Published in final edited form as:

Plast Reconstr Surg. 2014 March ; 133(3): 314e–321e. doi:10.1097/01.prs.0000438454.29980.f8.

Prophylactic Amifostine Preserves the Biomechanical Properties of Irradiated Bone in the Murine Mandible

Peter A. Felice, M.D., Salman Ahsan, B.S., Joseph E. Perosky, M.S., Sagar S. Deshpande, B.S., Noah S. Nelson, B.S., Alexis Donneys, M.D., M.S., Kenneth M. Kozloff, Ph.D., and Steven R. Buchman, M.D.

Craniofacial Research Laboratory, Plastic Surgery Section, and the Orthopaedic Research Laboratory, Orthopaedic Surgery Section, University of Michigan; and the Department of General Surgery, University of South Carolina School of Medicine

Abstract

Background—The authors have previously demonstrated that amifostine prophylaxis mitigates the pernicious effects of radiation in settings of fracture repair and distraction osteogenesis. Expanding on these studies, the authors examined the biomechanical properties of uninjured bone exposed to both radiation and amifostine. The authors hypothesize that radiation will degrade the biomechanical properties of native bone, and further hypothesize that prophylactic amifostine will preserve biomechanical properties to levels of normal bone and protect against radiation-induced morbidities.

Methods—Rats were randomized into control, irradiated, and amifostine pretreatment plus radiation (amifostine-pretreated) groups. Irradiated animals received a fractionated dosing schedule of 35 Gy, with amifostine-pretreated animals receiving amifostine before irradiation. Hemimandibles were harvested at 8 and 18 weeks for biomechanical testing and micro-computed tomographic analysis.

Results—At 8 weeks, irradiated specimens displayed elevations above controls for all biomechanical properties. At 18 weeks, the biomechanical properties of irradiated specimens degraded in comparison with controls; at both time points, amifostine-pretreated specimens were maintained at levels comparable to controls. There was a significant decrease in tissue mineral density from 8- to 18-week irradiated specimens, whereas no such change existed for control and amifostine-pretreated specimens.

Conclusions—The authors' findings demonstrate paradoxical and transient elevations in the initial biomechanical properties of irradiated specimens that were not sustained through the later study time point. Amifostine pretreatment, however, provided uninterrupted preservation of the biomechanical properties of normal, native bone at both time points. This supports the contention that amifostine is capable of providing continuous protection to bone against the untoward effects of radiation therapy.

Copyright © 2014 by the American Society of Plastic Surgeons

Steven R. Buchman, M.D., University of Michigan Medical School, Pediatric Plastic Surgery Section, 4-730 C.S. Mott Children's Hospital, 1540 East Hospital Drive, Ann Arbor, Mich. 48109-4215, sbuchman@med.umich.edu.

Disclosure: None of the authors has a financial interest in any of the products, devices, or drugs mentioned in this article.

Head and neck cancer constitutes a group of several devastating diseases that accounted for over 52,000 new cases and 11,000 deaths in 2012.¹ Therapeutic strategies for patients with this disease often consist of tumor extirpation and subsequent reconstructive procedures. In addition to operative management, radiation therapy is often a necessary component of the treatment regimen used to decrease rates of local tumor recurrence²; however, radiation-induced injuries such as mucositis, xerostomia, and vessel obliteration are common adverse sequelae of therapeutic radiation administration.^{3–6} Furthermore, nearly one in four patients succumbs to long-term consequences such as pathologic fracture and osteoradionecrosis.⁷ Patients are thus vulnerable to functional and aesthetic impairments of the mandible and other components of the craniofacial skeleton, compromising the ability to eat, to communicate, and to engage in social interactions, all of which have a profound impact on patients' quality of life.

