Published in final edited form as:

J Biomech. 2014 February 7; 47(3): 645–652. doi:10.1016/j.jbiomech.2013.11.050.

An *In Vitro* Model for the Pathological Degradation of Articular Cartilage in Osteoarthritis

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Abstract

The objective of this study was to develop an *in vitro* cartilage degradation model that emulates the damage seen in early-stage osteoarthritis. To this end, cartilage explants were collagenase-treated to induce enzymatic degradation of collagen fibers and proteoglycans at the articular surface. To assess changes in mechanical properties, intact and degraded cartilage explants were subjected to a series of confined compression creep tests. Changes in extracellular matrix structure and composition were determined using biochemical and histological approaches. Our results show that collagenase-induced degradation increased the amount of deformation experienced by the cartilage explants under compression. An increase in apparent permeability as well as a decrease in instantaneous and aggregate moduli were measured following collagenase treatment. Histological analysis of degraded explants revealed the presence of surface fibrillation, proteoglycan depletion in the superficial and intermediate zones and loss of the lamina splendens. Collagen cleavage was confirmed by the Col II- 34 C_{short} antibody. Degraded specimens experienced a significant decrease in proteoglycan content but maintained total collagen content. Repetitive testing of degraded samples resulted in the gradual collapse of the articular surface and the compaction of the superficial zone. Taken together, our data demonstrates that enzymatic degradation with collagenase can be used to emulate changes seen in early-stage osteoarthritis. Further, our *in vitro* model provides information on cartilage mechanics and insights on how matrix changes can affect cartilage's functional properties. More importantly, our model can be applied to develop and test treatment options for tissue repair.

Keywords

osteoarthritis; articular cartilage; collagenase; mechanical properties

1. INTRODUCTION

Osteoarthritis is a pathological process involving matrix damage of articular cartilage. Tissue breakdown generally begins at the articular surface (Hollander et al., 1995), and is characterized by the degradation of the two principal macromolecular components of

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to disclose.

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cartilage extracellular matrix (ECM): the type II collagen fibrillar network and the proteoglycan (PG) aggregates (Brocklehurst et al., 1984). Two of the primary factors for the initial damage to the collagen at the articular surface are injurious or excessive mechanical loads and enzyme cleavage. Catabolic enzymes (i.e. metalloproteinases (MMPs) and aggrecanases) enter the tissue matrix, either from the synovial fluid through the articular surface or from chondrocyte secretion, and digest the extracellular matrix components (Mort and Billington, 2001). While this process can be idiopathic (Armstrong and Mow, 1982), it often occurs as a result of direct injury to the joint, such as with trauma (Von Porat et al., 2004) or secondary to joint inflammation (Hedbom and Hauselmann, 2002). As the surface becomes damaged, the surface porosity and permeability increase, resulting in increased interstitial fluid flow out of the tissue (Akizuki et al., 1987) and increased tissue deformation when the matrix is mechanically loaded (Torzilli, 1984; Lotke and Granda, 1971; Bank et al., 2000). Concomitantly, the components of the proteoglycan aggregate together with any of their fragments resulting from enzyme degradation, will be dragged through the matrix and out the surface, further softening the matrix (Broom and Poole, 1983). Even though the chondrocytes produce increased amounts of the proteoglycan components in response to matrix damage (McDevitt et al., 1977; McDevitt and Muir, 1976), there is limited proteoglycan aggregation and immobilization within the matrix due to the loss of the collagen structural integrity. Collagen damage at the articular surface has long been recognized as one of the earliest stages of the disease process (Weiss and Mirow, 1972; Panula et al., 1998) and is believed to be the point of no return for disease progression (Buckwalter and Mankin, 1998). With further matrix breakdown and component loss, the matrix becomes increasingly more porous and less stiff (Hwang et al., 1992; Akizuki et al., 1986), resulting in a complete loss in its functional properties (i.e., ability to resist joint compressive loads).

