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Amifostine Protects Vascularity and Improves Unions in a Model of Irradiated Mandibular Fracture Healing

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Abstract

Background—Pathologic fractures after irradiation (XRT) can be a devastating problem for cancer patients as XRT has a pernicious effect on bone healing in a large part due to impairment of vascularity. Our aim is to ascertain whether Amifostine (AMF), a radio-protective drug, will preserve the vascularity of the irradiated mandible, thereby improving bony healing and unions after exposure to a human equivalent dose of radiation (HEDR) in our murine model of mandibular fracture repair.

Methods—Rats were randomized into: Fx (n=9), XRT/Fx (n=5) and AMF/XRT/Fx (n=7). XRT/Fx and AMF/XRT/Fx underwent HEDR directed at the left hemimandible. AMF/XRT/Fx received AMF concomitantly with HEDR. All animals underwent unilateral left-mandibular osteotomy with external fixation set to a 2.1mm fracture gap. Fracture healing was allowed for 40 days prior to perfusion with Microfil. Vascular radiomorphometrics were quantified with micro-computed tomography.

Results—We observed a 100% rate of bony union in the non-irradiated Fx compared to 25% in XRT/Fx. Union rate in AMF/XRT/Fx more than doubled to 57%. We also saw substantial

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increase in Vessel Number (123%, $p < 0.05$) and a corresponding decrease in Vessel Separation (55.5%, $p < 0.05$) in AMF/XRT/Fx versus XRT/Fx and no differences between Fx and AMF/XRT/Fx.

Conclusions—We report that AMF prophylaxis maintains vascularity at levels seen in non-irradiated Fx specimens, correlating with a significant increase in bony unions after HEDR. Our results set the stage for exploration of this targeted therapy alone, and in combination with other treatments, to mitigate the harmful effects of XRT on fracture repair and bone healing in the clinical setting.

Introduction

In recent years, adjuvant radiation therapy (XRT) has proven to significantly increase survival rates in patients suffering from head and neck cancer (HNC).¹ However, patients who undergo XRT are often predisposed to the development of late pathologic fractures of the mandible, which can result in severe functional consequences and inoperable pain that are exceedingly difficult to manage.^{2–7} Despite current advances in radiation protocols aimed at minimizing the collateral damage to non-tumor tissues adjacent to the radiation bed, the occurrence of late pathologic fractures to the mandible remains at a rate of 4.8–6%.^{3,4,6} Kelishadi et al estimated that the relative cost associated with the treatment of such disabling injuries to approximate \$20,000 to \$30,000 per patient.⁸

Several noteworthy studies have delineated the destructive consequences that underlie the associated pernicious side effects of radiotherapy. In one particular study, Robert Marx famously characterized the pathophysiologic effects of radiation on tissue as the “three H’s”: hypocellularity, hypovascularity, and hypoxia — conditions that inevitably lead to tissue breakdown.^{9,10} XRT engenders significant pathological changes in the local cellular environment of the radiated bed, which is left devoid of fibroblasts, osteoblasts, and undifferentiated immunocompetent cells. Radio-damage further results in fibrosis and obliteration of blood vessels, which, in turn, produces tissue hypoxia.^{9,10} These induced effects are protracted, lasting months or even years after the completion of radiotherapy, leaving bone susceptible to further insult, including fracture.

In light of the “three H’s”, irradiated bone that subsequently fractures is abnormally slow to heal resulting in delayed union or, in a worst case scenario, persistent non-union and ORN.^{11,12} Several progressively invasive options currently exist for managing delayed fracture union that occurs in the setting of an irradiated bed, none of which, however, are uniformly successful. These management options include the indefinite maintenance of a soft diet, the placement of archbars and intermaxillary fixation, and the application of rigid internal fixation with bridging reconstruction plates.^{3,6,13} In addition, HBO and bone grafting have been attempted, but with limited success.^{14,15} Unfortunately, in these worst-case scenarios, the only currently proven solution is vascularized free-flap transfer to provide fresh substrate. However, this option is inevitably associated with a high rate of complications. A 2008 study of 25 patients who underwent vascularized free flap transfer due to ORN found an 88% postoperative complication rate for both donor and recipient sites.¹⁶ These complications included repeat Fx, donor site infection, nerve palsies, osteomyelitis, limb loss, and persistent non-union. Given this demonstrated difficulty in reliably achieving Fx union after radiotherapy with current techniques, it is vital to develop a therapeutic method to prevent the negative effects of radiotherapy on the fracture healing milieu.

