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Amifostine Prophylaxis on Bone Densitometry, Biomechanical Strength and Union in Mandibular Pathologic Fracture Repair

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Abstract

Background—Pathologic fractures (Fx) of the mandibles are severely debilitating consequences of radiation (XRT) in the treatment of craniofacial malignancy. We have previously demonstrated Amifostine's effect (AMF) in the remediation of radiation-induced cellular damage. We posit that AMF prophylaxis will preserve bone strength and drastically reverse radiotherapy-induced non-union in a murine mandibular model of pathologic fracture repair.

Materials and Methods—Twenty-nine rats were randomized into 3 groups: Fx, XRT/Fx, and AMF/XRT/Fx. A fractionated human equivalent dose of radiation was delivered to the left hemimandibles of XRT/Fx and AMF/XRT/Fx. AMF/XRT/Fx was pre-treated with AMF. All groups underwent left mandibular osteotomy with external fixation and setting of a 2.1mm fracture gap post-operatively. Utilizing micro-computed tomography and biomechanical testing, the healed fracture was evaluated for strength.

Results—All radiomorphometrics and biomechanical properties were significantly diminished in XRT/Fx compared to both Fx and AMF/XRT/Fx. No difference was demonstrated between Fx and AMF/XRT/Fx in both outcomes.

Conclusion—Our investigation establishes the significant and substantial capability of AMF prophylaxis to preserve and enhance bone union, quality and strength in the setting of human equivalent radiotherapy. Such novel discoveries establish the true potential to utilize pharmacotherapy to prevent and improve the treatment outcomes of radiation-induced late pathologic fractures.

Keywords

Pathologic fracture; osteoradionecrosis; Amifostine; bone density; biomechanical strength

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Disclosure Page

None of the authors have any conflict of interest.

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Introduction

Late pathologic fractures are one of the most debilitating and devastating sequelae related to adjuvant radiotherapy in the treatment of craniofacial malignancies.^(1–9) Pathologic fractures are also a part of a corrosive spectrum of clinical manifestations of osteoradionecrosis that include bone necrosis and fistula formation.^(1–9) Osteoradionecrosis takes place after irradiated bone undergoes devascularization and exposure through its overlying skin or mucosa and fails to heal over a three-month period in the absence of a local tumor.^(2–9) The injury occurs secondary to a cascade phenomenon of radiation induced-hypoxic milieu, cellular depletion or hypocellularity and blood vessels obliteration or hypovascularity.^(2,3,9,10)

In the craniofacial skeleton, the mandible is the most commonly affected bone with the incidence of mandibular osteoradionecrosis and late pathologic fractures reportedly as high as 8.2% and 6% respectively.^(4–6,8,11,12) The discrepancy in the incidence rates reported is based on the various doses of radiation delivered in those reports. The mandible is more susceptible to pathological fracture because of its particularly vulnerable set of anatomical features. It is a cortical and compact bone with a tenuous blood supply from the inferior alveolar artery and limited collateral circulation. Pathologic fractures are commonly reported in relation to osteoradionecrosis, which may occur as early as few months after the completion of radiotherapy to several years post-radiation or the so-called later onset osteoradionecrosis.^(4–6,8,11,12) The basis of the timeframe of pathologic fractures occurrence resides in many factors such as those inherent to the patient (oral hygiene, periodontal disease, infections, abscesses), tumor site, surgical extirpation during irradiation and radiotherapy delivery and dosage.^(9,10) Radiation inflicts multiple insults on both the cellular and the vascular constituents of bone; significantly escalating the risk for delayed fracture unions and severely undermines the chances to heal and achieve union.^(2,3,9–12)

Osteosynthesis and efforts at reconstruction of mandibular fractures secondary to radiation can be quite challenging; requiring both structural and functional considerations as well as strategies that can address the devastating effects upon the patient's quality of life. Surgeons have attempted a large variety of treatment options to address the noxious sequelae of radiation on fracture healing. These options have ranged from conservative approaches with local wound care, ultrasound treatment, and adjuvant hyperbaric oxygen therapy, to the insertion of large metal plates, the use of non-vascularized bone grafts and the use of vascularized osseous free flaps.^(6–8,11,12) Unfortunately, each of those options have drawbacks such as plate extrusion, infections, donor site morbidity, high cost and modest to poor outcomes.⁽⁸⁾ Consequently, the development of late pfx presents the clinician with an exceptionally difficult infirmity to successfully treat necessitating the discovery of effective therapies that would thwart the corrosive and detrimental effects of radiation on bone healing and repair.

