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Effects of Low Dose X-Ray Irradiation on Porcine Articular Cartilage Explants

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Abstract

Ionizing radiation therapy is a crucial treatment for cancer, but can damage surrounding normal tissues. Damage to articular cartilage leading to arthropathy can occur at irradiated sites. It is unclear whether this response is due to damaging surrounding skeletal structures or direct effects on cartilage. In this study, we showed that irradiation with 2 Gy of X-rays causes a significant reduction in the stiffness of porcine explants 1 week post-irradiation. By using both microindentation and indentation-type atomic force microscopy, ionizing radiation reduces stiffness in both the superficial zone and throughout the entire thickness of the tissue. Young's modulus values were 75% and 60% lower in 2 Gy irradiated samples when compared with controls using microindentation and nanoindentation, respectively. Glycosaminoglycans (GAGs) released into the culture media of irradiated samples was nearly 100% greater at 24 hours after exposure. While collagen content in the tissue is similar between groups, GAG content is 55% lower in irradiated explants compared with controls by one week. Therefore, the irradiated explants are unable to recover from the initial loss of GAGs by one week. This acute loss of GAGs is a likely contributor to the reduction in modulus seen after exposure to ionizing radiation.

Keywords

articular cartilage; radiation exposure; cartilage mechanics; atomic force microscopy; glycosaminoglycans

INTRODUCTION

Ionizing radiation has developed into a key treatment option to prevent tumor growth and metastasis in cancer patients. However, radiation can cause both acute and chronic damage to normal tissues through a dynamic process involving both cell death and altered cell and tissue function independent of reduced viability.[1, 2] Musculoskeletal tissues have historically been considered late-responding tissues,[3, 4] with bone damage and fractures of irradiated sites a well documented response to exposure for treatment of malignancies, especially in the femoral neck or sacrum for pelvic malignancy[5, 6] or ribs following stereotactic body radiation therapy.[7] However, recent studies have shed new light onto the sensitivity of skeletal tissues to low doses of radiation, with early skeletal deficits occurring after exposure resulting from elevated osteoclast activity.[4, 8] Joint injury, including degenerative arthritis or arthropathy within synovial joints, are also considered late consequences of radiation exposure.[9] The arthropathy observed in the hip and knee (commonly termed "post-

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irradiation osteoarthritis") are generally attributed to osteonecrosis rather than chondrocyte-induced cartilage degradation.[10, 11] Little information is known regarding early effects on articular cartilage metabolism or mechanical properties following exposure to radiation.

While the radiation response of articular cartilage from embryonic or very young animal models remain inconsistent,[12–14] articular cartilage from adult humans or large animal species appears to degrade following exposure.[12, 15] This response is characterized by an active degradation of cartilage matrix and reduced proteoglycan production in pigs,[15] dogs,[12] and from human donors.[15] Collagen II synthesis following radiation exposure has been shown to be lowered in articular chondrocytes harvested from a large animal species (bovine).[16] If radiation alters cartilage matrix metabolism, including active degradation of proteoglycans or lowered proteoglycan or collagen II synthesis, a reduction compressive modulus of the irradiated cartilage is imminent. In our pilot study, we showed a significant decrease in Young's modulus of articular cartilage in mice one week after a 2Gy, whole-body X-ray irradiation.17

The goal of this study was to characterize the alterations in mechanical properties and matrix composition in porcine articular cartilage explants following direct exposure to X-rays, a model that has been shown to respond similarly to human tissues.[15] No studies have addressed the possible mechanical alterations that might occur as a result of the direct damage to cartilage following radiation exposure. While our prior studies showed a marked decrease in modulus following radiation exposure, the scope of these results is limited. Due to the small sample size of murine articular cartilage, indentation-type atomic force microscopy (IT AFM) was used to measure the Young's modulus of the tissue. However, the limitations of IT AFM only allow for the tissue to be indented 1–2μm, while the measured thickness of our articular cartilage samples was found to be ~70μm. Therefore, in this study, we ran mechanical tests at multiple length scales to determine if radiation exposure reduces the stiffness of articular cartilage only in the superficial zone, or if it affects the bulk properties of the tissue.

