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Amifostine Remediates the Degenerative Effects of Radiation on the Mineralization Capacity of the Murine Mandible

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Abstract

Background—Although radiotherapy is a cornerstone in head and neck cancer treatment it causes substantial morbidity to the normal adjoining bone. Our specific aim is to assess the radioprotective effect of Amifostine (AMF) therapy on the mineralization of the mandible using micro-computed tomography (μ CT). We hypothesize that AMF will safeguard the mandible from radiation-induced disruption of the mineralization process and the associated failure of new bone creation.

Methods—Male Sprague Dawley rats were randomized into 3 groups: Control (n=8), XRT (n=5) and AMF (n=8). XRT and AMF underwent human bioequivalent radiation of 70Gy in 5 fractions to the left hemimandible. 56 days post-radiation, the hemimandibles were harvested for radiomorphometric analyses.

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Results—AMF exhibited less alopecia, mucositis and weight loss as well as increased cortical density in comparison to XRT. Bone and Tissue Mineral Densities showed statistically significant improvement in AMF versus XRT and no difference was observed between AMF versus Control. Detailed μ CT analysis further demonstrated significant differences in the mineralization profile when comparing XRT and AMF. AMF maintained regions of lower mineralization consistent with the preservation of normal remodeling.

Conclusion—We have successfully demonstrated the ability of AMF pre-treatment to protect the natural mineralization profile of bone. This reflects the capacity of AMF prophylaxis to safeguard the normal surrounding mandible from the impediments of collateral damage imposed by radiation. Further study can now be directed at correlating these findings with the potential use of AMF to prevent the devastating associated morbidities of radiotherapy such as pathologic fractures and osteoradionecrosis.

Introduction

Adjuvant radiation therapy for head and neck cancer (HNC) has significantly improved with the advent of fractionation of the radiation dose. Fractionation provides much better tumor control and has also been shown to decrease the associated morbidity than a single large dose of radiation.¹ Despite the advantages that fractionation has brought about to improve treatment protocols, acute toxicity reactions still remain the limiting factors in the institution of accelerated radiation schedules. The need to further diminish the ravaging effects of radiation on normal tissue has led to the development of chemical radioprotectants.² Amifostine or AMF, *S*-2-[3-aminopropylamino]ethylphosphothioic acid or WR-2721 or its active metabolite WR-1065 was evaluated by the Walter Reed Army Institute of Research among thousands (over 4000) of sulfhydryl-containing drugs with various degree of radioprotective properties and was far superior, displaying tolerable toxicity.² AMF functions as a free radical scavenger selectively sparing normal cells from the damaging effects of ionizing radiation. The significant potential of AMF as pharmaceutical agent for the protection of normal surrounding tissues during HNC radiotherapy has been bolstered after a Phase III *randomized control trial* that demonstrated its efficacy in protecting against radiation-induced xerostomia.³ In addition, the American Society of Clinical Oncology (ASCO) has approved and instituted guidelines for AMF prophylactic use for xerostomia in HNC.⁴

Unfortunately, the few animal studies that focused on the beneficial effects of AMF to protect osseous tissue only looked at endochondral long bones following radiation.^{5–7} Currently the literature lack sufficient data on the use of AMF as a protective drug against the destructive effects of HNC adjuvant radiotherapy in the exposed underlying bone of the craniofacial skeleton.

Previous studies in our laboratory have demonstrated that AMF administration in an irradiated murine model of mandibular DO resulted in the restoration of complete bridging and bony unions when compared to the failures of regenerate healing in distracted irradiated mandibles alone.⁸ Our model quantitatively analyzed the effects of radiation on the structural integrity and function of a cortical membranous bone after exposure to a fractionated human equivalent dose of radiation (HEDR) and subsequent unilateral distraction. Specifically, we demonstrated a significant increase in bone mineral density, tissue mineral density and bone volume fraction in AMF-pretreated mandibular regenerate compared to the irradiated and distracted hemimandible. These important findings led our lab to question whether the protective effects of AMF could be extended and applied to a much larger clinical problem, namely the detrimental effects of radiation on bone and the unacceptably high incidence of devastating wound healing complications as well as the

associated morbidity of late pathologic fractures and osteoradionecrosis. The specific aim of this report, therefore, is to assess the radioprotective effect of AMF therapy on the mineralization of the murine mandible after receiving HEDR utilizing micro-computed tomography (μ CT). We hypothesize that AMF will safeguard the irradiated mandible from radiation-induced degradation of the mineralization process and the associated failure of new bone creation. The use of AMF as a radioprotectant has the potential to address the ravaging effects of radiation on normal tissue and the significant biomedical burden of radiation therapy imposed as a consequence of its well-intended treatment goals.

