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Osteophyte formation after ACL rupture in mice is associated with joint restabilization and loss of range of motion

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Abstract

Osteophytes are a typical radiographic finding during osteoarthritis (OA). Osteophytes are thought to form in response to joint instability, however the time course of osteophyte formation and joint stabilization following joint injury is not well understood. In this study, we investigated the time course of osteophyte formation and joint function following non-invasive knee injury in mice. We hypothesized that initial joint instability following knee injury would initiate osteophyte formation, which would in turn restabilize the joint and reduce range of motion (ROM). Mice were subjected to non-invasive anterior cruciate ligament (ACL) rupture. Anterior-posterior (AP) joint laxity, ROM, and chondro/osteophyte formation were measured immediately after injury, and 2, 4, 6, and 8 weeks post-injury. Chondrophyte areas at each time point were measured with histology, while mineralized osteophyte volume was determined using micro-computed tomography. Immediately after ACL rupture, AP joint laxity was increased 2-fold, while ROM was increased 11.7%. Chondrophytes appeared by 2 weeks post-injury, corresponding with a decrease in AP joint laxity and ROM. By 8 weeks post-injury, considerable osteophyte formation was observed around the joint, AP joint laxity returned to control levels, and joint ROM decreased to 61% of control values. These data support a role for chondro/osteophytes in joint restabilization after injury, and provide crucial insight into the time course and pathology of joint degeneration during OA development in the mouse.

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Author contributions:

AWH collected osteophyte volume, area, and ossification data and was primarily responsible for data analysis and manuscript writing. MJA assisted with study design and performed joint laxity and ROM measurements. MAH assisted with joint laxity data collection. EPL performed osteophyte area and ossification measurements. RZ collected qualitative data on joint pathology. BAC coordinated all analyses, assisted with study design, and contributed to manuscript writing. All authors have read and approved the final submitted manuscript.

The authors have no conflicts of interest to disclose.

Keywords

post-traumatic osteoarthritis; osteophytes; knee injury; joint laxity; range of motion

Introduction

Approximately 12% of osteoarthritis (OA) patients, translating to roughly 5.6 million people in the United States, suffer from post-traumatic osteoarthritis (PTOA) due to a previous joint injury, most commonly of the knee [1–3]. OA has traditionally been regarded as an articular cartilage degenerative disease, but in fact OA is a whole-joint disease, with all tissues contributing to symptoms. Cartilage outgrowths, or chondrophytes, appear and undergo endochondral ossification as OA progresses [4, 5]. These bony outgrowths, called osteophytes, are often found at joint margins and are a typical radiographic finding of OA [6]. Despite the prevalence of osteophyte formation during OA, the role of osteophytes in disease progression is not well understood. Mechanical stabilization has been proposed as a possible role, as osteophyte removal has previously led to increased joint mobility [7–9] and osteophytosis is associated with decreased joint range of motion (ROM) [10]. Furthermore, a cyclic compression mouse model showed that cartilage compression alone was insufficient to induce osteophyte formation, suggesting that mechanical instability induced by ACL rupture is required for osteophyte formation [11].

In a previous study, we found that mechanically-induced non-invasive ACL rupture in mice resulted in a greater than 2-fold increase in anterior-posterior (AP) joint laxity immediately after injury, but that AP joint stability is restored to control values by 12 and 16 weeks post-injury, with considerable osteophyte formation also observed at these time points [12]. A previous study also correlated joint instability in mice to long-term osteophyte formation [13], but no study has quantified this relationship over the full time course of OA development. Studying the time course of injury-induced joint instability, ROM, and osteophyte formation during joint restabilization could provide crucial information about osteophyte development and its role in joint function, and may help guide clinical interventions to attenuate osteophyte formation.

The current study investigated the time course of AP joint laxity, joint ROM, and osteophyte formation following non-invasive knee injury in mice. We hypothesized that initial joint instability following knee injury would lead to osteophyte formation, which would in turn restabilize the joint (decreased AP joint laxity toward uninjured values) and reduce joint ROM. These findings would contribute to current understanding of the roles of joint instability and osteophytes in OA progression, and provide crucial insight for the development of future treatments for osteophytes and OA.

Methods

Animals

A total of 30 skeletally mature (10 weeks old at injury; [14]) female C57BL/6 mice were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN). Mice underwent a 1-week

acclimation period in a housing facility before injury. Mice were caged in groups of four and were maintained and used in accordance with National Institutes of Health guidelines on the care and use of laboratory animals. All procedures were approved by the UC Davis Institutional Animal Care and Use Committee.

Non-Invasive Knee Injury

Mice were subjected to unilateral ACL injury via tibial compression overload as previously described [12, 15]. Briefly, mice were anesthetized using isoflurane inhalation, and lower right legs were positioned between two loading platens in an electromagnetic materials testing machine (Bose Electro-Force 3200, Eden Prairie, MN). A single dynamic axial compressive load was applied at 500 mm/s to produce ACL rupture, and buprenorphine analgesia was given immediately post-injury. We previously evaluated this injury model and found that this loading rate induces midsubstance ACL rupture with no obvious damage to the posterior collateral ligament, menisci, or other structures [12]. Left limbs served as uninjured, internal controls. Mice were euthanized immediately after injury (n=6), or 2, 4, 6, or 8 weeks post-injury (n=6/time point), and both knees were removed for analysis.

Micro-computed tomography analysis

Knees were scanned using micro-computed tomography (SCANCO, μCT 35, Brüttisellen, Switzerland) to quantify osteophyte volume around the knee joint. Dissected limbs were preserved in 70% ethanol. MicroCT scans were performed according to guidelines for rodent bone structure analysis (x-ray tube potential = 55 kVp, intensity = 114 μA, 10 μm isotropic nominal voxel size, integration time = 900 ms) [16]. Contours were drawn manually on 2D transverse slices of all non-native mineralized tissue attached to the distal femur and proximal tibia, as well as the entire patella, fabellae, and menisci. For uninjured joints, contours were drawn around the patella, fabellae, and menisci. Uninjured joints were assumed to have no osteophyte volume, so total osteophyte volume for injured knees was calculated as the difference in volume of mineralized tissue between injured and uninjured joints.