MATERIALS AND METHODS

Tissue Harvest and Culture

Articular cartilage was excised from fresh tibiofemoral condyles of 4–6 month old swine using aseptic techniques (Snow Creek Meat Processing, Seneca, SC). Explants were cut into disks 5 mm in diameter and ~2 mm thick using a dermal biopsy punch. All samples were measured for thickness using a digital caliper (Mitutoyo, Aurora, IL) and only explants 2.0 ± 0.1 mm in thickness were used. Care was taken to avoid collecting subchondral bone with the cartilage samples. Cartilage disks were rinsed in Hank's balanced salt solution (HBSS) supplemented with 1% penicillin/streptomycin and amphotericin B (Gibco, Carlsbad, CA). After 3 rinses, explants were individually placed in 24-well plates and separated into control and irradiated groups. All samples were cultured at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA), 1% nonessential amino acids, 1% penicillin G, 1% streptomycin, and 1% amphoticerin B (Gibco), 20 mM ascorbic acid, 10 mM HEPES buffer, and 0.4 mM proline (Sigma-Aldrich, St. Louis, MO).[18] Culture media was replaced every other day. For all studies, each animal was represented with 2 explants, 1 for the control group and 1 for the treatment group (2Gy X-ray irradiation).

X-Ray Irradiation

Cartilage disks were equilibrated for 24h in tissue culture conditions before irradiation. The covers of the 24-well plates were removed and the plates were placed in sterile plastic bags

to prevent attenuation of the X-rays (the X-ray source was above the samples). Explants in the irradiated group were exposed to 2 Gray (Gy) of 125 peak kilovoltage (kVp) X-rays using a 150kV industrial portable X-ray unit (Philips Medical Systems, Bothell, WA). Control samples were placed in plastic bags and transported to the X-ray facility (but not irradiated) to prevent discrepancies in culture conditions.

AFM Nanoindentation Testing

Samples from both groups ($n = 5$) were cultured for either 24h or 168h after radiation exposure (or sham irradiation for control group). Each explant was placed on a glass slide, hydrated with culture media, and securely mounted on the atomic force microscope (AFM). Standard indentation testing in fluid was performed on an Asylum Research MFP-3D (Goleta, CA). A borosilicate glass cantilever with a 0.12 N/m nominal spring constant and a 5μm diameter spherical indenter was utilized for the indentation testing. Samples were indented 1μm at a speed of 1μm/s.

Microindentation Testing

Control and irradiated samples ($n = 5$) were cultured and tested using microindentation at the same time points as the AFM indentation. Each samples was placed in a Petri dish and covered in culture media. Cartilage disks were indented using a CETR Universal Mechanical Tester with a 20N load cell and a 1mm diameter stainless steel spherical indenter (Campbell, CA). Explants were pre-loaded to a force of 0.15N for 20s, then indented 300μm at a speed of 5μm/s.

Indentation Data Analysis

Both the nanoindentation and the microindentation curves were fit the to Hertz model, which assumes an infinitely hard sphere indents a flat, linear elastic, infinite half-space:

$$F=\frac{4}{3}\frac{E}{1-\upsilon^2}R^{\frac{1}{2}}\delta^{\frac{3}{2}}$$

where F is the measured force (N), E is apparent Young's modulus (Pa), υ is Poisson's ratio, and R is the spherical indenter radius (R = 2.5 μm), and δ is the indentation depth (m).[19] Since biological tissue is nearly incompressible at the indentation rates used in the present study, the Poisson's ratio (υ) was assumed to be 0.5.[20] Since cartilage is viscoelastic in nature and the Hertz model assumes linear elastic behavior, only the first 250nm and 100μm of indentation were used to fit the Hertz model to the nanoindentation and microindentation curves, respectively. Each explant was indented at 3 different locations and AFM samples were indented 5 times at each location.

