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Raman spectroscopy delineates radiation-induced injury and partial rescue by amifostine in bone: a murine mandibular model

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Abstract

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Conflict of interest All authors declare they have no conflict of interest.

Despite its therapeutic role in head and neck cancer, radiation administration degrades the biomechanical properties of bone and can lead to pathologic fracture and osteoradionecrosis. Our laboratories have previously demonstrated that prophylactic amifostine administration preserves the biomechanical properties of irradiated bone and that Raman spectroscopy accurately evaluates bone composition *ex vivo*. As such, we hypothesize that Raman spectroscopy can offer insight into the temporal and mechanical effects of both irradiation and amifostine administration on bone to potentially predict and even prevent radiation-induced injury. Male Sprague–Dawley rats (350–400 g) were randomized into control, radiation exposure (XRT), and amifostine pre-treatment/radiation exposure groups (AMF-XRT). Irradiated animals received fractionated 70 Gy radiation to the left hemi-mandible, while AMF-XRT animals received amifostine just prior to radiation. Hemi-mandibles were harvested at 18 weeks after radiation, analyzed via Raman spectroscopy, and compared with specimens previously harvested at 8 weeks after radiation. Mineral ($\rho 958$) and collagen ($\rho 1665$) depolarization ratios were significantly lower in XRT specimens than in AMF-XRT and control specimens at both 8 and 18 weeks. amifostine administration resulted in a full return of mineral and collagen depolarization ratios to normal levels at 18 weeks. Raman spectroscopy demonstrates radiation-induced damage to the chemical composition and ultrastructure of bone while amifostine prophylaxis results in a recovery towards normal, native mineral and collagen composition and orientation. These findings have the potential to impact on clinical evaluations and interventions by preventing or detecting radiation-induced injury in patients requiring radiotherapy as part of a treatment regimen.

Keywords

Raman spectroscopy; Amifostine; Mandible; Pathologic fracture; Osteoradionecrosis

Introduction

Head and neck cancer encompasses a series of disease process that are collectively responsible for over 52,000 new cases and 11,000 deaths yearly [1]. The mainstay for treating these patients often consists of surgical extirpation of tumor burden with subsequent reconstruction to achieve functional and aesthetic recovery. In lieu of, or in addition to operative intervention, radiation therapy is another facet of the treatment regimen for these patients, the purpose of which is to assist in locoregional disease control and decrease the risk of subsequent recurrence [2–4]. However, radiation administration is responsible for inflicting multiple short-term and long-term morbidities; xerostomia, mucositis, impaired collagen synthesis, and blood vessel obliteration are common negative sequelae seen in soft tissues, while substantial damages to bone include pathologic fracture and osteoradionecrosis [5, 6]. These injuries leave patients susceptible to devastating impairments of the mandible and craniofacial skeleton and have a profound impact on the quality of life.

The cytoprotectant amifostine is on formulary for prophylaxis against radiation-induced mucositis and xerostomia in patients with squamous cell head and neck carcinoma [3, 7]. Amifostine is a pro-drug, free-radical scavenger that facilitates normal genetic expression, cell cycle progression, and facilitates repair of damaged DNA. Amifostine does not impair

the therapeutic efficacy of radiation treatment regimens, nor does it increase tumor recurrence rates or decrease disease-free and overall survival [3, 7, 8]. In addition to providing cytoprotection in soft tissues, amifostine is also capable of protecting bone and preserving new bone formation in irradiated murine mandibles in the settings of both distraction osteogenesis and fracture repair [9–11].

Over the past decade, there has been an increasing interest in the use of Raman spectroscopy to study a range of bone-related problems [12, 13]. Like Fourier transform infrared (FTIR) spectroscopy, used in bone studies since the early 1980s, Raman spectroscopy provides chemical signatures of both the mineral and matrix of bone tissue [13, 14]. Furthermore, Raman spectroscopy has proven to be a useful tool in quantifying the mineral and matrix chemical composition properties of bone; these include the bone mineral crystallinity and carbonate content, the amount of mineral relative to collagen, the state of collagen cross-linking, and the orientation of mineral crystallite and collagen fibrils. Alterations in these parameters can be easily monitored by Raman spectroscopy and could potentially help elucidate radiation-induced changes in bone material behavior and evaluate the efficacy of radioprotective therapies.