Amifostine has a well-established clinical profile as a radioprotectant currently on formulary for prevention of mucositis and xerostomia in patients with head and neck squamous cell carcinoma.^{8,9} It is a prodrug, free-radical scavenger that regulates cell cycle checkpoints and gene expression, and facilitates DNA repair. Data demonstrate no impairment of the therapeutic efficacy of radiation therapy, no increase in tumor recurrence, and no decrease of overall and disease-free survival associated with administration of amifostine.^{8–10} In our laboratory, we have demonstrated that the cytoprotective properties exhibited by amifostine in soft tissues are also translatable to bone, as prophylactic amifostine administration was shown to preserve new bone formation in irradiated murine mandibles after distraction osteogenesis and fracture repair.^{11,12}

The ability of amifostine to protect distracted and fractured bone from the pernicious effects of radiation led us to question whether uninjured irradiated bone would also benefit from amifostine prophylaxis. We hypothesized that radiation administration would compromise the biomechanical properties of bone, whereas amifostine pretreatment before irradiation would preserve biomechanical properties to levels of normal, uninjured, and nonirradiated bone. We investigated this premise by developing an animal model using biomechanical testing and micro-computed tomography to evaluate hemimandibles at short- (8-week) and long-term (18-week) periods after radiation therapy.

The use of amifostine as a radioprotectant for normal, uninjured bone in the setting of irradiation has the potential for tremendous translational benefit and may prevent the ruinous complications of radiation-induced bone injury. Such a benefit would potentially have an impact on the approach and use of any radiation protocol in which normal bone exists within the irradiated field. Our goal is to ameliorate the adverse sequelae that radiation imposes on the structural integrity of native bone, thus alleviating the threat of pathologic fracture and the morbidities associated with radiation treatment regimens that would otherwise cause substantial collateral damage to bone within an irradiated field.

MATERIALS AND METHODS

All components of our project pertaining to the use of animal subjects were performed in compliance with, and approved by, the University of Michigan's Committee for the Utilization and Care of Animals.

Experimental Grouping

Male Sprague-Dawley rats (350 to 400 g) were assigned randomly into 8-week or 18-week experimental groups. Animals were then further divided and assigned randomly to one of three subgroups: control specimens receiving neither radiation nor amifostine pretreatment, irradiated specimens receiving radiation alone, and amifostine-pretreated specimens receiving amifostine pretreatment before irradiation (Table 1).

Radiation and Amifostine Administration

We collaborated with the Experimental Irradiation Core of the University of Michigan Comprehensive Cancer Center and calculated a head and neck cancer human-equivalent dose of radiation consisting of five fractions at 7 Gy per fraction for a total of 35 Gy.¹³ Fractions were administered once daily over 5 consecutive days using a Philips RT250 orthovoltage unit (250 kV x-rays, 15 mA; Kimtron Medical, Oxford, Conn.). Specimens receiving amifostine were administered a 100-mg/kg dorsal subcutaneous injection 45 minutes before irradiation, a dose previously developed by our laboratory and used in published studies.^{11,12} Animals underwent closed-chamber induction with inhalational isoflurane before being placed in the radiation chamber and covered with a protective lead shield that exposed only the left hemimandible. Low-flow isoflurane and oxygen were administered continuously during irradiation, which lasted approximately 5 minutes for each fractionated dose. Animals were then placed singly in cages to wake under continuous supervision.

Recovery and Harvesting

Animals underwent an 8- or 18-week recovery and care period in accordance with their experimental grouping. During this time, animals were housed, provided food and water, and cared for in compliance with the University of Michigan's Committee for the Utilization and Care of Animals. Daily weights were recorded to monitor nutritional status, and daily inspection of the animals was performed to evaluate for any complications of radiation or amifostine administration. At the end of each recovery period, animals were killed with drop isoflurane and left hemimandibles were harvested for biomechanical testing and micro-computed tomographic analysis.