Numerous *in vitro*, *ex vivo* and *in vivo* models have been used to study the pathological process involved in cartilage degeneration and to test potential treatment modalities. Animal models are commonly used because they permit the study of physiological and metabolic factors, as well as long-term transient changes in tissue structure and joint organization (Pritzker, 1994). Typical examples include naturally occurring osteoarthritis models (Nordling et al., 1992; Arzi et al., 2012), transgenic models (Helminen et al., 2002), joint instability models (Setton et al., 1993; Setton et al., 1999; Leroux et al., 2000), displaced biomechanical load models (Reimann, 1973; Johnson and Poole, 1988), and structural alteration models (Van der Kraan et al., 1990; Elford et al., 1992; O'Byrne et al., 1990). Although extremely useful, animal models are very complex and alternative *ex vivo* models such as the tissue model presented in this study can provide relevant information on isolated events or mechanisms within cartilage.

Indeed, *in vitro* degradation models using a variety of enzyme types have been used in the past to simulate the ECM degradation seen in human osteoarthritis. To investigate the potential use of imaging as a diagnostic tool for osteoarthritic diseases, Saarakkala et al. (2004) and Wang et al. (2008) enzymatically digested bovine patellar cartilage with purified collagenase, trypsin or chondroitinase ABC to evaluate the capacity of high-frequency ultrasound to detect spatial and temporal changes in matrix composition and structure. Wagner et al. (1999) demonstrated that collagenase-induced damage to cartilage can be visualized using high-resolution nuclear magnetic resonance microscopy. Broom and Poole (1983) used a technique of simultaneous microdeformation and interference light microscopy to investigate the changes in cartilage structural response after enzymatic digestion of proteoglycans using hyaluronidase. They found that the loss in compressive strength observed in proteoglycan-depleted tissue was directly related to an abnormal structural response of the collagen network. As an alternative to enzyme cleavage, investigators have applied injurious or excessive mechanical loads to articular cartilage

explants in order to initiate damage. In a recent study performed by De Vries-van Melle et al. (2012), an *in vitro* osteochondral model was developed to study mechanisms involved in cartilage repair by creating defects of different depths using a dermal biopsy punch and scalpel. Lin et al. (2004) observed progressive changes in cell viability, collagen cleavage and proteoglycan loss by cyclically loading cartilage explants with 1 and 5 MPa for 24 hr. Thibault et al. (2002) subjected cartilage explants to high but physiological cyclic load levels and characterized the resulting damage using a sequence of unconfined compression stress relaxation tests. This mechanically-induced *in vitro* degradation model resulted in collagen cleavage with a concomitant increase in matrix permeability, however without any change in the aggregate compressive modulus.

To date, limited *in vitro* models exist for studying the effect of surface damage on the mechanical properties of articular cartilage. Physical removal of the superficial zone of articular cartilage has been shown to increase ECM permeability and deformation (Torzilli et al., 1983; Torzilli, 1984; Setton et al., 1993; Gannon et al., 2012), both changes found in the early stages of osteoarthritis (Setton et al., 1994). The objective of this study was to develop an *in vitro* cartilage degradation model with comparable ECM damage at the articular surface (i.e. fibrillation, increased porosity and matrix permeability, proteoglycan loss) as that observed in the early stages of osteoarthritis. To this end, bacterial collagenase was used to induce enzymatic degradation of superficial collagens and proteoglycans. Functional and material properties of articular cartilage, before and after the collagenase treatment, were determined via biomechanical, biochemical and histological analyses. Our *in vitro* cartilage degradation model was able to produce superficial matrix damage similar to that seen in the early stages of osteoarthritis, with concomitant reductions of the tissue's mechanical properties. This model will be useful for developing and testing resurfacing treatments for the repair of early-stage cartilage damage.

2. MATERIALS AND METHODS

2.1 Tissue Preparation

Eight-millimeter diameter cylindrical plugs of articular cartilage, without subchondral bone, were harvested from the trochlea of mature bovine knee joints, incubated in a broad spectrum of MMPs, serine, cysteine and calpains inhibitors (Complete Mini, Roche Diagnostics, Indianapolis, IN) for 1 hr and stored frozen. Each explant was mounted surface down on a freezing stage sledge microtome, sectioned through the deep zone to obtain a surface parallel to the intact articular surface, and rinsed several times in fresh phosphate buffered saline (PBS) to remove the inhibitors prior to mechanical testing and collagenase degradation. A biopsy punch was then used to reduce the explant's diameter to 5 mm and the specimen was immediately mounted in a 5 mm diameter confined chamber, with the articular surface facing upward. The outer rings were stored and later processed as controls for histology.

