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Quantitative Histologic Evidence of Amifostine Induced Cytoprotection in an Irradiated Murine Model of Mandibular Distraction Osteogenesis

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Abstract

Background—Head and neck cancer (HNC) management requires adjuvant radiation therapy (XRT). The authors have previously demonstrated the damaging effect of a human equivalent dose of radiation (HEDR) on a murine mandibular model of distraction osteogenesis (DO). Utilizing quantitative histomorphometry (QHM), our specific aim is to objectively measure the radio-protective effects of Amifostine (AMF) on the cellular integrity and tissue quality of an irradiated and distracted regenerate.

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Methods—Sprague Dawley rats were randomly assigned into 2 groups: XRT/DO and AMF/XRT/DO, which received AMF prior to XRT. Both groups were given HEDR in 5 fractionated doses and underwent a left mandibular osteotomy with bilateral fixator placement. Distraction to 5.1mm was followed by a 28-day consolidation period. Left hemimandibles were harvested. QHM was performed for osteocyte count (Oc), empty lacunae (EL), Bone Volume/Tissue Volume (BV/TV) and Osteoid Volume/Tissue Volume (OV/TV) ratios.

Results—AMF/XRT/DO exhibited bony bridging as opposed to XRT/DO fibrous unions. QHM analysis revealed statistically significant higher Oc and BV/TV ratio in AMF-treated mandibles compared with irradiated mandibles. There was a corresponding decrease in EL and the ratio of OV/TV between AMF/XRT/DO and XRT/DO.

Conclusion—We have successfully established the significant osseous cytoprotective and histoprotective capacity of AMF on DO in the face of XRT. AMF-sparing effect on bone cellularity correlated with an increase in bony union and elimination of fibrous union. We posit that the demonstration of similar efficacy of AMF in the clinic may allow the successful implementation of DO as a viable reconstructive option for HNC in the future.

Introduction

Head and neck cancer (HNC) poses a significant biomedical burden despite the consequential advances made in treatment regimens. Notably, according to the 2011 Cancer Statistics estimate there were 7,900 deaths per 39,400 new cases of HNC.¹ Conventional treatment of HNC involves surgical resection of the tumor followed by adjuvant radiotherapy and reconstruction.^{2,3} Unfortunately, facial reconstructive options are very limited. Free tissue transfer is the standard reconstructive strategy in most centers.^{4,5} It provides a substrate for reconstruction from the transfer of soft tissue and bone harvested on a vascular pedicle from a donor site outside of the affected area. The operation is a lengthy and extensive process and the composite flaps often provide substantial bone at the expense of tenuous soft tissue coverage. The operations are expensive and require extended lengths of stay. In addition, associated wound healing complications, such as fistula formation, often result in the delay of the initiation of the radiation treatment (XRT), thus further jeopardizing the patient's quality of life and prognosis.^{6,7}

Distraction osteogenesis (DO) is an innovative source of endogeneous tissue regeneration that stimulates the growth of new bone by the gradual separation of two osteogenic fronts. In the craniofacial region, DO was initially employed to correct congenital mandibular anomalies, however, it has since evolved to a more sophisticated tool that is now an important part of the reconstructive surgeon's armamentarium for an ever widening array of applications.^{8–13} This important technique provides the advantages of generating robust endogeneous tissue, avoiding donor site morbidity, and inducing the simultaneous production of bone and soft tissue in order to attain a structurally and functionally successful reconstruction. Furthermore, patients undergoing DO can often recover in the outpatient setting, resulting in a decrease in overall cost and morbidity.¹⁴ Elderly and infirmed patients are often not candidates for extensive free tissue transfer operations because of their overall poorer health, in addition failed free flaps may often require strategies for reconstruction that do not include another free tissue transfer. The utilization of DO for tissue replacement after oncologic resection or as a reconstructive option for deformations secondary to irradiated bone could have immense potential therapeutic ramifications. Unfortunately the ravages of radiation-induced damage, which include the diminution in essential osteogenic cells, the destruction of blood vessels and the creation of an hypoxic milieu, have all conspired to preclude the predictable and reliable use of DO in this patient population. This important conundrum has led us to investigate the use of a radioprotective drug, Amifostine (AMF or WR-2721, Ethylol, MedImmune, Gaithersburg, MD), that would be given to the patient prior