Biomechanical Testing

Left hemimandibles were hand-machined with a diamond cutting saw to isolate a 15-mm-long, 3-mm-wide, 2-mm-high area from the inferoposterior aspect of the mandibular angle. Figure 1 illustrates the whole, harvested hemimandible and the isolated region of interest extending a distance of 2 mm posteriorly from the third molar, an area chosen because of its bony consistency, relatively free from molar, incisor, or marrow pocket involvement. Machined hemimandibles were loaded to failure in four-point bending at 0.5 mm/second

using a servohydraulic testing machine (858 Minibiox II; MTS Systems Corp., Eden Prairie, Minn.). Crosshead displacement was recorded using an external variable differential transducer (Lucas Schavitts, Hampton, Va.), and load data were collected with a 50-lb load cell (Sensotec, Columbus, Ohio). Data were sampled at 2048 Hz (TestStar IIs System version 2.4; MTS Systems). Load-displacement curves were analyzed for yield load, ultimate load, and stiffness using custom computational code (MATLAB 7.11; Mathworks, Inc., Natick, Mass.). Bone tissue material properties were predicted using standard mechanics of material equations, assuming a bending mode of failure. The moment of inertia over which the specimen was tested in bending, the thickness of the specimen, and the bone volume were compared to ensure proper machining and to test that the specimens were uniform in size and geometry. Specimens that were nonuniform or had any type of defect were excluded from biomechanical testing and subsequent analysis, resulting in smaller group sizes ultimately subjected to biomechanical testing (Table 1 and Fig. 1).

Micro-Computed Tomography

Micro-computed tomographic (eXplore Locus SP; GE Healthcare PreClinical Imaging, London, Ontario, Canada) images were obtained using an 80-kVp, 80-mA, and 1100-msec exposure at a resolution of 45- μ m voxel size. After initial calibration of known density standards, machined specimens were scanned four at a time in water using the Parker method (180 degrees plus a 20-degree fan angle) of rotation, and added filtration in the form of both an acrylic beam flattener and a 0.02-inch aluminum filter was used to minimize beam hardening artifacts. Individual scans were reconstructed and reoriented with the sagittal sections following a posterior-to-anterior, left-to-right orientation along the x plane. The region of interest for analysis was identified and centered at the location of failure during four-point bending. A splined region of interest was isolated spanning 1 mm anteriorly and 1 mm posteriorly from this point of failure. Computational software (MicroView v2.2; GE Healthcare PreClinical Imaging) was used to calculate tissue mineral density after application of a uniform threshold of 1250 HU.

Statistics Analysis

Statistical analysis was calculated using IBM SPSS Version 20 (IBM Corp., Armonk, N.Y.). Significance for biomechanical testing was determined using a one-way analysis of variance test. Significance for micro-computed tomography was determined using a two-way analysis of variance test. Homogeneity of variances was verified with the Levene test; as the Levene test was not significant for any variables, Tukey post hoc tests were used to determine individual significance between groups. Significance was taken at $p < 0.05$, and a trend toward significance was defined as a value between $p < 0.05$ and $p < 0.1$.

RESULTS

Biomechanical Testing

For all metrics evaluated at the 8-week time point in our study, we observed that the amifostine-pretreated specimens were not significantly different from levels of controls. Irradiated specimens in the irradiated group, however, were significantly elevated above levels of controls for ultimate load ($p = 0.013$) and stiffness ($p = 0.048$), and approaching a

trending increase above controls for yield load ($p = 0.104$). These results are represented graphically in Figure 2.

Biomechanical testing for the 18-week specimens also demonstrated no significant difference between control and amifostine-pretreated specimens for yield load, ultimate load, and stiffness. However, at this later time point, irradiated specimens demonstrated a degradation of biomechanical properties in comparison with the amifostine-pretreated specimens, exhibiting a significantly lower yield load ($p = 0.047$) and a trend toward a significantly lower ultimate load ($p = 0.092$). Degradation in all biomechanical properties of the irradiated specimens could be appreciated graphically in comparison with controls, but this decrement in structural integrity did not reach statistical significance. These results are represented graphically in Figure 3.