Methods

All studies were approved by the University of Michigan's Committee for the Utilization and Care of Animals (UCUCA). Twenty-one adult male Sprague Dawley rats (400 g) were obtained through the University of Michigan's Unit for Laboratory Animal Medicine (ULAM). These animals were randomly assigned to three experimental groups. Group 1 (n=8), served as standard Control and did not receive any radiation and/or treatment. Group 2, XRT (n=5) underwent radiation; Group 3, AMF (n=8) was pre-treated with AMF (100mg/kg; Ethyol, MedImmune, Gaithersburg, MD) subcutaneously 45 minute prior to radiation.⁸ The animals were paired in cages in a restricted, pathogen-free facility and maintained on a 12-hour light/dark schedule. Prior to handling and radiation, the rats had a 7-day acclimation period.

Radiation

An Oxygen-Isoflurane mixture was used for anesthesia during radiotherapy. The rats were positioned with the right side down and a lead shield designed to solely expose the left posterior mandible while protecting the remainder of the body.

The left hemimandibles underwent a 5-day fractionated external beam irradiation at the human equivalent radiation dosage of 70 Gy via a Philips RT250 orthovoltage unit (250 kV, 15 mA) (Kimtron Medical, Woodbury, CT). Our laboratory has previously established the protocol to deliver fractionated radiation that is equivalent to 70 Gy, the conventional dosing regimens for humans with HNC.⁸⁻¹² In addition this general radiation protocol has been performed for several years in the department of Radiation Oncology under ULAM and UCUCA approved protocols.⁸⁻¹⁶

Following radiation, the rats were provided soft chow and water ad libitum for 56 days. Rats received subcutaneous injection of Buprenorphine (0.15mg/kg) with 10 cc Lactated Ringer solution when showing signs of radiation-induced discomfort and/or pain.⁸⁻¹⁶

Tissue harvesting

All animals survived and the left hemimandibles were harvested and frozen at -20°C until imaging.

Micro-Computed Tomography (μ CT) Imaging

μ CT was performed using eXplore Locus SP Micro-CT (GE Healthcare, Fairfield, CT) with the images reconstructed at $45\mu\text{m}$ voxel size. The region of interest (ROI) selected for analysis was located 5.1mm posterior to the third molar, and was analyzed for the following parameters:

Bone Mineral Density or the ratio of bone mineral content over the ROI total volume. This represents the amount of bone within the entire volume of interest (which also includes empty spaces, water, and marrow).

Tissue Mineral Density or the ratio of tissue mineral content over the bone volume of the ROI. This represents the average mineral density for only the tissue identified as bone within the volume of interest, excluding the spaces in the volume of interest i.e. excluding empty spaces, water, and marrow)

Comparative histograms, representing the frequency of occurrence of voxels at calibrated density values, were also generated utilizing Microview 2.2 software and provided additional Bone Mineral Density Distribution¹⁷ (BMDD) metrics as follows:

Mean Total mineralization volume (MTNV) or total number of mineralized voxels, which represents the overall volume of bone being analyzed.

5th percentile of mineralization represents the corresponding Hounsfield Units of mineralization at the 5th percentile for a standard control. That marker was used as a reference HU to look at the volume of minerals (in voxels) or the area under the curve (M_{Low} Integral) for the experimental groups.

Statistics

One-way ANOVA with post-Tukey test was utilized for analysis of the aforementioned radiomorphometrics (PASW version 18; SSPS, Inc., Chicago, IL). Statistical significance was considered at p value less than 0.05.

Results

The post-radiation recovery period was uneventful and all animals survived. Initially, AMF pre-treated animals underwent some weight loss (13%) during the radiation week. However, the AMF animals significantly recovered compared to the untreated radiated animals by the end of the experimental time. Less alopecia and mucositis were observed in the AMF group compared to the radiated animals. Gross inspection of the hemimandibles at the time of harvest demonstrated significant atrophy of the bone. Specifically, the XRT group had thinning and loss of cortical density particularly noticeable at the angular process of the mandible when compared to both Control and AMF group (Fig.1). Such findings were reproduced on the Faxitron scans of the specimens (MX20, Faxitron X-Ray, Lincolnshire, IL) (Fig.2).

