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Assessment and Prevention of Cartilage Degeneration Surrounding a Focal Chondral Defect in the Porcine Model

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Abstract

Focal defects in articular cartilage are unable to self-repair and, if left untreated, are a leading risk factor for osteoarthritis. This study examined cartilage degeneration surrounding a defect and then assessed whether infilling the defect prevents degeneration. We created a focal chondral defect in porcine osteochondral explants and cultured them *ex vivo* with and without dynamic compressive loading to decouple the role of loading. When compared to a defect in a porcine knee four weeks post-injury, this model captured loss in sulfated glycosaminoglycans (sGAGs) along the defect's edge that was observed *in vivo*, but this loss was not load dependent. Loading, however, reduced the indentation modulus of the surrounding cartilage. After infilling with *in situ* polymerized hydrogels that were soft (100 kPa) or stiff (1 MPa) and which produced swelling pressures of 13 and 310 kPa, respectively, sGAG loss was reduced. This reduction correlated with increased hydrogel stiffness and swelling pressure, but was not affected by loading. This *ex vivo* model recapitulates sGAG loss surrounding a defect and, when infilled with a mechanically supportive hydrogel, degeneration is minimized.

Keywords

cartilage; focal defect; degeneration; hydrogel; in vivo; ex vivo

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1. Introduction

Acute injuries sustained to articulating joints can lead to focal defects in articular cartilage. Cartilage has a limited capacity to regenerate and on its own is unable to facilitate repair. When defects span into the underlying subchondral bone, the formation of a blood clot induces fibrocartilage repair. However, defects that are limited to the articular cartilage are often untreated and remain empty. Since injury to articular cartilage is a leading risk factor for developing osteoarthritis [1], it is reasonable to postulate that a chondral defect in an otherwise healthy joint may represent a point source for early stages of cartilage degeneration. Several critical questions remain regarding the degeneration of cartilage tissue immediately surrounding an empty defect such as the time course of degradation and whether degeneration can be prevented.

Chondral defects in articular cartilage are particularly vulnerable due to daily physical activity. Under mechanical loading, the tissue surrounding an empty chondral defect will be subjected to abnormal loads [2]. For example, an experimental whole joint model showed unusually large deformations in the cartilage adjacent to an empty defect [3]. *Ex vivo* studies demonstrated abnormally high strains that were mapped around empty focal chondral defects [4]. Computational modeling of focal defects located in regions of direct cartilage-cartilage contact also indicated supraphysiological strain levels [5]. The magnitude of these reported strains are associated with cell-mediated cartilage degradation, cell death, and even tissue failure [6]. Indeed, these regions of high contract stresses have been linked with symptomatic osteoarthritis in at-risk human patients [7]. These studies raise the question if the defect is filled with a mechanically supportive material, can degeneration be prevented within a loading environment?

The goals for this study were two-fold. First, we aimed to develop an *ex vivo* experimental model of a focal chondral defect that captures cartilage degeneration under physiological loading similar to that observed in an animal model, and enables the role of loading to be decoupled. To accomplish this goal, porcine focal chondral defects were created either *in vivo* in the knee of a pig or in explants of porcine osteochondral plugs. The latter was cultured under free swelling or subjected to compressive mechanical loading to emulate aspects of the physiological environment. We next evaluated the health of the adjacent cartilage when infilled with an *in situ* forming hydrogel and subjected to daily mechanical loads. Poly(ethylene glycol) (PEG) hydrogels were chosen for their tunability including achieving compressive moduli similar to that of articular cartilage [8–11]. Two hydrogel formulations were investigated that were softer or of similar stiffness to cartilage. In both studies, the cartilage adjacent to the defect was analyzed for loss of sulfated glycosaminoglycans (sGAGs). In the *ex vivo* studies, mechanical properties of the cartilage adjacent to the defect were analyzed by atomic force microscopy.