Micro-Computed Tomography

Analysis of tissue mineral density for 8-week specimens demonstrated a significant elevation of both irradiated ($p = 0.00$) and amifostine-pretreated ($p = 0.004$) specimens above controls. In the 18-week specimens, however, irradiated tissue mineral density levels remained significantly elevated above controls ($p = 0.013$), but the tissue mineral density in amifostine-pretreated specimens was no longer significantly different from controls. In addition, a two-way comparison of tissue mineral density from 8- to 18-week irradiated specimens demonstrated a significant degradation of mineralization, whereas amifostine-pretreated and control groups demonstrated no such significant change in mineralization over time ($p = 0.040$). These results are represented graphically in Figure 4.

DISCUSSION

Radiation therapy is often a necessary constituent of treatment regimens for patients with head and neck cancer. Although the known benefits of radiation therapy include increased disease-free survival and decreased recurrence rates, radiation administration poses substantial risks and deleterious consequences to patients.^{3–6} The aim of this proposal was to determine whether amifostine prophylaxis could ameliorate or prevent such negative sequelae altogether. This experimental model compares biomechanical properties and micro-computed tomographic metrics for control, irradiated, and amifostine-pretreated specimens to investigate the hypothesis that radiation will significantly alter the biomechanical properties of otherwise uninjured bone. We further tested the hypothesis that prophylactic amifostine will preserve the biomechanical properties to levels of normal bone and protect the mandible from the morbidities associated with irradiation.

Data collected from analysis of 8-week specimens demonstrated that values for yield load, ultimate load, and stiffness were of comparable levels for both amifostine-pretreated specimens and controls. Interestingly, we noted a significant increase in these same metrics for irradiated specimens above controls. The former finding supported the supposition that amifostine is capable of preserving biomechanical properties of irradiated bone to levels comparable to uninjured controls, whereas the increase in biomechanical properties of irradiated specimens enigmatically suggested that irradiated bone was somehow stronger than controls. These findings were also supported by micro-computed tomographic analysis,

evidenced by increased levels of tissue mineral density in irradiated specimens above both controls and amifostine-pretreated specimens at the 8-week time point.

The paradoxical strengthening of biomechanical properties and tissue composition in irradiated bone is a finding at odds with complications that are expected and observed in the clinical setting. Other investigators, however, have also encountered similar “perplexing” findings with respect to bolstered irradiated bone strength, specifically, as an increase in torsional rigidity of bone after radiation exposure.¹⁴ Although the observation is made that some properties of irradiated bone appear to be more robust over a short-term period following irradiation, a specific mechanism to account for this finding is not elucidated. Previous investigations posit the role of radiation-induced variations in collagen content or crystalline structure, or changes in calcium metabolism as possible causes of these counterintuitive results.^{14–16} These interesting suppositions will be the focus of upcoming studies in which we will use Raman spectroscopy to evaluate radiation-induced changes in collagen content and cross-linking. With this future avenue of research, we hope to answer the questions of previous investigations and uncover a mechanism responsible for the curious increases in biomechanical properties in irradiated bone. Nevertheless, what is apparent from this study is that amifostine pretreatment reversed this change back to baseline levels, proving its efficacy in protecting bone from both radiation-induced decrease, and the transient perplexing increase, in biomechanical properties.

Results from harvested 18-week specimens more closely supported our hypotheses, as the biomechanical properties of irradiated specimens were degraded in comparison with control and amifostine-pretreated specimens for all metrics. This was in stark contrast to the elevated biomechanical properties observed in the 8-week irradiated specimens, suggesting that this increase was merely a transient phenomenon reversed by the 18-week time point. Corroborative findings were observed for micro-computed tomographic analysis, and the tissue mineral density in 18-week irradiated specimens exhibited significantly lower mineralization than that in 8-week irradiated specimens. These findings were congruent with the experimental hypothesis and are in sync with the clinical outcomes expected over a long-term period after subjecting bone to irradiation. Other studies have also noted similar findings with respect to diminishing properties of irradiated bone at time points farther out from completion of irradiation.¹⁴

