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Radioprotection with Amifostine Enhances Bone Strength and Regeneration and Bony Union in a Rat Model of Mandibular Distraction Osteogenesis

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Introduction

Irradiation of the craniofacial skeleton is virtually inescapable in the clinical management of head and neck cancer.¹ Radiation has tangible side effects on both soft tissue and bone that are exposed within the target field. Radiation injury begins with the formation of free radicals that lead to a hypoxic milieu, the diminution of cellular populations, the devascularization and atrophy of the tissues, including bone, and ultimately generalized fibrosis.^{1–4}

The harmful sequelae of radiation treatment can also result in the progression of bony destruction to the point of osteoradionecrosis (ORN), which drastically hinders any chance for osteosynthesis and post-oncologic reconstruction.^{5–7} In such cases, craniofacial surgeons are left with the limited option of a mandibulectomy and subsequent free tissue transfer to

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fill in the gap.⁷⁻⁹ Although advantageous for the provision of a good blood supply and an ample source of soft tissue and bone, microvascular procedures such as free tissue transfers remain lengthy and involved operations that are associated with extended hospitalizations.⁷⁻⁹ As such, these procedures are often not a sound option for the elderly and infirm.⁸ Ironically, the patient population most afflicted with HNC is the infirm and elderly, who unfortunately are left with few suitable reconstructive options. Reconstruction utilizing distraction osteogenesis (DO) may serve as a viable solution to this clinical conundrum. The essence of DO is the generation of endogenous, well-vascularized soft tissue and bone from local substrate. The technique is much less complex and time consuming to perform than a free flap, is less expensive, and can often be done on an outpatient basis with much more rapid recuperation.¹⁰⁻¹² DO has been utilized for the reconstruction of congenital mandibular deformities and traumatic injury of the craniomaxillofacial skeleton;¹⁰⁻¹² thus making its use for oncologic reconstruction axiomatic. Unfortunately, the effects of radiation on the loco-regional tissue have heretofore prevented the suitability of such an attractive technique in patients with HNC.

Our laboratory has documented the deleterious effect of radiation damage on bone regeneration and DO in a murine model of mandibular DO¹³ after exposure to a human equivalent dose of radiation (HEDR),^{14,15} which mimics the regimen encountered by HNC patients. In our attempt to remediate the damage of radiation on bone and optimize DO for its use in post-oncologic repair, we investigated therapies directed at circumventing the devastating side effects of radiation treatment. One such therapy is Amifostine (AMF, WR-2721, Ethiol MedImmune, Gaithersburg, MD), an FDA-approved compound that has been successful in the prevention of radiation-induced mucositis and xerostomia in HNC patients.^{16,17} AMF is a free radical scavenger that preferentially stabilizes the cellular DNA of normal surrounding tissue, thereby ameliorating some of the key injurious effects of attendant radiation damage.^{18,19} AMF acts more favorably to safeguard normal cells in part due to their high level of the activating enzyme alkaline phosphatase and pH in contradistinction to the malignant cells.^{18,19} Utilizing our *in vivo* murine model of DO, we previously demonstrated that AMF successfully prevented radiation's harmful effects on both osteocyte number²⁰ and bone mineral density.^{21,22} In this study, we posit that the protection afforded by AMF in soft tissues, bone cellularity, and mineral composition will be reflected in the important clinical correlates of mechanical strength and bony union, thus allowing for successful DO with quantifiable enhancements in the bony biomechanical parameters.

Methods

All animals' experiments were approved by the University of Michigan's Committee on Use and Care of Animals in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, protocol number PRO00001267.

Radiation Protocol

Fifty-two Sprague-Dawley rats weighing approximately 400g (XRT/DO, n=22 and AMF/XRT/DO, n=30) were acclimated for a week prior to radiotherapy. All animals were

anesthetized with a mixture of oxygen and isoflurane. The animals were shielded with a customized lead that exposed their left hemimandible to a fractionated HEDR over 5 days via the Philips RT250 orthovoltage unit (250kV, 15mA-Kimtron Medical, Woodbury, CT). Specifically, a total of 35 Gy of radiation were administered in 7-Gy fractions over 5 days. This HEDR approximates the 70 Gy received by humans in 35 sessions of 2 Gy during the treatment of HNC.

The AMF/XRT/DO group was prophylactically treated with a subcutaneous (SQ) injection of 100mg/kg of AMF forty-five minutes before each daily irradiation. During a 2-week recovery period, animals showing any signs of mucositis or irritation were adequately provided analgesia with Buprenorphine (0.15mg/kg) mixed in 10cc of Lactated Ringer solution given SQ.

