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The effects of displacement rate and proteoglycan digestion on the fracture resistance of tissue grown from chondrocyte culture

BRENDAN E. KOOP, JACK L. LEWIS*, MICHELLE M. FEDEWA, THEODORE R. OEGEMA, JR.
Departments of Orthopaedic Surgery and Mechanical Engineering, University of Minnesota, USA
E-mail: Lewis00l@tc.umn.edu

Fracture toughness of cartilage and cartilage replacement tissues is important in injury and disease. For example, cartilage is thought to weaken before it fibrillates in the disease osteoarthritis. Since both loading rate and proteoglycan content affect viscoelastic properties, they may both affect fracture toughness of cartilage and cartilage analogs. In this study, fracture toughness of tissue grown in chondrocyte culture was measured as a function of loading rate and proteoglycan digestion. Control tissue and tissue digested with chondroitinase ABC (cABC) to remove proteoglycans were tested at displacement rates of 0.1 and 0.5 mm/sec. Displacement rate had no effect on fracture toughness for either control or digested tissue. Proteoglycan digestion reduced tissue thickness by 30% and when evaluated on a material basis increased fracture toughness. There was no interaction between digestion and loading rate. When the fracture toughness was normalized to collagen content, which removed the effect of tissue shrinkage, there was no effect of proteoglycan digestion on fracture toughness. These data suggest that proteoglycans do not contribute to tissue toughness, other than by reducing thickness and increasing collagen density.
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Introduction

A tear test is a convenient method for characterizing the tear toughness properties of soft tissues [1–4]. In this type of test, the work of fracture per unit of new crack area is the computed measure of tear toughness. The work done at the tear site is the quantity of interest. However, the total work done includes work done within the entire specimen. Since the tissues are highly viscoelastic, the irreversible viscoelastic work occurring away from the tear is also included in this total work and must be subtracted from the total reversible work done in order to obtain the work due to crack propagation. From this calculation, a tear toughness value for the crack propagating in the specimen can be computed.

One use of tear tests is to determine the effect of various material constituents on the tear toughness. For example, in the present paper, the effect of proteoglycans on tear toughness is evaluated. In a similar fashion, the effect of loading rate on tear toughness is also examined. A change in the rate of loading may change the tear toughness as well as the amount of viscoelastic energy dissipated. There is also a question if these effects interact, i.e. if removal of proteoglycans also changes the effect of loading rate on tear toughness. Thus, it is desirable to know the relative and separate effects, and their interactions, of loading rate and proteoglycan removal on tear toughness, separate from the viscoelastic work. In this paper, experiments and results are described in which both proteoglycan removal and loading rate are varied in order to assess the effect of both factors on the tear toughness of tissue generated from chondrocyte culture.

Previous interest in the effect of proteoglycans on cartilage mechanical properties has been mainly focused on the pre-failure viscoelastic properties [5]. This is probably due to the assumed roles of the various constituents of cartilage on mechanical properties. It is thought that collagen is the primary determinant of strength, and that proteoglycans, with their interaction with water, are responsible for cartilage's ability to withstand compressive stress and reduce hydraulic permeability. It is also thought that proteoglycans do not contribute to strength. However, recent biochemical evidence questions this assumption. Eyre and Wu [6] have shown the existence of covalent crosslinks between Type II and Type IX collagen. Type IX contains cation domains in the Col2 domain that could bind to proteoglycans. Also, the small leucine-rich proteoglycan

*Author to whom all correspondence should be addressed: Department of Orthopaedic Surgery, University of Minnesota, 420 Delaware Street, S.E., Box 289, Minneapolis, MN 55455, USA.

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decorin binds to collagen and its dermatin surface chains may bind to each other. In either case, the proteoglycans would play the role of a ''glue'' between collagen fibrils and would be expected to contribute to tissue strength. Since loss of proteoglycans is one of the earliest signs of osteoarthritis, this might indicate a weakening of the cartilage, and its increased susceptibility to fracture. Consequently, it would be important to determine the role of proteoglycans on cartilage strength.