Range of motion testing

Joint ROM was quantified using a custom-made goniometer to measure maximum joint flexion and extension during application of a 1.47 N-mm torque. The center of the joint was rested on a support pin below the knee, with the tibia held parallel to the ground. A 10 g mass was supported by the tibia at a distance 15 mm from the center of the joint, and the subsequent joint angle was recorded (Fig. 1). Joint ROM was determined as the difference between joint angles during extension and flexion.

Anterior-posterior joint laxity testing (Anterior Drawer test)

AP stability of knees was quantified as previously described [12, 17]. Briefly, knee joints were rehydrated in phosphate buffered saline (PBS) prior to mechanical testing and femoral heads and soft tissues were removed, keeping the joint capsule intact. Femurs and tibias were embedded in brass tubes using polymethylmethacrylate (PMMA), allowing full ROM of the knee joint. Tibias were secured to the load cell, with the long axis of the tibia

perpendicular to the axis of loading. Tibias were allowed to translate and rotate about the longitudinal axis of the bone. Femurs were fixed so that the angle of the knee was 60°. Five anterior-posterior (AP) loading cycles were applied normal to the longitudinal axis of the tibia to a target force of +/−1.5 N at a loading rate of 0.5 mm/s. Total AP joint laxity was determined as the difference between displacement at +0.8 N and −0.8 N [17].

Histology

Whole joint histology was performed to visualize chondro/osteophyte composition and non-mineralized fibrous formations around the joint. Knees were decalcified for 3 days in 15% formic acid and processed for standard paraffin embedding. Sagittal 6 μm sections were cut across the medial aspect of the joint, separated by 250 μm (6 sections for each joint), then stained with either Hematoxylin and Eosin (H&E) or Safranin-O and Fast Green (SafO/FG). Comparable sections from the mid-medial aspect of the joint were chosen for analysis.

Chondro/osteophyte areas were quantified and graded for ossification by three blinded researchers. Freehand selections were made around chondro/osteophytes in three locations: the anterior femur, the anterior horn of the medial meniscus, and the posterior tibia (ImageJ, National Institutes of Health, Bethesda, MD) (Fig. 2). Total chondro/osteophyte area was determined as the sum of the chondro/osteophyte areas in each location. Grades from 0 to 4 were assigned according to the extent of ossification, with 0 indicating no ossification and 4 indicating 76–100% ossification. Additionally, joint pathology was qualitatively examined by a veterinary pathologist.

Statistical analysis

Various statistical methods were used to compare injured and uninjured limbs, examine differences between time points, as well as evaluate the correlation between osteophyte formation and changes in joint function. AP joint laxity, ROM testing, and mineralized osteophyte volume for injured and uninjured knees were compared at each time point using paired t-tests (JMP 11, SAS Institute, Inc., Cary, NC). Each time point for each outcome measure, including chondro/osteophyte areas and ossification grades, was compared using one-way ANOVA. Post hoc comparison of means was performed using the Tukey-Kramer test. Mean ± standard deviation is presented for all data. Simple linear regression was used to determine Pearson correlation coefficients to relate osteophyte formation with AP joint laxity and ROM. Significance was defined as $p<0.05$ for all tests.

Results

Micro-computed tomography analysis of mineralized osteophytes

Mineralized osteophyte volume increased over time after injury. No significant mineralized osteophyte volume appeared immediately after injury (Fig. 3), and little osteophyte volume was observed at 2 weeks post-injury. However, mineralized osteophyte volume significantly increased in a fairly linear manner at 4, 6, and 8 weeks post-injury (5.9-fold, 14.1-fold, and 20.8-fold increases, respectively, compared to 2 weeks post-injury).

Range of motion testing

Knee joint ROM was slightly increased immediately following injury, but significantly decreased within two weeks post-injury (Fig. 4A). Immediately after ACL rupture, injured joints displayed 11.7% greater ROM than contralateral limbs (134 vs. 120 degrees, respectively). However, by 2 weeks post-injury, injured joints exhibited a 45.6% decrease in ROM compared to contralateral joints. This trend continued to progress throughout the study; by 8 weeks post-injury, ROM of injured knees was 61.4% less than that of contralateral knees (49 vs. 127 degrees, respectively).

Joint laxity testing

Non-invasive knee injury resulted in an initial increase in AP joint laxity compared to contralateral knees similar to our previous study (Fig. 4B). Immediately post-injury, injured knees demonstrated a 2-fold increase in AP laxity (1.36 mm vs. 0.67 mm for injured and contralateral knees, respectively). However, by two weeks post-injury, AP joint laxity in injured knees was largely restored back to control levels with only a 1.3-fold increase in laxity (0.79 mm and 0.62 mm for injured and contralateral knees, respectively), and was not significantly different from contralateral knees. By 8 weeks post-injury, AP joint laxity was fully restored to control levels.

Histology

Chondro/osteophyte area measurements revealed significant chondro/osteophyte formation within 2 weeks post-injury, with further increases at later time points. No chondrophyte formation was observed immediately post-injury. Thus, the most drastic change in chondro/osteophyte formation occurred in the first 2 weeks after injury, when the total outgrowth area significantly increases to 0.918 mm^2 (Fig. 5A). At later time points, chondro/osteophyte area continued to increase; chondro/osteophyte areas after 4, 6, and 8 weeks were 1.256 mm^2, 1.130 mm^2, and 1.417 mm^2, respectively.

Chondro/osteophyte mineralization also significantly increased with time (Fig. 5B). At 2 and 4 weeks post-injury, developing osteophytes displayed ossification grades of 1.21 and 1.79, respectively. At 6 and 8 weeks post-injury, we observed gradually increasing ossification grades, though they were not significantly different from week 4: 2.34 and 2.75, respectively.