2.2 Collagenase Degradation

To induce matrix degradation from the articular surface downwards, cartilage explants remained within the confined chamber and the surface was treated with 0.1% type II bacterial collagenase (*Clostridium histolyticum* collagenase, Worthington Biochemical Company, Freehold, NJ) in serum-free Dulbecco's modified eagle medium at 37°C. Treatment duration was selected based on preliminary tests showing minimal changes in mechanical properties for degradation times under 45 min. Therefore, collagenase treatment was applied for 45, 90 and 120 min. To stop the collagenase digestion, the explants were rinsed in PBS for 5 min, washed in a broad spectrum of MMPs, serine, cysteine and calpains

inhibitors (Complete Mini, Roche Diagnostics, Indianapolis, IN) for the same amount of time as the collagenase treatment, and rinsed again in PBS for 30 min.

2.3 Mechanical Testing

Previously mounted intact cartilage explants were immersed in PBS with the articular surface facing a 35 μm porous plane-ended load platen (5 mm diameter) and subjected to a series of compression creep tests using a previously described custom-built test apparatus (Torzilli, 1990). Briefly, a small tare-load of 2 g was applied to detect contact with the articular surface, followed by a predisplacement of 20 μm to ensure uniform contact with the load platen. This also provided a measure of specimen thickness (h) and was used as the zero-strain, zero-stress state. After a 30 sec relaxation period, the explant was loaded at a ramp speed of 0.1 mm/sec until a 50 g load was reached, as recorded using a 250 g load cell (resolution = 0.25 g, Sensotec, Columbus, OH). Creep deformation (resolution = 40 nm), load and time were collected at a frequency of 20 Hz for the first 60 sec and at 0.2 Hz thereafter. The 50 g load was selected to insure that the total strain (instantaneous + creep) was within the physiological range of 20 to 25%.

The intact specimens were subjected to a series of four consecutive creep tests; three 100 sec tests (tests 1–3) followed by one 45 min test (test 4). Sufficient unloaded time was allocated between each creep test to allow the specimen to fully recover from the creep deformation. After the intact specimens were tested, the articular surface was subjected to collagenase treatment (degraded state) and an identical series of four creep tests was performed (tests 5–8).

Functional mechanical properties were determined for each cartilage specimen (before and after treatment) from the loading and creep phases as indicated in Fig. 1. The instantaneous or dynamic modulus was calculated by fitting an exponential function to the stress-strain response during the loading phase (time ~ 1 –2 sec) and calculated at a strain of 5%. The instantaneous displacement (U_0) and strain (U_0/h) were determined at the end of the loading phase. The experimental creep response (after the loading phase) was numerically fitted to the analytical solution for linear biphasic creep of articular cartilage in confined compression (Mow et al., 1980). Creep displacement U and time t were adjusted (zeroed) by subtracting U_0 and time t_0 , respectively, and the specimen thickness reduced by U_0 . The resulting creep strains were thus within the $\sim 25\%$ strain limit of the linear biphasic theory.

In confined creep, the equilibrium aggregate modulus is a measure of the compressive resistance of the solid phase of the ECM at equilibrium (time $\sim 10,000$ sec). The intact and degraded aggregate moduli were both *approximated* using the 45 min (2,700 sec) creep test data (Fig. 2A, tests 4 and 8). The permeability k is a measure of the ability of cartilage to exude fluid through the articular surface and is associated with the initial curvature of the creep response ($\sim 1,000$ sec). The apparent permeability and apparent modulus were both measured after 100 sec for each test (tests 1–8). The 100 sec time period was selected because it enabled a better and repeatable curvature fit between the experimental creep response and the analytical solution for biphasic creep of cartilage, especially for the permeability, as illustrated in Fig. 2B. The apparent permeability does not correspond to the permeability determined from a full equilibrium creep test ($\sim 10,000$ sec), and therefore is referred to as an apparent permeability for the first 100 sec of fluid exudation. This definition also applies to the apparent modulus. However, this technique allows us to track the relative change in permeability between the intact and degraded states in a consistent and reliable manner. All moduli and permeabilities were calculated using the reduced specimen thickness ($h_0 = h - U_0$).

