Zone-Specific Changes in Micromechanical, Biochemical, and Structural Properties in Articular Cartilage from a Rabbit Joint Flexion Model

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ABSTRACT

Metacarpophalangeal (MCP) joint proximal bone-cartilage specimens from the fourth digit were collected from repetitively flexed and non-flexed (control) paws of four New Zealand White rabbits. The specimens were cryo-fractured to reveal a sagittal cut containing the cartilage zones of different collagen microstructure. Nanoindentation, Fourier Transform infrared microspectroscopic imaging (FTIRMI), and histology were performed on a region of interest (ROI) ~400 microns wide and through the thickness of the cartilage with two goals in mind: (1) to examine the effect of collagen network structure (random in the mid zone versus organized in the deep zone) on the biomechanical and biochemical properties of cartilage; and (2) to understand the changes in these properties due to physical forces. We found that zone microstructure significantly affected the measurement of the local relaxed modulus measured by nanoindentation. The deep zone had a higher modulus than the mid zone (Wilcoxon paired test, $p<0.05$). We also found that flexion significantly decreased the proteoglycan content in both the mid and deep zones (Wilcoxon paired test, $p<0.05$), suggesting indirect repetitive loading in the rabbit paw can be damaging to the joint via down regulating proteoglycan synthesis in the mid and deep zone cartilage. This is the first study to simultaneously report the local zone-specific mechanical and biochemical properties in the rabbit joint flexion model.

INTRODUCTION

Nanoindentation is becoming a valuable mechanical characterization tool for both biologists and engineers due to its ability to measure *in situ* local properties of small, irregularly shaped, heterogeneous, and anisotropic biological specimens. The rabbit metacarpophalangeal (MCP) joint, with a surface area ~16 mm^2 and an uncalcified cartilage thickness ~100 μm, is an example of such a specimen. In the present study, nanoindentation of rabbit joint cartilage is applied in conjunction with Fourier Transform infrared microspectroscopic imaging (FTIRMI), and histology to implement a comprehensive composition-structure-property characterization of the zonal cartilage.

This study is part of a larger study in which we have used the MCP model to investigate the effect of motion and load from repetitive hand tasks on the changes in cartilage biochemical and biomechanical properties [1]. In a preliminary study of nanoindentation of rabbit MCP cartilage, the indenter tip applied a force perpendicular to the articular surface and measured the cartilage properties at various locations on this surface of one control joint. The results suggested that the superficial zone is important in determining how joint cartilage responds to loading. Specifically we found that indentation stiffness correlated positively and significantly with the superficial zone thickness [2]. However, the stiffnesses of the mid and deep zones were not successfully determined. This was due to the fact that the superficial zone dominated the contribution to the mechanical properties measured (considering the superficial zone thickness of ~10 μm and the depth of tip indentation of ~3 μm).

The primary objective of this study is to investigate the properties of the mid zone cartilage (with random collagen organization) and deep zone cartilage (with aligned collagen organization), and in particular, the relationships between mechanical property and tissue microstructure and biochemical composition. The second objective is to measure possible effects of *in vivo* repetitive flexion on these properties within specific cartilage zones.

EXPERIMENT

Experimental design

Specimens were 4th digit rabbit MCPs which had undergone repetitive flexion at 1 Hz for 80 cumulative hours and their contralateral non-flexed control MCPs. Four rabbits were used (n=4), with one being a non-experimental rabbit (neither digit was flexed). We estimated the joint contact pressure of these experiments to be < 0.08 MPa. Specimens were first snap frozen in liquid N_2, and then cryo-fractured down the middle with a sharp razor blade in a sagittal section. The fracture surface revealed the zones of cartilage containing the region of interest (ROI)—a rectangular region ~400 μm x 100 μm.