Standard radiomorphometrics

Standard radiomorphometrics of bone mineralization were generated using α CT for the Control, AMF and XRT groups and are shown in Table 1, Figures 3 and 4. The average values for BMD were 769 mg/cc, 807 mg/cc, and 773 mg/cc Control, XRT and AMF respectively. No difference was observed between AMF versus Control ($p=0.936$). There were statistically significant differences between Control versus XRT ($p=0.018$) and AMF versus XRT ($p=0.033$; Fig.3).

Similar significant differences were observed in the averages of the TMD of Control versus XRT (830 vs 857 mg/cc; $p=0.001$) and AMF versus XRT (818 vs 857 mg/cc; $p=0.018$); while there continued to be no difference between Control versus AMF (830 vs 818 mg/cc; $p=0.284$; Fig.4).

Bone Mineral Density Distribution metrics

Microdensitometry histograms were generated and compared among the 3 groups (Table 1, Fig. 5, 6 & 7). The mean total mineralization volumes (M_{TNV}) were: 266218, 210871, 251758 voxels for Control, XRT and AMF respectively. The XRT group was significantly

different from the AMF group and from the Control ($p=0.005$, Fig.6). Again, there continued to be no difference between Control and AMF. The 5th percentile of mineralization occurred at the following Hounsfield units (HU): 1036, 1349, 1162 for Control, XRT and AMF respectively (Table 1). Statistical significant differences in the 5th percentile of mineralization mirrored the ones that were observed in the M_{TNV} . The M_{Low} or the integrals generated at the 5th percentile of the control (1036 HU) were the following: 1.23×10^7 , 2.93×10^6 , 6.74×10^6 voxels for Control, XRT and AMF respectively. M_{Low} of the XRT group represented 23.7% of that of the Control group whereas M_{Low} of the AMF group was 54.5% of that of the Control group (Fig.7).

Discussion

Technological advances in the delivery of radiation, the total amount of radiation and the radiation fractionation regimen have significantly contributed to minimizing injury to adjacent normal tissue after radiation therapy. In spite of the aforementioned progress, irradiation induced injury to surrounding bone and soft tissue in HNC treatment remains a devastating reality. To date, the literature reports of radiation injuries to bone are limited to the axial skeleton and endochondral long bones.⁵⁻⁷ Unfortunately, few if any studies have focused on the damaging effects of irradiation to the membranous craniofacial skeleton, such as the vulnerable cortical mandible, where osteoradionecrosis and pathologic fractures can be a particularly vexing conundrum to the clinician.

Our lab has previously demonstrated radiation-induced dose response damage to the murine mandible and also established a protocol that delivers a human equivalent dose of radiation (HEDR) that mirrors the total dosage patients receive in the clinical management of HNC.⁸⁻¹² In addition, our lab has also investigated the effect of AMF pretreatment in a murine mandibular model of DO after HEDR. We established that AMF was able to preserve new bone formation by demonstrating that AMF pre-treated hemimandibles exhibited strong bridging and bony union of the regenerate. Furthermore, these gross findings translated to quantifiable volumetric and mineralization radiomorphometrics, where BVF, BMD, and TMD were significantly different when an AMF pre-treated group was compared to an irradiated distracted group.⁸ These results directed further inquiry into the application of such findings in a broader clinical context, specifically: Could AMF be employed prophylactically in the craniofacial skeleton to protect against the adverse sequelae of radiation-induced pathologic fractures and mandibular osteonecrosis? To test this question we aimed to demonstrate the ability of the selective radioprotectant, AMF, to preserve the normal mineralization profile of bone in the setting of radiation, utilizing bioequivalent radiation dosing in the same animal model without DO (on normal bone alone).