Qualitative Histological Assessment of Joint Pathology

Osteophytes formed in a predictive and progressive pattern at the joint margins, but histology also indicated additional degenerative changes that may contribute to changes in joint function. The normally elastic joint capsule was gradually replaced by thicker fibrocartilaginous and later fibrous stroma (Fig. 6a). The synovial hypertrophy and hyperplasia resulted in increased joint effusion and contributed to expansion of the joint capsule. The loss of congruent motion between the tibia, femur, and meniscus was due to fibrous pannus variably covering the articular cartilage, and regional loss of the articular cartilage and subchondral bone (Fig. 6b). Large osteochondral formation within the anterior meniscus and osteophytes at the joint margins also interfered with normal motion. The formation of new articulation between juxtaposing surfaces of the anterior femoral

osteophyte and the meniscal osteochondral formation support the chronic abnormal anterior movement of the tibia (Fig. 6c). Osteolysis and periosteal expansion of the posterior metaphysis of the distal femur was noted throughout the time course of the experiment (Fig. 6d). Subchondral sclerosis was observed most prominently at the distal femur, and was most likely caused by abnormal stress distribution in the joint due to degeneration of the meniscus and articular cartilage, as well as synovial fluid changes.

Injury resulted in inflammation and neovascularization within the joint capsule that subsided with time. The mononuclear cells characterized by lymphocytes, plasma cells, macrophages, and mast cells were found in the joint capsule and persisted throughout all time points, likely sustaining the inflammatory cytokine environment in the joint. Neutrophils were noted in the earliest time point after injury.

Correlations between osteophyte formation and ROM or AP laxity

Mineralized osteophyte volume correlated relatively poorly with outcome measures compared to chondro/osteophyte area measurements (Fig. 7). In fact, mineralized and developing osteophyte measurements did not even exhibit a very strong relationship to each other, with a Pearson correlation coefficient (r) of 0.590 ($p<0.002$). Joint ROM displayed a strong inverse correlation to total chondro/osteophyte area ($r= -0.8891$, $p<0.0001$), but correlated poorly with mineralized osteophyte volume ($r= -0.6048$, $p<0.0001$) (Fig. 7). Similarly, AP joint laxity displayed a strong inverse correlation with total chondro/osteophyte area ($r= -0.7294$, $p<0.0001$), but a poor correlation with total osteophyte volume ($r= -0.6072$, $p<0.0001$).

Discussion

In this study we investigated the time course of changes in AP joint laxity, joint ROM, and osteophyte formation following non-invasive ACL injury in mice. We found that joint injury led to an initial increase in joint laxity and ROM, but that the joint rapidly restabilized by 2 weeks post-injury. This restabilization was accompanied by a rapid formation of chondrophytes that may act to restore mechanical stability to the joint. By 8 weeks post-injury, AP laxity of injured joints was fully restored to control levels, and ROM was considerably less than control limbs. These data support our hypothesis that initial joint instability following knee injury leads to osteophyte formation, which in turn restabilizes the joint and reduces joint ROM.

In a previous study, intra-articular (IA) collagenase injections were used to induce knee OA in mice, and AP joint laxity was examined after 3 and 42 days to yield a similarly strong relationship between osteophyte formation and changes in joint stability [13]. In that study, the main effects of PTOA (e.g., cartilage damage and osteophyte formation) were also more prevalent on the medial side of the joint than the lateral, and osteophyte area correlated highly with AP laxity (r=0.92) [13]. However, invasive methods like IA injections, though able to lead to degenerative joint changes, do not mimic human disease conditions and can lead to confounding factors [18]. Thus, we have approached this problem with the noninvasive ACL rupture mouse model we previously developed, and that follows human

disease etiology [12]. Additionally, our data encompass a range of weeks, allowing us to observe the gradual transition from chondrophyte to osteophyte.

Tracking the time course of osteophyte development in relation to changes in joint stability and ROM is important because of compositional changes that occur during osteophyte maturation that may affect joint function. Osteophytes arise in either the periosteum or at tendon insertions (enthesophytes) [19]. In this study, we also documented that the injured perichondrial ring and the growth plate contribute to formation of the osteophytes as well (posterior tibial osteophyte). However, an important consideration is that the growth plate in mice never closes as it does in humans, which may present a difference between OA development in mice and humans [14]. Osteophytes typically begin as proliferative cells that undergo chondrogenesis to form chondrophytes. As these cartilaginous structures mature, their central chondrocytes differentiate and hypertrophy, leading to endochondral ossification and the shift from chondrophyte to osteophyte. Mature osteophytes integrate with the subchondral bone and are covered with cartilage [4, 19, 20]. We were able to better understand the role of the chondrophyte before mineralization because we studied osteophyte formation throughout the entirety of its development.

Other studies examining the relationship between osteophytes and joint stability drew their conclusions from human radiograph data, limiting longitudinal study and investigation of early pre-osteophyte influence on joint stability [7, 10, 21]. Additionally, there are a lack of studies examining both joint laxity and ROM. Loss of mobility, which eventually leads to loss of joint function, is a major symptom of OA and warrants further investigation [22]. Our results, which show a correlation between chondro/osteophyte development and loss of ROM, suggest that decreased joint mobility during OA may be due to not only mature osteophytes, but also chondrophytes. Future examinations of osteophytosis during OA should take this into account, and shift the focus to earlier time points. However, extrapolation of these results for clinical use must take the shorter lifespan of mice into account. Mice develop PTOA much more quickly than humans, so the rate of restabilization may be faster than can be anticipated for humans.