2.4 Histological and Immunohistochemical (IHC) analyses

Following mechanical testing, the degraded specimens and intact untested outer rings were fixed at 4°C in 10% formalin for 24 hr, dehydrated, and embedded in paraffin for histological analysis. Six-micrometer-thick sections were deparaffinized and rehydrated for Safranin-O and Picrosirius Red staining using standard protocols. For IHC detection of enzyme-cleaved collagen, sections were deparaffinized, hydrated, treated with hyaluronidase, quenched for endogenous peroxidase activity, and incubated overnight at 4°C with a specific polyclonal antibody (Col II-3/4C_{short}, IBEX Technologies Inc, Montreal, PQ, dilution 1:200) against the neopeptide generated by collagenase matrix MMP cleavage of collagen type II at the Gly⁷⁷⁵-Leu/Ile⁷⁷⁶ site (Billinghurst et al., 1997). For negative controls, isotype-matched IgG (Santa Cruz Biotechnology, Dallas, TX) was used in place of the primary antibody. The Vectastain ABC Elite kit (Vector Laboratories Inc, Burlingame, CA) was used as described by the manufacturer.

2.5 Biochemical Assays

A 1.5 mm diameter plug was extracted from the degraded specimens and intact untested outer rings. The plugs were weighed wet and digested in a 0.5 mg/ml proteinase K (MP Biomedicals LLC, Solon, OH) solution containing 30mM Tris HCl (pH = 8.0) in a 56 °C water bath overnight. The sulfated glycosaminoglycan (S-GAG) concentration was determined using the dimethyl-methylene blue (DMMB) method, as described (Enobakhare et al., 1996), using a standard curve of chondroitin 6-sulfate from shark cartilage (Sigma-Aldrich, St.Louis, MO) to determine the amount of s-GAG in tissue digests. Absorbance was measured at 535 nm using a Tecan SpectraFluor Plus plate-reader (Tecan Group, Durham, NC). To quantify collagen content, the release of hydroxyproline was measured using a simplified method (Reddy and Enwemeka, 1996) and standards of hydroxyproline (Sigma-Aldrich, St.Louis, Missouri). All samples were quantified spectrophotometrically at 535 nm absorbance.

2.6 Statistical Analysis

Two-way repeated measures ANOVA with Tukey post tests were used for statistical analysis ($\alpha = 0.05$) of mechanical testing and biochemical assays.

3. RESULTS

3.1 Mechanical Properties

Analysis of the intact specimens, individually or as groups (45, 90 and 120 min), found no statistical difference in any of the measured parameters or between tests (sequence independent). Thus, the intact parameters were averaged (tests 1–4) and given in Table 1. The mean intact specimen thickness h was almost 1 mm. As expected, during the rapid loading phase, there was a small instantaneous compression ($U_0/h \sim 6\%$) of the cartilage ECM and a higher instantaneous modulus ($D_0 = 1.04 \pm 0.68$ MPa at 5% strain) compared to the lower apparent (0.22 ± 0.08 MPa) and aggregate (0.13 ± 0.06 MPa) moduli. Finally, the apparent permeability averaged $\sim 4.7 \times 10^{-14}$ M⁴/N-sec.