Our laboratory has previously investigated a radio-protective drug Amifostine (AMF, WR-2721, Ethiol MedImmune, Gaithersburg, MD) on irradiated murine mandibles. AMF is a pro-drug that gets dephosphorylated by the cell membrane alkaline phosphatase into an active metabolite that scavenges radiation-induced free radicals; thereby protecting cellular DNA from an avalanche of significant deleterious effects.⁽¹³⁾ AMF selectively guards normal cells because they have a significantly higher content of the key activating enzyme alkaline phosphatase in contrast to the tumor cells.^(13,14) AMF has been FDA-approved for the prevention of radiotherapy-induced xerostomia and mucositis in head and neck cancer patients.^(13,14) It has also shown successful remediation of radiation damage on osteocytes⁽¹⁵⁾, microvasculature^(13,14) and bone mineralization^(17–19) in our in vivo experimental model of murine mandibular distraction osteogenesis.⁽²⁰⁾

We posit that AMF prophylactic therapy will significantly prevent radiation-induced non-union and maintain bone quality and strength in our murine mandible model of pathologic fracture repair. Our goal is to establish a successful pharmacologically based treatment strategy aimed at the prevention of late pathologic fracture in any patient where bone is in the target field. Achieving such objective holds the promise to significantly impact the scourge of radiation associated morbidity for the tens of thousands of patients diagnosed with cancer every year.

Materials and Methods

Sprague Dawley male rats (~400 g) were randomized into three experimental groups: Fx (n=5), XRT/Fx (n=14), and AMF/XRT/Fx (n=10). The University of Michigan's Committee for the Utilization and Care of Animals approved all animals' experiments as performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Radiotherapy

A human equivalent dose of radiation (or bioequivalent radiation dose of 70Gy in human) was delivered in a 7Gy daily fraction over five days to the left hemimandible of both XRT/Fx and AMF/XRT/Fx animals, utilizing the Philips RT250 orthovoltage unit (250kV, 15mA-Kimtron Medical, Woodbury, CT), in accordance to a protocol performed and previously described in our laboratory.^(16–19,21–23) A subcutaneous injection of 100mg/kg of AMF was prophylactically administered to AMF/XRT/Fx forty-five minutes prior to irradiation. Fx did not undergo any radiation or AMF pre-treatment.

Surgical and Postoperative Procedures

Following preoperative analgesia and antibiotic administration, all animals underwent a unilateral left mandibular osteotomy with bilateral external fixator placement as previously described.^(15,16,19–24) To prevent peri-operative blood loss, a 4 to 8-hour interval was observed prior to setting a 2.1mm fracture gap. On post-operative day forty, animals were euthanized and left hemimandibles were harvested devoid of soft tissue and evaluated for bony bridging or complete union versus non-union.

Micro-Computer Tomography (μCT)

Hemimandibles were scanned with eXplore Locus SP μCT (GE Healthcare Bioscience, Fairfield, CT) utilizing 80kVp, 80mA and 1100ms exposure, where 392 projections were taken at a resolution of 45μm voxel size. The μCT machine was calibrated prior to scanning each specimen into a chilled dH₂O solution. Each individual scan was reconstructed and reoriented in a 3-dimensional (x, y, z) plane until all axial, coronal and sagittal sections were alignment with a +X representing the mandibular length from posterior to anterior, +Z representing the mandibular height from inferior to superior, and +Y representing the depth (or lingual to buccal thickness) of the mandible. The region of interest (ROI) is delineated posteriorly by the third molar, extends 2.1mm and corresponds to the fracture gap. Utilizing the spline tool of the MicroView 2.2 software (GE Healthcare, Milwaukee, WI), several contours were generated on serial sagittal sections that highlighted the ROI, with selective inclusion of bone. For uniform data analysis of the ROI, rotations, cropping of non-bone space and exclusion of the incisor, canal, tooth and periodontal tissue were performed. Microview assigns a grayscale value to each voxel and uses an algorithm that converts the grayscale to mineral content.