There are some limitations to this study worth mentioning. The process of hand-machining mandibles to obtain regions of interest subjected to four-point bending led to some inconsistency when evaluating our biomechanical testing metrics. To ensure that specimens both within and across experimental groups were consistent, we included only those specimens in which the moment of inertia, thickness, and bone volume were of uniform in size and geometry, as noted previously in Table 1. Although this resulted in a lower experimental population, it was necessary for standardization across the 8-week and 18-week specimens. Interestingly, statistical analysis still showed significance or trending for our metrics; we can assume this would be further buttressed by increasing the number of specimens within each experimental group. These issues can be remedied for future studies by adding more specimens to each subgroup and adopting more precise methods of machining the regions of interest.

CONCLUSIONS

To our knowledge, this study is the first of its kind to directly investigate the temporal pathophysiology of irradiated mandibular bone in the quantifiable and clinically relevant metrics of biomechanical testing and micro-computed tomographic analysis. Our findings have tremendous potential clinical benefit for mitigating radiation-induced degradation of bony biomechanical properties. We believe this study is an important first step toward taking our work from the bench to the bedside in an effort to alleviate the morbidities associated with radiation therapy protocols in which normal bone sustains collateral exposure in the irradiated field.

Acknowledgments

Funding was provided by the following grant: "Optimization of Bone Regeneration in the Irradiated Mandible," NIH-R01#CA 125187-01 (principal investigator, Steven R. Buchman, M.D.). The authors thank Ted Lawrence, M.D., Ph.D., Mary Davis, Ph.D., David Karnak, Ph.D. from the Experimental Irradiation Core of the University of Michigan Comprehensive Cancer Center for help with calculating their radiation doses and assisting with radiation administration. They also express thanks to the Orthopaedic Research Laboratory at the University of Michigan for technical assistance with performing biomechanical testing, micro-computed tomography, and result analysis.

References

1. American Cancer Society. Cancer Facts & Figures 2012. Atlanta: American Cancer Society; 2012.
2. Marx RE. Osteoradionecrosis: A new concept of its pathophysiology. *J Oral Maxillofac Surg.* 1983; 41:283–288. [PubMed: 6572704]
3. Brown RK, Pelker RR, Friedlaender GE, Peschel RE, Panjabi MM. Postfracture irradiation effects on the biomechanical and histologic parameters of fracture healing. *J Orthop Res.* 1991; 9:876–882. [PubMed: 1919851]
4. Jacobsson M, Albrektsson T, Turesson I. Dynamics of irradiation injury to bone tissue: A vital microscopic investigation. *Acta Radiol Oncol.* 1985; 24:343–350. [PubMed: 2994392]
5. Jacobsson MG, Jonsson AK, Albrektsson TO, Turesson IE. Short- and long-term effects of irradiation on bone regeneration. *Plast Reconstr Surg.* 1985; 76:841–850. [PubMed: 4070452]
6. Sugimoto M, Takahashi S, Toguchida J, Kotoura Y, Shibamoto Y, Yamamuro T. Changes in bone after high-dose irradiation: Biomechanics and histomorphology. *J Bone Joint Surg Br.* 1991; 73:492–497. [PubMed: 1670456]
7. Coletti D, Ord RA. Treatment rationale for pathological fractures of the mandible: A series of 44 fractures. *Int J Oral Maxillofac Surg.* 2008; 37:215–222. [PubMed: 18023145]
8. Brizel DM, Wasserman TH, Henke M, et al. Phase III randomized trial of amifostine as a radioprotector in head and neck cancer. *J Clin Oncol.* 2000; 18:3339–3345. [PubMed: 11013273]
9. Wasserman TH, Brizel DM, Henke M, et al. Influence of intravenous amifostine on xerostomia, tumor control, and survival after radiotherapy for head-and-neck cancer: 2-year follow-up of a prospective, randomized, phase III trial. *Int J Radiat Oncol Biol Phys.* 2005; 63:985–990. [PubMed: 16253773]
10. Andreassen CN, Grau C, Lindegaard JC. Chemical radioprotection: A critical review of amifostine as a cytoprotector in radiotherapy. *Semin Radiat Oncol.* 2003; 13:62–72. [PubMed: 12520465]
11. Tchanque-Fossuo CN, Donneys A, Razdolsky ER, et al. Quantitative histologic evidence of amifostine-induced cytoprotection in an irradiated murine model of mandibular distraction osteogenesis. *Plast Reconstr Surg.* 2012; 130:1199–1207. [PubMed: 22878481]
12. Tchanque-Fossuo CN, Donneys A, Deshpande SS, et al. Amifostine remediates the degenerative effects of radiation on the mineralization capacity of the murine mandible. *Plast Reconstr Surg.* 2012; 129:646e–655e.
13. Tchanque-Fossuo CN, Monson LA, Farberg AS, et al. Dose-response effect of human equivalent radiation in the murine mandible. *Plast Reconstr Surg.* 2011; 128:480e–487e.

