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Viscoelastic Characterization of Murine Articular Cartilage through Nondestructive Dynamic Microindentation

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Abstract:

Transgenic and biochemical mouse models of osteoarthritis have become increasingly popular. However, the microscale thickness of mouse cartilage complicates mechanical characterization of these models. Existing methods are time-prohibitive and difficult, especially for life scientists. We developed a rapid, nondestructive method for viscoelastically characterizing murine articular cartilage, and validated it. We dissected tibial plateaus from adult mouse knees, and subjected them to trypsination and ribosylation treatments. Dynamic mechanical testing was performed using a BioDent testing apparatus (ActiveLife Scientific, Santa Barbara, CA). Viscoelastic parameters were extracted, and repeat testing was conducted to check for testing-induced damage. This protocol should demonstrated significant mechanical differences between the control and osteoarthritic models. Additionally, we verified using histology and finite element analysis.

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Author's Note:

I wrote this paper with the intention of eventually submitting it to the Journal of Mechanical Behavior of Biomedical Materials. The journal has a 2,500-word limit for technical notes, which I have observed here.

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1. Introduction

Knee osteoarthritis is a common disease among the elderly. It is caused by degradation of articular cartilage at the knee joint. Specifically, tibial plateau and femoral condyle cartilage are completely destroyed, causing painful bone-on-bone contact (Figure 1). To explore underlying mechanisms and potential cures of osteoarthritis, animal models are important. Transgenic and biochemical mouse models for osteoarthritis are popular among life scientists. However, microscale thickness of mouse cartilage complicates mechanical evaluation of osteoarthritic cartilage. Since murine cartilage is less than 100 microns thick even when healthy, exceptional precision is required. The complex interaction of chondrocytes, proteoglycans, and collagen in cartilage also produces unique viscoelastic behavior. As a result, cartilage cannot be characterized by traditional mechanical testing. Creep, stress-relaxation, and biphasic tests that account for this time-dependent behavior take hours, if not days. They also require custom testing apparatuses that limit these methods to engineers, rather than the life scientists who would benefit most. There is an obvious need for a rapid, standardized protocol to test murine articular cartilage. To address this need, we propose a dynamic sinusoidal test protocol.

(Image removed)

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Figure 1. Exposure of bone by human cartilage degradation.

2. Materials and Methods

Four male Balb/c mice (9 weeks old) were sacrificed with Washington University Animal Studies Committee approval. Two tibial plateaus were obtained from each animal, for a total of eight plateaus. The dissection was conducted with assistance of a dissection microscope. A scalpel cut was made at midshaft to separate each plateau from the rest of the tibia. The cut was made parallel to the medial plateau surface, ensuring a level surface during mechanical testing. Another scalpel cut was made between the femoral condyles and tibial plateau, isolating the plateau. The meniscus was also removed to prevent interference with mechanical testing. All samples were wrapped in gauze soaked with PBS (Sigma-Aldrich, St. Louis, MO), and stored at -80°C until ready for use.

2.1 Biochemical Treatment

We utilized two biochemical modifications, trypsination and ribosylation, to induce degradation of the articular cartilage. Trypsin is a serine protease that selectively cleaves proteoglycans, resulting in their degradation. Cartilage with damaged proteoglycans loses its ability to retain water, causing loss of viscoelasticity and stiffness. Ribose cross-links collagen proteins, stiffening the extracellular environment of the cartilage. Like trypsin, ribose also decreases cartilage viscoelasticity.

Four of the tibial plateaus were removed from -80°C storage and allowed to thaw for 30 minutes. We then mechanically tested them as a control group (Section 2.2). Upon completion of mechanical testing, the same samples were submerged in 1x trypsin-EDTA solution (Sigma-Aldrich, St. Louis, MO) and incubated at 37°C. At 3, 7, and 10 hours of total treatment time, they were removed from trypsin solution and washed with PBS to stop trypsin degradation. They then

underwent mechanical testing. In the case of the 3 and 7-hour time points, the samples were placed back into fresh trypsin solution and incubated at 37°C until the next time point (not including mechanical testing time).