Surgery and Post-operative Care

All animals received preoperative analgesia and antibiotic prior to undergoing our surgical procedure as previously described.¹³ Following a unilateral osteotomy to the left mandible posterior to the third molar, a customized bilateral external distraction device was placed. Animals had water available ad libitum along with moist chow and Hill's calorie diet supplementation (Columbus Serum, Columbus, OH). Distraction was carried out after a 4-day latency at a rate of 0.3mm every 12 hours for 8 days to a maximum gap of 5.1mm.¹³ During the consolidation period, animal care consisted of daily records of weights, trimming of overgrown upper incisors, and pin sites evaluation and treatment for any signs of infection. On post-operative day 40, all animals were euthanized. The left hemimandibles were retrieved and all the soft tissue was removed prior to storage at -20°C until testing.

Biomechanical Testing

All specimens were thawed in water at room temperature, then reinforced with stainless steel wires (~1.5 cm in length) that were placed at a 90° angle through both anterior and posterior hemimandible regions. The potting medium, Cerrobend (Cerro Products, Bellefont, PA) was carefully poured within a cylindrical pot containing the posterior region to a depth that excluded the region of interest. Once the Cerrobend had solidified, the anterior region was potted with the last molar concealed into the medium. Following the Cerrobend solidification and cooling, all potted hemimandibles were loaded for uniaxial tension testing at a displacement rate of 0.5mm/s utilizing a servohydraulic machine (858 Mini Bionix II; MTS System Corporation, Eden Prairie, MN). A 50lb load cell (Sensotec, Columbus, OH) was applied while an external linear differential transducer (LVDT, Lucas Schaevitz Inc., Pennsauken, NJ) recorded grip-to-grip displacement. A sampling frequency of 2000 Hz on a TestStar II System (version 2.4;MTS) allowed for data acquisition and generation of load-displacement curves (LDCs). Biomechanical properties of each specimen, breaking load (BL) and yield strength (Y) were extracted from the LDCs and analyzed.

Statistical Analysis

Analyses were performed using SPSS version 23 (SPSS, Chicago, IL). Due to small sizes, Fisher's exact test was used for the assessment of frequency of animals experiencing alopecia, infection, death and the complete bony union at the end of consolidation.

Biomechanical data comparison was completed with ANOVA with Tukey's post hoc method and two-tailed Student's t-test with significance set at $p < 0.05$.

Results

During the recovery period that follows radiotherapy, the AMF-treated animals exhibited fewer signs and symptoms of radiation-induced oral mucositis and severe alopecia (6/30 animals, $p = 0.001$; Fig. 1). On the other hand, the animals from the XRT/DO group required more treatment for those radiation-related discomfort (15/22 animals).

During the consolidation period, infection and overt necrosis of the soft tissues were frequently encountered in the XRT/DO group, however not statistically significant (7 vs 5, $p = 0.318$; Fig. 1). Similarly, complications from infection resulting in animal deaths were comparable in both groups with 7 deaths in the AMF/XRT/DO group versus 5 in the XRT/DO group ($p = 0.318$; Fig. 1). As a result, the subsequent analyses were based on the remaining animals with 22 rats in the AMF/XRT/DO group and 13 rats in the XRT/DO group.

Upon harvest, all the left hemimandibles were examined grossly for complete bony union, in particular across the region of interest behind the third molar. AMF/XRT/DO group clearly demonstrated strong bony union within the gap as opposed to the XRT/DO group, which showed incomplete bony bridging across the DO gap (Fig. 2). This clinical observation was reproduced in the statistical analysis with significantly more bony union in the AMF-treated group (21/25 animals) compared to the untreated group (6/15 animals; $p = 0.004$; Fig. 2).

A total of thirty-five hemimandibles were loaded for uniaxial tension testing and LDCs were generated. The LDCs are a graphical depiction of the correlation between the force or load applied on an specimen and the ensuing deformation or mechanical behavior (Fig. 3). Accordingly, there are two areas defined by the LDC: an elastic (pre-yield) and a plastic (post-yield) that mimic the structural properties of the material. The load is plotted on the y axis in Newton (N) and the corresponding displacement is represented on the x axis in millimeters (mm).

The yield (Y) corresponds to a 10% deflection from linear regression fit at which a permanent deformation of the material is observed and cannot be reversed. The mean Y for XRT/DO was significantly reduced ($21.78 \pm 17.85\text{N}$) compared to the mean Y of AMF/XRT/DO ($39.41 \pm 27.45\text{N}$, $p = 0.023$; Fig. 3, Table 1).