To prevent the occurrence of XRT induced damage to the vascular supply and to enhance the healing capacity of adjacent bone in the radiation field, we investigated the FDA-approved, free-radical scavenging drug, Amifostine (AMF).^{17,18} AMF is a radio-protective,

antioxidative drug that is activated within cells by alkaline phosphatase.^{17,18} The drug's requirement for intracellular activation accounts for its selective protection of normal cells, which express more alkaline phosphatase than cancer cells.^{17,18} AMF is well known clinically for its efficacy in preventing xerostomia in patients undergoing XRT for HNC.¹⁷⁻¹⁹ It has also demonstrated a consistent ability to protect osteoprogenitor cells in the face of XRT, both *in vivo* and *in vitro*.

As we have previously demonstrated AMF's efficacy in protecting osteocytes in an *in vivo* model of bone healing²⁰, the drug would also purportedly protect endothelial cells and angiogenesis by the same mechanism. However, current literature regarding AMF's effect on vascularity provides contradictory and equivocal results. The purpose of this study is to explore the potential protective effects of AMF on mandibular vascularity and bony union following exposure to a human equivalent dose of radiation (HEDR)²¹⁻²³ in our previously established murine model of irradiated Fx repair. We posit that the administration of AMF concomitantly with HEDR will serve to protect the vascular supply of the mandible from the detrimental effects of radiation, thereby allowing for reliable fracture healing and improved bony unions following radiation.

Methods

Animals were randomly assigned to 3 surgical groups: Group 1, Fx (n=9), Group 2, XRT/Fx (n=5) and Group 3, AMF/XRT/Fx (n=7). All animal procedures were approved by the University of Michigan's Committee for the Utilization and Care of Animals (UCUCA) and performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Radiation/Amifostine Therapy

Following the acclimation period, Groups 2 and 3 received a HEDR of 35Gy (bioequivalent to 70Gy in a human) fractionated over 5 days, as previously described²¹⁻²³, via a Philips RT250 orthovoltage unit (250kV, 15mA) (Kimtron Medical, Woodbury, CT). Prior to irradiation, animals were anesthetized with an isoflurane-oxygen mixture and placed right-side down beneath a lead-shield custom designed to expose only the left-hemimandible. Group 3 received concomitant AMF (100mg/kg) via a subcutaneous (SQ) injection 45 minutes prior to irradiation. Animals had 14 days for recovery, and those exhibiting any signs of radiation-related stress received buprenorphine (0.03 mg/kg) in a solution of 5cc Lactated Ringers (LR).

Peri-Operative/Surgical and Postoperative Procedures

All animals received SQ injections of buprenorphine (0.03mg/kg), and gentamicin (5mg/kg) prior to surgery. Anesthesia was induced using an inhaled isoflurane-oxygen mixture. Animals underwent unilateral left mandibular osteotomy with external fixator placement, as previously described.²¹⁻²³

Four hours after surgery, each animal's external fixator was set to a gap of 2.1mm via 7-clockwise turns of the distraction screw. The four-hour delay in setting the fracture gap was to prevent peri-operative bleeding.

Animals were maintained one per cage with *ad libitum* access to water and moist chow with Hill's high-calorie diet (Columbus Serum, Columbus, OH). They received two postoperative doses of gentamicin (5mg/kg every 12 hours) and buprenorphine (0.03mg/kg) every 12 hours through post-operative day (POD) 4, and as needed thereafter. Animal weight and pins were monitored daily, and overgrown incisors clipped as needed. Pin site care was

performed with Silvadene (Monarch Pharmaceuticals, Inc., Bristol, TN). All animals were sacrificed on POD40 via perfusion.

Perfusion Protocol

Following induction of anesthesia, animals underwent thoracotomy, left-ventricular catheterization, and ligation of the descending aorta. The vasculature was flushed with heparinized normal saline (1000U/mL), followed by formalin fixation and perfusion with Microfil® MV-122 (Flow Tech, Carver, Mass.), a radio-opaque silicone rubber contrast agent. To determine adequate perfusion, the tongue and eyes were grossly examined for color change to that of Microfil®. Carcasses were kept at 4°C for 24 hours to allow Microfil® to cure completely within the vessels.

