsequently causes the increased transcription of VEGF and other downstream angiogenic molecules, resulting in a variety of effects on the growth of new blood vessels. *In vitro* studies have confirmed augmentation in angiogenesis utilizing HUVEC tubule-formation assays, metatarsal endothelial sprouting assays, and immunostaining with anti-CD31 monoclonal antibodies [11,12]. *In vivo*, investigators have shown significant increases in vascular density and bone quality utilizing Micro-Computed Tomography (μ CT), and a trend towards increased stiffness and maximum torque with biomechanical testing in mouse femur fractures treated with DFO when compared to untreated controls [10].

In order to investigate the therapeutic potential of DFO to remediate radiation-induced damage to both angiogenesis and fracture site vascularity, we examined its utility to restore angiogenesis *in vitro* using tubule-formation assays, and *in vivo* using an established model of pathologic fracture healing in the setting of high dose radiotherapy. We posit that the addition of DFO will allow for significant remediation of angiogenesis *in vitro* and foster a physiologic environment capable of successfully healing a fracture in the setting of radiotherapy *in vivo*.

(1.2) Materials and Methods

(1.2.1) In Vitro Experimental Design

Human Umbilical Vein Endothelial Cells (HUVEC) were obtained from Cell Applications, Inc. (San Diego, CA). Cells were grown in Cell Applications' proprietary Endothelial Cell Growth Medium. All cells were utilized at passage two through four.

(1.2.2) Cellular Radiation Protocol

HUVEC cells near confluence were radiated using a Philips RT250 orthovoltage unit (250 kV X-rays, 15 mA; Kimtron Medical, Woodbury, CT), which delivers ionizing radiation through a filtered system. The cells were exposed to a dose of 5 Gy of radiation in a single fraction according to previously established protocols proven to successfully impair the growth and development of cellular cultures [13].

(1.2.3) Tubule Formation Assay

Matrigel (BD Biosciences, Franklin Lakes, NJ) was thawed and placed in eight-well chamber slides at 37°C for 30 minutes to allow for solidification. Then, control, radiated, or DFO-radiated HUVEC cells (48,000 cells per well) were plated alone on Matrigel with 200 μ L of 25% HUVEC proprietary media and 75% RMPI 1640 media. Cells were then incubated at 37°C under 23% oxygen for 12 hours. Plates receiving DFO received either 25 μ M (low-dose) or 50 μ M (high dose) doses at the time of incubation. Tubule formation was defined as a structure exhibiting a length four times its width. Experiments were performed with a sample size of six. Tubule counts were determined in 10 random fields per well using an inverted Leica DMIL light microscope (Leica Microsystems, Wetzlar, Germany) at 100 $\times$ magnification as described previously [14]. Tubule formation was time-lapse recorded for 10 hours from the start of incubation and photographed every four hours over the 12-hour period. Quantification of tubule formation between the groups was compared using ANOVA with $p < 0.05$ considered statistically significant. All analyses were conducted using PSAW 18 software.

(1.2.4) In Vivo Experimental Design

Animal experimentation was conducted in accordance with the guidelines published in the *Guide for the Care and Use of Laboratory Animals: Eighth Edition*. Protocols were approved by the University of Michigan's Committee for the Utilization and Care of Animals (UCUCA) prior to implementation. All animals were subjected to radiotherapy and osteotomy surgery with select animals also receiving Deferoxamine (DFO) therapy.

(1.2.5) Animal Radiation Protocol

All radiation procedures were conducted in the Irradiation Core at the University of Michigan Cancer Center. After transient induction of anesthesia with an oxygen/isoflurane mixture, left hemi-mandibles were radiated using the Philips RT250 orthovoltage unit. Our selected region of interest (ROI) spans a 2 mm distance posterior to the third molar and correlates to the future site of surgically created osteotomy. Lead shielding is provided to ensure localized delivery and protection of surrounding tissues. A previously described Human Equivalent Dose of Radiation (HEDR) developed with the guidance of the department of radiation oncology at the University of Michigan was utilized [15,16]. Briefly, a fractionated dose of 7 Gy per day was administered over 5 days for a total of 35 Gy. This is comparable to 70 Gy in human mandibular high-dose radiotherapy. This dose was designed to predictably replicate pathologies analogous to those observed in the setting of clinically advanced mandibular ORN, while taking the diminutive size of the mandible and surrounding tissues into consideration.