Quantification of GAG Content

Glycosaminoglycan (GAG) degradation and synthesis was analyzed by measuring the sulfated GAG (sGAG) quantity in the cartilage explants and in culture media. Conditioned media was collected immediately before irradiation (Day 0) and 1, 3, and 7 days after radiation exposure. Articular cartilage tissue explants were digested 7 days post-irradiation using previously described methods.[21] Briefly, explants were individually placed in 500μL papain solution consisting of 125μg/mL papain, 0.1M sodium acetate, 5mM EDTA, 5mM L-cysteine-HCl and heated at 60°C for 12h (Sigma-Aldrich). Media and digests were tested for sGAG content using the dimethylmethylene blue colorimetric assay.[22, 23] Using a 96-well plate, 180μL of dimethymethylene blue was added to 20μL of each sample. The standard curve was created using increasing concentrations of chondroitin sulfate (Sigma-Aldrich). Absorbance was read immediately at 525nm using a Synergy 3 microplate reader and each reaction was performed in triplicate (BioTek, Winooski, VT). Sulfated GAG content for each sample was determined by substituting its absorbance measurement into the linear

regression equation determined using the standard curve, then normalized to each cartilage explant's wet weight.

Quantification of Collagen Content

Collagen concentrations were assessed in the explants 7 days after radiation exposure using the hydroxyproline (HYP) colorimetric method.[24, 25] Following papain digestion as described before, 100μL of the tissue lysates were hydrolyzed by adding 900μL of 6M HCl and heated at 110°C for 18h. Following hydrolysis, the lysates were neutralized using NaOH and diluted to 5mL to minimize salt concentrations. In a 96-well plate, 100μL of Chloramine T was added to 10μL of each sample and incubated for 5 minutes at room temperature. Then, 100μL of Ehrlick's reagent (DMAB) was added to each well and incubated for 90m at 60°C. *Trans*-4-hydroxy-L-proline in increasing concentrations was used to create the standard curve (Sigma-Aldrich). All reactions were performed in triplicate. The absorbance was read at 560nm on the microplate reader. HYP content was calculated by using the measured absorbance and the linear regression equation fit to the standard curve, then normalized to tissue wet weight.

Histology

Histological techniques were used to examine alterations to cell morphology and matrix composition in the explants following radiation exposure. Control and irradiated cartilage samples were fixed with 10% neutral buffered formalin 1 and 7 days after radiation exposure. Cartilage disks were embedded in paraffin wax and cut into 6μm cross sections using a microtome. Explants were then stained with 1 of 3 stains. Hematoxylin and Eosin (H&E), Safranin O-Fast Green, and Masson's Trichrome were used to assess cell viability, GAG integrity, and collagen content, respectively.

Statistics

All data are shown as mean ± standard deviation. Significance was determined using SigmaStat version 3.5 (Systat Software, Inc.; Richmond, CA). For both the microindentation and nanoindentation data, a Student's *t*-test was used to compare Young's modulus between control and irradiated groups (n=15 per group). One-way analyses of variance (ANOVAs) were used to test for inter-animal variability within each group (n=3 per animal). Student's *t*-tests were used to test for significance in GAG content between control and irradiated samples for the DMB assay on the culture media (n=6 per group) and tissue (n=9 per group). Additionally, one-way ANOVAs followed by Tukey's *post hoc* tests were used to compare GAG content in conditioned culture media between different days within the same treatment group (n=6 per day). For the hydroxyproline assay, a Student's *t*-test was used to test for significance in collagen content control and irradiated cartilage explants (n=9 per group).

RESULTS

Mechanical Testing

One week after radiation exposure, the average Young's modulus in the articular cartilage calculated from both the microindentation and AFM curves was significantly lower in the irradiated groups when compared to the non-irradiated groups ($p < 0.001$, Figures 1 & 2). The Young's modulus values were ~75% and 60% lower in the irradiated cartilage when compared with the control cartilage for microindentation and nanoindentation, respectively. For both mechanical testing modalities, there were no significant differences in Young's modulus between animals within each group ($p > 0.05$).