to radiotherapy to mitigate the damaging effects of ionizing radiation. AMF is a FDA-approved drug found to selectively protect the normal tissues from both radiation-induced xerostomia in HNC and cisplatin-induced nephrotoxicity in ovarian cancer or non-small-cell lung cancer. Its action resides in the dephosphorylation of the prodrug WR-2721 to its active sulfhydryl metabolite WR-1065 by the membrane-bound alkaline phosphatase. WR-1065 scavenges the free radicals generated by ionizing radiation on cellular DNA and donates hydrogen molecules to repair damaged target molecules.^{15–18} In fact, previous work in our laboratory has demonstrated that AMF pre-treatment on normal bone resulted in a quantifiable remediation of the mineralization profile of bone.¹⁹ Subsequent microcomputed tomography of irradiated and distracted mandibles revealed AMF's preservation of bone mineral density and complete bony bridging of the regenerate.²⁰ We hypothesize that the inability of DO to produce a strong regenerate in the irradiated mandible is the result of the depletion of osteogenic cells, which could be prevented by the prophylactic utilization of AMF prior to radiation. Specifically, our aim is to objectively measure the radioprotective effects of AMF on the cellular integrity and tissue quality of the irradiated and distracted regenerate utilizing quantitative histomorphometry (QHM). We posit that AMF will protect the constitutive cells of the distraction gap that are critical for bone regeneration and healing from the damaging effects of radiation. We also posit that AMF's protection will quantitatively be reflected upon the ratio of bone volume/tissue volume and the ratio of osteoid volume/tissue volume. Our long-term goal is to mitigate the pathologic effects of radiation via AMF prophylactic therapy and optimize bone regeneration in the setting of adjuvant radiotherapy for HNC patients.

Methods

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. Eighteen Sprague Dawley rats weighing approximately 400 g were obtained through the University of Michigan's Unit for Laboratory Animal Medicine (ULAM). The animals were randomly assigned to 2 groups: Group 1, XRT/DO (n=8) and Group 2, AMF/XRT/DO (n=10).

Radiation protocol

All animals left hemimandibles received a human bioequivalent radiation dose of 70Gy fractionated over 5 days via a Philips RT250 orthovoltage unit (250 kV, 15 mA, Kimtron Medical, Woodbury, CT) according to established ULAM and UCUCA approved protocols in our laboratory and the department of Radiation Oncology.^{21–23} Group 2 was pre-treated with a subcutaneous injection of AMF (100 mg/kg) 45 minutes before radiation. The animals were anesthetized using a mixture of Oxygen and Isoflurane. The rats were placed with the right side down under a custom-lead shield designed to expose exclusively the left hemimandible. The animals were housed in a pathogen-free facility on a 12-hour light/dark schedule where they were fed soft chow and water for two weeks. Rats exhibiting any signs of radiation-related stress were administered Buprenorphine (0.03mg/kg) with 10 cc Lactated Ringer (LR) solution.^{21–23}

Surgical procedure, post-operative animal care and DO

The surgical procedure has been described previously using a technique developed and reported in our laboratory.^{20,24–28} Following, pre-operative antibiotic and analgesia, customized bilateral titanium external fixator devices were placed along with a left unilateral vertical osteotomy. Animals were given buprenorphine with 10cc LR SQ every 12 hours through post-operative day (POD) #4 and prn thereafter. Animals' weights were recorded daily and pin sites were monitored and cleaned. Both groups underwent DO, which

consisted in 0.3mm half turn of the distraction device every 12 hours to a maximal distraction gap of 5.1mm. The animals had a 28-day consolidation period followed by euthanasia and harvest of all left hemimandibles.

Tissue harvest

All specimens were processed and embedded following a protocol the authors have previously described.^{22,26,27} Blocks were sectioned coronally from anterior to posterior into 7 μ m thick sections taken on a Leica Reichert-Jung microtome (Model 2030 Biocut, Germany) and mounted on glass slides. Every 7th slide was selected to uniformly represent the distraction gap, i.e. our region of interest (ROI), which spanned a distance of 5.1 mm posterior to the 3rd molar. The selected sections were surface-stained with Gomori's "One step" Trichrome. Two slides representative of the mid-gap of the ROI were chosen per specimen for histomorphometric analysis.