Treating the articular surface with bacterial collagenase increased the amount of matrix deformation of cartilage explants when loaded under confined creep compression (Fig. 3). Longer incubation times resulted in higher strains, increased permeability, and lower moduli (Fig. 4 and Tab. 1). While the specimen thickness h for the intact state remained constant throughout testing (tests 1–4), collagenase treatment resulted in a statistically significant gradual decrease in thickness (tests 5–8) as shown in Figure 4A. The matrix apparent permeability (Fig. 4B) of degraded specimens (test 5) was significantly higher than the intact specimens (tests 1–4) for all degradation times, and then slowly decreased with

subsequent testing (tests 6–8). Degrading the articular surface with collagenase also caused a decrease in the apparent (Fig. 4C) and instantaneous moduli (Fig. 4D) in test 5, however both moduli slowly increased with each subsequent test (tests 6–8). In contrast, the instantaneous strain (Fig. 4E) of degraded specimens (test 5) was initially the same as intact specimens (tests 1–4) but gradually decreased thereafter (tests 6–8). A statistically significant decrease in the aggregate moduli (Fig. 4F) was also observed in the 90 and 120 min degraded specimens compared to the intact specimens.

3.2 Histological and IHC findings

Changes in surface morphology of the superficial zone were observed after collagenase treatment. While intact specimens had smooth articular surfaces, surface fibrillation was microscopically observed in degraded specimens (Fig. 5). Safranin-O staining showed proteoglycan depletion in the superficial and intermediate zones of degraded specimens as opposed to more uniform staining throughout all zones of intact specimens. Bright field histological analysis using Picrosirius red indicated that collagen was still present in degraded specimens, however changes in birefringence were observed. Indeed, polarized light microscopy showed that collagenase treatment resulted in the compaction of the superficial zone and the loss of lamina splendens after 90 and 120 min. Moreover, collagen cleavage was confirmed using the Col II- α 1C short antibody. Intact specimens showed weak positive immunostaining for collagen cleavage neoepitopes that was restricted to the articular surface, whereas collagenase-treated specimens had a more intense positive staining that penetrated throughout the superficial and into the intermediate zones.

3.3 Biochemical analysis

Collagenase treatment had a significant effect on the total amount of sulfated GAGs present within the cartilage explants (Fig. 6A). Sulfated GAG content for intact specimens accounted for ~3–4% of wet weight whereas degraded specimens had a statistically significant decrease in sulfated GAG content (~1–2% of wet weight). Total collagen content of intact specimens accounted for ~10–15% of the wet weight of explants, and no statistically significant difference in collagen content was observed when compared to the degraded state for all degradation time points (Fig. 6B).

4. DISCUSSION

Osteoarthritis has been well documented as a process of simultaneous surface and matrix degradation resulting from mechanical damage and enzymatic action (Englund, 2010; Mort and Billington, 2001; Smith, 1999). MMPs, such as the collagenases-1, 2 and 3 (MMP-1, 8 and 13) play a key role in mediating the initial steps in matrix breakdown (Wu et al., 2002; Mort and Billington, 2001). In the current study, we developed an *in vitro* cartilage degradation model by treating the articular surface with bacterial collagenase. Our characterization of the functional and material properties via biomechanical, biochemical and histological analyses showed that collagenase cleaved the collagen fiber network, resulting in the loss of proteoglycan (GAG) content and biomechanical properties. These changes in matrix structure and composition created fibrillation at the articular surface and matrix softening, hence lowering the moduli and increasing the apparent permeability of the degraded samples. The higher apparent permeability values observed in degraded specimens were likely due to an increase in surface porosity, which resulted from the disrupted collagenous meshwork and decreased concentration of proteoglycans in the superficial zone. These changes facilitated water and solute transport through the superficial zone and further contributed to an increase in permeability concomitant with proteoglycan loss, as typically seen in early-stage osteoarthritis (Buckwalter and Mankin, 1998). Interestingly, repetitive mechanical testing of degraded cartilage resulted in the gradual collapse of the articular

surface and compaction of the superficial zone. In turn, tissue compaction had a minor effect on biomechanical properties in subsequent tests (tests 6–8), where there was a small decrease in apparent permeability and instantaneous strain, and a small increase in instantaneous and apparent moduli. This is likely due to a reduction in pore size and change in pore geometry. In a recent study, Den Buijs et al. (2009) demonstrated that pore shape and size greatly influenced solute transport rate and spatial distribution in cyclically-deformed scaffolds.