Twelve-week-old male Sprague Dawley rats weighing approximately 400g were acclimated for seven days in light and temperature controlled facilities and given food and water ad libitum. Radiotherapy was administered over a five day-period followed by a recuperation period of 14 days prior to surgery. During recovery, animals were acclimated to a soft chow high-calorie diet (Hills-Columbus Serum; Columbus, Ohio) to ensure adequate food intake and nutrition in the post-radiation and post-operative periods. Subsequently, animals underwent osteotomy, DFO injection and a 40-day consolidation period as outlined prior to perfusion with Microfil, dissection, decalcification and μ CT imaging (Figure 1).

(1.2.6) Peri-operative Care

Gentamycin (30mg/kg SQ) was given prophylactically before surgery and twice postoperatively. To ensure adequate analgesia, hydration and anesthesia, rats were given Buprenorphine (0.15mg/kg SQ) along with subcutaneous Lactated Ringers solution (25cc/kg SQ), and then anesthetized using an inhalational isoflurane/oxygen mixture throughout the surgical procedure. Post-operatively, animals were placed on warming blankets and monitored for heart and respiratory rates. Post-operative analgesia with Buprenorphine was continued twice daily until POD 4, and as needed thereafter. Weight gain, porphyrin staining, food and fluid intake were assessed to determine the need for continued analgesia.

(1.2.7) Osteotomy Surgery and Fracture Repair

After standard preparation and draping a 2cm midline incision was placed ventrally from the anterior submentum to the neck crease. A custom titanium fixator device was placed as previously described [17]. After stabilization of the fixator, a vertical osteotomy was created using a reciprocating saw blade directly behind the third molar on the left hemi-mandible. After reduction of the osteotomy edges, wounds were irrigated, hemostasis verified and incisions were closed in layers. Four hours after osteotomy, the fixator device was set to a 2 mm fixed distance for the length of the experiment.

(1.2.8) Deferoxamine Injection

The dose and delivery method of DFO used in these protocols was derived from an extensive literature search regarding its use in long bone animal models and the subsequent advancement of our experimentation with this drug over recent years [10–12,18,19]. We have modified the reported dose to accommodate the larger volume of the mandible in our animal model. DFO (200 μ M in 300 μ L NS) was given as a local injection directly into the fracture site every other day starting on post-operative day 4 and continuing through post-operative day 12. This time frame was chosen to correlate with the reasonable time period for initiation of angiogenesis in a murine fracture model [5,20,21].

(1.2.9) 3D Angiographic Modeling

Rats were anesthetized prior to thoracotomy and underwent left ventricular catheterization. Perfusion with heparinized normal saline, followed by pressure fixation with normal buffered formalin solution ensued and ensured euthanasia. After fixation, the vasculature was injected with Microfil MV122 (Flow Tech; Carver, MA). Mandibles were harvested en bloc and demineralized using Cal-Ex II solution (Fisher Scientifics; Fairlawn, NJ). Leeching of mineral was confirmed with serial radiographs to ensure adequate demineralization prior to scanning. μ CT images were obtained using 80kVp, 80mA and 1100 ms exposures. 392 projections were taken at a resolution of 18-micron voxel size [22,23]. Utilizing GE's Microview 2.2 software, scans were reconstructed and reoriented in a 3-D x, y and z plane. The region of interest (ROI) was then cropped, and splined for analysis. Due to demineralization only the vessels perfused with Microfil appeared on the μ -CT scan. Vessel Volume (VV), Vessel Thickness (VT), Vessel Number (VN) and Vessel Separation (VS) were assessed. All variables were compared using ANOVA with $p < 0.05$ considered statistically significant. All statistical analysis was conducted using PSAW 18 software.

(1.3) Results

(1.3.1) Tubule Formation Assay

We observed a severe diminution in tubule formation in response to ionizing radiotherapy that was evident at all time-points (Figure 2).

The corresponding means for each group and p values comparing all experimental groups to 5Gy XRT alone are demonstrated in Tables 1 and 2 respectively.