Nanoindentation was first performed in the ROI as visualized under the indenter optics. The mechanical data collection generally took several hours to complete. Next, the specimen was gently retrieved with a pair of forceps and placed in 10% formalin for fixation for 24 h. Then it was decalcified in Decal® (Decal Chemical Corporation, Tallman, NY) for 48 h, followed by rinsing with running tap water, de-ionized water, dehydration through graded ethanol solutions, and finally embedded in paraffin blocks following standard tissue processing procedures. A microtome was used to obtain 6 μm thick intact osteochondral sections. The first intact section was mounted on IR transparent BaF_2 slides for FTIRMI of biochemical composition. Consecutive sections within 300 μm thickness from the first tissue slice were mounted on glass slides for structural analysis with histology and microscopy. In certain cases, a section from the complimentary surface to the indented surface was used for histology or for FTIRMI. Upon completion of data collection, data analysis proceeded with structural characterization.

Histology, light and polarized light microscopy

Histology sections were deparaffined and stained with safranin-O and hematoxylin to reveal the layers of the cartilage by biochemical composition. The stained tissues were characterized using a light microscope (BX 51, Olympus America Inc., San Diego, CA) and analyzed using Image Pro Plus v6.0 (Media Cybernetics, Inc., Silver Spring, MD). The same section was then visualized under cross polarizers to reveal the three structural zones within the uncalcified cartilage. Organized collagen microstructure appeared birefringent. The mean thicknesses for the total uncalcified cartilage layer, the mid zone (here combined with the superficial zone), and the deep zone were measured with ImagePro. These thicknesses were used to assign nanoindentation measurements to determine the average zone-specific stiffnesses. These thicknesses were also used to construct masks used for averaging zone-specific IR absorbance due to the presence of proteoglycan and collagen.

Nanoindentation

A Hysitron Triboindenter (Hysitron, Inc. Minneapolis, MN) was used to measure the mechanical stiffness. The specimen was prepared following slightly modified protocols developed previously [2]. Briefly, the specimen half was oriented so that the fracture surface containing ROI faced the indenter tip. The specimen was immobilized in polymethyl methacrylate dental cement enclosed in a polymer well mounted on a metal stub. Tissue was immersed in phosphate buffered saline during testing. A conospherical diamond tip with radius

of curvature of 100 μm was employed for each indent. The indentation process was executed in displacement feedback control. The load function consisted of a loading and unloading rate of 400 nm/s, and a hold period of 30-60 s, which allowed creep to dissipate and the sample response to reach near equilibrium. The tip first detected the sample surface during initial approach at ~1-2 μN of preload. The tip was then lifted off from contact with the surface before load-displacement curves were recorded.

To evaluate the relaxed modulus, the Hertz contact model coupled with a stress relaxation selection criterion was used. The (pseudo) equilibrium load was defined as the load reached at the end of the hold period in the load vs. time plot. The relaxed modulus, $E_{H,}$ was evaluated as

$$E_H = \frac{3P_{eq}}{4\sqrt{R}h_{eq}^{\frac{3}{2}}} \qquad (1)$$

where P_{eq} is the (pseudo) equilibrium load, h_{eq} is the corresponding displacement, and R is the radius of curvature of the spherical indenter. The elastic modulus is related to E_H as

$$E = E_H(1-\nu^2) \qquad (2)$$

where ν is the Poisson's ratio of the specimen. The load vs. time relaxation data was fit to a power law function and the theoretical equilibrium load was determined from the curve fit. The relaxation parameter was defined as P_{eq} normalized to the theoretical equilibrium load. Only sufficiently relaxed data (relaxation parameter < 3) were included in the analysis.

Fourier Transform infrared microspectroscopic imaging

The FTIRMI data were collected using a Michelson-type interferometer (ThermoNicolet 870, Thermo Electron Corporation, Waltham, MA, USA) coupled to an ImageMax infrared microscope (Thermo Electron Corporation) and a 64 × 64-pixel mercury cadmium telluride focal plane array detector under N_2 purge. All data were collected at the Musculoskeletal and Quantitative Imaging Research Center, University of California (San Francisco, CA, USA). Each ROI (400 μm in length and through the thickness of the cartilage) was imaged with a pixel resolution of 6.25 μm at 16-cm^{-1} spectral resolution and 4 scans per pixel. One tissue section from each specimen was imaged. Each image contained information on the amounts and distribution of proteoglycan and collagen, and took approximately 10 min to collect. The spectroscopic images were background-corrected and baseline-subtracted. The amount of proteoglycan was measured as the mean integrated area of the sugar peak (1,150 - 950 cm^{-1}) in the ROI defined above for each image [3]. The amount of collagen was measured as the mean integrated area of the amide I peak (1,710 - 1,600 cm^{-1}) for each ROI [3]. Spatial masks constructed based on the mean zone thicknesses were then superimposed on the entire ROI to determine zone-specific collagen and proteoglycan contents. Spectral data processing was performed using the Isys software package version 2.1 R1247 (Spectral Dimensions, Inc., Olney, MD, USA).