Microdensitometry parameters including bone volume fraction (BVf), bone mineral density (BMD) and tissue mineral density (TMD) were quantitatively measured. BVf is defined as

the volume of newly formed bone divided by the total volume of the gap or region of interest ROI (which includes air, soft tissue, water and marrow). BMD is defined as the ratio of bone mineral content (mass) of newly remodeled bone over the total volume of the ROI. BMD represents the amount of bone within the entire ROI (which includes air, soft tissue, water and marrow).

Tissue mineral density (TMD) is defined as the ratio of tissue mineral content over the bone volume of the ROI. TMD represents the average mineral density for only the tissue identified as bone within the ROI (which excludes air, soft tissue, water and marrow). Maximum intensity projections (MIPs) of each hemimandible were obtained for qualitative illustration. MIPs allow for instant volume representation of a volumetric data set into a three-dimensional visualization of the specimen scanned.

Biomechanical Testing

Hemimandibles were mounted on cylindrical fixtures with initial potting of the posterior hemimandible in bismuth alloy medium (Cerrobend, Cerro Products, Bellefont, PA) to a depth that was excluding the ROI, followed by the potting of the anterior hemimandible. Once the potted medium and specimens have solidified and sufficiently cooled down, they were loaded to failure for uni-axial tension testing at a displacement rate of 0.5mm/s. Grip to grip displacement was recorded and data were acquired at a 2000Hz sampling frequency. Load-displacement curves were generated and analyzed for breaking load (BL) and yield strength (Y).

Statistics

One-Way ANOVA with post-Tukey (SPSS 19.0;SPSS, Inc., Chicago, IL) was performed and results were considered statistically significant at $p<0.05$.

Results

In the immediate post-radiation and recovery interlude, all radiated animals showed signs and symptoms of oral mucositis and alopecia. In contradistinction, the animals pre-treated with AMF exhibited much less signs of radiation-induced stress. Similarly, during the consolidation and healing hiatus, the majority of XRT/Fx animals demonstrated evidence of the late sequelae of radiotherapy such as soft tissue and mucosal necrosis, exposure of the underlying bone and in the worst-case scenario, bone necrosis. Gross examination of the specimens after sacrifice and dissection clearly revealed remarkable differences among the groups. All hemimandibles (100%) from both Fx and AMF/XRT/Fx showed complete union across the fracture gap; whereas, the specimens from XRT/Fx demonstrated a bony union rate of only 20% within the same ROI (Fig. 1). Qualitative imaging with MIPs unambiguously confirmed both bridged and non-healed fracture gaps (Fig. 1).

Radiomorphometric parameters demonstrated significant differences among the groups (Fig. 2;Table 1). Specifically, the mean BVF (unitless) was significantly decreased in XRT/Fx compared to both Fx and AMF/XRT/Fx. (0.35 vs 0.80 and 0.76; $p<0.001$). The mean BMD (mg/cc) also demonstrated significant reduction in the irradiated fracture group compared to Fx and AMF/XRT/Fx by 56% and 51.6% respectively ($p<0.001$). Along the same line, the mean TMD (mg/cc) of XRT/Fx was also quantitatively lower than that of the standard fracture and the AMF pre-treated groups. (643.85 vs 805.45 and 750.93mg/cc; $p=0.001$). Conversely no differences were observed between Fx and AMF/XRT/Fx in all three microdensitometrics (BVF, $p=0.954$; BMD, $p=0.732$; TMD, $p=0.454$).