We produced ribose solution (all reagents Sigma-Aldrich, St. Louis, MO) with final concentrations of 25mM e-amino-n-caproic acid, 5mM benzamidine, 10mM N-ethylmaleimide, 30mM Hepes, and 0.6M ribose. 0.5M NaOH and 0.5M HCl were used dropwise as needed to maintain a solution pH between 7.2 and 7.6. The four tibial plateaus not trypsinated were incubated for 24 hours in this solution at 37°C. Upon 24 hours of incubation, the tibial plateaus were washed in PBS and mechanically tested.

2.2 Mechanical Testing

After thawing, we superglued each sample to a 10 mm by 10 mm metal platen. Each glued sample was then moved into a PBS-filled petri dish to simulate in-vivo conditions. To test a sample, we placed the dish onto a balance (Mettler-Toledo, Oakland, CA). The balance was then placed below a BioDent testing apparatus (Active Life Scientific, Santa Barbara, CA). The BioDent probe (BP1, 0.375 mm diameter) was lowered until it made contact with the lateral tibial plateau (Figure 2). A sustained preload of 0.5 grams was maintained for thirty seconds, verifying contact of the probe with cartilage. The BioDent then performed 20 cycles of sinusoidal indentation, at 1 Hz frequency. Each indentation makes a 20 micron indentation, representing a 25% cartilage strain. After the indentations are completed, another 20 cycles of indentation are performed at the same site. This data is required to verify lack of probe-induced mechanical damage. The microindentation process requires only 1 minute, making it a convenient method for large sample sizes. Fast testing also prevents degradation of the thawed cartilage, ensuring sample quality is maintained.
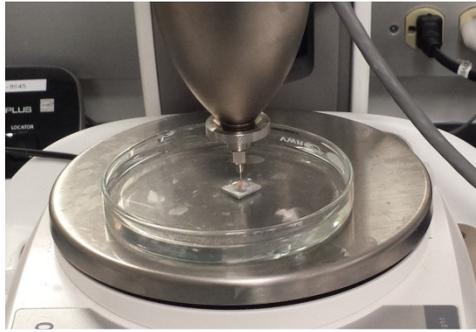
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Figure 2. Contact of the BioDent probe and left tibial plateau.

2.3 Histology

To evaluate the dose-dependent effect of trypsination, we conducted histology. Four additional tibial plateaus were acquired from the same age Balb/c mice, and incubated for either 0, 3, 7, or 10 hours in trypsin at 37°C. The histology specimens were decalcified for 10 days in Immunocal (StatLab, McKinney, TX). They were then fixed in 3% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) for 24 hours, and then dehydrated in increasing concentrations of ethanol. The specimens were embedded in paraffin and cut into frontal sections 10 microns thick. Finally, Safranin-O and Fast Green stains were used to visualize the extent of proteoglycan degradation.

2.3 Finite Element Analysis

Finite element analysis solves a system of differential equations that underlie a physical phenomenon. Hence, it is a powerful tool for simulating the contact scenario in our experiment. A finite element simulation was constructed in Ansys, with a 30 micron mesh and sliding contact. A 90 micron thickness was used to simulate the control case, whereas a 30 micron thickness was used for the trypsinated case. This represents the reduced effective thickness of cartilage due to proteoglycan loss. An infinite layer of bone was placed underneath to account for any amplification effects. The simulation allows for comparison between theoretical and experimental outcomes.

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3. Results

3.1 Mechanical Testing

All data analysis was performed in the statistical package Prism (Graphpad, La Jolla, CA). Two parameters were used to determine the viscoelastic behavior of the samples. The first, peak dynamic modulus, is the non-static equivalent of elastic modulus. Due to inertial effects, peak dynamic modulus is usually larger than the elastic modulus. The second parameter is loss tangent, which represents the phase lag between the displacement and force curves. Viscoelastic materials, due to underlying microarchitecture, do not exert a force immediately upon deformation.