2. Materials and Methods

2.1. In Vivo Porcine Studies of Focal Chondral Defect.

All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Massachusetts General Hospital. All anesthetics, antibiotics, and analgesics

were obtained from Patterson Veterinary Supply, Fort Devens, MA. Female Yorkshire swine were sedated with an intramuscular injection Telazol (4.4 mg/kg), Xylazine (2.2 mg/kg) and atropine (0.04 mg/kg), intubated, and anesthesia was maintained with inhaled Isoflurane at 1-5% titration. A single dose of Cefazolin 40 mg/kg IV was administered prior to incision. Buprenorphine (0.01 – 0.05 mg/kg IM) was given 30 minutes prior to surgery. The area around the stifle joint was washed with surgical scrub followed with betadine and sterile draped. A paramedian incision was made and the patella displaced laterally to allow for visualization of the trochlear groove. Four, 6 mm defects were made in the cartilage down to the underlying subchondral bone and hemostasis was achieved. The patella repositioned and stabilized in place using two, number 2 nylon sutures. The capsule, muscle and skin were closed. Fentanyl patch (1 – 4 µg/kg/hr) was applied and kept in place for 72 hours for analgesia. Animals were euthanized at four weeks and defects processed by histology.

2.2. Culture of Osteochondral Plugs with Chondral Focal Defects.

Osteochondral explants were obtained from the trochlear groove and femoral condyle of a 3-month-old female Yorkshire swine using a biopsy punch (8.5 mm diameter x 10 mm height). Full thickness focal chondral defects (3 mm diameter) were created centrally without disrupting the underlying bone (total height of ~2-3 mm). Explants were cultured individually in 24-well plates for four weeks under free swelling or under free swelling for one week followed by three weeks of unconfined dynamic compression in custom bioreactors (n = 4) [8,12]. Explants were compressed (0.1 mm min^{-1}) to 20% strain and held for 15 minutes, then subjected to sinusoidal dynamic compression at 2% peak-to-peak strain at 1 Hz for one hour [13], and finally cultured under a constant 2.5% strain for 23 hours. This protocol was repeated five days each week. Explants were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum, 0.05 mg ml^{-1} l-ascorbic acid, 0.4 mM L-proline, 0.1 nM non-essential amino acids, 0.01 M HEPES, 0.02 mg ml^{-1} gentamicin, 4 mM glutaGRO, 100 U ml^{-1} penicillin, and 50 µg ml^{-1} streptomycin. Medium was changed every 2-3 days.

2.3. Hydrogel Formation and In Situ Filling of Focal Defects.

Poly(ethylene glycol) dimethacrylate (PEGDM) was synthesized from PEG (4600 g mol^{-1}) and methacrylic anhydride via microwave methacrylation [14]. Hydrogel precursors consisted of 10 (g/g)% (soft) or 40 (g/g)% (stiff) PEGDM in phosphate buffered saline (PBS, pH 7.4) with 0.05% (g/g) photoinitiator Irgacure 2959 (I2959) and were polymerized under 352nm light at 5 mW cm^{-2} for 10 minutes. For infilling, the defect was dried with 0.2 µm filtered CO_2 for 1 minute. Approximately 50 µl of filter-sterilized precursor solution was injected into each defect and photopolymerized. Filled explants were cultured (n = 4 per group) as described previously.

2.4. Mechanical Characterization of Hydrogel.

Compressive modulus testing: equilibrium swollen hydrogels (n = 3-4) of 3 mm diameter, 3 mm height in PBS were strained (0.5 mm min^{-1}) in a mechanical tester (MTS Insight II) and compressive modulus determined from the linear region of the stress strain curve between 15 and 22%. Equilibrium mass swelling testing: hydrogels (n = 3) of 5 mm diameter, 2.5 mm height were immediately weighed, placed in de-ionized water and mass measured every

hour, for eight hours, and at 24 and 48-hours. Hydrogels were lyophilized and their dry polymer mass measured. Equilibrium mass swelling ratio (swollen / dry polymer masses) was determined. Swelling pressure experiments: soft and stiff hydrogels ($n = 3$ per group) were either immediately placed in a mechanical tester (BOSE Electroforce TestBench, Model 66-601B) and submerged in de-ionized water or first swollen in de-ionized water for 24 hours (i.e., pre-swollen control) and then placed in the mechanical tester. A pre-load of 0.2 N was applied and load required to maintain the platen's initial position was recorded every two seconds for 24-hours. Load was normalized to the initial area of the hydrogel.

2.5. Histology.

After 4 weeks, the cartilage was vertically bisected. One-half was prepared for histology by removing the underlying bone. Samples were fixed in 10% formalin for two days at room temperature, embedded in paraffin and processed following standard protocols. Sections (20 μm) were stained for sulfated glycosaminoglycans (sGAGs) by Safranin O/Fast green and imaged at 100 $\times$ by light microscopy (Zeiss Pascal, Olympus DP70). Quantitative analysis of sGAG loss was performed (NIH Image J) by measuring the width of degenerated tissue, defined by an absence of red stain. A total of $n = 10$ line measurements was made perpendicular to the defect's edge per image for four images per side and two sides per specimen. All measurements for each specimen were averaged ($n = 4$ per group).