Dimethylmethylene Blue Assay

Using the DMB assay, normalized sulfated GAG (sGAG) content was significantly lower in the 2 Gy irradiated cartilage tissue when compared with the control cartilage 7 days after radiation exposure ($p < 0.001$, Figure 3). The DMB assay also showed significantly higher sGAG released into the culture media in irradiated samples when compared to control samples at Day 1 ($p < 0.001$), while there was no significant difference in sGAG content released between treatment groups at the Day 0, 3, or 7 time points ($p > 0.05$) (Figure 4). Sulfated GAG concentrations in the conditioned culture media at Day 1 were approximately 100% greater in the irradiated group when compared with the control group. Moreover, GAG concentrations in the culture media of the irradiated cartilage samples were significantly higher at Day 1 than Days 0, 3, and 7 ($p < 0.001$). Sulfated GAG content in the media of the control group was significantly higher at Day 1 compared to Day 3 ($p < 0.01$).

Hydroxyproline Assay

Normalized HYP concentrations in the cartilage tissue 1 week post-irradiation averaged at 12.62 ± 1.43 and 12.96 ± 3.35 mg HYP/g total tissue for control and irradiated samples, respectively (Figure 5). HYP content between control and irradiated cartilages explants was similar.

Histology

Qualitatively, no differences were seen between control and irradiated samples for all 3 histological stains (Figure 6). For Hematoxylin and Eosin stained sections, there were no signs of significant apoptosis or necrosis in either the control or irradiated samples, and chondrocyte density and morphology were similar between groups. No signs of osteoarthritis were observed histologically using using the Osteoarthritis Research Society International (OARSI) scoring system.[26]. Collagen content as determined histologically was similar between control and irradiated explants.

DISCUSSION

Joint degradation following exposure to ionizing radiation is an understudied issue that may face radiation therapy patients, though evidence indicates late arthropathy or joint failure following cancer treatment at irradiated sites.[9, 27] Arthopathy in the hip and knee have often been attributed to osteonecrosis rather than chondrocyte-induced cartilage degradation.[10, 11] Recent work of explanted cartilage and cells from adult humans and large animal (pig) models indicate that radiation of cartilage in isolation can induce an active and early degradation of proteoglycans coincident with reduced proteoglycan synthesis and IGF-1 sensitivity, all characteristic of an osteoarthritic phenotype.[15] However, the effect of radiation on cartilage mechanical properties was previously unknown.

In this study, we demonstrated that low doses of radiation causes a significant decrease in the compressive stiffness of articular cartilage at multiple length scales (Figures 1 and 2). In addition, the Young's modulus values for our control tissue are comparable to those found in the literature using similar testing parameters for both microindentation and IT AFM.[28, 29] With Young's modulus values around 75% and 60% less in irradiated samples compared with controls using microindentation and nanoindentation, respectively, we believe that radiation affects the entire thickness of articular cartilage, not just the superficial zone. Such a drastic reduction in mechanical properties following irradiation suggests that alterations are occurring in the matrix.

Results from the DMB assay suggest that an acute release of GAGs occurs early after radiation exposure, in agreement with others.[15] Samples exposed to radiation showed

significantly higher sGAG levels in the culture media at Days 1 when compared with Days 0, 3, and 7. Also, sGAG release in the control samples was significantly higher at Day 1 when compared with Day 3, but this is usually seen in cartilage explants cultures.[30] At Day 1, irradiated samples released around twice as many sGAGs in the media when compared with controls. In both control and irradiated samples, sGAG concentrations in the media decrease to around Day 0 levels by Day 3 and maintain this concentration through Day 7. However, the concentration of sGAGs in the irradiated tissue is over 50% less than the concentration found in control tissue. Thus, GAGs present in the tissue at exposure were likely degraded and released. A direct reduction of proteoglycan synthesis has been shown from pig and human chondrocytes early after direct exposure at similar doses.[15] New GAG synthesis may have been insufficient to replenish these degraded and released GAGs after 1 week post-irradiation, though direct GAG synthesis was not measured in this study.