The effect of proteoglycan removal on mechanical properties has been studied in cartilage. Kempson [7] used one-dimensional tensile tests of bovine cartilage specimens cut from strips parallel to the articulating surface and showed that tensile strength did not change after digestion with cathepsin D, an enzyme that mainly removes proteoglycans. He reported that specimens shrunk after digestion, but did not give quantitative values. Schmidt *et al*. [5] performed similar tests on bovine cartilage. They also reported no change in tensile strength after digestion with chondroitinase ABC (cABC), an enzyme that removes the chondroitin sulfate from proteoglycans and the aggrecan hyaluronic acid backbone. They also noted specimen shrinkage after treatment. It appears they used specimen geometry after digestion, but before loading, as their reference geometry for defining stress. If the original undigested geometry were used to define stress, which would be equivalent to normalizing to collagen content, the structural tensile strength (maximum load carried by the tissue) would have gone down with cABC treatment, suggesting that proteoglycans do play a role in tissue strength. Schmidt *et al*. [5] also found that the pre-failure viscoelastic properties were changed by proteoglycan removal, causing the tissue to become less viscoelastic. Purslow [1] measured the tear toughness of rat skin as a function of strain rate and found a slight, statistically insignificant, increase with strain rate. There appears to have been no studies in which both proteoglycan content and loading rate were varied.

Tissue tearing is a failure mode more similar to actual failure modes of cartilage, *in vivo*, compared to tensile tests [8]. Cartilage fails, or fibrillates, mainly by crack propagation processes. It is, therefore, preferable to assess the effect of proteoglycans on failure properties using a crack or tear propagation test, rather than a tensile test. Since proteoglycan removal alters viscoelastic behavior, and this, in turn, may interact with failure properties, there is a need to determine the separate effects of proteoglycan removal and loading rate on tear toughness. The purpose of this paper is to describe the effect of proteoglycan removal and loading rate on tear toughness of a tissue generated from chondrocyte culture.

Experimental methods

Method of growing tissue

Experimental tissue was prepared by a previously reported method [9] using chondrocytes in culture. Briefly, male New Zealand white rabbits approximately 2–3 lb in weight were sacrificed and the cartilage was removed from the femoral heads, tibial plateaus, humeral heads, and glenoid cavities. The cartilage was minced into 1 mm^3 and the chondrocytes were isolated from the cartilage by means of a sequential digestion using 0.05% trypsin in EDTA (Gibco BRL, Grand Island, NY), followed by incubation with 0.8 mg DNAse II (Sigma Chemical Co., St Louis, MO) and 40 mg of collagenase II (Sigma Chemical Co., St Louis, MO) in 15 ml of F-12 media. The cell suspension was centrifuged and the pellet re-suspended in 10 ml of Hank's balanced salt solution (Gibco BRL, Grand Island, NY). The pellet was re-suspended in Ham's F-12 medium containing 15% fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY) and 0.05 mg/ml gentomycin to obtain the desired cell concentration. Aliquots of 2.5×10^6 cells per 25 cm^2 flask were plated on Type I collagen coated plates (0.033% Vitrogen for 2 h at 37 °C) in 5 ml of medium.

Beginning the day after harvest, the 5 ml of cell culture medium was changed three times each week according to the following schedule. The cells were fed F-12 medium containing 15% FBS. During the first week, the medium contained 2.5 μg/ml of amphotercyn B and 0.05 mg/ml gentomycin. During the second week, the feeding media amphotercyn B was deleted. For the third and subsequent weeks, the medium contained 30 μg/ml ascorbic acid (Sigma Chemical Co., St Louis, MO). Culturing was continued for 8 weeks. Proteoglycans were then removed from half of the cultured tissue by incubating with 0.25 U/ml of cABC in buffer for 24 h at 25 °C. Buffer consisted of 0.05 M Tris–HCl, 0.06 M sodium acetate, pH 8.0 containing protease inhibitors 2 m MEDTA, 5 mM benzamidine HC1, 10 mM N-ethylmaleimide, and 2 mM phenylmethyl sulfonyl fluoride. Collagen content at harvest was determined by analysis of hydroxyproline [10].

Specimen preparation

Strips of tissue were prepared for testing by cutting open the culture flask and transferring the tissue to a petri dish with phosphate buffered saline (PBS). Once the tissue was in the dish, a template made of two razor blades spaced about 5 mm apart was used to cut eight to ten 5 × 20 mm specimens from each flask and kept in PBS.