14. Maeda M, Bryant MH, Yamagata M, Li G, Earle JD, Chao EY. Effects of irradiation on cortical bone and their time-related changes: A biomechanical and histomorphological study. *J Bone Joint Surg Am.* 1988; 70:392–399. [PubMed: 3346264]
15. Malaska-Waniewska I. Microdensitometric analysis of the influence of x-irradiation on mature bone in humans. *Acta Med Pol.* 1971; 12:357–380. [PubMed: 5112453]
16. Finston RA, Woodard HQ, Laughlin JS. 18 effects of external irradiation on mineral metabolism in the bones of adult dogs. *Clin Orthop Relat Res.* 1966; 45:183–201. [PubMed: 5915108]

Fig. 1.
(*Above*) Three-dimensional micro-computed tomographic image of a left hemimandible, viewed from the buccal side with the anterior incisor facing to the right. (*Below*) Isolated, machined area taken from the inferoposterior aspect of the mandibular angle. The area filled in *black* and noted by the *arrow* is the 2-mm region of interest subjected to biomechanical testing analysis.

Fig. 2. Graphic representation of metrics derived from four-point bending biomechanical testing in 8-week specimens. There was a significant difference (*solid bracket*) above control levels in irradiated specimens for ultimate load and stiffness, with trending significance (*dashed bracket*) above controls for yield load. There is no difference between controls and amifostine-pretreated specimens across all metrics. *XRT*, irradiation; *AMF*, amifostine.

Fig. 3. Graphic representation of metrics derived from four-point bending biomechanical testing in 18-week specimens. There is significant (*solid bracket*) elevation of amifostine-pretreated above irradiated specimens for yield load, with a trend (*dashed line*) toward increased difference in ultimate load. No statistical difference is seen between or among groups for stiffness. *XRT*, irradiation; *AMF*, amifostine.

Fig. 4.

Comparison of tissue mineral density micro-computed tomographic metrics for 8-week and 18-week specimens. Both irradiated and amifostine-pretreated specimens are significantly elevated above controls at 8 weeks, but only irradiated specimens remain significantly different from controls at 18 weeks. There is also a significant decrease in tissue mineral density for irradiated specimens at 18 weeks compared with 8 weeks, denoting that there is a diminished measure of tissue mineral density in irradiated specimens over time. *XRT*, irradiation; *AMF*, amifostine.

Table 1

Experimental Group Sizes and Ultimate Sample Sizes for Biomechanical Testing after Moment of Inertia Uniformity Screening for Both 8-Week and 18-Week Time Points

	8-Week	18-Week
Experimental group sizes		
Control	6	10
XRT administration	11	10
AMF-XRT administration	9	10
Ultimate sample sizes		
Control	3	4
XRT administration	2	3
AMF-XRT administration	4	4

AMF, amifostine; XRT, radiation therapy.