Harvest, Decalcification and Imaging

Left hemimandibles were harvested, cleaned completely of soft tissues, and stored in 70% ethanol at 4°C until further use. Hemimandibles were decalcified in Cal-Ex II® solution (Fisher-Scientific, Fair Lawn, NJ). Serial, daily Faxitron® radiographs were used to confirm complete demineralization. Specimens were scanned with μ CT at 18 μ m voxel size, as previously described.^{24–26} Briefly, μ CT was used to image the region of interest (ROI), measuring 2.1mm posterior to the third molar, which corresponds to the site of osteotomy and set Fx gap. MicroView 2.2 software (GE Healthcare, Milwaukee, WI) was utilized to quantitatively assess the vessel volume and vessel separation of the ROI. Maximum intensity projections (MIPs) of the ROI vasculature were generated for qualitative comparison.

Statistical Analysis

One-Way ANOVA and post-hoc Tukey test was used to analyze differences between vascular radiomorphometrics (SPSS version 19.0; SPSS, Inc., Chicago, IL), with statistical significance considered at $p < 0.05$.

Results

During the 14-day recovery post-radiation, both radiated groups exhibited clinical signs of radiation-induced mucositis and alopecia, but these effects were less apparent in the AMF pre-treated group. During the 40-day consolidation interval, XRT/Fx animals demonstrated clinical signs of ORN, including bone exposure, fistula formation, and bony necrosis. Again, these effects were rarely seen in the AMF/XRT/Fx animals, and, if present, were far less severe.

Upon harvest, left hemimandibles were assessed for bony union, as defined by gross bony bridging and lack of motion across the fracture gap upon palpation. We observed a 100% rate of bony union in Fx compared to 25% in XRT/Fx. With AMF treatment, the rate of bony union after radiation more than doubles, improving to a rate of 57% (Fig.1).

Representative MIPs of each group demonstrated a visibly appreciable increase in vascular density within the ROI of both Fx and AMF/XRT/Fx as compared to XRT/Fx, with contributions from central medullary vessels and surrounding periosteal networks (Fig.2).

Stereological analysis of the fracture gap revealed statistically significant differences in all vascular metrics between Fx and XRT/Fx as well as between AMF/XRT/Fx and XRT/Fx.

The mean Vessel Number (VN) of the irradiated XRT/Fx group was $0.272 \pm 0.118 \text{ mm}^{-1}$, demonstrating a 47% decrease in VN in comparison to the control, or Fx, group ($0.576 \pm$

0.271 mm⁻¹, $p = 0.049$; Fig.3). Similarly, XRT/Fx had a 123% decrease in mean VN compared to AMF/XRT/Fx (0.608 ± 0.271 mm⁻¹, $p = 0.037$; Fig.3). No significant difference in mean VN was observed between Fx and AMF/XRT/Fx ($p = 0.951$; Fig.3) indicating a return of vascular density to, at least, the same levels seen in the non-irradiated specimens.

A corroborating pattern was appreciated upon stereological analysis of the mean Vessel Separation (VS), a metric that reflects how distant the vessels are from each other within the ROI. Analysis of Fx versus XRT/Fx demonstrated a significant increase in vessel separation due to irradiation (1.857 ± 0.6 mm vs. 4.418 ± 2.428 mm; $p = 0.008$; Fig.4). This metric was decreased with AMF treatment, as seen upon comparison of XRT/Fx versus AMF/XRT/Fx (4.418 ± 2.428 mm vs. 1.959 ± 1.017 mm; $p = 0.016$; Fig.4). Again we saw no differences between non-irradiated Fx and AMF/XRT/Fx (1.857 ± 0.6 mm vs. 1.959 ± 1.017 mm; $p = 0.987$; Fig.4).

Discussion

In accordance with Marx's "three H's, the pathologic effects of radiation on bone are partly mediated through the progressive obliteration of small blood vessels.^{9,10,27} Bone that is exposed to XRT is therefore left susceptible to, and inadequately equipped to recover from, further insult such as fracture. This study was performed in order to investigate the potential efficacy of AMF, an established radio-protectant drug, to remediate radiation-induced tissue hypovascularity and the potential implications regarding bony union in mandibular fracture repair.

The early stages of fracture healing are highly dependent on the intricate association of osteogenesis and vascularization.²⁸ Several studies confirm the highly vascular nature of fracture repair, demonstrating that this increase in vascularity is a key element in ensuring the adequate and timely repair of fractured bone.^{28,29} Hausman et al dramatically demonstrated the detrimental effect of angiogenic impairment on fracture healing of rat long bones.³⁰ By employing an angiogenic inhibitor, fracture healing was completely prevented, resulting in "atrophic nonunions." Their study defines the central role of angiogenesis in early events of fracture healing, namely, callus and periosteal woven bone formation. Their work further demonstrates the deleterious impact of inadequate vascularization on both intramembranous and endochondral pathways of osteogenesis. Any disruption in angiogenesis, caused for example by a lack of fracture site stability, excessive inflammation, or irradiation, can result in delayed union, fibrous bridging or persistence of the fracture gap.