Quantitative Histomorphometry (QHM)

Digital Color Analysis—Using the imaging analysis software program Bioquant®NOVA Osteo version 7 (R&M Biometrics, Nashville, TN), digital images of each slide were obtained for the placement of a standard template over the ROI and color thresholding. The digital color analysis thresholds/attributes a blue/green color to the mature, mineralized bone color, and a red/pink color to the osteoid, or immature bone.

The following were obtained by three independent reviewers:

- Tissue volume (TV) or the total volume of the ROI template;
- Bone volume (BV), or the volume of mineralized bone within the ROI;
- Osteoid volume (OV), or the volume of non-mineralized, immature bone and vacuoles within the ROI.

The ratios of BV/TV and OV/TV were subsequently calculated the Bioquant® software.

Point counting of Osteocytes and Empty lacunae—Using a light microscope interfaced with a digital camera connected to a computer and the Bioquant software, the ROI was superimposed on the digital image. Nine high power field (HPF) images were randomly selected per ROI at the 16 $\times$ magnification and stored as TIFF files. Point counting of osteocytes (Oc) and empty lacunae (EL) was performed by three independent reviewers.

Statistics

Values were analyzed with the independent Samples t-test using SPSS for Windows version 19.0 (Chicago, IL) and statistically significance considered at $p = 0.05$.

Results

All animals tolerated the surgery and recovered successfully. No significant weight loss or wound infections were observed. Complete bony bridging was observed in all AMF pre-treated animals, whereas the irradiated group exhibited fibrous unions and incomplete bridging in 75% of the specimens (Fig.1). Qualitative analysis of the histologic slides revealed consistent differences between the AMF pre-treated group and the irradiated group in the region of interest (Fig.2) Specifically, the AMF treated specimens showed a much greater degree of new bone formation represented by a substantial increase in turquoise staining woven bone compared to the irradiated slides (Fig.2, right). Precise directional patterning could not be seen in the microscopic examination of the healing bone in either group. Non-mineralized immature bone or red staining osteoid bone was more

predominantly observed in XRT/DO slides (Fig.2, left). Examination under $16\times$ magnification further demonstrated differences in cellularity between both groups (Fig.3). More mature osteocytes were consistently detected within the bone matrix in the AMF pre-treated group; whereas fewer osteocytes were found irregularly scattered within the regenerated gap of the irradiated group (Fig.3, left). There was a corresponding decrease in the presence of empty lacunae in the AMF pre-treated group (Fig.3, right).

QHM analysis

Quantifiable differences in cellular integrity and tissue quality were demonstrated between those animals treated with AMF and those that were not. The mean osteocyte count per high power field (HPF) was statistically significantly higher in the AMF/XRT/DO group compared to the XRT/DO (68.62 ± 9.6 vs. 43.8 ± 1.4 osteocytes; $p=0.000$; Fig.4, left), demonstrating a 57% increase in the amount of mature bone cells. There was also a corresponding decrease in the number of EL between the AMF-treated and irradiated groups (3.95 ± 1.5 vs. 11.4 ± 6.1 EL; $p=0.002$; Fig.4, right). The AMF pre-treated specimens demonstrated a significant difference in mature bone as anticipated by an increase in the ratio of bone volume/tissue volume ($74.24 \pm 6.44\%$ vs. $53.05 \pm 11.49\%$; $p=0.000$; Fig.5, left) when compared to the irradiated distracted specimens. A resultant decrease in the percentage of immature bone was also demonstrated in the ratio of osteoid bone volume/tissue volume in the AMF/XRT/DO comparatively to the XRT/DO group ($25.83 \pm 5.55\%$ vs. $42.23 \pm 5.05\%$; $p=0.000$; Fig.5, right).