We observe 75% and 60% decreases in Young's modulus after radiation exposure with microindentation and nanoindentation, respectively; it is unlikely that this large decrease is solely due to the observed loss of proteoglycan. Proteoglycans and their associated GAGs are only estimated to be responsible for about 50% of the compressive stiffness of articular cartilage.[31] Therefore, we believe GAG loss is not the only factor affecting the modulus post-irradiation, though other causes remain undefined. While we observe no significant difference in collagen content between control and irradiated groups, the hydroxyproline assay is unable to detect the integrity of collagen in the tissue. As a result, it was not possible to determine if changes in cross-linking or other structural damage are contributing to the reduction in modulus post-irradiation. However, there is no observable fibrillation in our irradiated histology samples (Figure 6). Additionally, reduced cell viability or necrosis is unlikely to have contributed to the observed results. Our histological data indicate no evidence of necrosis or apoptosis after radiation exposure. These observations are in in agreement with others who have examined cell viability in pig, human, and growing rabbit chondrocytes at similar time points following 10 Gy exposure.[15, 32] Thus, while reduction in GAGs within pig articular cartilage occurs after exposure and likely contributes to our observed lowering of stiffness, the entirety of the cause remains unclear.

Explant culture is a commonly used method to observe the metabolic activity of articular cartilage. However, interactions between individual tissues within the joint (e.g., synovium, synovial fluid, subchondral bone) can affect cartilage morphology, as observed during conditions such as rheumatoid arthritis.[33] The entire joint would absorb dose during cancer therapy and thus would likely affect several joint tissues. Direct irradiation of synovial joints *in vivo* is necessary to identify whether the degradation of articular cartilage occurs in a similar manner after exposure, and identify possible tissue interactions. Furthermore, determining the nature of the cartilage degradation following exposure was not a component of this study. Further research (both *in vivo* and *ex vivo*) will identify potential molecular targets for the functional deficits in cartilage after exposure.

In conclusion, the Young's modulus of articular cartilage was found to decrease after exposure to low doses of ionizing radiation, regardless of mechanical testing length scale. Therefore, we believe that radiation affects the bulk mechanical properties of the cartilage, not only the superficial zone. The acute release of GAGs is a likely contributor to this change in stiffness, as irradiated samples had a significantly higher release of GAGs 24 hours after irradiation. Therefore, further investigation should be performed to determine if radiotherapy causes long-term damage to articular cartilage.

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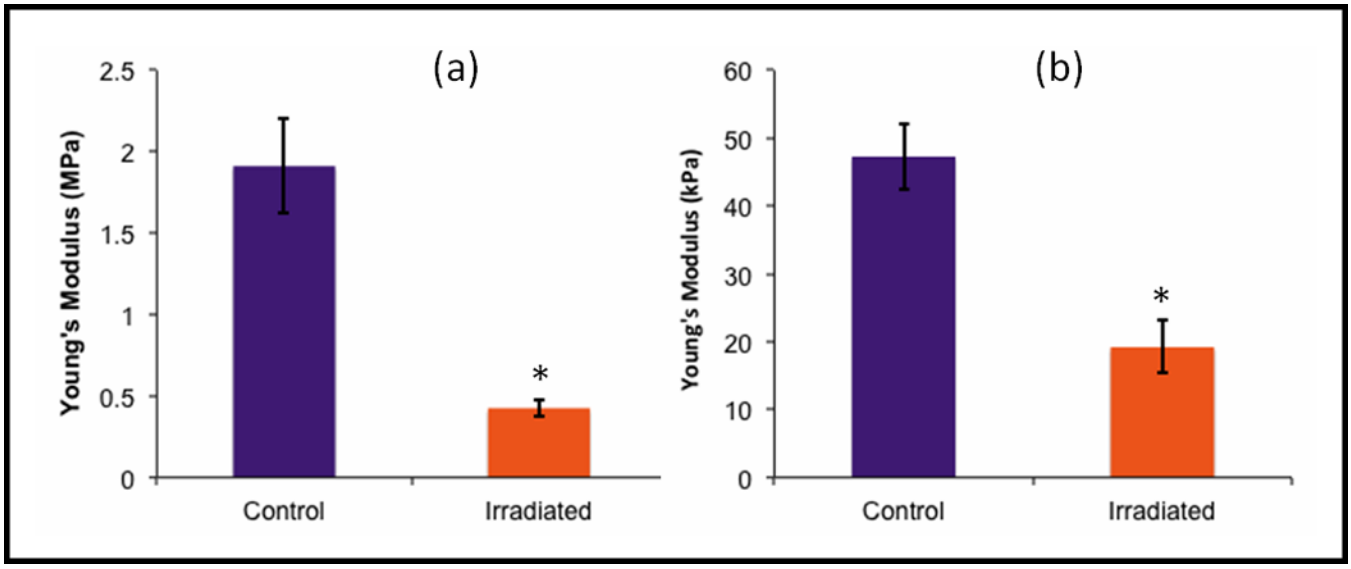