The breaking load (BL) corresponds to the maximum force or ultimate load that can be sustained by the specimen before it breaks. We found that the mean BL for the radiated and distracted group was significantly lower ($34.77 \pm 30.78\text{N}$) than the mean BL of the pre-treated AMF group ($61.74 \pm 48.49\text{N}$, $p = 0.044$; Table 1). Once again, both quantitative observations correlated with the clinical findings described earlier.

The macroscopic analysis of the potted hemimandibles following the biomechanical testing revealed unusual findings. Some of the specimens from either group which appeared to have a complete union of the regenerate area showed an outer cortical envelope of well-formed

bone and a cylindrical-like core that was not completely filled up by bone tissue (Fig. 4). However, their biomechanical parameters were consistently higher than the specimens with non-unions.

Discussion

The clinical management of mandibular defects following tumor extirpation remains challenging. The reconstructive surgeon must fill the gap, restoring cosmesis and function to an already debilitated patient. Distraction osteogenesis (DO) may be an important vehicle for post-oncologic restoration, as it creates healthy new bone from the stretching of a pre-existing local substrate. However, the presence of an irradiated surgical milieu fosters complexity in any prospective reconstruction. Hence, the benefit of a selective radioprotectant therapy such as AMF to thwart radiation's damaging effects while still allowing for adequate cancer treatment. We previously published on the successful cytophylactic and histoprotective ability of AMF on DO in a similar radiation setting with our *in vivo* murine DO model.¹³ The AMF pre-treated specimens demonstrated osteogenesis maintenance and increase bone volume-to-tissue volume ratio that correlated with a higher bony bridging capacity compared with the loss of osteocytes and the increase of immature bone present in the irradiated and distracted mandibles.²⁰

Utilizing, once again, our rodent model of DO, we have also demonstrated AMF-sparing effect on bone mineral distribution.^{21,22} All the radiomorphometric parameters of bone mineral density, tissue mineral density and bone volume fraction were significantly improved with AMF preventive therapy. Correspondingly a higher number of specimens showed complete bony union in the AMF-treated mandibles in contradistinction to the non-treated ones. The aforementioned results led us to confirm our findings with the gold standard of determining bone quality: one that would actually test the strength of the regenerate and ultimately pilot the translation of our bench study to the clinical arena and provide another venue for the treatment of HNC patients.

Our experimental findings validate our hypothesis, whereby the strategic application of AMF successfully protected the soft tissues and strengthened the bony regenerate with quantifiable improved biomechanical parameters. First, our gross findings corroborate with those of our previously published research.^{20–22} We demonstrated that AMF prophylaxis is associated with stronger and more complete bony bridging at the site of the regenerate. Radiation-induced effects consistently altered the biomechanical parameters of Y and BL. Such findings not only correlate with the literature in long bones but also with the authors' prior research. Currey et al demonstrated a considerable reduction of the human femora bending strength at high (94.7kGy) dose of ionizing radiation.²³ Similarly, Nyaruba showed a diminution in the maximum load of Wistar rats tibia after single dose of irradiation (10-60Gy).²⁴ Hamer's investigation of femora cortical allograft after gamma irradiation at 28kGy revealed a 64% decrease in biomechanical strength.²⁵ What distinguishes our work from those studies is that we have formerly established a fractionated human equivalent dose of radiation-HEDR in our *in vivo* murine mandible that mirrors the regimen experienced in the clinical setting by HNC patients.^{14,15} We have also successfully demonstrated how HEDR negatively impacts the mechanical behavior of the bone.²⁶

There have been reports on the decrease biomechanical strength of the regenerate after mandibular DO, along with the decrease in cellularity and bone mineral density parameters.^{27–29} None of those reports, to our knowledge, have any reference to AMF. In fact, the remediation of bone mechanical parameters by AMF in distraction osteogenesis following radiation is an exclusive and innovative application of AMF therapy.

AMF is currently FDA-approved for the protection against radiation-induced xerostomia and mucositis for HNC individuals. AMF selectively assuages the corrosive effects of radiation in normal cells without protecting the tumor cells as they have a higher pH and lack the alkaline phosphatase enzyme warranted to efficiently metabolize the pro-drug WR-2721 to its active metabolite WR-1065.^{18,19} AMF's role on bone cells have been reported by Damron et al who showed that AMF reduced limb loss in the irradiated long bones of Sprague Dawley rats;³⁰ however, their fractionation regimen did not mimic that observed in humans. Gevorgyan's study of gamma radiation (0-10Gy) exposure of mouse calvarial MC3T3-E1 cells or osteoblast-like cells *in vitro* demonstrated that AMF pre-treated cells had significant improvement in cell survival.³¹ Their study limitation is inherent to the use of an *in vitro* experimental model along with a radiation regimen that does not translate to humans' HNC treatment approach.