Control and high-dose DFO cultures were significantly higher than XRT-alone at all time points. High-Dose DFO peaked earlier than control, to approximately equivalent levels. At later time-points, smaller tubules consolidated into larger ones, lowering the overall number of Tubules/High Power Field. In all cases, control and high dose DFO values were significantly greater than XRT alone. All comparisons were performed within a time-point cohort. While no statistical restorative effects were observed with low-dose DFO, high-dose treated cultures demonstrated a superior ability to generate tubules that was comparable to non-radiated controls. Despite a lack of statistical differences with low-dose DFO, time-lapse recording demonstrated a considerable increase in cellular organization, activity and locomotion in response to this low-dose of therapy (See e-pub videos 1,2 and 3). Further observations demonstrated that fewer tubules were seen in culture at the late (12 hr) time-point than at the middle (8 hr) time-point across all groups. However, the tubules in culture at the late time-points were typically thicker, and were comprised of more cells than those at the middle time-point indicating a consolidation of tubule formation (Figure 3).

In summary, we demonstrate that at low doses, DFO increases cellular movement and organization. At high doses, DFO remediates radiation induced impairments to angiogenesis

in a threshold dependent manner (Figure 4). Furthermore, the time to peak angiogenesis and consolidation were significantly reduced with high dose DFO.

(1.3.2) 3D Angiographic Modeling

The XFx group demonstrated a significant decrease in Vessel Volume when compared to Fx. Vessel Number, Thickness and Separation also evidenced similar significant deleterious effects. The DFO treated group demonstrated a significant increase in Vessel Volume when compared to XFx. This vascular density was not statistically different than the normal fracture repair. No significant restorative effects were appreciated for Vessel Thickness. However, Vessel Number and Separation did evidence similar significant restorative effects (Table 3). These results therefore quantify significant diminutions of vascular metrics in response to radiotherapy and the efficacy of DFO to remediate those detriments *in vivo*. These observations are visibly appreciated with CT angiograph reconstructions (Figure 5).

The fracture and radiated fracture group showed significant differences in all metrics and the radiated fracture and DFO treated groups showed statistical differences in all but vessel thickness. The XFxDFO and Fx groups showed no statistical differences, indicating that the DFO restored the vasculature to pre-radiation levels of Vessel Volume, Number, Thickness, and Separation.

(1.3.3) Gross Bone Quality, Union and Vascular anatomy

Mandibles exposed to radiotherapy exhibited grossly evident structural damage and a substantial decrease in the formation of bony unions. Anatomic distortions in size and geometry were visible, as well as bony defects in radiated samples when compared to fractured controls. Control fractures exhibited bony union in all cases (100%), radiated fractures achieved a 25% union rate and DFO treatment allowed for a union rate of 67%. The restorative effect of DFO was grossly and radiographically appreciated when comparing the the XFxDFO group to the Fx group (Figure 6 A and B). Further, detriments to vascular microarchitecture were also apparent with radiotherapy and restored with the addition of DFO (Figure 6C).

(1.4) Discussion

In the present study we investigated the therapeutic potential of Deferoxamine to remediate radiation-induced detriments of angiogenesis and pathologic fracture healing. Our first aim was to quantify impediments to endothelial cell proliferation and tubule formation *in vitro*, and diminution of fracture site vascularity *in vivo*. Ultimately, we posited that the addition of DFO would allow for a significant remediation of angiogenesis *in vitro* and foster a physiologic environment capable of successfully healing a fracture in the setting of radiotherapy *in vivo*. We observed a severe diminution in endothelial cell proliferation and tubule formation after radiotherapy. Cultures exposed to a single dose of ionizing radiation demonstrated vastly impaired tubule formation that was diminished to 30%, 34%, and 27% of control values (at 4, 8, and 12 hours respectively). The pernicious effect of radiation was remediated by treatment with a high-dose of DFO. The remediation of tubule formation did not scale between low-dose and high-dose DFO in a linear fashion, indicating a threshold effect for the activity of DFO. Finally, DFO appeared to accelerate the angiogenic process, lowering the time required to achieve peak tubule formation. This finding was clearly demonstrated with time-lapse recording. High-dose DFO cultures appeared to organize within 2 hours of incubation and achieved a robust network that was visibly superior to all other experimental groups in an accelerated fashion. Although we did not observe a restorative effect with the addition of low-dose DFO, time-lapse recording demonstrated a

visible increase in cellular organization, activity and locomotion in response to therapy (See Figures 7,8,9 corresponding to e-pub videos 1, 2, 3 respectively).