Figure 1.

Young's modulus values for control (purple) and 2Gy-irradiated samples estimated using the Hertz model tested by a) microindentation and b) nanoindentation. The modulus values for irradiated samples were significantly lower than control samples using both mechanical tests (*, $p < 0.001$, $n = 15$). Error bars show ±standard deviation.

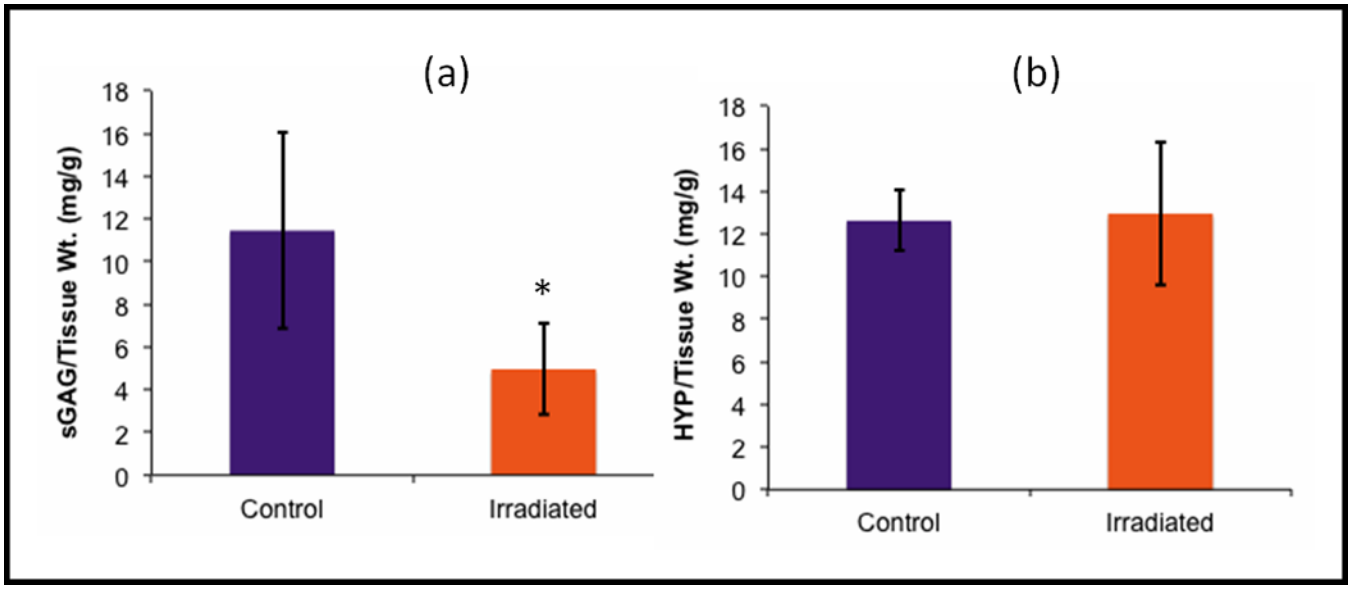

Figure 2.

Normalized a) sGAG and b) HYP content in the tissue at Day 7, the time point used for mechanical testing. Irradiated samples had significantly lower sGAG content when compared to control groups (*, $p < 0.05$, $n = 9$). HYP content was similar between groups. Error bars indicate ±standard deviation.