Although this study found strong correlations between osteophyte formation and decreasing AP joint laxity and ROM, our measurements present a set of limitations that must be taken under consideration. In this study, we used female mice to examine OA progression; however, a previous study has found that OA progression in female mice is significantly less severe than joint degeneration in male mice [23]. While OA severity may be sex-dependent, we have not found any significant changes between female and male mice using our particular PTOA mouse model, and do not expect using female mice to have impacted our results [24]. Additionally, in this study and previous studies, osteophytes were quantified volumetrically using μCT, which is only able to measure mineralized tissue volume. These measurements did not take chondrophytes into consideration, and resulted in expectedly poor correlations. However, this measurement was necessary to better understand the importance of these fibrous formations in decreasing AP joint laxity and ROM. The chondro/osteophyte combined measurements correlated strongly with changes in ROM and AP joint laxity, but the actual measurements themselves are quite limited. Chondro/osteophytes are three-dimensional asymmetrical structures and cannot be accurately

measured using area. However, sections from similar locations were compared, so cross-sectional areas are an appropriate measure of the general osteophyte size.

Additionally, we did not investigate the early joint restabilization response. Joint restabilization begins rapidly, and already begins manifesting itself at 2 weeks post-injury. Future studies will investigate earlier time points, as we anticipate that the prompt fibrosis and chrondrophyte formation act to stabilize the joint, similarly to bone callus formation during fracture healing. The observed arthrofibrosis, in addition to chondro/osteophyte formation, may also contribute to changes in joint mobility, but we are currently unable to separate the mechanical contributions of arthrofibrosis and osteophytosis. The importance of this soft tissue in joint kinematics post-injury must be taken into consideration; results from previous studies may be limited due to reliance on micro-CT data, and future studies should aim to include soft tissues in their measurements.

In this study we have shown that osteophyte formation in strongly correlated with changes in joint laxity and ROM after knee injury in mice. However, this study did not directly show a cause-effect relationship between osteophyte formation and joint stability, but rather a correlative relationship. Additionally, this study did not investigate specific mechanisms leading to osteophyte formation. Other studies have investigated a variety of contributors to osteophyte formation, including growth factors, oxygen tension, and dynamic fluid pressure [20]. Future studies will aim to understand how osteophytes develop, how they change joint kinematics, and how these joint changes may influence osteoarthritis initiation and progression. Despite this limitation, our results suggest that osteophyte formation leads to permanent joint changes that potentially contribute to pain and decreased mobility, and hold high value as a future therapeutic target for knee OA patients.

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Statement of Clinical Significance

Results from this study increase understanding of conditions leading to osteophyte formation.

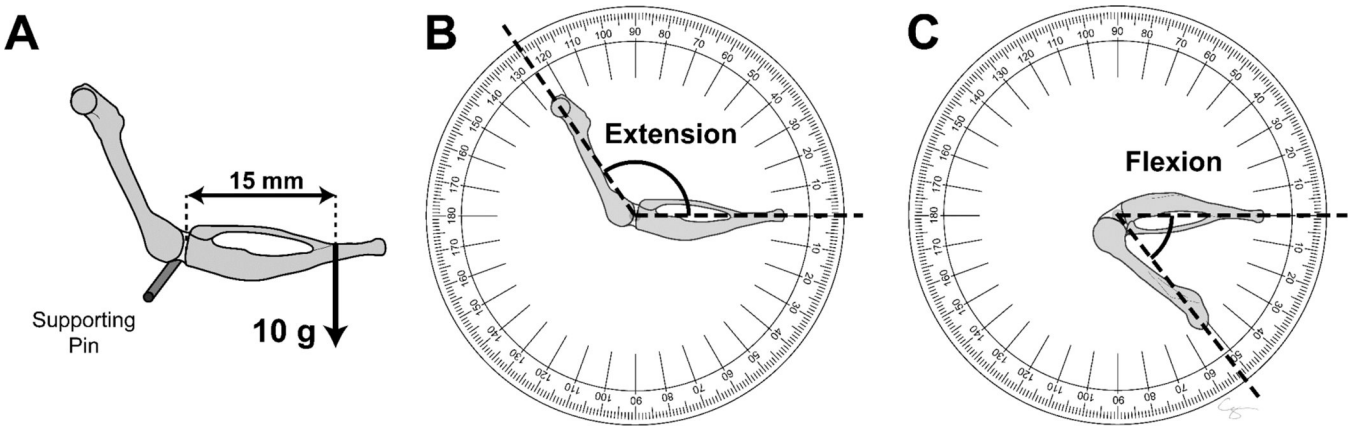

Figure 1.

A custom goniometer was used to quantify range of motion (ROM). The tibia was held parallel to the ground with a 10 g weight 15 mm from the center of the joint, which was placed on top of a support pin (A). Joint angles were measured in extension (B; medial view) and flexion (C; lateral view). ROM was calculated as the difference between joint extension and flexion angles.