We quantified the damage caused by the collagenase treatment in our *in vitro* model using the OARSI grading system (Pritzker et al., 2006) which is based on six grades that represent the severity and depth progression of the disease in cartilage. Based on the key features and associated criteria for each grade, our *in vitro* cartilage degradation model is equivalent to a grade 2 osteoarthritis. Briefly, this grade is characterized by discontinuities at the superficial zone (fibrillations) and loss of Safranin-O staining (PG depletion) within the upper one-third of the cartilage matrix. Both of these features were present at all collagenase treatment times (45, 90 and 120 min). However, the loss of the lamina splendens observed in the two longer treatment times indicates a slightly more advanced grade (grade 2.5) and demonstrates how the damage due to collagenase treatment can be easily adjusted by selecting different incubation periods.

Immunostaining with a specific antibody that recognizes neoepitopes for collagenase-induced cleavage of type II collagen revealed that bacterial collagenase successfully cleaved collagen fibrils located at the articular surface and within the superficial zone. Changes in collagen birefringence were also observed suggesting disruption or decreased organization of the collagen network. A previous study in our laboratory also reported changes in the collagen fibril orientation of collagenase-treated articular cartilage using Fourier transform infrared spectroscopy (West et al., 2005). The obtained polarization images and spectra showed an inverse relationship between collagen orientation and collagenase treatment times. For treated specimens, the amide I/amide II area ratio, an indicator of collagen orientation, decreased indicating a reduction in collagen fibrils having an orientation parallel to the articular surface. While the collagen network in our study was evidently damaged and altered, the concentration of collagen remained constant for all degradation times, as measured by hydroxyproline assay. This phenomenon, according to Buckwalter and Mankin (1998), is typical of early-stage osteoarthritis. Other studies performed by Panula et al. (1998) and Appleyard et al. (1999) reported collagen disorientation in the superficial zone of animal osteoarthritis models using optical-path polarized-light birefringence techniques. Changes in collagen birefringence were observed at the surface, but the volume fraction of superficial collagen fibrils remained constant, indicating collagenase-driven changes in the collagen microstructural orientation/conformation independent on the amount of collagen. In agreement with the mechanically-induced *in vitro* degradation model of Thibault et al. (2002), our *in vitro* enzymatic degradation model measured an increase in matrix permeability and collagen cleavage without changes in the total amount of collagen before and after degradation. However, in contrast to our study, they did not observe a change in the aggregate modulus, which was likely due to the fact that the mechanical loads did not affect the proteoglycan content within the extracellular matrix.

In our study, we used bacterial collagenase to degrade the ECM components of articular cartilage and emulate the early stages of cartilage damage observed in post-traumatic and aging osteoarthritis. In contrast to mammalian MMPs-1, 8 and 13 which cleave collagen at a single site in each chain of the triple helix, bacterial collagenase initially cleaves all three chains at multiple domains along the triple helix, followed by continued cleavage of the collagen fragments into multiple smaller fragments (Mookhtiar and Van Wart, 1992). Once cleaved, the collagen fragments unwind to expose the neoepitope site for antibody

recognition, as we found in this study and elsewhere (Billinghurst et al., 1997; West et al., 2005). In addition, bacterial collagenase contains small amounts of proteases which are capable of degrading the proteoglycans within the ECM (Herring, 1977; Krueger et al., 1990; Mookhtair and Van Wart, 1992), the loss of which can be detected by histological staining as found here. Thus, bacterial collagenase as used in our study was found to produce similar collagen degradation and proteoglycan loss at the articular surface and in the superficial zone as that found in human and animal models of osteoarthritis.

Overall, our *in vitro* cartilage degradation model produced matrix damage comparable to that seen in the early stages of osteoarthritis. This model provided novel information on cartilage mechanics and insights on the mechanisms for changes in material and functional properties associated with cartilage degeneration. By calculating the permeability after 100 sec of confined creep deformation, we were able to improve the fit between the experimental response and the analytical solution, generating more consistent and reliable results. In contrast to animal models, our *in vitro* model provides more standardization as it is better controlled and induces specific changes in cartilage structure and composition. Changes at the articular surface, in particular the gradual collapse and compaction of the superficial zone, could potentially be monitored to detect the early manifestation or initiation of the disease process. Moreover, our model can be used to study and develop treatment options for repairing injured cartilage to avoid or limit further osteoarthritis progression. It is especially convenient as one can monitor different states of a cartilage sample, from intact to degraded to repaired, and determine whether surface treatment can restore the mechanical properties (i.e., permeability, moduli, strain) and reduce susceptibility to enzymatic degradation.