Nonetheless, the enhancement of bone cellularity and function likely elucidate the changes observed in the mechanical parameters of bone quality and strength that are generated following AMF therapy. We have demonstrated AMF's beneficial influence in a murine mandibular model of pathologic fracture repair.³² In that model, the fracture gap is 2.1 mm and our investigation unequivocally revealed a significant increase in biomechanical properties for the AMF-treated specimens compared to the radiated and fractured group. Those data also imply that the mechanical biology of fracture and DO are different beyond the proliferation of osteoprogenitor cells. The differential response of these two models can be partially attributed to both the greater regenerative challenge of the 5.1mm DO gap in comparison to the fracture space of 2.1mm gap, and additionally differences in biologic response. DO is inherently linked to a substantially higher vascularization than that observed in fracture healing. In addition, the osteosynthesis in DO occurs primarily via an intramembranous process in contradistinction with the endochondral bone formation encountered in fracture repair.³³ There are also significant osteoconductive growth factors and molecules involved in the DO process.³³

In the present study, the findings of a partially filled regenerate cavity after the mechanical test may signify that a complete consolidation of the bone through the regenerate core did not happen, suggesting that perhaps a longer consolidation time would have been necessary to fill in the distraction gap. It is also possible that the cavity is a result of remodeling as the regenerated bone attempts to restore a marrow cavity. However, this has also previously been investigated in our laboratory and bone mineralization and strength were substantially increased by the 28th day of consolidation.³⁴ In fact, since the development of our initial murine model of distraction osteogenesis,¹³ our laboratory has refined the model over the past decade from the establishment of a human equivalent radiation dosage^{14,15,35} to the optimal consolidation period.³⁴ There are limitations inherent to our experimental studies. Although our biomechanical system has been polished to become a pertinent and

quantifiable tool,^{13,27,28,32,34} it is not perfect. In this case the load is applied in a uniaxial direction that is parallel to the fixation device. This setting may not reflect all the intricate forces experienced by the human mandible *in vivo* where multidirectional tensile and compressive loading might be taking place. However, given that the bony union observed from gross inspection parallel the biomechanical data, our hope is that these results will serve as a foundation for successful clinical translation.

The intricacy of the irradiated local substrate and its related prevention of robust wound healing continue to hinder the immense potential of DO as an attractive and reliable reconstructive alternative for HNC treatment. Our previously published data along with the trends demonstrated in our current data undoubtedly indicate that AMF has the capacity to circumvent radiation-induced bone damage. It also strongly implies that AMF might benefit from combination with other therapies designed to augment vascularity and/or reinforcing the organic matrix of the bone. Such studies are currently underway in our laboratory.

Conclusion

The use of DO for post-oncologic reconstruction of HNC is an approaching horizon. The novel application of AMF prophylaxis could establish the potential for optimization of DO in the irradiated surgical bed and help to make this important technique a clinical reality.

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Figure 1. Graph of the number of animals who suffered from radiation-induced severe alopecia (left,) infection (middle) and death (right) (Asterisk (*) indicates statistical significance at $p < 0.05$). XRT-DO: radiated and distracted group. AMF-XRT-DO: Amifostine pre-treated, radiated and distracted group.

Figure 2.

Gross illustration of hemimandibles upon harvest with complete union of the regenerate (dashed area) in a specimen from the Amifostine pre-treated, radiated and distracted group (AMF-XRT-DO, left) compared to the incomplete union observed in a specimen from the radiated and distracted group (XRT-DO group, middle). Graph of the number of animals who had complete bony union of the regenerate area (right). Asterisk (*) indicates statistical significance at $p<0.05$.

Figure 3.

Graph depiction of the load displacement curve (left) with biomechanical parameters Breaking Load or ultimate load and Yield. Biomechanical graphs depicting the mean Yield (middle) and mean Breaking Load (right) for the XRT-DO, radiated and distracted group. AMF-XRT-DO, Amifostine pre-treated, radiated and distracted group (Asterisk (*) indicates statistical significance at $p < 0.05$. Error bars denote $\pm$ one standard deviation).

Figure 4. Photograph of solid appearing regenerate after uniaxial tension testing (left). Note the cortical envelope and the incompletely filled core of the hemimandible (right).

Table 1

Mean values for the biomechanical parameters of Yield and Breaking Load in Newton (N) for the XRT-DO, radiated and distracted group. AMF-XRT-DO, Amifostine pre-treated, radiated and distracted group.

	Yield (N)		Breaking Load (N)		P value
	XRT/DO	AMF/XRT/DO	XRT/DO	AMF/XRT/DO	
Mean	21.78	39.41	34.77	61.74	0.044