2.6. Nanomechanical Analysis.

The other half of each osteochondral explant ($n = 3$ per group), designated for mechanical property assessment, was stored at -80°C . Each specimen was thawed immediately prior to testing. Slices (200 μm) spanning the entire cartilage thickness were obtained using a vibratome, fixed to a glass slide using cyanoacrylate, submerged in PBS, and mounted on the stage of an atomic force microscope (AFM) (Keysight 5500 AFM). One sample (free swelling, soft infill group) was contaminated with cyanoacrylate and removed from analysis. Cantilevers with a nominal stiffness of 16 N/m determined using Sader's method [15] were affixed with a colloidal probe with a nominal diameter of 25 μm determined by optical microscopy. Cantilever deflection sensitivity was re-calibrated immediately prior to testing. A minimum of 80 indents were performed in the middle zone of each slice and centrally placed at half width using a peak voltage corresponding to an approximate peak force of 500 nN and a piezo displacement rate of 50 $\mu\text{m/s}$, which was chosen to avoid poroelastic relaxation during loading. Custom MATLAB script was used to convert voltage vs. piezo displacement data into load (μN) vs. indentation depth (nm). Data were corrected for the point of contact [16] and a simple Hertzian analysis was used to determine indentation modulus, E^* . The indentation modulus for all indents measured in a given sample were averaged to determine an overall E^* .

2.7. Statistical Analysis.

Data are reported as mean with standard deviation as error bars or parenthetically in the text. Statistical analysis was performed using Real Statistics add-on for Excel. Statistical analyses included unpaired two-sample t-test assuming equal variance, an oneway ANalysis Of VAriance ($\alpha = 0.05$), or two-way ANOVA ($\alpha = 0.05$). Data were confirmed to be normally

distributed and exhibit a homogeneous variance. Follow-up analyses were performed using a Tukey's post-hoc. $P = 0.05$ was considered statistically significant.

3. Results

Tissue surrounding the empty focal chondral defect *in vivo* was evaluated four weeks post-injury (Fig. 1A). The defect remained largely empty with limited fibrous tissue formation adjacent to the calcified zone. The adjacent hyaline cartilage showed signs of fissures and an irregular border with the calcified zone. A region of reduced sGAGs that spanned a distance of approximately 200 μm from the edge of the defect was evident. This finding indicates that within four weeks, substantial damage occurs to the tissue immediately surrounding the defect.

To create an *ex vivo* model, full depth focal chondral defects were created in explants of osteochondral plugs that accounted for ~12% of the surface area (Fig. 1Bi). The explants were cultured for four weeks under free swelling or under dynamic compressive loading for one hour per day (Fig. 1Bii). Signs of degeneration in the cartilage adjacent to the defect were evident by reduced staining for sGAGs within the middle zone (Fig. 1Biii). The distance of degeneration from the defect's edge was 210 (60) μm under free swelling and, whose mean was lower, but not statistically significant at 150 (50) μm under loading. AFM assessment of cartilage modulus was performed in the middle zone of cartilage where sGAG degeneration was most pronounced. While histological assessment showed the greatest sGAG loss at the defect edge, the indented regions were placed centrally within each cartilage explant to avoid experimental variability associated with both edge effects and the steep sGAG gradient that was observed adjacent to the defect. This approach also gave an assessment of the quality of cartilage away from the defect that supports loads applied to the articular surface. The indentation modulus was 1700 (170) kPa under free swelling and decreased ($p = 0.008$) by 47% under loading (Fig. 1Biv).

The defects were infilled with either a soft or stiff hydrogel. After infilling, the hydrogels adhered to the cartilage and remained in place after swelling (Fig. 2A). The hydrogels were first characterized on specimens alone (i.e., not within the defect). Representative stress-strain plots for soft and stiff hydrogels are shown along with the resulting compressive modulus of 100 kPa and 1 MPa for the soft and stiff hydrogels, respectively (Fig. 2B). Because the hydrogels are formed *in situ* in the defect, a swelling pressure is generated as the hydrogels reach equilibrium. The mass swelling ratio for the soft hydrogel increased from 11 to 13 over ~eight hours and then did not significantly change thereafter, indicating that they had reached equilibrium (Fig. 2Ci). The mass swelling ratio for the stiff hydrogel increased from 2.3 to 5.2 over ~eight hours and then remained constant (Fig. 2Ci). The volume increase from the polymerized state to equilibrium was 15% for the soft hydrogel and 130% for the stiff hydrogel (Fig. 2Cii). The swelling pressure increased over time corresponding with the temporal swelling response (Fig. 2Di). Contrarily, the pre-swollen hydrogels exhibited a small amount of stress relaxation. The maximum stresses were 13 kPa and 310 kPa for the soft and stiff hydrogels, respectively (Fig. 2Dii). Both were higher than the equilibrium stress of the pre-swollen hydrogels.