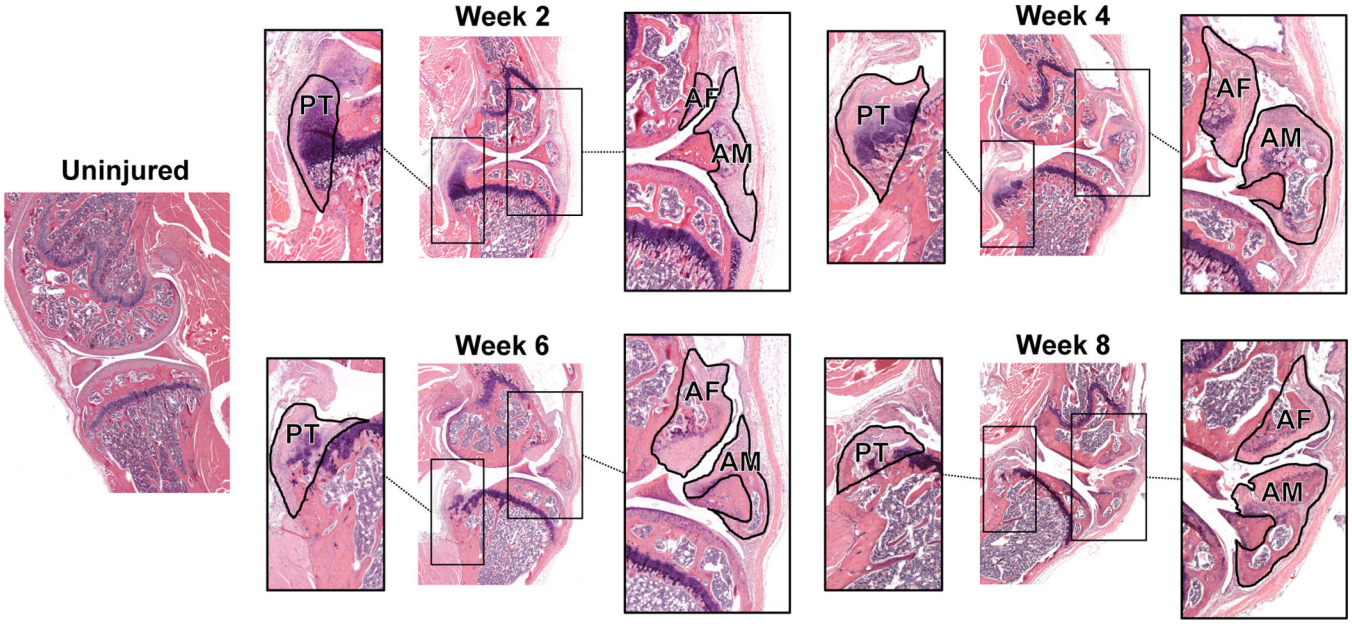

Figure 2.

The medial compartment of knee joints were sectioned sagittally and stained with hematoxylin and eosin (H&E). Chondrophyte/osteophyte areas were measured in 3 areas of each sagittal section: posterior tibia (PT), anterior femur (AF), and anterior meniscus (AM). Injured limbs were analyzed 2, 4, 6, and 8 weeks after injury.

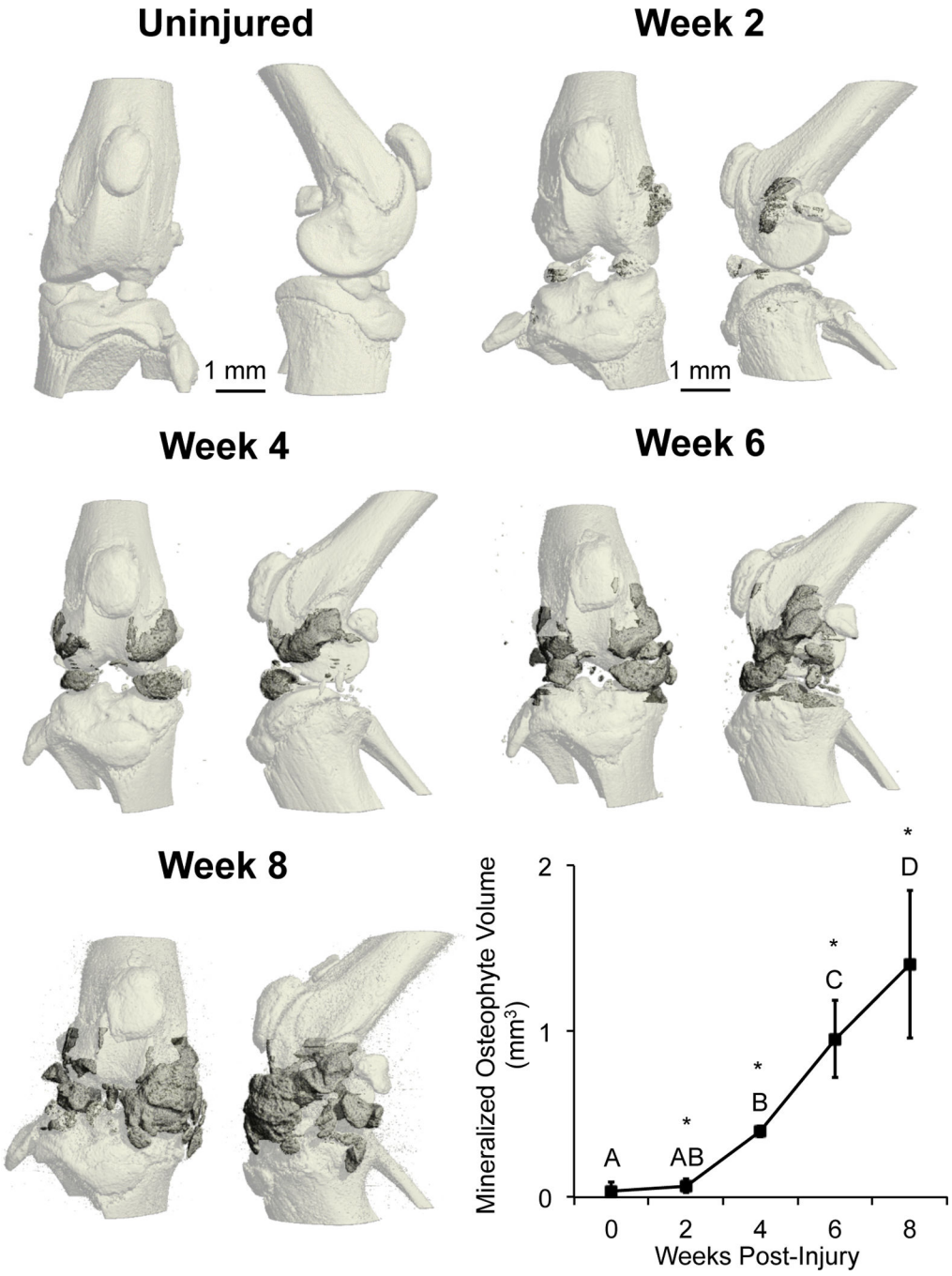

Figure 3.