Evidence exists to show that bone which has been radiated undergoes changes in the remodeling process because of radiation-induced death of osteogenic cells.¹⁸⁻²¹ Typically, bone formation during remodeling involves several steps. In fact, one of the key biological contributing factors of mineralization is the rate of turnover.²²⁻²⁷ Therefore, when bone turnover is slowed or blunted, the lack of resorption results in a regional shift towards hypermineralization.²⁴⁻³⁰ Since mineral growth continues to occur after initial mineralization, this shift towards a regional hypermineralization represents the combined effect of a loss of remodeling (therefore, no newly formed low mineralized bone) and continued growth of mineral in existing bone. Before we established a HEDR, our laboratory demonstrated significant alterations in volumetric bone mineralization patterns in irradiated-distracted murine mandible after a fractionation radiation regimen of a total dose of 36 Gy divided into 10 days.¹³ However, we did not find any statistically significant differences in BMD when compared to the control. This confounding result is likely due to

the fact that our densitometric analysis was conducted on a much narrower spectrum of HU of mineralization, specifically ranging from 800 to 2000 HU instead of the full mineralization spectrum of 800 to 4000 in our current series. Furthermore, the radiation dose we previously utilized was two-third of the current bioequivalent radiation dosing used in this study and therefore, in keeping with other dose response studies in our lab, inflicted less overall damage to the underlying bone.^{9–12}

In fact, the conventional thought that radiation "weakens" the underlying exposed skeleton by decreasing bone mineral density is now in doubt and under further inquiry in present-day studies, again a careful look at dosages in all of these studies as the effect of radiation varies significantly with dose as well as mode of delivery.³¹ Margulies et al demonstrated an increase in BMD following irradiation of the murine tibia at a single radiation fraction of 17.5 Gy. In addition, they also showed that AMF pre-treated animals consistently demonstrated a BMD close to that of control animals.²⁸ Similarly, Verdonck et al reported an increase in BMD at 3 months after a total radiation dose of 24 Gy delivered to the alveolar bone of minipigs when compared to non-irradiated bone.²⁹ They attributed the increase in BMD to radiation-induced bone damage, specifically bone sclerosis, and emphasized the importance of BMD as a parameter of bone quality. The most recent clinical study by Dhakal et al which evaluated changes in bone density following radiation for extremity sarcomas found that radiation did not routinely decrease bone density and that possible variations in bone remodeling could result in stable or even increased bone density.³² Our results corroborate these findings of hypermineralization as outlined above, confirming in our model what happens clinically using HEDR. Furthermore our results demonstrate the ability of AMF to protect the membranous bone of the murine mandible from the ravages of hypermineralization and function to keep the radiomorphometric parameters of bone density normalized. Research studies have also established that the heterogeneity of mineralization is a reflection of bone quality and strength.^{22–26} The heterogeneity is created as a result of normal turnover of bone within the volume of interest. When remodeling is blunted, that heterogeneity is lost. Again, there is a higher degree of mineralization, which leads to an average hypermineralized bone. As bone becomes more highly mineralized, it becomes more brittle. In fact, remodeling is a mean by which bone regains its normal, less brittle mechanical properties. In our study, the mean total mineralization volume or M_{TNV} in the irradiated group became higher and more homogeneous compared to the Control and AMF groups. This was characterized by the narrowing of the histogram curve and its shift to a higher level of HU of mineralization. On the other hand, the M_{TNV} for the AMF group showed a more heterogeneous degree of mineralization which paralleled that observed in the Control group. This is also confirmed by the broadening of the histogram curve and its distinctive shift to the left towards the Control HU of mineralization. The aforementioned shift to the right of the XRT group is further demonstrated by the 5th percentile of mineralization, which occurred at a significantly higher HU (1349) as compared to the Control group (1036) and the AMF group (1162). In line with the above observation is the fact that radiation also causes a quite specific loss of mineralization in the lower HU spectrum. This is evidenced by the significantly diminished level of the M_{Low} at the 5th percentile of mineralization that is found in the XRT group in contrast to the Control and AMF groups. This illustrates radiation-induced blunting of remodeling and the inhibition of new bone formation. Conversely, AMF clearly demonstrated a quantifiable increase in the amount of mineralization in the lower HU spectrum, which is emblematic of a remediation of the radiation-induced inability to recruit the osteogenic cells responsible for bone turnover. AMF brought the level of mineralization to more than half of the Control (54.5%), therefore protecting the bone from the pernicious pathologic changes by a substantial extent. The maintenance of remodeling brought about by AMF prompts greater heterogeneity in bone, which in turn can function to preserve its natural beneficial mechanical properties. Those

more resilient and less brittle mechanical characteristics can then act to prevent the dreadful complication of late pathologic fractures. Optimal homeostasis mandates a balance between the alterations of bone turnover and the process of mineralization to reach an ideal equilibrium to be able to maintain the most advantageous configuration of the bone.^{30,33,34} Our study demonstrated in all cases, the preservation of mineralization metrics, thwarting the hypermineralization process and maintaining levels of mineralization that were not significantly different when compared to controls.