The thickness of each specimen was measured using a thickness tester based on a vertically oriented linear slide, linear variable differential transformer (LVDT), and force transducer. The LVDT and force transducer were attached to the linear slide so that the distance between the tip of the force transducer and the plate that held the specimen could be measured. As the force transducer was lowered using the linear slide, a voltmeter was set to indicate the signal from the force transducer. The voltmeter read zero until the specimen was touched. Thus, the linear slide was adjusted until the force transducer was just touching the specimen. Once this was done, the LVDT value was recorded. The same procedure was then done without a specimen. The voltage difference was converted to a distance in μm, which was recorded as the thickness of the specimen at the point being measured. This process was repeated for at least five points in different areas of the specimen and the thickness values were averaged. The methods of cutting samples and measuring their thickness were used

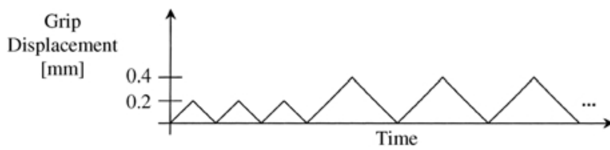

Figure 1 Grip displacement for fracture test.

for all control and proteoglycan digested specimens in this study.

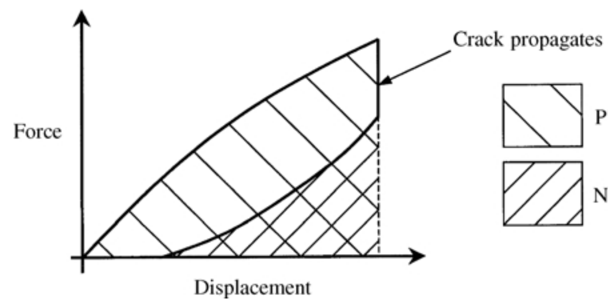

Figure 2 Schematic of force–displacement curve for loading–unloading cycle with crack propagation.

Mechanical testing

After specimen preparation, a tensile tear test was performed using a Microbionix (MTS, Inc.) mechanical test system. The tear propagation was observed with a video camera and recorded on videotape. Each specimen was bathed in PBS during the test to ensure it remained hydrated and that material properties did not change over time. When a specimen was secured in the grips of the tensile tester, the gauge length was recorded (approximately 11 mm) for each specimen. Once the magnification on the video camera was at its desired level, recording was activated on the VCR and a ruler with centimeter and millimeter marks was placed in view of the camera at the level of the specimen. This provided a reference for data reduction after testing. Any time the magnification was altered on the camera, this step was repeated to ensure an accurate length reference. A label corresponding to the specimen being tested was also briefly placed in view of the camera so that the identity of each specimen would be known during data reduction.

The tissue was pre-conditioned by holding a 1.5 mm displacement for 5 min, and then ramping down until zero force was reached. The grips were then stopped and held for 30 s. During the 30-s hold, a scalpel was used to cut a notch in the specimen, spanning approximately 1/3 of the width. The fracture test cycle then began by moving the grips in a triangular load-time wave at a rate of either 0.1 mm/sec or 0.5 mm/sec to a maximum displacement of 0.2 mm. This was done three times.

The maximum displacement was then increased to 0.4 mm and the displacement was repeated three times at the same rate. This three-cycle procedure continued by increasing the maximum displacement in 0.2 mm increments and repeating three times at each displacement (Fig. 1). As the displacement increased, the crack propagated a short distance and stopped. The crack continued to propagate across the specimen over the course of the displacement cycles until complete failure of the tissue strip.

The loading rates were chosen to accommodate the test method. The fastest rate for which the crack propagation could be read from the video image was 0.5 mm/sec and 0.1 mm/sec was the practical slow rate that would not result in too long a test. Tests were first run on control tissue at the two displacement rates and then on tissue digested with cABC, at the two displacement rates.

Method of data reduction

Upon completion of testing, the data was reduced using both video analysis and the force–displacement data. Using the ruler that was recorded before every specimen test as a reference, the video of each specimen was analyzed frame by frame. Both the length and width at the specimen center were measured after the 5-min hold, as well as the length of crack propagation during each cycle. Then, the force–displacement data was converted to Microsoft Excel and all specimens were analyzed using a programmed macro.