Previous findings from our laboratory suggest that amifostine is capable of protecting bone against negative radiation-induced negative sequelae; we have also shown that Raman spectroscopy is a viable means by which to evaluate the chemical composition and ultrastructure of irradiated bone, having studied the effects of therapeutic-dose radiation in both the mandible and the tibia [15, 16]. However, as these two sites of bone are different in physiology and in biomechanical properties, our results are not directly transferable from one case to the other. For this reason, we are continuing our studies on both.

Expanding on this knowledge, we hypothesize that Raman spectroscopy will offer insight into the temporal and mechanical consequences of both irradiated and amifostine pre-treated mandibular bone. These findings could substantially impact clinical evaluation and interventional practices by detecting and even preventing radiation-induced injury in patients undergoing treatment regimens utilizing radiotherapy.

Materials and methods

All aspects of this project pertaining to the use of animal subjects were performed in compliance with, and with the approval of, the University of Michigan's Committee for the Utilization and Care of Animals.

Experimental grouping

Male Sprague–Dawley rats (350–400 g) were randomly assigned into subgroups of animal controls ($n = 5$), animals receiving radiation (XRT) ($n = 5$), and animals receiving amifostine pre-treatment prior to radiation (AMF-XRT) ($n = 5$).

Radiation and amifostine administration

We collaborated with the Experimental Irradiation Core of the University of Michigan Comprehensive Cancer Center to calculate a head and neck cancer human-equivalent dose

radiation regimen of 70 Gy total [17]. Fractions were administered once daily over five consecutive days using a Philips RT250 orthovoltage unit (250 kV X-rays, 15 mA; Kimtron Medical, Oxford, CT, USA). Specimens receiving amifostine were administered a 100 mg/kg dorsal subcutaneous injection 45 min prior to irradiation, a dose developed by our laboratory and utilized in published studies [9–11]. Animals underwent induction with inhalational isoflurane and were placed in the radiation chamber and covered with a lead shield that exposed only the left hemi-mandible. During irradiation, isoflurane and oxygen were continuously administered. Animals were individually placed in cages and allowed to wake under continuous supervision upon completion of radiation administration.

Recovery and harvesting

Animals underwent a recovery and care period of 18 weeks after the completion of radiation. During this time, animals were housed, provided food and water, and cared for in compliance with the University of Michigan Committee for the Utilization and Care of Animals. Daily weights were recorded to monitor nutritional status and daily animal inspection was performed to evaluate for any complications from radiation or amifostine administration. At the end of the 18-week recovery period, animals were euthanized with drop isoflurane and left hemi-mandibles were harvested for analysis via Raman spectroscopy.

Raman spectroscopy

Raman spectra of male Sprague–Dawley left hemi-mandibles were recorded using a custom-built Raman microscope system. A 2 mm region of interest located just posterior to third molar of the hemi-mandible was excited with 100 mW of 785 nm laser radiation (Innovative Photonics Laser, Monmouth Junction, NJ, USA) (Fig. 1). The back-scattered Raman light from the hemi-mandible was collected via a 10X/0.5NA microscope objective (S Fluor, Nikon Instruments, Inc., Melville, NY, USA) and dispersed with a spectrograph (HoloSpec, Kaiser Optical Systems, Inc. Ann Arbor, MI, USA) and recorded with a CCD (Newton, Andor Technology, Belfast, Northern Ireland) with acquisition times of 120 s. A total of 8 spectra were collected from each hemi-mandible surface over the irradiated region of interest. For polarized Raman measurements, additional optical parts including a half-wave plate (WPMH05 M-780, Thorlabs, Inc., Newton, NJ, USA), a polarization analyzer (LPNIR050, Thorlabs, Inc.), and a wedge depolarizer (DPU-25-B, Thorlabs, Inc.) were added to the Raman microscope system as described previously [18].