Load-displacement curves depict the mechanical response or deformation of the hemimandible regenerate after it has been subjected to a load. The load-displacement curve

is a plot of the force applied (in Newtons) to the specimen and the ensuing displacement (in millimeters) (Fig. 3). It has two distinct areas: elastic (pre-yield) and plastic (post-yield), which reproduce the properties of the cortical mandible in situ that are influenced by its thickness and its mechanical quality.^(22,24) The biomechanical parameters derived from the load-displacement curves include Y and BL (Fig. 3; Table 1). The Y is defined as the point/load at which deformation of the specimen occurs (10% deviation from linear regression fit).^(22,24) Mean Y obtained in XRT/Fx demonstrated striking difference compared to Fx (23.55 vs 81.92N; $p=0.005$). In addition, the XRT/Fx mean Y was also significantly lower than that of AMF/XRT/Fx (23.55 vs 69.97N; $p=0.005$). Interestingly, no discrepancy could be appreciated between the mean Y of Fx and AMF/XRT/Fx ($p=0.779$).

BL is the maximum load sustained by the mandible that results in breakage of the specimen.^(22,24) The mean BL of XRT/Fx was significantly decreased by 64.7% compared to Fx ($p=0.018$) and diminished by 61.4% compared to AMF/XRT/Fx ($p=0.01$). On the other hand, the AMF treatment group demonstrated a mean BL that was indistinguishable from that of Fx ($p=0.931$).

Discussion

In spite of advances in the delivery and the optimization of radiation protocols, radiotherapy of head and neck malignancies is associated with substantial injuries to the oral mucosa, macro and microvasculature, muscle and osteoprogenitor cells.^(9,10) Pathologic fractures and the associated osteoradionecrosis tend to settle more favorably within the mandible in more than 60% of the cases because of the mandible's vulnerable blood supply.⁽¹⁻⁸⁾ The delayed non-unions remain a significant source of severe disability among the already infirmed head and neck cancer population.⁽²⁻¹²⁾ Unfortunately, little success has been achieved in the formulation of therapeutic regimens that can successfully and predictably treat radiation-induced non-unions secondary to pathologic fractures. This clinically relevant reconstructive dilemma has prompted our investigation to both elucidate the underpinnings of radiation-induced pathologic fractures and to develop efficacious therapies that will function to optimize bone repair in the setting of radiotherapy. The FDA-approved antioxidant compound AMF has successfully proven its free-radical scavenger role in the prevention of radiation-induced xerostomia and mucositis.^(13,14) Furthermore, AMF has shown a prophylactic role in the safeguard of bone osteoprogenitor cells in both an *in vivo* and *in vitro* model.^(15,25)

The work of Damron et al is among the few *in vivo* reports on AMF influence on bone cells. They harvested both the tibia and femur of Sprague Dawley rats after an irradiation exposure to three equal radiation fractions of 25Gy along with incremental injections of AMF. They determined that AMF doses of 100 and 200mg/kg successfully reduced limb loss beyond the results observed with the use of fractionation alone. Their study limitation, which they acknowledge, resides in a fractionation regimen that was not equivalent to that used in humans.⁽²⁵⁾

In that regard, our laboratory has established a human bio-equivalent fractionation radiation dosimetry regimen⁽²¹⁻²³⁾ utilizing an *in vivo* murine mandibular model of fracture healing and repair. We have since utilized this model to mirror what occurs in the human clinical scenario in order to remediate the changes induced by radiotherapy.

There have been conflicting reports in the literature on the effects of radiation on bone mineralization. Intuitively, radiation causes degradation in the bone mineral composition.^(26,27) Yet, Stutz 'evaluation of patients following orchidectomy and radiation treatment for seminoma did not reveal any differences in BMD between irradiated and non-

irradiated hips.⁽²⁸⁾ Nevertheless, there have been clinical studies that have reported the diminution of bone mineral content in the lumbar spine of cervical cancer patients following radiation.⁽²⁹⁾ Hamada et al described disturbances in bone mineral metabolism of the lumbar vertebra that might be occurring in the early phase of radiation.⁽³⁰⁾

On the other hand, Reinbold et al have assessed bone density of vertebral metastases after a 40Gy total dose of radiation delivered over 4 weeks. Interestingly, they showed an initial 24.7% decrease in bone density followed by a 60.6% increase at the end and 3 months after the radiation treatment.⁽³¹⁾