3D reconstructions of micro-computed tomography (μCT) scans depict a gradual increase in mineralized osteophyte formation, with light areas indicating native (normal) bone and dark areas representing osteophytes (non-native mineralized tissue). Considerable mineralized osteophyte volume was noticeable by 4 weeks post-injury, and continued to increase at 6 and 8 weeks post-injury. Time points that do not share a letter are significantly different from one another ($p<0.05$). Asterisks indicate significant differences in non-native mineralized

tissue between injured limbs and contralateral limbs ($p<0.05$). Data is presented as mean ± SD.

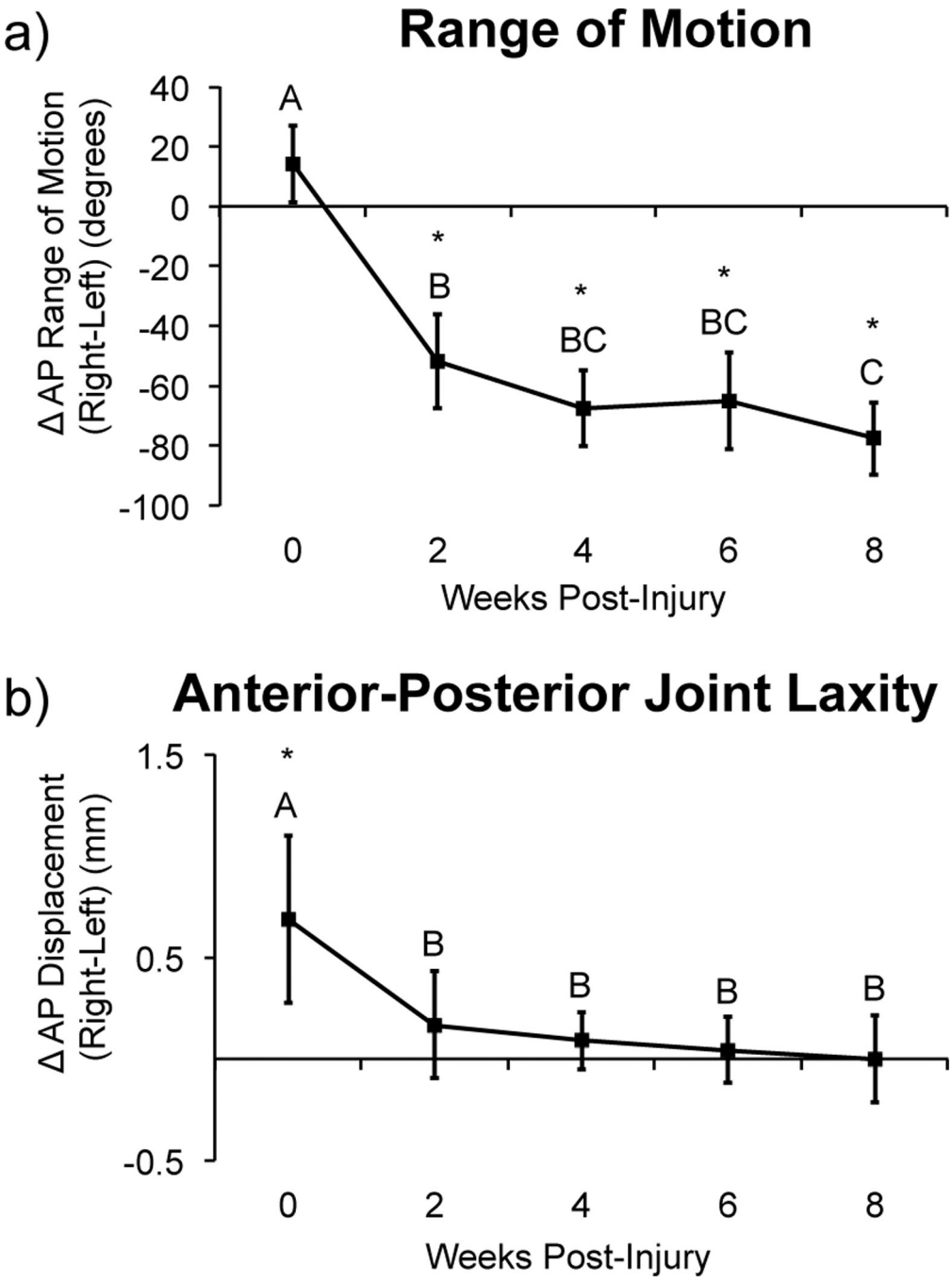

Figure 4.

Range of motion (ROM) increased immediately after injury, but decreased considerably by 2 weeks post-injury; ROM further decreases throughout the duration of the study (a). Similarly, AP joint laxity exhibited a ~2-fold increase immediately after injury, but decreased toward contralateral values at later time points. No differences in AP joint laxity between injured and contralateral limbs were detected by 8 weeks post-injury (b). Time points that do not share a letter are significantly different from one another ($p<0.05$).

Asterisks indicate significant differences between injured limbs and contralateral limbs ($p<0.05$). Data is presented as mean ± SD.