Discussion

DO has emerged as an innovative and attractive tool for endogenous bone tissue regeneration. It has evolved over many years from an esoteric technique that found use in the repair of congenital mandibular anomalies to a more sophisticated one that is increasingly performed for the reconstruction of craniofacial defects in many clinical centers. The translation of such a powerful tool to the application in patients affected by HNC remains challenging because of the serious impediments brought about by adjuvant radiotherapy. Our previously published studies have shown a significant decrease in the number of osteogenic cells in radiated distracted murine mandibles compared to control DO and corresponding changes in the empty lacunae numbers.²⁶ Although the studies were done at lower radiation dosage, they still reflected radiation deleterious effect on cellular integrity. The need to overcome the major limitations of radiation-induced injury to the surrounding normal tissue of the irradiated field has prompted our laboratory to look into radiation protectors. Amifostine emerged as such compound, with limited toxicity and superior radioprotective properties. The biochemical basis of AMF selective cytoprotective activity in normal versus neoplastic tissues lies on the tissue membrane phosphatase activity that is higher compared to that of the tumor cells. In addition, the hypovascularity and low interstitial pH of tumor cells contribute to slower metabolism and uptake of the WR-1065 in cancer cell membranes.^{15–18} On the basis of these findings, we anticipated that AMF would optimize distraction osteogenesis in the setting of irradiation by safeguarding and recruiting the osteogenic cells essential for bone regeneration. To our knowledge, no study has evaluated nor quantified the direct in-vivo effect of AMF on the murine craniofacial bone histology following in vivo human equivalent radiation. In fact, most reports looking at the efficacy and mechanism of AMF have made only indirect inferences on the effects of the drug on bone cellularity. Those reports have focused on models ranging from endochondral long bones to in vitro osteoclast and osteoblastic-like cell lines without radiation. In the rare case where the effect of AMF in bone was evaluated it was performed either after the delivery of a single irradiation dose or non-human bioequivalent fractionation schedules. Weiss et al investigated the in vivo and in vitro effects of AMF on hypercalcemia in fetal rat

long bones organ culture without any radiation and concluded that AMF exerted direct inhibition on osteoclast-mediated bone resorption.²⁹ Damron et al focused mainly on a murine model of the growth plate quantifying the number of osteoclasts and chondroclasts following AMF pre-treatment in a single irradiation dose of 17.5 Gy.³⁰ Conversely to Weiss, Damron reported minimal radioprotective effect of AMF on the osteoclasts but significantly higher number of chondroclasts.^{29,30} Gevorgyan et al investigated young mouse calvarial MC3T3-E1 cells or osteoblast-like cells in vitro, which had received 0–10 Gy of gamma radiation with or without AMF. Their study revealed AMF radioprotection with significant improvement of osteoblast-like cell survival.³¹ Yurut-Caloglu et al studied the left femora of Wistar albino rats, which they irradiated at a single dose of 20 Gy following AMF pre-treatment. Their histopathological analysis of bone and cartilage revealed a significant reduction in radiation-induced bone and epiphyseal cartilage damage in the AMF pre-treated specimens compared to irradiated specimens alone.³² Although these studies have reported AMF remediation of radiation-induced skeletal defects via the impairment of the activity of the constituent bone cells, none of the above studies and models has mimicked the radiation dosage administered for HNC patients like our irradiated murine model of distraction osteogenesis. In addition, the aforementioned studies have not delineated any effects on the osteocytes, which are the cells essential for “bone maintenance”. Indeed, osteocytes are well known to function as the gatekeepers for both bone formation and remodeling. They are the active players in bone turnover as they establish an extensive syncytial network, which allow great ion exchange and communication with the deepest areas in the bone and extra-osseous regions.^{33–36} Lastly, osteocytes are proposed to be bone essential mechanosensory cells with a central role in the functional adaptation of bone.^{33–37} They may respond to any stimuli functioning to sense and control the need for bone remodeling, especially in processes such as DO.³⁷ In that regards, the osteocytes are thought to be capable of sensing the intermittent micromechanical forces generated during DO and may act to auto-regulate their viability by sending signals directly to osteoblasts and osteoclasts.^{38–41} Our study has successfully demonstrated for the first time, an AMF sparing effect on the osteocytes, protecting them from radiation-induced cellular depletion.