RESULTS AND DISCUSSION

Mechanical properties

The median relaxed modulus for the mid zone was 291.2 kPa for the flexed group and 146.5 kPa for the control group. The median relaxed modulus for the deep zone was 1474.6 kPa for the flexed group and 908.4 kPa for the control group. The deep zone cartilage with aligned collagen microstructure was significantly stiffer than that of the mid zone cartilage with random collagen microstructure (Wilcoxon paired test, $p<0.05$ for the flexed group and for the control group). The deep zone data (both groups) also showed a larger variation in the stiffness measurement (Figure 1). In these indentations, the tip-specimen contact diameter was approximately 30 µm, and since the largest characteristic dimension (length) of the collagen fibers are only hundreds of nm, the fiber itself is unlikely to contribute directly to the measured mechanical property. However a prior study on rabbit cartilage structure has indicated the presence of collagen columns—collagen network structure surrounding the chondrocytes that are approximately 10 µm in diameter [4]. This higher level microsctructure was more likely to directly contribute to the mechanical property measured. The representative volume of interest probed consisted of the collagen column-reinforced proteoglycan gel composite matrix. This microstructural effect of collagen columns on stiffness was absent in the mid zone cartilage which is explained by its randomly organized collagen fiber network. Furthermore, the distribution of collagen columns likely explains the larger variation in the deep zone data than that of the mid zone.

The flexed group was found to be stiffer than the control group with a 99.3% and a 62.4% increase in the relaxed modulus for the mid and deep zones respectively; however, this effect of flexion on the relaxed modulus was not statistically significant, which is attributed mainly to large inter-specimen variations.

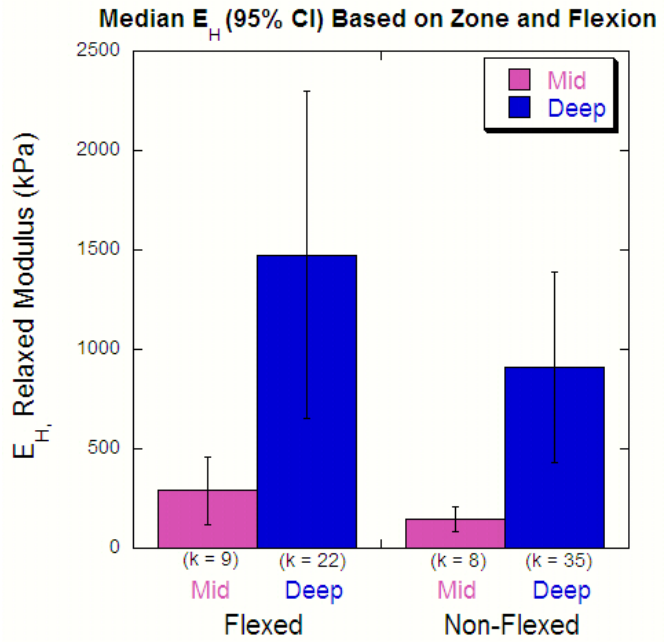

Figure 1. Medians of the relaxed modulus, E_H, measured for each group with 95% confidence intervals. K is the number of indents used to calculate the confidence intervals.