A remarkable increase in cellular organization and movement was apparent for the duration of the 10 hour recording when comparing radiated cells to those treated with 25 μ M DFO. This interesting finding leads us to further posit that this localized micromotion, in addition to the known angiogenic capacity of DFO, may be contributing to the enhancement in osteogenesis and fracture repair seen *in vivo*. The key findings from this experiment were that 1) radiation impairs angiogenesis in an *in vitro* Matrigel Assay 2) high-dose DFO remedies this radiation-induced impairment of angiogenesis, 3) DFO demonstrates a threshold effect for the remediation of angiogenesis, 4) DFO increases cellular movement and organization between endothelial cells at low-doses, 5) DFO accelerates the organization and establishment of peak tubule formation *in vitro*.

In vivo, radiated fractures demonstrated a significant 80% depletion in Vessel Volume within the fracture-healing region. This volume depletion could be quantifiably attributed to both a 70% decrease in the number of vessels, and a 60% decrease in the thickness of existing vessels. These findings were corroborated with a corresponding increase in the separation between the vessels that was 19 times greater in the radiated group when compared to fractured controls. The addition of DFO markedly improved metrics of Vascular Volume, Number, and Separation to control levels, and ultimately evidenced a 42% enhancement in the clinical appreciation of bony unions over radiated fractures without therapy. Interestingly, Vessel Thickness was not affected by the addition of DFO, indicating that the effect on the restoration of the Vessel Volume was impacted by an increase in the number of small vessels, and not necessarily by the maturity and growth of those vessels.

Our selection of DFO was based on a review of the literature concerning the therapeutic augmentation of fracture repair by a means of angiogenic augmentation [10–12]. Due to the inherent detriments to fracture vascularity imposed by radiotherapy, agents that would replenish this necessary substrate were considered. The understanding of the role of VEGF during angiogenesis and fracture repair has triggered a scientific curiosity concerning the ability to manipulate angiogenesis to improve bone healing. Its demonstrated capacity to augment angiogenesis, osteogenesis and fracture healing has resulted in a line of inquiry concerning the therapeutic optimization of fracture healing and delivery strategies with potential clinical translation.

Utilizing a soluble neutralizing VEGF receptor Street et al. demonstrated that inhibition of VEGF dramatically decreased angiogenesis, bone formation, and callus mineralization in mouse femur fractures, and inhibited bony bridging of segmental gap defects in rabbit radii. In contrast, the administration of exogenous VEGF enhanced blood vessel formation, ossification, and callus maturation; and further, promoted bony bridging of those respective fracture and segmental defect models. In regards to these findings, he proposed that a slow-release formulation of VEGF, given locally at the site of bone healing might prove to be an effective therapeutic to optimize human bone repair [12]. Despite these findings, the feasibility of clinical translation of VEGF as a therapeutic means has raised concerns due to the need for recombinant protein or gene therapy approaches.

The work of Wan et al. offered an important contribution to these developments by considering a more global activation of VEGF and angiogenesis through triggering the HIF 1- α pathway. His work demonstrated that this pathway could be exploited genetically and therapeutically to improve bone healing. Utilizing mice designed to constitutively activate HIF 1- α in osteoblasts (pVHL mutants), he observed markedly increased vascularity and increased bone production in response to distraction osteogenesis in mice femurs. In

contrast, mice lacking HIF 1- α had impaired angiogenesis and bone regeneration. Utilizing VEGF receptor antibodies he subsequently demonstrated that the positive findings in the pVHL mutants were VEGF dependent and could be eliminated in the presence of these antibodies. The most clinically relevant contribution from this work was the idea that prolyl hydroxylase inhibitors such as DFO exhibited a phenomenon analogous to the genetically altered model, causing markedly improved angiogenesis and bone regeneration. Similar to the genetic pVHL models, prolyl hydroxylase inhibitors upregulate the transcription of VEGF and other angioproteins by favoring the accumulation of HIF 1- α in osteoblasts. Based on these findings, the authors suggested the application of prolyl hydroxylase inhibitors as a superior and more readily translatable alternative to the direct addition of VEGF for these purposes [11].