Data was reduced using the method of Oyen-Tiesma and Cook [4], which was a modification of the original method by Fedewa *et al*. [3]. Briefly, the work done over a load–unload cycle was determined from the area in the force–displacement cycle, which created a closed loop on a force–displacement plot. The work done during the load phase (P) and the work done during the unload phase (N) were calculated independently (Fig. 2).

The ratio $(P-N)/P$ was formed and plotted as a function of cycle. These points tended to follow a smooth curve, except at those cycles during which the crack propagated. For those points, the fracture work was estimated from the difference between the measured ratio and the estimated ratio for that cycle if the fracture would not have occurred, i.e.

$$\left(\frac{P-N}{P}\right)_{\text{fracture}} - \left(\frac{P-N^*}{P}\right)_{\text{non-fracture}} = \frac{-N+N^*}{P}$$

N* was estimated from a curve fit of the points before the crack propagation cycle on the ratio vs. cycle plot (Fig. 3).

$(P-N^*)$ is the work that would have been done during the cycle if fracture had not occurred, i.e. the viscoelastic-dissipated work. Multiplying by P gives an estimate of the work done during the fracture propagation, E_f.

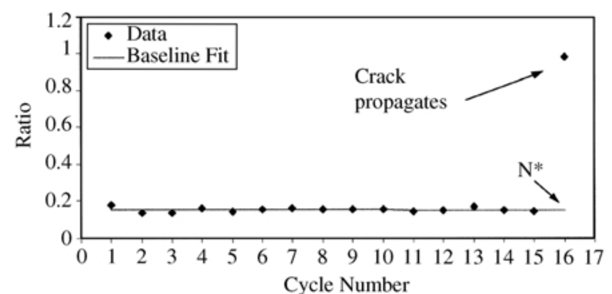

Figure 3 Example of the change of ratio $= (P-N)/P$ with cycle number. Note the increase in the ratio when the crack propagates.

$$\left(\frac{-N+N^*}{P}\right)P = (-N+N^*) \approx E_f \text{ (work of fracture)}$$

The tear toughness (T) was calculated by dividing E_{f} by the change in crack area during fracture propagation (ΔA_c):

$$T = E_f / \Delta A_c$$

Here, ΔA_c is equal to Δl_c, the increment in crack length, multiplied by the thickness of the specimen, t. Typically, three or four of these values were obtained for each specimen, depending on how many times the crack propagated a measurable amount.

One of the main factors influencing tear toughness, a material parament, was the change in specimen geometry due to digestion and the initial 5-min preconditioning. The specimen cross-section was reduced as a result of both of these actions, while the amount of collagen within the specimen remained the same. Thus, the collagen density increased, leading to an increase in work per unit area of crack extension. To eliminate this effect, and thus assess the possibility that factors other than an increase in collagen density could change tear toughness, the tear toughness was divided by the ratio of final to initial collagen density. Since cABC does not affect collagen, the amount of collagen before digestion was assumed to be the same as that after digestion; i.e. the mass of collagen in a specimen was the initial collagen density, ρ_0, multiplied by the specimen volume.

$$\text{Collagen mass in specimen} = (\rho_0)\,(\text{initial specimen volume})$$

$$\text{Initial specimen volume} = L_0\, w_0\, t_0$$

where ρ_0 is the initial collagen density, L_0 is the initial specimen length between clamps, w_0 is the initial specimen width, t_0 is the initial specimen thickness.

After initial specimen cutting, the specimens were either digested with cABC or left untreated as controls. Thickness was measured for controls and after the digestion. Each specimen then underwent the 5-min preconditioning tensile stretch. This caused negligible geometry change in controls, but caused significant elongation and narrowing of the cABC digested specimens. Therefore, the geometry after the digestion and the 5-min hold was used for all specimens. Because thickness could not be measured directly after the specimen was mounted in the test machine, the thickness change during the 5-min hold for the cABC digested specimens was assumed to change in the same ratio as the width change. Therefore, the specimen volume after the 5-min hold, and at the start of the fracture test, was assumed to be

$$\text{Test specimen volume} = Lwt$$

where L is the specimen length after the 5-min hold, w is the center width of the specimen after the 5-min hold, t is the digested (or control) specimen thickness $\times$ (w/w_0).