Verdonck et al quantified the effect of Cobalt radiation (three doses of up to 8Gy with seven days intervals for a total dose of 24Gy) on the bone mineral density of the maxilla and mandibles of minipigs utilizing μ CT. They observed higher values of BMD in the irradiated minipigs compared to the non-irradiated control, though not statistically significant; that they suggested was due to a bone sclerosis effect of radiation on BMD.⁽³²⁾

Herein, we found a relative inhibition of bone formation by radiation that was readily reflected upon the gross examination of the specimens. Subsequently, the microdensitometry results of our study quantifiably demonstrated radiation effects on bone mineralization. Specifically, the irradiated and fractured samples showed diminution in all radiomorphometrics in comparison to Fx and AMF/XRT/Fx. AMF pre-treatment of the radiated and fractured mandibles, however, demonstrated microdensitometric results that paralleled those of the standard control, non-irradiated fracture mandibles. Our findings in this report are in line with our previous studies which capitalized on the exceptional analytical capacity of μ CT to assess the different facets of bone mineral density distribution^(17,19,33) in response to radiation. In fact, our previous findings not only demonstrated significant alterations in radiomorphometrics but also showed that those metrics became normalized with the prophylactic use of AMF.^(17,19) From related experiments in our laboratory, using the human equivalent dose of radiation, we also determined that radiation might cause either a low or a high (even a hyper-) pattern of mineralization that might subsequently be severely damaging to the material properties of bone. In the context of low mineralization, there was reduced level of bone biomechanical parameters.⁽²²⁾ On the other hand, high or hypermineralization might also result in reduced bone strength, as the heterogeneity in mineralization is lost.⁽¹⁷⁾ Our data successfully revealed the ability of AMF to effectively uphold bone osteoprogenitor cells, which was likely associated with the maximization of bony unions within the AMF pre-treated specimens in our present study, further delineating the correlation between radiation, bone constituent cells, mineralization, and bone regeneration and healing.

The second part of this study involved the evaluation of bone quality and strength, which ultimately is one of the essential rights of passage from the bench to the bedside in order to be able to truly accomplish clinical translation. Yet, no other study, to our knowledge has evaluated the biomechanical properties of irradiated bone pre-treated with AMF. Numerous studies, however, have been published on the pernicious effect of radiation on bone strength. Research in humans has shown that fracture risk was consistently increased in postmenopausal women who had received pelvic radiation treatment⁽³⁴⁾ and in post-radiotherapy patients treated for non-Hodgkin's malignant lymphoma of the bone.⁽³⁵⁾

Sugimoto's rabbit model of long bone, specifically tibia, exposed to a single radiation dose of 50Gy of external beam radiation, utilized a three-point bending strength test and showed a decrease in bending strength by 50%.⁽³⁶⁾ Currey et al looked at the impact of both a low (17kGy) and a high (94.7kGy) dosage of ionizing radiation on the mechanical properties of femora from human cadavers. They demonstrated significant reduction in bending strength

with the more damaging effect at the higher radiation dosage.⁽³⁷⁾ All of these previous studies corroborate each other on the diminution of the mechanical strength of the bone secondary to radiation-induced change in the structural aspect of bone matrix. Such alterations ultimately bring a new dynamic in the marriage of the organic matrix and the inorganic mineral that previously gave the bone its unique quality and strength.^(38–40) A drawback of many of the aforementioned studies concern the disparate and wide-ranging methods relating to the type, delivery and dosing of radiation. In our attempts to reproduce radiation effects at the human equivalent dose of radiation, we have previously demonstrated a substantial dose-dependent reduction in bone parameters Y and BL.⁽²²⁾ In this study, the load-displacement curves showed a clear discrimination in the derivatives obtained during the linearly elastic and the plastic (or non-linear) mechanical behavior of the cortical mandible before and after the yield point. In XRT/Fx, the Y was significantly lower than that obtained in either Fx or AMF-treated groups. However, no difference in Y was demonstrated between Fx and AMF/XRT/Fx specimens. The changes observed in the mean Y between all the groups were reproduced in the mean BL results across all the groups. Consequentially, we could deduce that radiation has induced impairment of the ability of bone to resist loads beyond the yield point. This impairment is most likely explained by the negative impact radiation has on bone mineralization (BVF, BMD, TMD); as bone strength and quality is strongly dependent on bone mineral density.^(41,42) Conversely, AMF prophylaxis prevented the damaging effect of radiation and significantly restored the hemimandible's ultimate strength, which paralleled that of the Fx group.