Shen et al. extended this application to a model of murine femur fracture repair and examined three prolyl hydroxylase inhibitors. His results demonstrated that prolyl hydroxylase inhibition increased vascularity at 14 days and increased callus size at 28 days in normal femoral fracture repair. From those studies it was apparent that Deferoxamine, one of three compounds tested, exhibited the most predictable *in vivo* effects on vascular augmentation and fracture healing thereby producing the proof of principal on which our studies are based. Further, he proposed that prolyl hydroxylase inhibitors might be more broadly applied in bone injury and tissue regeneration in the setting of vascular compromise [10].

Based on these studies, our experiment was designed to investigate the potential of Deferoxamine to restore angiogenesis and normal fracture healing with the addition of radiation in our *in vitro* and *in vivo* models. Our results expand and extend the previous findings in more rigorous models of radiation induced impairments to endothelial cell function *in vitro* and pathologic fracture healing where non-unions are the expected outcome in 75% of cases. We found a substantial restoration in endothelial cell function and a restitution of vascular density at the time of consolidation. Finally, our *in vivo* investigation revealed a clinically relevant increase in the rate of bony unions. Despite this, these findings must be appreciated in the context of the proposed clinical applications. We envision the utility of this therapy in a select population of patients who are at risk for the development of radiation induced pathologic fractures, or in those who have sustained pathologic fractures to prevent the development of non-unions. Investigation regarding the tumorigenic safety of this therapy, given its use in cancer patients, will need further clarification. Based on previous studies, we do not believe DFO will promote tumor recurrence, as investigators have demonstrated that DFO has an anti-tumorigenic effect in select cancers [24–27]. Nonetheless, a closer look at the juxtaposition between HNC tumor recurrence and Deferoxamine's demonstrated angiogenic and osteogenic potential in this setting is warranted.

(1.5) Conclusions

We conclude that for the purposes of augmenting angiogenesis and promoting fracture healing after radiotherapy, DFO is a therapeutic option with a clear potential for clinical translation. Our findings suggest a significant remediation of angiogenesis and vascularity that ultimately demonstrated a 42% increase in the number of bony unions observed in our pathologic model. Further, our *in vitro* experiments suggest that early micromotion and organization are apparent with the addition of this therapy, and that this may be contributing to the enhancement in osteogenesis and fracture repair seen *in vivo*. Although a complete restoration of bony unions was not observed *in vivo*, further experimentation may be warranted regarding the role of combination therapies designed to restore other vital substrates affected by radiotherapy. Given its broad acceptance in the current clinical arena,

its known and limited side effect profile, and its affordability, we cautiously support the scientific optimization and clinical translation of this promising therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(Top): Experimental timeline showing 7 days of acclimation, radiation over a 5 day period, a 14 day recovery period, surgery, and a 40 day healing period. Note that DFO is given to animals in the experimental group after surgery on POD 4–12. (Middle): Schematic left hemi-mandible demonstrating the region of interest highlighted in white. (Bottom): A perfused, dissected and decalcified hemi-mandible showing blood vessel infiltration with yellow Microfil.

Figure 2.
Graph shows tubules per high power field for each time point for each group.

Figure 3.

Matrigel tubule assay of HUVEC cells after radiation with or without DFO treatment. Representative high power field microscopy images of the following groups: 1) control (0GY XRT), 2) 5GY XRT, 3) 5GY XRT+ 25uM DFO and 4) 5GY XRT+50uM DFO at 4, 8 and 12 hours. At all time points, 5 Gy XRT images show sparse tubule formation with many inactive cells. This is not observed in control or high-dose DFO cultures. At late time points, there are fewer tubules, however, these tubules are larger, more robust, and are comprised of more cells than those at the early and middle time-points.

Figure 4.

Plot showing threshold effect of DFO on HUVEC tubule formation. Low-dose DFO was much less effective than high-dose DFO. The 8 hr plot appears linear; however, this is not due to the effect of low-dose DFO, but rather due to the fact that high-dose DFO peaked at 4 hrs and is in the consolidation phase at 8 hrs, while low-dose DFO is undergoing a more “normal” peak at 8 hrs. The 4 and 12 hr plots validate the threshold effect that we observed. Control data is provided for visual comparison.