Acknowledgments

Support for this study was made possible by Grant Number R21-AR059203 (PAT) from the National Institutes of Health - National Institute of Arthritis and Musculoskeletal and Skin Diseases. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIH-NIAMS. This investigation was conducted in a facility constructed with support from Research Facilities Improvement Program Grant Number C06-RR12538-01 from the National Center for Research Resources, National Institutes of Health.

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Figure 1.

Schematic representation of the functional response of cartilage showing the loading phase from time 0 to t_0 and the creep phase from t_0 to t_f .

Figure 2.
Comparison between experimental creep responses and theoretical fits after A) 45 min and B) 100 sec of confined creep compression.

Figure 3.

Experimental values for permeability, instantaneous strain, and aggregate modulus were determined using the biphasic theory. Predicted theoretical creep deformations for intact and degraded cartilage explants were generated from these average experimental values.

Figure 4.

Normalised cartilage explant properties (y-axis) versus series of consecutive creep tests (x-axis). The instantaneous modulus was calculated by fitting an exponential function to the stress-strain response during the loading phase and the slope calculated at a strain of 5%. The instantaneous strain was determined at the end of the loading phase. The aggregate modulus is a measure of the compressive resistance of the solid phase of the ECM at equilibrium and was approximated at the end of the 45 min creep test data (tests 4 and 8). The apparent permeability and apparent modulus were both measured after 100 sec for each creep test (tests 1–8). Two-way repeated measures ANOVA with Tukey post-hoc multiple comparisons were used for statistical analysis ($\alpha=0.05$, $N=10$). Statistically significant differences were not observed between intact values (tests 1–4) for every graph, therefore the degraded values (tests 5–8) were compared to the average of tests 1–4 (average intact value). * indicates statistically significant difference with the average intact value. + indicates statistically significant difference with test 5. ° indicates statistically significant difference with test 6. ° indicates statistically significant difference with test 7.

Figure 5. Representative micrographs (10X) of Safranin-O, Picrosirius red, & Col II-^{3/4}Cshort staining of intact, 45, 90 and 120 min-degraded cartilage explants.

Figure 6. Normalised (A) sulfated glycosaminoglycans and (B) collagen content for intact and degraded cartilage specimens. Two-way repeated measures ANOVA with Tukey post-hoc multiple comparisons were used for statistical analysis ($\alpha=0.05$, mean $\pm$ SEM).

Mechanical properties of intact (average of tests 1–4) and 90 min-degraded (test 5–8) specimens (mean $\pm$ standard deviation).

Table 1

	Intact	Test 5	Test 6	Test 7	Test 8
Thickness (μm)	948 $\pm$ 134	909 $\pm$ 143	854 $\pm$ 162	831 $\pm$ 167	816 $\pm$ 170
Apparent Permeability ($\text{M}^4\text{N}\cdot\text{sec} \times 10^{-14}$)	4.73 $\pm$ 1.43	8.25 $\pm$ 2.24	6.88 $\pm$ 1.81	6.24 $\pm$ 1.57	5.96 $\pm$ 1.66
Apparent Modulus (MPa)	0.22 $\pm$ 0.08	0.10 $\pm$ 0.04	0.11 $\pm$ 0.04	0.11 $\pm$ 0.04	0.11 $\pm$ 0.04
Instantaneous Modulus (MPa)	1.04 $\pm$ 0.68	0.49 $\pm$ 0.25	1.14 $\pm$ 0.67	1.17 $\pm$ 0.63	2.12 $\pm$ 2.34
Instantaneous Strain (%)	6.27 $\pm$ 3.30	7.09 $\pm$ 2.46	5.70 $\pm$ 2.25	4.98 $\pm$ 1.64	5.03 $\pm$ 2.06
Aggregate Modulus (MPa)	0.13 $\pm$ 0.06				0.06 $\pm$ 0.03