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Prevention of radiation-induced bone pathology through combined pharmacologic cytoprotection and angiogenic stimulation☆

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ABSTRACT

Pathologic fractures and associated non-unions arising in previously irradiated bone are severely debilitating diseases. Although radiation is known to have deleterious effects on healthy tissue cellularity and vascularity, no clinically accepted pharmacologic interventions currently exist to target these destructive mechanisms within osseous tissues. We utilized amifostine—a cellular radioprotectant—and deferoxamine—an angiogenic stimulant—to simultaneously target the cellular and vascular niches within irradiated bone in a rat model of mandibular fracture repair following irradiation. Rats treated with combined therapy were compared to those undergoing treatment with singular amifostine or deferoxamine therapy, nontreated/irradiated animals (XFx) and non-treated/non-irradiated animals (Fx). 3D angiographic modeling, histology, Bone Mineral Density Distribution and mechanical metrics were utilized to assess therapeutic efficacy. We observed diminished metrics for all outcomes when comparing XFx to Fx alone, indicating the damaging effects of radiation. Across all outcomes, only the combined treatment group improved upon XFx levels, normalized all metrics to Fx levels, and was consistently as good as, or superior to the other treatment options ($p < 0.05$). Collectively, our data demonstrate that pharmacologically targeting the cellular and vascular environments within irradiated bone prevents bone injury and enhances fracture healing.

1. Introduction

Adjuvant radiotherapy is an effective treatment modality that is utilized by approximately half of the cancer patient population [1]. Despite its benefits, radiotherapy is known to have caustic effects on healthy tissues through mechanisms that disrupt normal tissue vascularity and cellularity [2,3]. Bone is particularly susceptible to these detrimental effects because of a baseline metabolic turnover rate that is comparatively slower than that of other tissue types [4]. This slow metabolism can help to mask clinical symptoms until pathologies progress beyond the point of prevention or early intervention. These aberrant effects can lead to debilitating pathologies such as osteoradionecrosis, pathologic fractures and associated non-unions [5].

Although the underlying mechanisms of radiation injury have been studied extensively, currently, no clinically accepted medical therapies exist to prevent the deleterious effects of radiation on normal osseous tissues [6]. Pharmacologic strategies designed to manipulate and optimize the cellular and vascular environments within irradiated bone are therefore warranted.

Previously, our laboratory has utilized amifostine—a radioprotectant—and deferoxamine—an angiogenic stimulant—as targeted interventions to selectively preserve osteocyte viability and augment vascularity, respectively, in an animal model of mandibular fracture repair following radiation exposure. Our results demonstrated the ability of these singular therapies to partially temper the effects of radiation on mechanisms of fracture healing as measured with 3D angiographic modeling, histology, radiomorphometrics and mechanical testing [7–10].

Although our results with singular therapies are promising, complete restoration of our outcome measures and clinical assessments to that of normal, non-irradiated bone has yet to be achieved. The purpose of this study was to improve upon the success of singular therapies in an effort to reach more consistently normalized outcome measures by combining these targeted therapeutic interventions. We hypothesized that the cellular radio-protective nature of amifostine, in combination with the angiogenic stimulation of deferoxamine would act in a complementary manner to improve upon irradiated fracture metrics and normalize outcome measures to reach non-irradiated fracture levels. Here we report 3D angiographic modeling, histology, Bone Mineral Density Distribution (BMDD) and biomechanical metrics of bone healing.

☆ Disclosures: All authors state that they have no conflicts of interest.

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2. Materials and methods

2.1. Study design

All animal experimentation was approved by the University of Michigan's Committee for the Utilization and Care of Animals (UCUCA), and conducted in accordance with the guidelines published in the Guide for the Care and Use of Laboratory Animals: Eighth Edition. In order to facilitate the incorporation of destructive outcome measures, two cohorts of animals undergoing identical experimentation (with the exception of outcome testing) represent each group. Animals in cohort 1 underwent 3D angiographic modeling and histology, while animals in cohort 2 underwent μCT imaging for BMDD analysis and mechanical testing.

Twelve-week-old male Sprague Dawley rats (n = 117) were divided into 5 groups: fracture (Fx), irradiated fracture (XFx), irradiated fracture treated with deferoxamine alone (DFO), irradiated fracture treated with amifostine alone (AMF), and irradiated fracture treated with amifostine plus deferoxamine combination therapy (Combined). In Cohort 1 (n = 60), animals were equally divided between groups (n = 12/group). Cohort 2 (n = 57) consisted of: Fx (n = 5), XFx (n = 14), DFO (n = 15), AMF (n = 10) and Combined (n = 13). All irradiated groups received a previously established human equivalent dose of radiotherapy (HEDR) two weeks prior to surgery. AMF and Combined groups received an injection of subcutaneous amifostine immediately prior to each radiation therapy session. Following a two-week recovery period, all groups received an osteotomy posterior to the 3rd molar of the left hemi-mandible, along with the placement of an external fixator device. The DFO and Combined groups then received injections of deferoxamine directly into the fracture callus every other day from post-operative days 4–12 for a total of 5 doses. Following a 40-day healing period, animals were sacrificed, and mandibles were dissected for outcome testing (see Fig. 1).