Figure 5. Three-dimensional reconstructed μ CT angiograms of rat mandibles showing the fracture sites from fractured (*top*); radiated-fractured (*middle*); and Deferoxamine treated radiated-fractured mandibles. Vessels are highlighted in the region of interest and the surrounding mandible is superimposed for visual localization and clarity. Note the efficacy of DFO in augmenting vascularity in the radiated fracture.

Figure 6.

(A) Four selected gross specimens from each group. (Top row): Fx, (Middle row): XFx and (Bottom row): XFxDFO. The first mandible in each row was clinically appreciated as a bony union. This distinction is more readily appreciated when comparing each bone to its corresponding radiograph in Figure 6 (B). The unions in the XFx group appeared to be due to small islands of bone that had healed across the fracture site rather than to the complete bony bridging of control and DFO treated counterparts. (C) Corresponding microangiographic reconstructions for each mandible are shown. Note the apparent diminution of vascular volume that is restored to near normal levels with the addition of therapy.

Figure 7. Snapshot for video 1: Simultaneous video recording at 100X magnification of four cultures of HUVEC cells in Matrigel. Control (*top left*), XRT (*top right*), XRT/LD-DFO (*bottom left*) and XRT/HD-DFO (*bottom right*). Ten hours of video was captured and rendered in 20 seconds.

Figure 8. Snapshot for video 2: The XRT group in video 1 was enlarged and slowed to a one minute rendering for visual appreciation. Clumping of endothelial cells due to the effects of XRT is evident from this clip.

Figure 9. Snapshot for video 3: The XRT/HD-DFO group in video 1 was enlarged and slowed to a one minute rendering for visual appreciation. Notice the enhanced motion, organization and superior tubular network structure of the endothelial cells exposed to DFO despite their exposure to XRT.

Table 1

Means $\pm$ SD for tubules per high power field in each of the treatment groups at 4, 8, and 12 hours using an *in-vitro* Matrigel angiogenesis assay.

Tubules/High Power Field				
	Control	5 Gy XRT	5 Gy XRT + 25 μ M DFO	5 Gy XRT + 50 μ M DFO
4 hr	14.56 $\pm$ 5.46 *	4.33 $\pm$ 2.87	7.44 $\pm$ 3.64	19.33 $\pm$ 11.42 *
8 hr	18.44 $\pm$ 6.21 *	6.22 $\pm$ 2.82	9.56 $\pm$ 3.05	13.33 $\pm$ 6.65 *
12 hr	12.78 $\pm$ 5.83 *	3.44 $\pm$ 2.83	4.44 $\pm$ 2.74	11.00 $\pm$ 4.95 *

* indicates $p < 0.05$.

Table 2

P values for *in vitro* angiogenesis assay utilizing one-way ANOVA with Tukey post-hoc test. All comparisons were performed with respect to 5 Gy XRT and within the respective timepoint cohort.

<i>P</i> -Values for <i>in-vitro</i> Angiogenesis Assay in Comparison to 5 Gy XRT			
	Control	5 Gy XRT + 25 μ M DFO	5 Gy XRT + 50 μ M DFO
4 hr	.018	.776	.000
8 hr	.000	.500	.024
12 hr	.000	.960	.004

Table 3

Means + standard deviations for angiographic metrics between each experimental group.

3D Angiographic Metrics			
	Fx	XFx	XFxDFO
Vessel Volume (mm ³)	1.72 ± 0.95	0.24 ± 0.3	1.13 ± 0.98
Vessel Number (mm)	0.50 ± 0.22	0.15 ± 0.14	0.48 ± 0.33
Vessel Thickness (mm)	0.05 ± 0.02	0.03 ± 0.01	0.4 ± 0.01
Vessel Separation (mm)	2.34 ± 1.03	45 ± 15	3.05 ± 1.91

Table 4

Shows the p-values for the statistical comparison of angiographic metrics between groups.

P-Values for Angiographic Metrics			
	Fx vs. XFx	XFxDFO vs. XFx	XFxDFO vs. Fx
Vessel Volume (mm ³)	0.001	0.029	0.192
Vessel Number (mm)	0.004	0.007	0.947
Vessel Thickness (mm)	0.006	0.228	0.243
Vessel Separation (mm)	0.016	0.018	0.999