Surprisingly, the peak dynamic modulus of trypsinated cartilage increased significantly from control ($P < 0.05$) for all time points (Figure 3). The peak dynamic modulus of ribosylated cartilage also increased significantly from control. As expected, the loss tangent of both ribosylated and trypsinated cartilage significantly decreased from control (Figure 4). The repeat testing at each site showed no significant differences from control (paired t-test, $P > 0.05$).
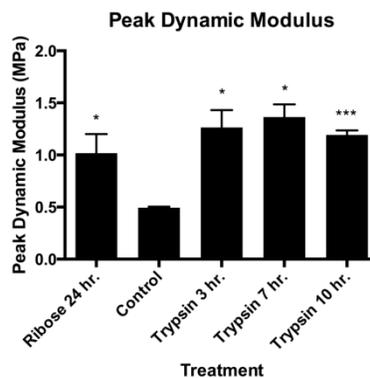

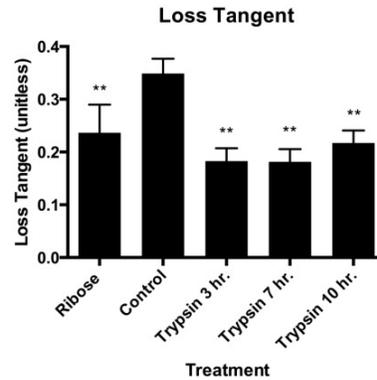Figure 3. Peak dynamic modulus results.

Figure 4. Loss tangent results.

3.2 Histology

Histology of the trypsinated samples indicated a time-dependent movement of the tidemark (Figure 5). Additionally, the strength of color decreased over time, as indicated by the faded pink regions in the 7 and 10-hour time points. Histology was not conducted for ribosylated samples, since the treatment does not induce proteoglycan loss and cannot be visualized.
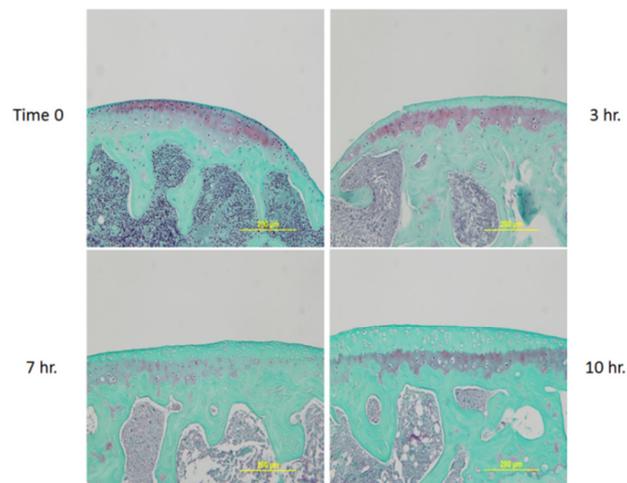

Figure 5. Histology showed loss of proteoglycans over time.

3.3 Finite Element Analysis

The results of the finite element analysis were elucidating. In control model, almost all the stresses from indentation were from the cartilage itself. However, almost all the stresses in

the trypsinated model came from the bone layer. (Figure 6). This had immense implications in interpreting our results.
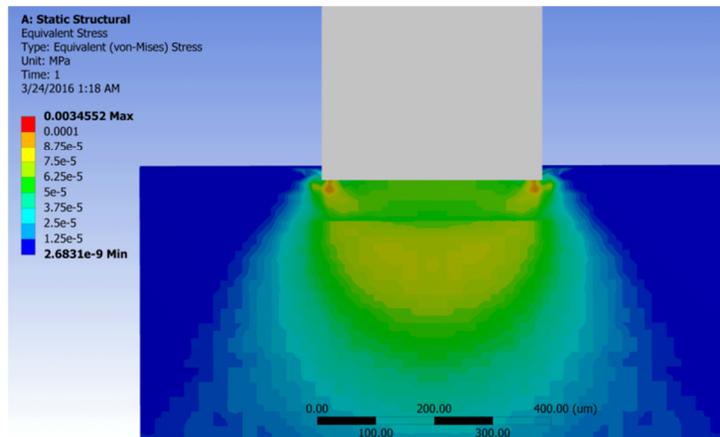

Figure 6. Finite element model of our experimental setup.

