Direct Effects of *Ex Vivo* Radiation on Microdamage Accumulation During Fatigue Loading

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INTRODUCTION: Radiation therapy (RTx) has been shown to weaken and embrittle bone, rendering it susceptible to fragility fractures. Depending on cancer type, treatments, and location, 4-30% of cancer survivors will suffer post-RTx fractures. To date, most studies of bone irradiated *in vivo* have utilized monotonic loading in test protocols, and little is known about *how* irradiation may affect bone fragility, damage propagation, or failure following cyclic loading during activities of daily living. The goal of this study was to use a cadaveric rat model to quantify direct effects of therapeutic radiation dose on the accumulation of microdamage during fatigue loading of femurs, and relationships between fatigue testing properties and damage formation. Data from this study will inform future *in vivo* work using small animal models to study how post-radiotherapy femur fatigue strength and microdamage propagation change longitudinally following radiation treatment.

METHODS: Paired femurs were excised from fresh frozen rat cadavers (Layne Labs). The right femurs were irradiated with 20 Gy (x-rays, 1 Gy/min) in a saline bath to maintain hydration. The left femurs constituted the non-irradiated Sham group. Femurs were stored frozen at -80°C, wrapped in saline soaked gauze, until testing. Freeze-thaw cycles were minimized and kept consistent within femur pairs. Immediately prior to testing, pre-existing bone microdamage was labeled with 0.02% alizarin complexone. Post-staining femurs were rinsed for 1 hour in running diH_2O and then positioned into a 3-point bending fixture (14.5 mm support span, anterior femur contacting the central loading point, in a saline bath) for fatigue testing (MTS 585 Mini Bionix II). A preliminary 100-cycle pre-load was applied to determine the specimen stiffness (-5 N to -35 N). Pre-load stiffness data were then used to calculate forces required to achieve the target strain levels (either 0.6%, 0.8%, 1.0%, or 1.2% strain, n = 5 bones/strain/treatment group, except for n = 4 for the 1.0% strain). Femurs were fatigued at the designated strain level until failure (fracture) or an upper limit of 500,000 cycles was reached. Immediately post-testing, femurs were stained in 0.005% calcein green to label loading-induced damage, embedded in Spurr's resin, sectioned and imaged under fluorescence microscopy. Three sections of tissue were made from each bone, one located at the central loading point and one each midway between the loading point and the proximal and distal ends of the femur. Diffuse damage was quantified by manually tracing areas of intense calcein staining on fluorescence microscopy images in Affinity Photo software. Outcome measures included number of cycles to failure, apparent strain, stiffness, and diffuse damage area.

RESULTS: While there was a strong inverse relationship between apparent strain and cycles to failure ($p < 0.001$, Wald Test), RTx was not a significant independent predictor of cycles to failure ($p = 0.1719$). However, the interaction term (strain*treatment), which tests whether the slopes of the strain-cycles to failure relationship differ, was significantly different between treatment groups ($p = 0.0167$) and there was divergence between RTx and Sham femurs in the number of loading cycles to failure with increasing strain. Preliminary analysis indicated that the distal-most tissue section was the source of greatest intra-sample variability, which was not entirely surprising given the compliance of the physeal tissue at this location. Because of this variability, the distal-most section was excluded from further data analysis. Evaluation of diffuse microdamage area from sections at the loading point and proximal from the loading point demonstrated a negative correlation between microdamage area and increasing number of cycles to failure, and a positive correlation between microdamage area and increasing apparent strain at 1,000 cycles. The r^2 values for these correlations were stronger for RTx femurs (0.24-0.45) than for Sham group femurs (0.05-0.18). While ANOVA did not indicate a significant effect of treatment (RTx vs. Sham), both number of cycles to failure (log[n]) and strain at 1,000 cycles were significantly correlated with diffuse damage area ($p \leq 0.0348$).

DISCUSSION: Fatigue testing allows us to recapitulate the cyclic, atraumatic loading of bone that can lead to fragility fractures in post-RTx patients. The relationship between bone microdamage and strength is incompletely understood, and can be altered in disease states (drug treatments, diabetes, disorders of the extracellular matrix). To date, *in vivo* models have shown that localized therapeutic doses of radiation induce loss of both bone strength and fracture toughness accompanied by only minor changes in collagen crosslinking and no measurable collagen fragmentation [1]. Data presented here suggest that radiation can directly induce functional changes in fatigue strength— RTx femurs are more likely to fail at low cycle numbers (and high strains) than contralateral Sham femurs. However, these changes which are likely small compared to the post-RTx bone modifications that occur during *in vivo* remodeling activity. This is evidenced by the RTx-induced disruption in the relationship between microdamage area generated during testing and biochemical parameters (strain or cycles to failure). Future work will include use of an *in vivo* rat model to overcome the limitations of the short femurs in the feeder rats used here, as well as to assess how longitudinal factors may affect the post-RTx relationship between bone strength and microdamage.

SIGNIFICANCE: Following irradiation, atraumatic fragility fractures occur in 4 – 30% of cancer survivors. These complex fractures are difficult to predict, prevent, and treat. Developing a better understanding of the fatigue properties of irradiated bone will help us to characterize failure mechanisms and develop preventative interventions for future clinical use.

REFERENCES: [1] Bartlow CM, Mann KA, Damron TA, Oest ME. (2018) *PLOS ONE* 13(10):e0204928, PMID 30281657.

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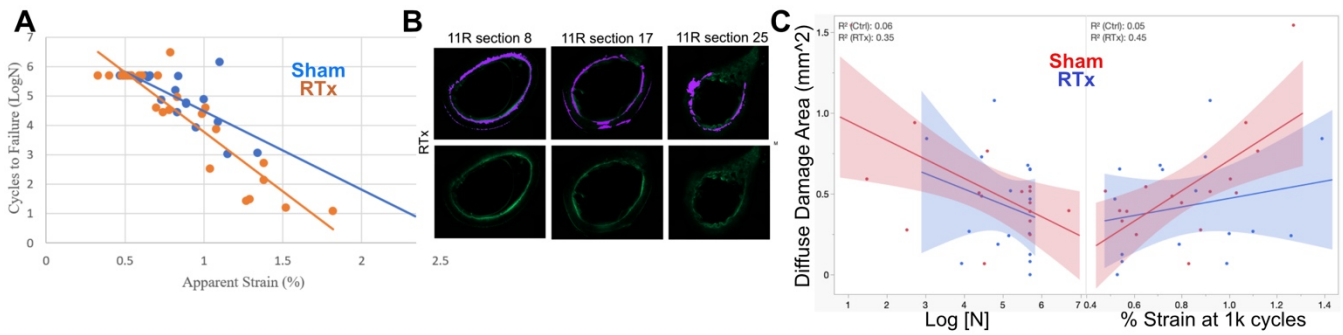

A) S-N curves (cycles to failure vs. apparent strain) for Sham (blue) and RTx (orange) femurs. **B)** Representative images of the microdamage quantification process. **C)** Diffuse damage area (mm^2) at the loading point vs. cycles to failure and strain at 1,000 loading cycles.