Under free swelling, the cartilage adjacent to the filled defects showed visibly less sGAG loss along the edge of the defect over the empty defect under free swelling (compare Fig. 3A to Fig. 1Biii). This distance was 110 (48) μm and 57 (25) μm for the soft and stiff infilled hydrogel, respectively. The width of sGAG loss decreased by 47% and 73% for the soft and stiff infilled hydrogel, respectively, from the empty defect (Fig. 3A). The indentation modulus was not affected by the infilled hydrogel. Under loading, there was also visibly less sGAG loss when compared to the empty defect (compare Fig. 3C to Fig. 1Biii). This distance was 80 (31) μm and 16 (9) μm for the soft and stiff infilled hydrogel, respectively. The width of sGAG loss decreased by 45% and by 89% for the soft and stiff infilled hydrogels, respectively, from the empty defect (Fig. 3C). A trend of increased indentation modulus was evident in the infilled hydrogels under loading, but this difference was not ($p = 0.068$) statistically significant.

4. Discussion

This study demonstrates that cartilage adjacent to a chondral defect *in vivo* in porcine articular cartilage shows substantial signs of physical damage and tissue degeneration within four weeks post-injury. The *ex vivo* focal chondral defect also showed signs of cartilage degeneration adjacent to a defect. However, it did not capture the physical damage of the cartilage that was observed *in vivo*; a finding attributed to the absence of sliding *ex vivo*. The *ex vivo* model, however, enabled decoupling of the role of loading in cartilage degeneration and investigation into the prevention of degeneration by infilling the defect with a hydrogel. Our results show that degeneration was prevalent regardless of loading and that infilling the defect significantly reduced degeneration with hydrogels of increased stiffness and swelling pressures having an even greater beneficial effect. Overall, this study provides evidence that degeneration occurs along the defect regardless of loading, but which can be prevented by infilling due to the effects of hydrogel swelling pressure and stiffness.

Our *in vitro* results indicate that degeneration, based on sGAG loss, is prevalent regardless of loading. When collagen fibers are damaged after an acute injury, the collagen network no longer resists the swelling pressures of proteoglycans, leading to cartilage swelling [17,18]. This effect was most pronounced *ex vivo* in the middle zone where sGAG concentration is highest [19]. This sGAG loss may be attributed to cell-mediated degradation, where chondrocytes respond to increased cartilage swelling, and/or to physically-mediated degradation, where swelling effectively increases porosity and diffusive transport. sGAGs are localized in large aggrecan aggregates with dimensions of several microns [20]. It seems less likely that increased porosity could lead to loss of aggrecan aggregates alone without concurrent aggrecan degradation. This conjecture is supported by the observation that sGAG loss was not immediate, as infilling twenty-four hours post-injury reduced sGAG loss long-term. Moreover, studies have reported an increase in swelling in the middle zone without sGAG loss [17]. While mechanical injury can lead to physical loss within the first twenty-four hours, extended sGAG loss after injury occurs by enzyme-mediated degradation [21]. *In vivo*, sGAG loss was apparent throughout the cartilage thickness and was accompanied by evidence of mechanical damage. We surmise that *ex vivo*, sGAG loss observed along the middle zone is due to cell-mediated degradation resulting from localized tissue swelling that alters the chondrocyte environment. However, the *in vivo* environment is more complex with

uniaxial loading combined with sliding and the presence of inflammatory mediators. The sGAG loss observed *in vivo* is likely due to both mechanical injury and cell-mediated degradation.