The mandible, as compared to long bones, has a compact bony structure and limited vascular supply, rendering it particularly susceptible to the pathologic effects of radiation.^{9-12,31} This bone is often within the radiated bed during treatment for HNC. If further insult should occur after XRT, patients can potentially suffer devastating debilitation. The radio-protective effect of AMF on bone-forming cells has previously been shown to improve the quality of bony regenerate.²⁰ Thus, it was reasonable to expect that this mechanism of protection would also be conferred to cells that contribute to blood-vessel formation, thereby calling for a focused line of inquiry into the protective effect of AMF on the vascular supply of the mandible in the setting of high-dose XRT.

In this study, we were able to demonstrate the vascular-protective effect of AMF in the irradiated, fractured murine mandible. Not only did the concomitant administration of AMF with XRT dramatically increase the vascularity within the fracture gap as compared to the non-protected animals, but vascularity was preserved at levels seen in non-irradiated mandibles. The AMF induced preservation of the blood supply correlated with a drastic

increase in bony unions in the AMF treated group, further defining the relationship between XRT, vascular supply, and bone healing.

AMF has a demonstrated capacity both clinically and at the bench to limit the effects of XRT on healthy cutaneous and soft-tissues by selectively protecting non-tumor tissues from the effects of XRT.^{17–19,32–35} However, the majority of published studies that explore the effect of AMF on vascularity are carried out *in vitro*, via assays that indirectly evaluate angiogenesis through the culture of endothelial cells or through the expression of biochemical signals and molecules implicated in the angiogenic process. Despite numerous published studies, findings remain contradictory and unclear.

Several studies characterize AMF as a pro-angiogenic drug.^{36–40} Plasswilm et al conducted an *in vivo* study demonstrating the pro-angiogenic effect of a single dose of AMF to the area vasculosa of the fertilized egg, noting induced endothelial cell proliferation.³⁷ The pro-angiogenic effect of AMF was further described by Andreopoulos et al in the setting of XRT.³⁸ Their *in vitro* study exhibited a higher proliferation rate of AMF treated human endothelial cells after a single 10Gy dose of XRT as compared to irradiated, non-treated cells. Along the same line, Rubin et al demonstrated that AMF promotes the replication of endothelial cells *in vitro*, allowing for tissue recovery from XRT,³⁹ which was further corroborated by Mooteri et al,⁴⁰ who described the mechanism of this protective effect at the level of morphological changes in cell cytoskeleton and adhesion molecule expression.

Several other studies, however, demonstrated findings that directly contradict the pro-angiogenic properties attributed to AMF. Dedieu et al corroborated the well-known cytoprotective effects of AMF, while asserting that the drug has an additional, primarily anti-angiogenic effect on endothelial cells.⁴¹ Giannopoulou et al further demonstrated the anti-angiogenic effects of AMF both *in vitro*, on human umbilical vein endothelial cells, and *in vivo*, by measuring the expression of angiogenic genes and the composition of the extracellular membrane in the chicken embryo chorioallantoic membrane model.^{42,43} It is evident that all these previous investigations into AMF's effect on angiogenesis and vascularity utilized indirect measures to glean information, yielding unclear and equivocal findings regarding the drug's effects. Further, these studies lack a clinically translatable model.

Our *in vivo* study was designed to provide a more clinically applicable and direct determination of the vascular protection conferred by prophylactic administration of AMF. In our *in vivo* study of post-irradiation fracture, animals were subjected to a fractionated, human bioequivalent dose of radiation with concomitant AMF administration, directly mirroring clinical protocols. By perfusing animals with a radio-opaque compound, Microfil, followed by μ CT imaging and angiograph reconstruction, we directly visualized and quantified the vascularity of the fracture gap with and without AMF radio-protection. This method of vascular assessment yields more robust, accurate and objective data when compared to the classical but indirect methods of vascular quantification, such as immunohistochemistry.⁴⁴

Certainly, there are limitations that we must consider in the context of our experiment. Our study does not assess the process of angiogenesis due to AMF administration, per se; however, we do provide a direct measure of the outcomes that are of clinical interest. Namely, these are the persistence of blood vessels in the face of HEDR and, of utmost clinical import, an increased rate of bony unions in this scenario. Thus, even if the mechanism proposed in the aforementioned study by Dedieu et al³⁸ were indeed true, our results would readily establish that the vascular protection provided by AMF is able to

overcome the drug's purported anti-angiogenic properties, affording a net vascular boon to the irradiated wound bed, with a resultant clinical improvement in fracture repair.