For the first time, to our knowledge, we have successfully demonstrated the protective effect of AMF on bone mechanical quality, strength and function in a murine mandibular model of delayed Fx union. The results of our study may have immense clinical impact if our findings can be translated from the bench to the bedside. In fact, AMF demonstrates the potential to become one of the most effective radioprotective drugs found to date in the prevention of the unrelenting and debilitating effects of late pathologic fractures and osteoradionecrosis.

Conclusion

Our investigation establishes the significant and substantial capability of AMF to preserve and enhance bone quality and strength in the setting of human equivalent radiotherapy. In addition, our results also demonstrate the clinically significant finding of an immense reduction in radiation-induced non-union as a consequence of AMF prophylaxis. These novel discoveries establish the true potential to utilize pharmacotherapy to both prevent and vastly improve the treatment outcomes of radiation-induced late pathologic fractures. Further studies will now need to be instituted to begin to translate these findings from the bench to the bedside in order to assuage the pain and suffering of patients with head and neck cancer and the radiation-induced morbidity associated with their clinical management.

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Highlights

Amifostine's effect on quality and strength of mandible pathologic fracture

Radiated samples differ from Fracture Control and Amifostine-treated samples

No difference between Amifostine-treated specimens and Fracture in all metrics

Amifostine prophylaxis protects bone quality and strength in the setting of radiation

Figure 1.

Left panel-Gross examination of left hemimandible specimens of the fracture group (Fx, top), radiated and fracture group (XRT/Fx, middle), and Amifostine pre-treated radiated and fracture group (bottom). Notice the complete bony union across the fracture gap (red arrow) in both Fx and AMF/XRT/Fx compared to the non-union exhibited in XRT/Fx. Right panel-Maximum Intensity Projections. Observe the unambiguous lack of union within the fracture gap (red arrow) of XRT/Fx (middle) compared to Fx (top) and AMF/XRT/Fx (bottom).

Figure 2. Bone Volume Fraction (left), Bone Mineral Density (middle) and Tissue Mineral Density (right) means demonstrating Amifostine's therapeutic effect (*, denotes statistical significance at $p < 0.05$).

Figure 3. Load-displacement curve (left) with the distinctive biomechanical parameters of Yield and Breaking Load. Graphs of Yield (middle) and Breaking Load (right) means demonstrating Amifostine's therapeutic effect. (*, denotes statistical significance at $p < 0.05$).

Table 1

Radiomorphometric and biomechanical parameters means with standard deviations. ((*) denotes the p value between Fx and XRT/Fx; (†) denotes the p value between XRT/Fx and AMF/XRT/Fx; (‡), denotes the p value between Fx and AMF/XRT/Fx).

Radiomorphometrics	Means ± SD			p value		
	Fx	XRT/Fx	AMF/XRT/Fx	*	†	‡
BYF (unitless)	0.802 ± 0.098	0.350 ± 0.28	0.572 ± 0.05	0.001	<0.001	0.954
BMD (mg/cc)	710.27 ± 104.13	312.22 ± 207.83	645.03 ± 64.82	<0.001	<0.001	0.732
TMD (mg/cc)	805.45 ± 76.78	643.85 ± 97.35	750.93 ± 54.82	0.002	0.011	0.454
<u>Biomechanical parameters</u>						
Yield (N)	86.55 ± 25.75	30.56 ± 32.05	79.32 ± 45.91	0.005	0.005	0.779
Breaking Load (N)	81.92 ± 31.27	23.55 ± 28.23	69.97 ± 37.65	0.018	0.01	0.931