Raman data was processed with MATLAB (The Mathworks, Inc., Natick, MA, USA) using locally written scripts and GRAMS/AI software (Thermo Galactic, Madison, WI, USA). A typical Raman spectrum of a rat hemi-mandible with band assignments is shown in Fig. 2. The mineral to matrix ratio is defined as the intensity of phosphate ν_1 band ($\sim 958\text{ cm}^{-1}$) divided by the combined intensities of proline and hydroxyproline bands ($854 + 873\text{ cm}^{-1}$). The carbonate to phosphate ratio is defined as the intensity ratio of the carbonate band ($\sim 1070\text{ cm}^{-1}$) to the phosphate ν_1 band ($\sim 958\text{ cm}^{-1}$). Mineral crystallinity is inversely proportional to the full width at half maximum of the phosphate ν_1 band at $\sim 958\text{ cm}^{-1}$. The collagen cross-linking ratio was defined as the intensity ratio of two sub-component bands ($\sim 1660/\sim 1690\text{ cm}^{-1}$) in the amide I region. The depolarization ratios (ρ) of both mineral and

collagen were defined using conventional definition: $\rho = I(\perp)/I(\parallel)$, where $I(\perp)$ is the intensity of the perpendicular polarization component for either selected mineral band ($\sim 958 \text{ cm}^{-1}$) or selected collagen band ($\sim 1665 \text{ cm}^{-1}$), and $I(\parallel)$ is the intensity of the parallel polarization component for the same band.

Significant differences between all control, XRT, and AMF-XRT specimens were analyzed using one-way analysis of variance (ANOVA) with post-Tukey test (SPSS 20, IBM Corp. Armonk, NY, USA). Statistical significance was defined at $p < 0.05$.

Results

The methods of animal selection, radiation and amifostine administration, recovery and care, and evaluation via Raman spectroscopy for the current 18 week control, XRT, and AMF-XRT specimens were identical to our prior study utilizing Raman spectroscopy to analyze 8 week control, XRT, and AMF-XRT specimens [15]. As such, findings and results were tabulated and compared between 8 and 18 week specimens.

Raman metrics were calculated and analyzed for all specimens at both 8-week and 18-week time points post-irradiation (Figs. 3, 4; Table 1). In contrast to our previous 8 week results, there is no significant differences demonstrated for the mineral to matrix ratio, carbonate to mineral ratio, or crystallinity ratio and collagen cross-linking ratio amongst all 18 week control, XRT, and AMF-XRT specimens (Table 1). Polarized Raman results show that both mineral ($\rho 958$) and collagen ($\rho 1665$) depolarization ratios of XRT specimens were significantly lower than those of control and AMF-XRT specimen. While the $\rho 958$ value of mineral did not differ between controls at both 8-week and 18-week time points, the $\rho 1665$ of collagen of AMF-XRT specimens was significantly decreased relative to 8 week controls.

Discussion

While much attention has been paid to the clinical and qualitative aspects of radiation-induced bone damage, few investigations have focused on quantitatively analyzing the chemical composition or specific structural changes caused by radiation administration. We recently reported that the mineral compositions of irradiated rat hemi-mandibles, including mineral to matrix ratio and carbonate to mineral ratio and crystallinity, significantly differed from those of both control and AMF-XRT specimens at 8 weeks post-irradiation [15]. While 8 week AMF-XRT specimens showed mineralization patterns similar to controls, no difference was seen compared with 8 week XRT specimens in collagen cross-linking ratio. These findings indicate that prophylactic amifostine administration mitigates radiation-induced mineral damage, but not radiation-induced collagen damage, in bone. Expanding our post-irradiation duration from an 8 week period to the 18 week period of this current study, our results demonstrate that there is no significant difference for these Raman metrics across all three experimental groups. This loss of significance suggests that there is a recovery towards normal, native controls in the AMF-XRT specimens.

The mineral depolarization ratios of XRT specimens were significantly lower than those of controls and AMF-XRT specimens at both 8 and 18 weeks post-irradiation (Fig. 4; Table 1). The lower ratio indicates more order to the mineral orientation in XRT specimens relative to

AMF-XRT and control specimens. When the depolarization ratios of collagen fibrils were considered, ρ_{1665} values of XRT specimens were also significantly lower than controls and AMF-XRT specimens at both 8 week and 18 week periods. This observation was expected since the hydroxyapatite in bone is oriented with its long c-axis along the collagen fibril length, thus, both mineral and collagen fibril arrangements should be similar in the bony tissue [19].