In addition to the establishing a protective effect on osteocytes, we have also demonstrated a corresponding diminution in the mean empty lacunae count following AMF pre-treatment compared to the irradiated and distracted mandibles alone. A lacuna is the compartment in which an osteocyte resides. Osteocytes necrosis is reflected by the number of empty lacunae or the presence of lacunae with non-viable cells, in particular in pathologic processes such as radiation-induced cellular damage. Therefore, our finding again confirms the ability of AMF to reverse radiation-induced cellular depletion of the regenerate in the animals that were pre-treated, irradiated and then distracted. Our quantitative histologic metric to help to determine the effect of AMF on tissue quality was the ratio of bone volume/tissue volume. Here again, we demonstrated a significant increase in the ratio of BV/TV in the specimens prophylactically treated with AMF. The percent of BV/TV is a metric of bone quantity and a reflection of the maturity of the bony regenerate. Our findings are also in keeping with our gross results where complete bony union was observed at the end of the consolidation period in the majority (100%) of the AMF specimens compared to the irradiated ones. These findings obtained through the 2D quantitative histomorphometry are corroborated by our previously reported 3D micro-computed evaluation of the specimens where BV/TV is a proxy assessment of the tissue or bone density.²⁰ On the other hand, our experiments also clearly demonstrated a greater degree of regenerate immaturity as revealed by the higher ratio of the non mineralized osteoid volume/tissue volume observed in the irradiated specimens. This finding suggests that the increase in osteoid volume is most likely secondary to radiation-induced stunting or delay of the bone regenerative and remodeling processes, which can severely impair the ability of the bone to heal and produce a strong regenerate. We can speculate that the osteoid bone may well have gone on to consolidation

at a later date beyond our established timeline. Nonetheless, our findings corroborate other reports which have shown that radiated bone undergoes significant impairment in the remodeling process secondary to radiation-induced death of osteogenic cells.^{42–44} Again, these results were substantiated by our gross findings of 100% fibrous unions observed in the irradiated hemimandibles. This report clearly demonstrates, via quantitative histomorphometry, both the cytoprotective and histoprotective effect of AMF on the regenerate formed by distraction osteogenesis in the setting of human bioequivalent radiation and may have clinical ramifications in the care of patients with HNC in the future.

Conclusion

To our knowledge, the osseous cytoprotective and histoprotective capacity of AMF on distraction osteogenesis in the face of human equivalent radiation has been described for the first time. Our findings demonstrate the maintenance of bone forming cells and the regenerate cellular integrity and tissue quality that resulted from AMF prophylactic treatment, which was correlated to an increase in successful bony union and the complete elimination in the incidence of fibrous union. We believe that a similar demonstration of the efficacy of AMF in the clinical setting may aid in the successful implementation of distraction osteogenesis as a viable reconstructive option for patients afflicted with HNC in the future.

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Figure 1. Gross representative samples of left hemimandible from the radiated and distracted group, XRT/DO (left) and Amifostine pre-treated radiated and distracted group, AMF/XRT/DO group (right). Note the fibrous union exhibited in the region of the distracted gap (red arrow) of the XRT/DO specimen compared to the complete bony bridging observed in the AMF/XRT/DO regenerate. In addition, the XRT/DO specimen showed significant tissue loss over the coronoid process and the condyle (black arrow).

Figure 2.

Coronoid section of the region of the distracted gap stained with Gomori Trichrome. Non mineralized immature bone or osteoid highlighted in red by color thresholding was predominantly observed in the XRT/DO sample (red arrow), whereas mature bone highlighted in blue (black arrow) was abundant in the AMR/XRT/DO specimen.

Figure 3.

Digital photograph under $16\times$ magnification further demonstrating differences between both groups. More empty lacunae (black arrows) and less mature osteocytes (red arrows) were consistently detected within the bone matrix in the XRT/DO group (left) compared to the AMF/XRT/DO group (right).

Figure 4.

Graphs of the mean osteocyte count (left) and mean empty lacunae (right) per high power field. There were significantly more osteocytes and less empty lacunae in the AMF/XRT/DO group compared to the XRT/DO group (* denotes $p < 0.05$).

Figure 5.

Graphs of the percentage of mature bone (left) and immature bone (right) represented by the ratio of Bone Volume/Tissue Volume (BV/TV) and the ratio of Osteoid Volume/Tissue Volume (OV/TV) respectively. The AMF pre-treated specimens demonstrated a significant difference in mature bone as anticipated by an increase in the ratio of BV/TV and a resultant decrease in the percentage of immature bone compared to the XRT/DO specimens (* denotes $p < 0.05$).