Then, since collagen mass remains constant in the specimen:

$$\text{Collagen mass in specimen} = (\rho)\,(\text{test specimen volume})$$

$$\frac{\rho}{\rho_0} = \frac{L_0\, w_0\, t_0}{Lwt}$$

where ρ is the collagen density after the 5-min hold.

Tear toughness was then normalized for collagen density by multiplying by (ρ_0/ρ).

The number of times the crack propagated varied from specimen to specimen, and thus so did the amount of replicate data points taken for each specimen. Because of this fact, the data was not averaged for each specimen to avoid comparing values that represented averages of differing amounts of replicates. Instead, the data for all the specimens tested under each set of experimental conditions (i.e. displacement rate and cABC treatment) were grouped together and a two-way ANOVA was performed on this data. In order to group data from separate specimens together, it was assumed that the material properties of the tissue did not vary appreciably in specimens from the same flask. Plots of the residuals and normality plots showed this to be a reasonable assumption.

Results

The cABC caused approximately 30% reduction in thickness with respect to undigested specimens. The average thickness for all control specimens was 102.2 μm, whereas the average thickness for all cABC-digested specimens was 72.8 μm. The 5-min hold caused negligible changes in geometry of the controls, but caused a nearly 30% reduction in specimen width, with minimal change in length.

Summary data on tear toughness for each set of experimental conditions, though not adjusted for changes in collagen density, are shown in Table I and Fig. 4.

Analysis by two-way ANOVA showed that there was not a significant difference in tear toughness for specimens tested at the two different displacement rates, for either the control or cABC-digested samples. However, there was a significant difference ($p < 0.001$) in tear toughness between control specimens and those treated with cABC. The interaction of displacement rate and enzyme treatment was not significant at the $\alpha = 0.05$ level.

TABLE I Tear toughness ($T = E_f/\Delta A_c$) summary data

Rate (mm/s)	Digest	n	Mean T (N mm/mm^2)	Std. dev. (N mm/mm^2)	Std. error (N mm/mm^2)	Effect	p-value	Power
0.1	Undigested	16	0.154	0.0782	0.0196	Rate	0.236	0.205
0.1	Digested	9	0.450	0.1767	0.0589	Digestion	< 0.0001	0.999
0.5	Undigested	9	0.161	0.1666	0.0555	Rate*Digestion	0.185	0.247
0.5	Digested	12	0.330	0.2041	0.0589			

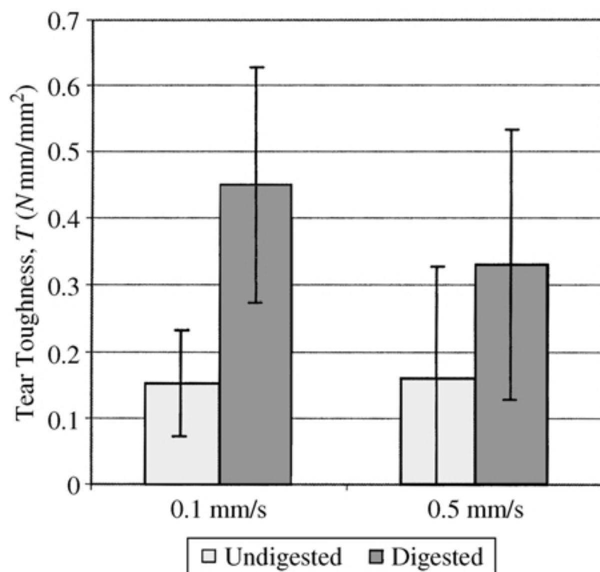

Figure 4 Tear toughness ($T = E_f/DA_c$) for control (undigested) and digested samples, for 0.1 and 0.5 mm/s displacement rates. Data is from Table I. Error bars are plus and minus one standard deviation.