Simply infilling the defect with a hydrogel significantly reduced degeneration along the defect. This finding points to an intrinsic property of the hydrogel that is capable of partially protecting the tissue. As the hydrogels were formed directly in the defect, hydrogel expansion due to swelling is restricted and instead, a swelling pressure is generated at the adjacent tissue surfaces. Interestingly, both hydrogels reduced sGAG loss suggesting that swelling pressures of 13 kPa may be sufficient to minimize degeneration, but that higher swelling pressures (e.g., 310 kPa) may provide an even greater reduction in degeneration. This magnitude is similar to those reported during hydrogel degradation where swelling pressures increased from 50 to 800 kPa [22]. These swelling pressures may serve to physically prevent the adjacent tissue from swelling and thus minimize cartilage swelling-induced sGAG loss and/or provide mechanical signals to maintain homeostasis. For example, studies have reported positive effects on chondrocytes when cartilage explants are exposed to static hydrostatic pressures [23]. Our data suggest that swelling pressures generated by the hydrogel may provide a mechanical stimulus that protects cartilage.

The mechanical properties of the cartilage distant to the empty defect were adversely affected by loading. This finding suggests that damage to cartilage may be more pervasive than that observed by sGAG presence. Finite element modeling of a focal chondral defect in a whole joint under loading indicated that elevated principal stresses and strains can extend large distances from a defect [5]. Infilling the defect with the hydrogels did not significantly alter the mechanical properties when compared to their empty counterpart. The hydrogels used in this study exhibit largely elastic behavior with minimal stress relaxation behavior. While cartilage exhibits large stress relaxation under static loading, the time-dependent properties become less pronounced under dynamic loading [24]. It remains to be determined if the infill material needs to recapitulate the time-dependent mechanical properties of cartilage to preserve the mechanical properties. It is also important to note that we did not control for the location from where the osteochondral explants were taken in the joint, which may introduce variability across specimen.

In summary, the *ex vivo* model captures sGAG degeneration along the edge of a defect, similar to that which was observed *in vivo*. This model indicates that the sGAG loss appears to be largely independent of loading, but can be prevented by infilling with a swelling hydrogel. While the *ex vivo* model does not capture the full complexity of the joint environment (e.g., shear loading), it allows for the investigation of the defect in a more controlled environment and the ability to decouple the effects due to an applied uniaxial compressive load. Our *ex vivo* dynamic mechanical loading paradigm may then serve as a first step towards identifying infilled materials that can minimize tissue degeneration and maintain the mechanical properties in the cartilage surrounding the defect. Pro-inflammatory cytokines could also be introduced into the *ex vivo* model to simulate an inflammatory joint. Testing *in vivo* within the joint environment is ultimately critical to establish whether any infill material can also protect against the shear forces that result in mechanical damage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- An *ex vivo* chondral defect recapitulated proteoglycan loss observed *in vivo*.
- Infilling the defect with an *in situ* polymerized hydrogel reduces degeneration.
- Cartilage-matched stiffness and exerted swelling pressures are beneficial.

Figure 1.

Evaluation of porcine cartilage adjacent to an empty focal chondral defect *in vivo* (A) and *ex vivo* (B). A) Representative images of Safranin-O/Fast green stained sections for sulfated glycosaminoglycans (sGAGs) surrounding the defect *in vivo* four weeks post-injury (*i*). A higher magnification of the boxed region is shown with arrows indicating decreased sGAGs (*ii*). B). Photographs of the *ex vivo* defect in osteochondral explants (*i*). Explants were cultured for four weeks under free swelling (FS) or under dynamic compressive loading (DL) (*ii*). Representative microscopy images for sGAGs are shown for full depth cartilage

adjacent to the defect and width of sGAG loss was quantified (*iii*). The indentation modulus E^* is presented (*iv*).

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Figure 2.

A) Schematic and photographs of infilled focal chondral defects. B) Representative stress-strain plots (*i*) and compressive moduli (*ii*) for the soft and stiff hydrogels. C) Temporal swelling behavior of the hydrogels is shown as a function of time under unconstrained conditions(*i*). Hydrogel volume increase from polymerized state to the equilibrium state is shown (*ii*). D) Swelling pressures were measured under constrained conditions as a function of time in soft and stiff hydrogels from the polymerized state to equilibrium (*i*). Maximum swelling pressures recorded at equilibrium are shown (*ii*).

Figure 3.

Evaluation of cartilage adjacent to *in situ* filled focal chondral defects in the *ex vivo* porcine model under free swelling, FS (A,B) and under dynamic compressive loading, DL (C,D). Representative microscopy images are shown for full depth cartilage adjacent to the filled defect and width of sGAG loss was quantified (A,C). The indentation modulus E^* is presented (B,D).