Our model is also limited by the use of an osteotomy to create the experimental fracture. Within the laboratory setting, it would be unreasonable to impart a true traumatic fracture, not only because such an unnecessary endeavor would not be justifiable to animal use committees, but also because this would introduce inconsistency between animals. An osteotomy ensures a reproducible cut in the left hemimandible and a constant fracture gap distance. It also mirrors the common clinical scenario wherein an HNC patient would require bony resection after XRT for tumor extirpation. Thus, the use of an osteotomy within the experimental setting provides an essential level of control for assessing healing capacity, while mirroring the clinical scenario as closely as possible.

From a translational standpoint, our findings provide evidence for the inclusion of this important radio-protectant into current XRT protocols. For HNC patients, the effects of adjuvant XRT manifests itself in a demonstrated diminution in the ability to heal in a normal and predictable manner. Even if XRT is administered using meticulous technique and cutting edge technology, these patients can still go on to develop pathologic Fx and ORN several years after XRT. In a case study of 44 patients with pathologic fracture, Coletti et al⁶ noted that the most common etiology was radiotherapy, implicated in 40% of their cases. Similarly, in a study of 25 patients with pathologic fracture conducted by Freidrich et al¹⁶, the investigators cited prior irradiation as the cause in 84% of the cases. It is apparent that despite recent advances in the administration of XRT, the detriment to normal physiologic processes involved in bone healing and repair have yet to be completely circumvented. The findings of our study are of great clinical import, as we demonstrate the potential efficacy of AMF to preserve the normal biological environment of bone, thereby allowing for improved healing outcomes and possibly, the complete prevention of the series of events leading to the devastating scenario characterized by pathologic fracture and ORN.

Conclusion

We report that prophylactic administration of AMF confers significant protection to the vascular supply of the mandible and correlates with a significant increase in bony unions after HEDR. Our results set the stage for exploration of this targeted therapy alone, and in combination with other treatment regimens, to mitigate the harmful effects of radiation on fracture repair and bone healing in the clinical setting.

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Figure 1. Fracture Unions: Gross and Radiographic Assessment

Representative gross samples (top) from each experimental group with corresponding Maximum Intensity Projection (bottom), illustrating the line of Fx union. The non-irradiated fracture group (Fx, left) demonstrated 100% rate of bony union. The irradiated, fracture specimens (XRT/Fx, middle) demonstrated a 25% rate of bony union. With AMF treatment, the AMF/XRT/Fx group (right) achieved an improved rate of bony union: 57% after radiotherapy.

Figure 2. Micro-Computed Tomography

Maximum Intensity Projections of representative hemimandibular samples demonstrate qualitative differences in vascularity within the fracture gap, highlighted in yellow. The irradiated, fracture specimen (XRT/Fx, middle) exhibits a paucity of vasculature within the ROI as compared to the robust vascular supply exhibited by both the non-irradiated fracture specimen (Fx, top) and the Amifostine treated, irradiated, fracture specimen (AMF/XRT/Fx, bottom). These findings were further quantitatively verified using μ CT vascular analysis.

Figure 3. Vessel Number

Stereological analysis of the vascular tree revealed a significant decrease in vessel number within the fracture gap due to XRT (* denotes statistical difference between Fx and XRT/Fx, $p < 0.05$). AMF protection conferred a 123% increase in vessel number as compared to untreated specimens († denotes statistical difference between XRT/Fx and AMF/XRT/Fx, $p < 0.05$). There was no significant difference in vessel number between Fx and AMF/XRT/Fx, indicating AMF's therapeutic ability to maintain vessel number at levels seen in non-irradiated Fx.

Figure 4. Vessel Separation

Vessel separation is a measure of the distance between individual vessels, providing an inverse measure of vessel density within a region of interest- here, the fracture gap. Stereological analysis of the vascular tree within the fracture gap revealed a statistically significant 55.5% decrease in vessel separation due to AMF protection, displaying AMF's efficacy in therapeutically maintaining vessel density within this region of interest at levels seen in non-irradiated fractured specimens. (* denotes statistical difference between Fx and XRT/Fx, † denotes statistical difference between XRT/Fx and AMF/XRT/Fx, $p < 0.05$).