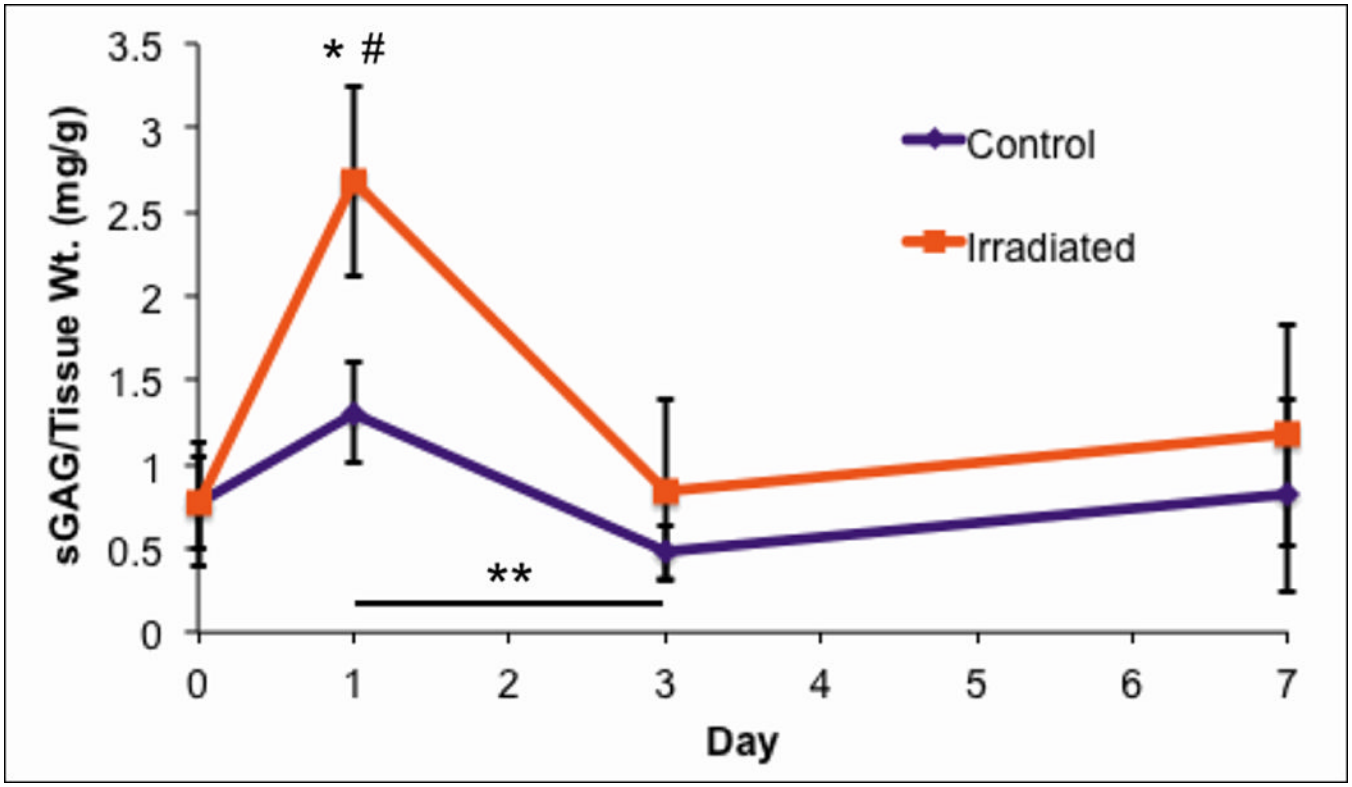

Figure 3.

Normalized sGAG content in the culture media over time ($n = 6$, error bars ±standard deviation). Significantly higher sGAG was released into the media in irradiated samples when compared to control samples at Day 1 (*, $p < 0.001$), while there was no significant difference in sGAG released between treatment groups at the Day 0, 3, or 7 time points. Concentrations of sGAG in the media of the irradiated samples were significantly higher at Day 1 when compared to Days 0, 3, and 7 ($^{\#}$, $p < 0.001$). Additionally, sGAG content in the media of the control samples was significantly greater at Day 1 when compared with Day 3 (**, $p < 0.01$).