There are very few reports that looked at AMF radioprotection of the craniofacial skeleton. Forrest et al used the infant rabbit orbitozygomatic complex to evaluate AMF role in craniofacial bone growth retardation following a single dose of radiation (35 Gy).³⁵ They found that AMF resulted in significant improvement of cephalometric parameters. In addition, bone volume and density were significantly diminished in the irradiated rabbits compared to the control and AMF-treated groups. Though they too demonstrated AMF protection their radiomorphometrics differed than ours. Again, we attribute the differences they saw in mineralization to their much smaller radiation dose as well as the fact that they did not fractionate and they used a growing animal model.

Conclusion

We have successfully demonstrated the ability of AMF pre-treatment to maintain the natural mineralization profile of bone. To our knowledge, this is the first investigation that reflects the capacity of AMF prophylaxis to safeguard the densitometric properties of the cortical murine mandible from the impediments of collateral damage of human bioequivalent radiation. Further study can now be directed at correlating these important findings with the potential use of AMF to prevent the disastrous morbidities associated with radiation therapy such as late pathologic fractures and osteoradionecrosis.

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Figure 1. Gross pictures of the hemimandibles. The XRT (middle) specimen demonstrates significant thinning and loss of cortical density of the angular process when compared to the Control (left) and the AMF (right) specimens.

Figure 2.

Faxitron scans of all 3 groups. The scans reproduced the gross results where the loss of opacity is demonstrated in the XRT (middle) specimen compared to the Control (left) and AMF (right) specimens.

Figure 3. Bone Mineral Density (BMD) mean values. There were significant differences between Control and XRT (†, $p=0.018$) and AMF and XRT (*, $p=0.033$). No difference was observed between Control and AMF ($p=0.936$).

Figure 4. Tissue Mineral Density (TMD) mean values. Significant differences were observed between Control and XRT (†, $p=0.001$) and AMF and XRT (*, $p=0.018$). No difference was observed between Control and AMF ($p=0.284$).

Bone Mineral Density Distribution Histograms

Figure 5. Comparative Bone Mineral Density Distribution Histograms. The Control group has a very heterogeneous distribution of minerals, which is shifted to the right in the XRT group towards a more homogeneous and higher degree of mineralization. The AMF's curve reverts back to the normal, heterogeneous profile of mineralization.

Mean Total Mineralization Volume- M_{TNV}

Figure 6.

Histogram and graph of the Mean Total Mineralization Volume (M_{TNV}). Control and XRT demonstrated significant differences (†, $p=0.001$) and AMF and XRT (*, $p=0.018$). No difference was observed between Control and AMF ($p=0.284$).

M_{Low} at the 5th percentile of Mineralization

Figure 7.

Histogram and graph of the M_{Low} Integral at the 5th percentile of mineralization of the Control (1036 HU). The M_{Low} from the XRT group represents 23.7% of that of the Control group ((†, $p=0$) group and was also different than the AMF group (*, $p=0.001$). The M_{Low} from the AMF group represents 54.5% of that of the Control group (‡, $p=0$).

Table 1

Mean values, standard deviations (SD) and p values obtained from the Standard radiomorphometrics and Bone mineral density distribution (BMDD) metrics.

Radiomorphometrics	Means			SD			p value		
	Control	XRT	AMF	Control	XRT	AMF	†	*	‡
BMD (mg/cc)	769	807	773	10	10	12	0.018	0.033	0.936
TMD (mg/cc)	830	818	857	9	9	11	0.001	0.018	0.284
MTNV (Voxels)	266218.125	210871.8	251758	8634.46	14953.57	24971.44	0	0.005	0.525
5th percentile of Mineralization (HU)	1035.75	1349.6	1162.125	18.97931	74.82179	56.97227	0	0.03	0.141
M Low (Voxels)	12361387	2933439	6742567	1220110	1798175	1628880	0	0.001	0

Bone Mineral Density, BMD; Tissue Mineral Density, TMD; Mean Total mineralization Volume, MTNV; Integral or Area under the curve obtained at the Control reference Hounsfield Unit or 5th percentile of mineralization, M_{Low}.

(†) p value between Control and XRT;

(*) p value between AMF and XRT;

(‡) p value between Control and AMF.