4. Discussion

The modulus of cartilage varies significantly depending on the underlying protocol. Atomic force microscopy measurements have found sub-MPa moduli, whereas Youn, et al. (2006) found a 2 MPa modulus. However, our 0.4 MPa control result falls within this range, and is very close to our findings in Tang, et al. (2011). Hence, we find that the sinusoidal indentation protocol produces reasonable elastic modulus results. The ribose modulus was nearly double that of control, indicating that ribosylation has a significant effect on the stiffness. However, the nearly three-fold increase of modulus with the trypsinated samples was a surprise. We expected the trypsin treatment to significantly decrease modulus, not increase. We were able to rectify this with the finite element analysis. Most of the forces in the trypsinated samples arise from the bone layer, not the cartilage itself. This is perhaps the biggest limitation of this protocol. In excessively thin cartilage, the system is unable to feel the cartilage only. While our results are fully explained, modulus is not a good mechanical discriminant. The inability of the system to distinguish between ribosylation and trypsination means another parameter is required.

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Fortunately, loss tangent was a very powerful viscoelastic parameter. The control loss tangent of 0.35 is nearly identical to our 2011 findings. Hence, our system is effective at detecting phase lag between displacement and force. The major decrease in loss tangent between the control and ribosylated samples was expected. Due to increased stiffness of the collagen network, force transmits more easily through the cartilage and will experience little delay. Likewise, we expected the major decrease in loss tangent for trypsinated samples. Since proteoglycans are essential in maintaining hydration of cartilage, trypsin cleavage reduces water content. Without the viscosity of water, the cartilage transmits force more elastically.

5. Conclusion

Mouse models will continue to dominate osteoarthritis research. While clinical studies are practical, the science of osteoarthritis is explored at the microscale. Hence, this experiment is of great relevance to life scientists working with mouse models. In summary, we developed a protocol that utilizes BioDent microindentation. In the future, we will explore the effects of reducing indentation depth on peak dynamic modulus. Perhaps with a lower strain, the apparatus will be able to detect the effects of trypsinated cartilage rather than the underlying bone.

References:

1. Abusara, Ziad, Markus Von Kossel, and Walter Herzog. "In Vivo Dynamic Deformation of Articular Cartilage in Intact Joints Loaded by Controlled Muscular Contractions." PLOS ONE PLoS ONE 11.1 (2016): n. pag. Web. 31 Mar. 2016.

2. Argatov, I., A.u. Daniels, G. Mishuris, S. Ronken, and D. Wirz. "Accounting for the Thickness Effect in Dynamic Spherical Indentation of a Viscoelastic Layer: Application to Non-destructive Testing of Articular Cartilage." European Journal of Mechanics - A/Solids 37 (2013): 304-17. Web. 31 Mar. 2016.

3. Hayes, W.c., L.m. Keer, G. Herrmann, and L.f. Mockros. "A Mathematical Analysis for Indentation Tests of Articular Cartilage." Journal of Biomechanics 5.5 (1972): 541-51. Web. 31 Mar. 2016.

4. Irianto, Jerome, Girish Ramaswamy, Rosa Serra, and Martin M. Knight. "Depletion of Chondrocyte Primary Cilia Reduces the Compressive Modulus of Articular Cartilage." Journal of Biomechanics 47.2 (2014): 579-82. Web. 31 Mar. 2016.

5. Liu, Jennifer W., Adam C. Abraham, and Simon Y. Tang. "The High-throughput Phenotyping of the Viscoelastic Behavior of Whole Mouse Intervertebral Discs Using a Novel Method of Dynamic Mechanical Testing." Journal of Biomechanics 48.10 (2015): 2189-194. Web. 31 Mar. 2016.

6. Richard, F., M. Villars, and S. Thibaud. "Viscoelastic Modeling and Quantitative Experimental Characterization of Normal and Osteoarthritic Human Articular Cartilage Using Indentation." Journal of the Mechanical Behavior of Biomedical Materials 24 (2013): 41-52. Web. 31 Mar. 2016.

7. Tang, Simon Y., Richard B. Souza, Michael Ries, Paul K. Hansma, Tamara Alliston, and Xiaojuan Li. "Local Tissue Properties of Human Osteoarthritic Cartilage Correlate with Magnetic Resonance T1rho Relaxation times." Journal of Orthopaedic Research J. Orthop. Res. 29.9 (2011): 1312-319. Web. 31 Mar. 2016.

8. Youn, Inchan. "Compressive Properties of Mouse Articular Cartilage Determined in a Novel Micro-Indentation Test Method and Biphasic Finite Element Model." Journal of Biomechanical Engineering J Biomech Eng 128.5 (2006): 766. Web. 31 Mar. 2016.

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