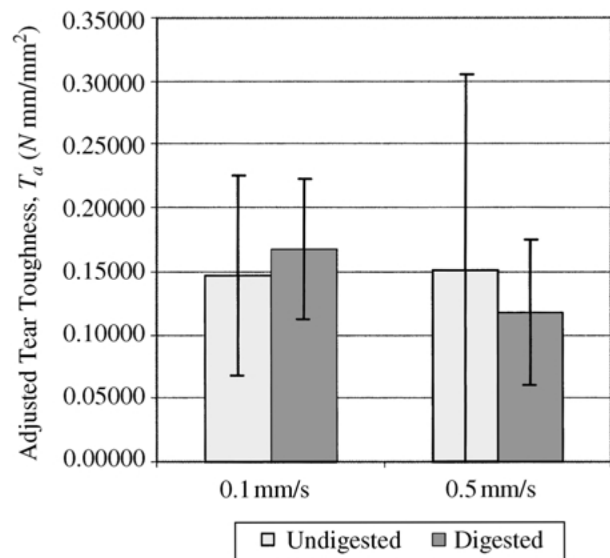

Figure 5 Adjusted tear toughness ($T_a = T\ r_0/r$) for control (undigested) and digested samples, for 0.1 and 0.5 mm/s displacement rates. Data is from Table II. Error bars are plus and minus one standard deviation.

Summary data on tear toughness normalized for collagen density is shown in Table II and Fig. 5.

Neither the effect of displacement rate nor enzyme treatment, nor the interaction effect, were significant at the $\alpha = 0.05$ level.

Discussion

The goal of this paper was to determine the effect of proteoglycan removal and loading rate, and their interaction, on tear toughness of tissue generated by chondrocyte culture. There was no effect of loading rate on tear toughness for either controls or tissue treated with cABC for the range of loading rate used in these experiments. Treatment by cABC caused a reduction in tissue thickness of 30%. Although there was an increase in tear toughness due to proteoglycan removal when changes in collagen density are not taken into account, there was no effect on the tear toughness when collagen density was accounted for. This latter value is the more important factor for determining the role of proteoglycans in tear resistance since there is the same amount of collagen in the control and cABC treated tissue, although the thickness of the cABC tissue is less and collagen density greater. This means it takes the same amount of work per unit of crack extension for the digested and undigested specimens, indicating that the proteoglycans are not contributing to the work of fracture or tearing.

When collagen density is not accounted for, the tear toughness, which is defined as the work per unit area of tear propagation, is increased after cABC digestion. However, this is only because the area is smaller for a unit of crack extension, due to the thickness decrease, so that the tear work per area, but not per extension, is greater than for controls. Thus, the tear toughness of the cABC treated tissue is higher because the same collagen is compressed into a thinner sheet. This conclusion is valid for both loading rates, suggesting there is no interaction between proteoglycan removal and loading rate on tear toughness or tear toughness normalized by collagen density.

The lack of effect of proteoglycan digestion on tear toughness normalized by collagen density suggests that proteoglycans play no role in tear toughness of this tissue. However, this may be misleading. Although the plastic deformation was not quantified, it appears from the permanent deformation after the 5 min pre-conditioning hold and the large first loop area for the cABC treated tissue that significantly more plastic deformation occurred with the cABC treated tissue compared to the controls. This would appear as dissipated energy in the loading–unloading loop and lead to an increased tear toughness. The primary source of the plastic deformation must be the collagen network, since there is little proteoglycan left. It has been suggested that the proteoglycans resist movement of the collagen fibrils [5]. If plastic deformation is occurring, there would have to be a compensating source of reduction of tear toughness to result in no change. Degradation of bonds

TABLE II Adjusted tear toughness ($T_a = T\,(\rho_0/\rho)$) summary data

Rate (mm/s)	Digest	n	Mean T_a (N mm/mm^2)	Std. dev. (N mm/mm^2)	Std. error (N mm/mm^2)	Effect	p-value	Power
0.1	Undigested	16	0.147	0.0781	0.0195	Rate	0.423	0.12
0.1	Digested	9	0.166	0.0562	0.0187	Digestion	0.787	0.058
0.5	Undigested	9	0.151	0.1535	0.0512	Rater*Digestion	0.335	0.151
0.5	Digested	12	0.117	0.0582	0.0168			

between collagen fibrils is a candidate source of toughness reduction after proteoglycan reduction.

Conclusions

Loading rate did not affect the calculated tear toughness of tissue grown in chondrocyte culture, within the range of 0.1–0.5 mm/s, either for the controls or for tissue treated with cABC. Tear toughness, a material parameter, was increased after proteoglycan removal, but tear toughness normalized by collagen density, a more relevant measure of the effect of the proteoglycans on tissue toughness, did not change. These results suggest that proteoglycans removed by cABC do not play a role in tear strength of tissue from chondrocyte culture regardless of displacement rate.

Acknowledgments

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