2.2. Amifostine injection

A subcutaneous amifostine injection (100 mg/kg) was given 40 min prior to radiation therapy once daily for five consecutive days according to the radiation therapy schedule outlined below. The dosage was derived from an extensive review of the literature and previous work in our laboratory. We further optimized these doses and dosing schedules for use in this animal model [11,12].

2.3. Radiation procedure

Induction of anesthesia was achieved with an oxygen/isoflurane mixture. Left hemi-mandibles were irradiated using a Philips RT250 orthovoltage unit (250 kV X-rays, 15 mA; Kimtron Medical, Woodbury, CT). Our selected region of interest (ROI) spanned a 2 mm distance posterior to the third molar, which corresponded to the future site of osteotomy. Lead shielding ensured localized delivery and protection of surrounding tissues. A previously described HEDR, developed with the guidance of the Department of Radiation Oncology at the University of Michigan, was utilized [13]. A fractionated dose of 7 Gy per day was administered over 5 days for a total of 35 Gy, which is comparable to 70 Gy in human mandibular high-dose radiotherapy. Animals were allowed a 14-day recovery period after radiation exposure prior to osteotomy surgery.

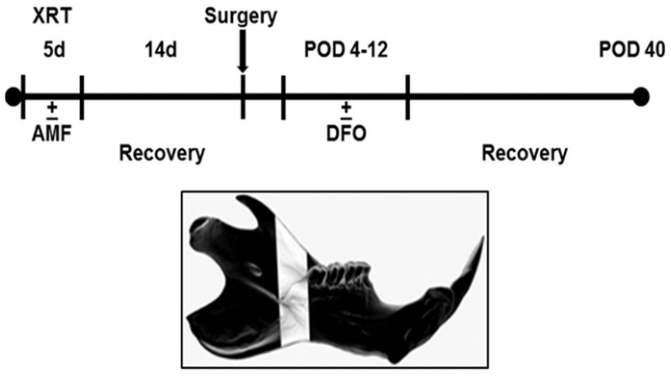

Fig. 1. (Top): Experimental timeline. (Bottom): Schematic left hemi-mandible demonstrating the region of interest (ROI) highlighted in white.

2.4. Surgical procedure

Animals were prepared for surgery and underwent external placement of a custom mandibular fixator device followed by osteotomy directly behind the third molar on the left hemi-mandible as previously described [14,15]. Four hours after osteotomy, the fixator device was set to a 2 mm fixed distance for the remainder of the experiment.

2.5. Deferoxamine injection

200 μM deferoxamine in 300 μL of normal saline was injected directly into the fracture site every other day starting on post-operative day 4 and continuing through post-operative day 12. This dosage was selected from a review of the literature concerning the use of deferoxamine in long bone animal models and modified according to our experimental use in the rat mandible [16–20]. The time frame for administration was chosen to correlate with the reasonable time period for the initiation of angiogenesis in a murine fracture model [21–23].

2.6. 3D angiographic modeling

Only rats in cohort 1 were anesthetized prior to thoracotomy and underwent left ventricular catheterization. Perfusion with heparinized normal saline followed by pressure fixation with normal buffered formalin solution ensued and ensured euthanasia. After fixation, the vasculature was injected with Microfil (MV-122, Flow Tech, Carver, Mass.), and mandibles were subsequently harvested en bloc and demineralized using Cal-Ex II solution (Fisher Scientific; Fairlawn, NJ). Leeching of mineral was confirmed with serial radiographs to ensure adequate demineralization prior to scanning. μCT images were obtained using 80 kVp, 80 mA and 1100 ms exposures. Three hundred and ninety-two projections were taken at a resolution of 18-μm voxel size. Utilizing GE's Microview 2.2 software, scans were reconstructed and reoriented in a 3D x, y and z plane. The ROI was then cropped and splined for analysis. Due to demineralization, only the vessels perfused with Microfil appeared on the μCT scan. Vessel Volume Fraction (VVF) and Vessel Number (VN) were assessed [24].

2.7. Histological analysis

All specimens underwent 70% ethanol fixation at 4 °C and were decalcified with Cal-Ex II solution. The specimens were then vacuum processed, filtrated, and embedded in Paraplast Plus (i.e., paraffin containing dimethylsulfoxide; McCormic Scientific, Richmond, Ill.) as previously described [39]. Embedding molds (22 × 40 mm) were used and stored overnight at 4 °C. Blocks were sectioned coronally from anterior to posterior spanning the ROI (a 2 mm distance posterior to the third molar, which corresponded to the site of osteotomy). 7 μm thick sections were taken through the ROI on a Leica Reichert-Jung microtome (model 2030; Biocut, Bensheim, Germany), and mounted on glass slides (Fisherbrand Superfrost Plus; Fisher Scientific). Sections were surface-stained with Gomori's one-step trichrome. Osteocyte count (OC) was performed with a light microscope interfaced with a digital camera connected to a computer. Our ROI was superimposed onto the digital image using Bioquant NOVA Osteo version 7 (R&M Biometrics, Nashville, Tenn.). Nine high-power field images were randomly selected per ROI