Although the nature of the pathological cross-links remains unknown, the more ordered arrangements of mineral and collagen fibrils formed in irradiated rat hemimandibles could be due to radiation-induced pathological cross-links between intermolecular and interfibrillar collagen molecules within collagen fibril bundles. We posit that once the collagen is damaged, the pathological cross-links would be poorly resorbed during remodeling; hence, any newly-formed tissue is constructed on a defective scaffold, ultimately leading to the deterioration of bone biomechanical properties. The radiation-induced abnormalities in orientation of mineral and collagen fibrils were fully rescued by prophylactic amifostine administration in the 18 week specimens, indicating a recovery or return towards the normal, native structure of irradiated bone when amifostine pre-treatment is utilized.

In the only peer-reviewed paper we could find, the reported differences in Raman spectra between irradiated human mandibular tissue and control subjects are even greater than what we observed in animal specimens [20]. Raman spectroscopy may potentially become an important modality for studying the effects of radiotherapy and adjuvant therapeutics in human subjects. While there is a small collection of literature on non-invasive measurements of Raman spectra of bone in animal models, no reports of human subject measurements have yet been published [21–23].

There are some limitations of this study worth mentioning. Firstly, we utilize skeletally-immature animals in our model. The recovery and rescue noted after inducing the stress of irradiation in amifostine pre-treated specimens may not be mirrored as precisely in a model utilizing older and potentially less resilient animals. Future studies can be performed to include varied age populations. Additionally, while we adhered to an identical protocol and environment for both the 8 week and 18 week specimen studies, it would be of benefit to examine experimental groups within both time points in parallel rather than in sequence to further eliminate variation and to validate our findings. Lastly, our experimental protocol examines ex vivo bone specimens, whereas in vivo methods of bone analysis would ultimately need to be utilized for real time, clinical evaluation.

Conclusion

In conclusion, we believe this study to be the first of its kind to elucidate the manner of radiation-induced damage to both the composition and ultrastructure of mandibular bone, complementing existing histological, imaging, and biomechanical information on the development of pathologic fracture and osteoradionecrosis. Furthermore, our results demonstrate the capacity for prophylactic amifostine administration to play a crucial role in protecting bone from the scourge of radiation-induced complications. By enabling recovery towards normal collagen and mineral composition, amifostine offers a translatable option to

protect mandibular bone from pathologic fracture and osteoradionecrosis. Our work may have tremendous potential impact on the clinical evaluation, prevention, and detection of radiation-induced injury in patients requiring radiotherapy as part of a treatment regimen for head and neck cancer.

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Fig. 1.
Representative image of a three-dimensional micro-computed tomography scan of the left hemi-mandible, viewed from the buccal side, with the anterior incisor facing to the right. The highlighted area is the 2 mm region of interest subjected to analysis via Raman spectroscopy

Fig. 2.
A typical Raman spectrum of a rat hemi-mandible with band assignments

Fig. 3. Comparison of Raman metrics at 18 weeks post-radiation: (*Top Left*) Mineral to matrix ratio, (*Top Right*) mineral crystallinity, (*Bottom Left*) carbonate to mineral ratio, and (*Bottom Right*) cross-link ratio. No significant differences were observed amongst all three groups

Fig. 4. Comparisons of depolarization ratios of mineral and collagen matrix for experimental groups at 8 weeks (*Top Left and Right*) and 18 weeks (*Bottom Left and Right*) panel post-radiation

Table 1

The results of statistical analysis among control, XRT and AFM/XRT groups at both 8 and 18 weeks post-radiation

Bone Raman parameters	Post irradiation time points	<i>p</i> (Control, XRT)	<i>p</i> (Control, AFM-XRT)	<i>p</i> (XRT, AMF-XRT)
Mineral/matrix	8 weeks	≤0.01	0.001	0.014
	18 weeks	0.217	0.907	0.111
Crystallinity	8 weeks	≤0.001	0.538	≤0.001
	18 weeks	0.614	0.801	0.282
Carbonate/mineral	8 weeks	≤0.001	0.157	≤0.001
	18 weeks	0.799	0.862	0.992
Collagen cross-linking ratio	8 weeks	0.003	0.004	0.994
	18 weeks	0.908	0.987	0.961
Depolarization ratio of mineral	8 weeks	0.003	0.525	0.039
	18 weeks	0.002	0.832	0.001
Depolarization ratio of collagen	8 weeks	≤0.001	0.025	≤0.001
	18 weeks	0.041	0.905	0.019