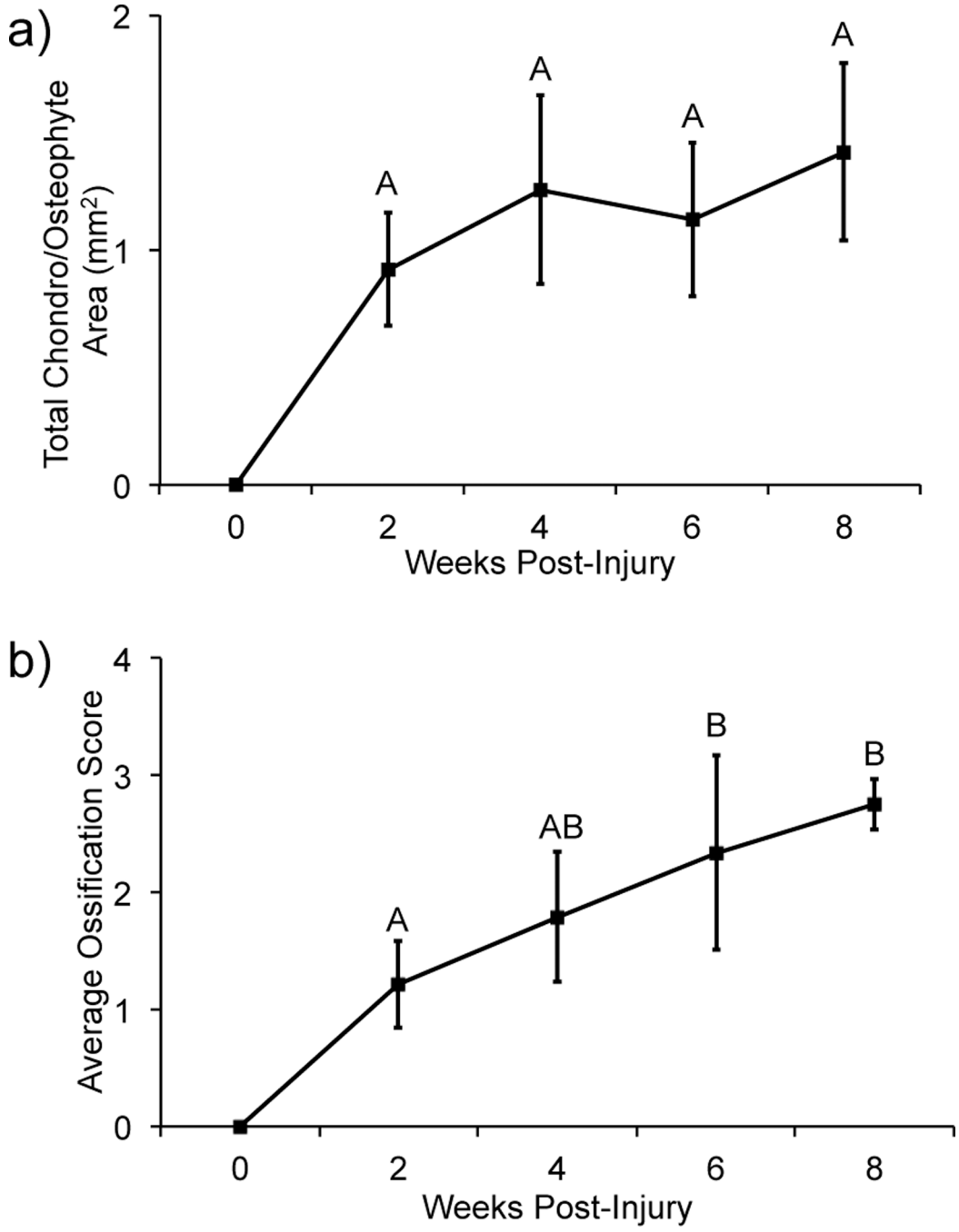

Figure 5.

Considerable chondro/osteophyte area (measured on 2D histological sections) was observed by 2 weeks post-injury. At later time points, total chondro/osteophyte area displayed an increasing trend, though not statistically significant (a). Average ossification score significantly increased between 2 and 6 weeks post-injury (b). Time points that do not share a letter are significantly different from one another ($p<0.05$). Data is presented as mean ± SD.