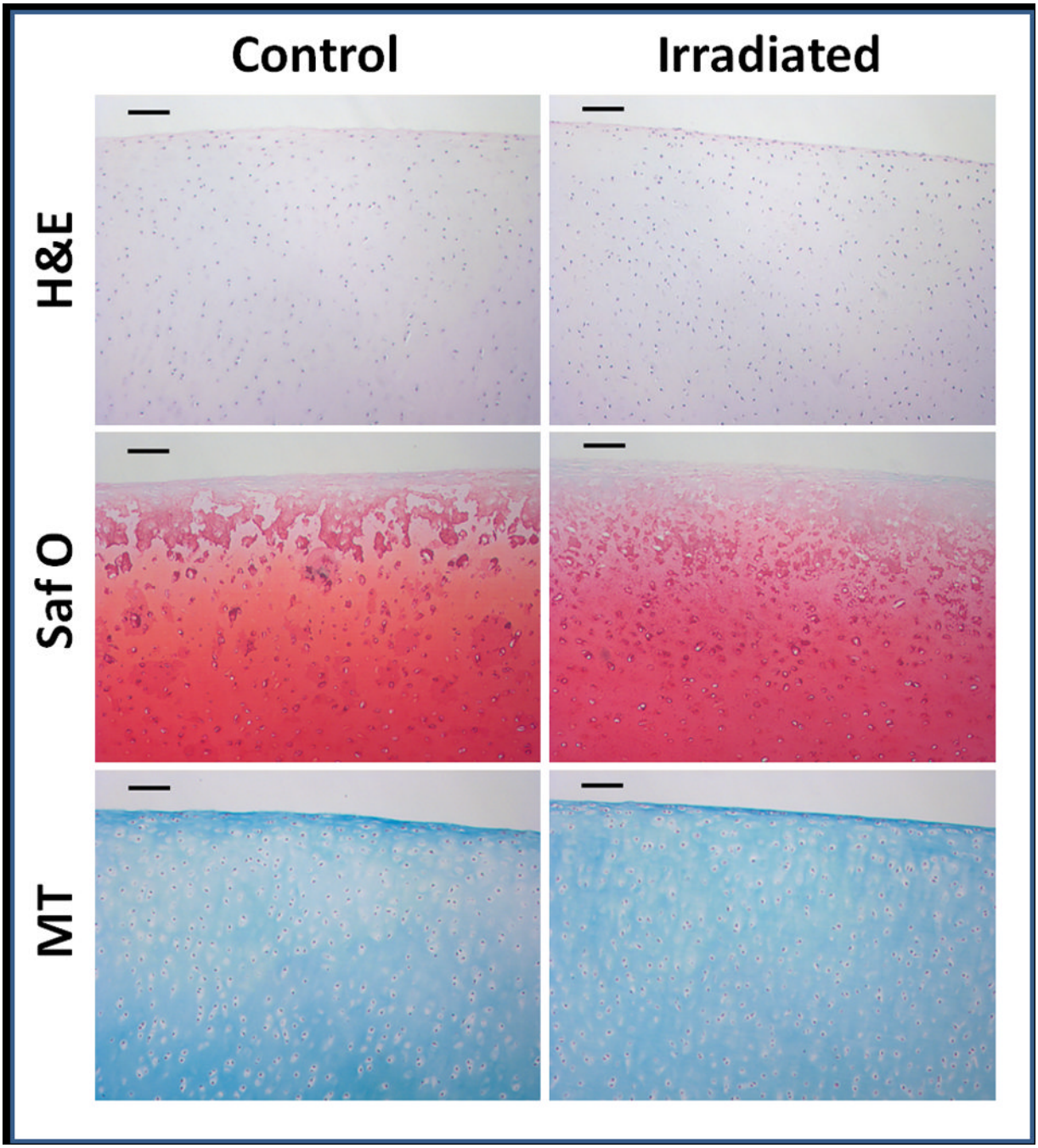

Figure 4.
Histological cross sections of articular cartilage explants stained with H&E, Safranin O, and Masson's trichrome. Qualitatively, there were no differences seen between control and irradiated samples. Scale bars represent 100μm.