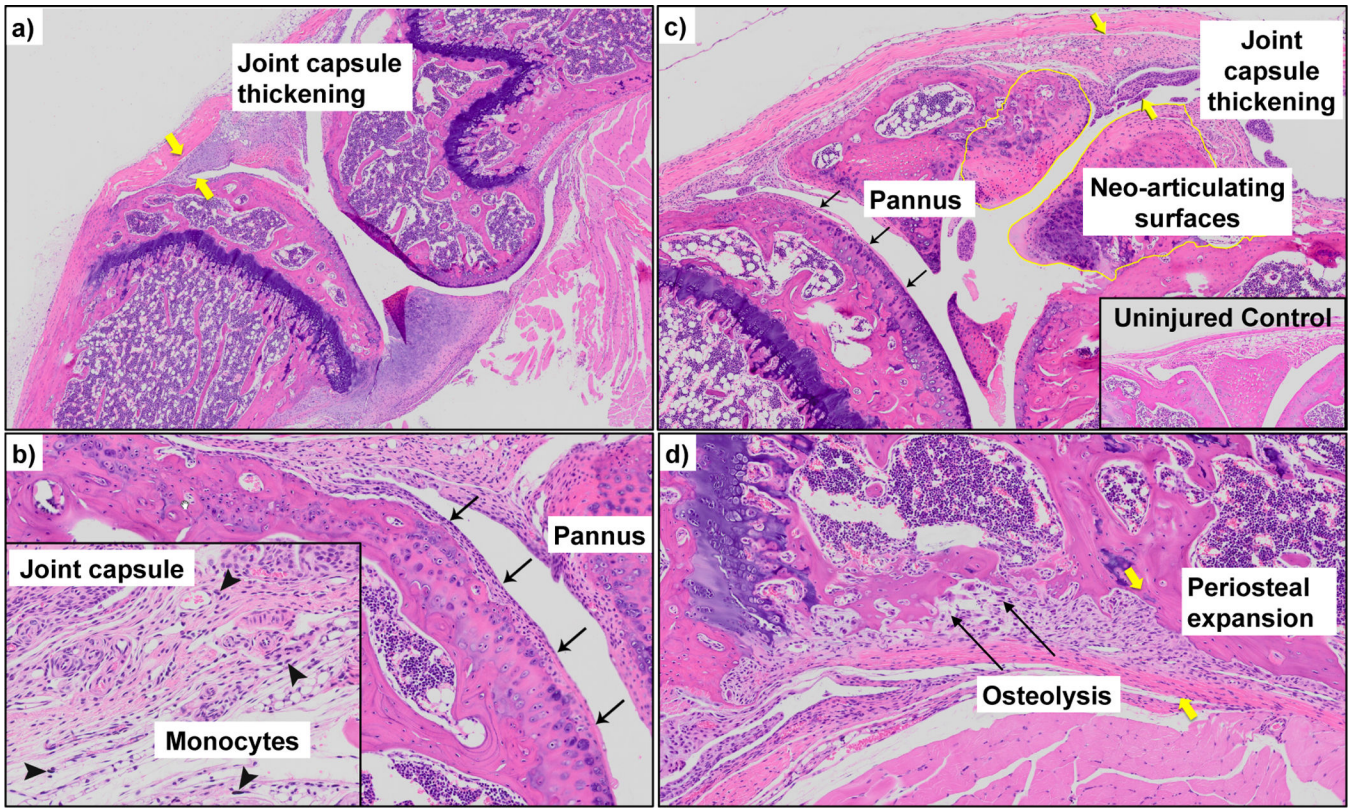

Figure 6.

Joint pathology was examined using H&E stained sections. The joint capsule exhibited fibrocartilaginous thickening (yellow arrows, a and c), while the articular cartilage showed regional loss and coverage by fibrous pannus (black arrows, b and c). Inflammatory mononuclear cells were located along the joint capsule (black arrowheads, b). Meniscal osteochondral formation and anterior femoral osteophytes developed new articulating surfaces (yellow region, c). The distal femur displayed osteolysis and periosteum expansion in the anterior metaphysis (black arrows, d).

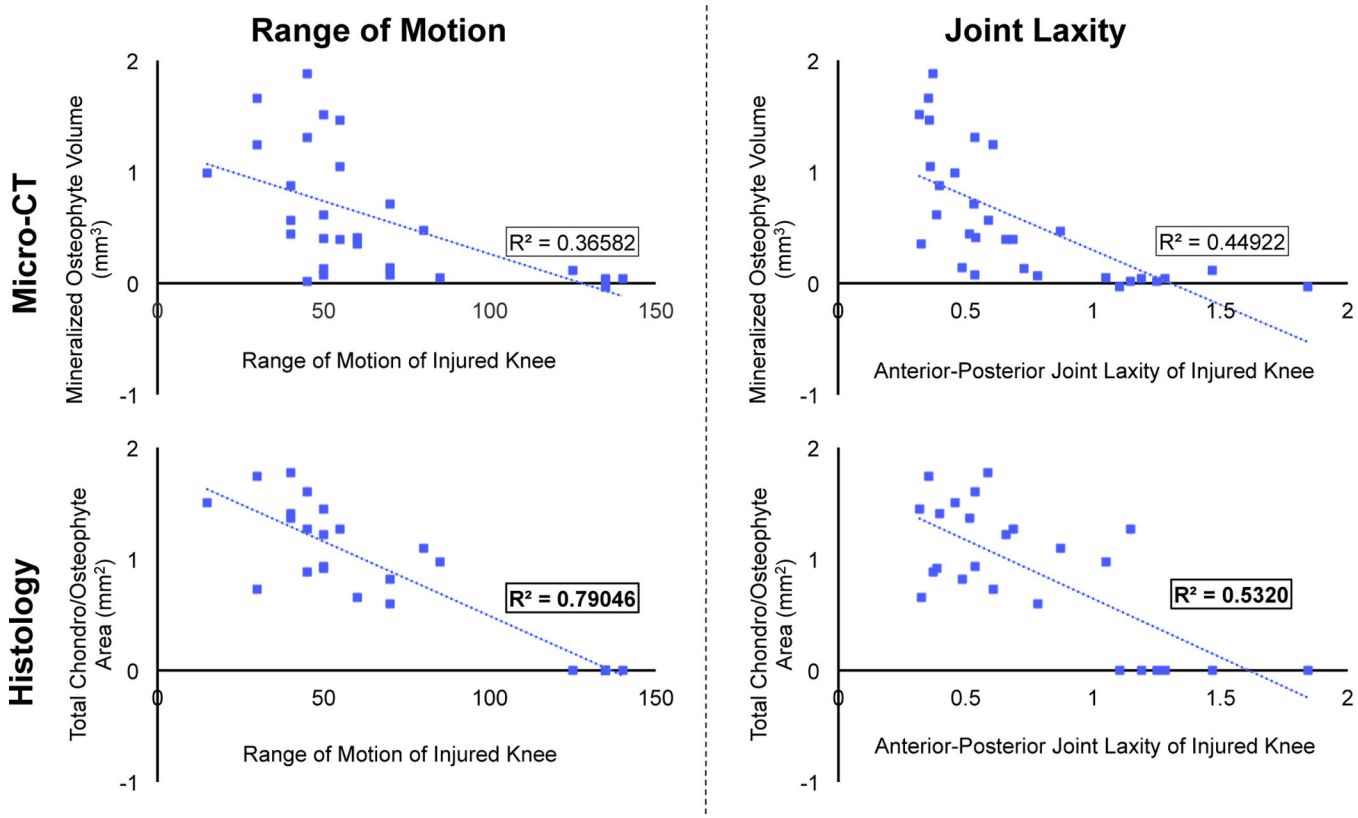

Figure 7.

Mineralized osteophyte volume (measured with μCT as the difference in mineralized tissue volume between injured and uninjured joints) displayed a weak inverse correlation to ROM (R^2=0.3658, p<0.0001) and AP joint laxity (R^2=0.4492, p<0.0001) of injured joints. However, total chondro/osteophyte area (measured on 2D histological sections) displayed a strong inverse correlation to ROM (R^2=0.7905, p<0.0001) and AP joint laxity (R^2=0.5320, p<0.0001).