Biochemical and structural findings

Collagen is known to have slow metabolic turnover and is found to be negligible for the duration of time considered in this study [5]. The mean integrated proteoglycan peak values (normalized to collagen content) for each of the groups were plotted (Figure 2). The amount of proteoglycan content decreased with flexion by 92% in the mid zone and 59.7% in the deep zone. This finding was significant for both zones (Wilcoxon paired test, $p<0.05$). The joint contact pressure of these experiments was estimated as < 0.08 MPa. In a previous study, it was found that in the directly loaded joints (at a much higher contact pressure of 1 MPa), loading with the same frequency and duration lead to an increase in the cartilage proteoglycan content localized to the deep zone [5]. A recent study also demonstrated no change in the proteoglycan content in joints undergone direct loading at the same frequency and duration but at a lower average contact pressure of 0.1 MPa [6]. Hence the results from this study indicate that 80 cumulative hours of near-zero stress flexion (lack of direct loading) may be a damaging loading regimen and can lead to chondrocytes down regulating proteoglycan synthesis in the mid and deep zones of the cartilage.

There was no significant difference between the mid and deep zone proteoglycan contents, with comparable median proteoglycan content found for the mid and deep zones in both the flexed and non-flexed groups. This suggests that repetitive flexion leads to changes in proteoglycan content independent of zone location. This finding together with the previous report by Saadat et al. that change in the proteoglycan content was localized to the deep zone only, suggest that different loading regimens may also elicit responses in metabolic activities that differ in the specific structural zone of the cartilage. These zone-dependent biochemical changes in response to different loading regimens are likely manifested through the unique stress environment in each cartilage zone, which in turn stimulate the resident chondrocytes to alter their metabolic activities. Further investigations will aim to measure tissue properties controlled for the types of loading as well as loading magnitude, frequency and duration.

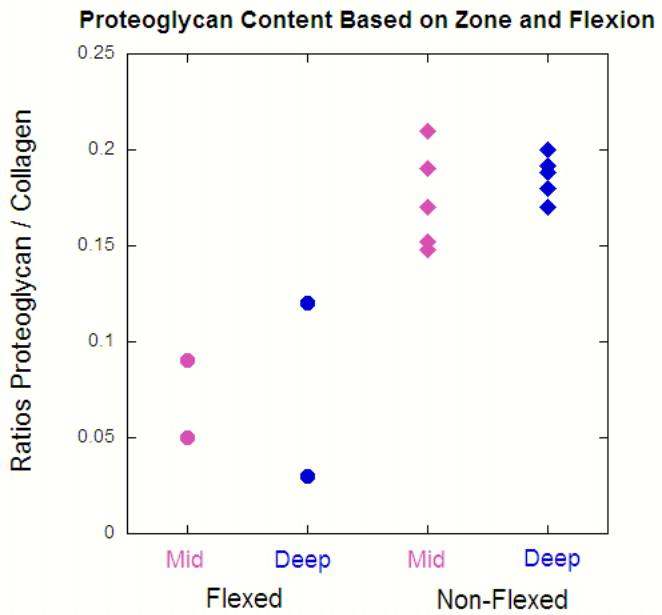

Figure 2. Mean proteoglycan content normalized to collagen content measured from FTIRMI based on mid and deep zone groups and flexion status.

There was no significant change in the mid and deep zone thicknesses as a result of flexion, which is consistent with a previous finding [5]. We noticed large intra- and inter-specimen variations in the property data. Some of these variations were attributed to the small sample size and having looked at data from specimen sections taken from different anatomical positions within the joint.

CONCLUSIONS

This is the first study to simultaneously report zone-specific mechanical and biochemical properties in the rabbit joint flexion model. We conclude that zone microstructure is the larger determining factor on local mechanical property as measured by nanoindentation, while flexion status is a more influential factor on the biochemical content of the tissue. We demonstrated that flexion significantly decreased the proteoglycan content in both the mid and deep zones, suggesting that 80 cumulative hours of indirect low-stress loading in the rabbit paw can be damaging to the joint via down regulating proteoglycan synthesis in the mid and deep zones.

The mechanisms by which cyclic loading ultimately leads to changes in cartilage mechanical properties is one that likely encompasses altered matrix microstructure. These microstructural changes can be manifested in the production/reduction of collagen and proteoglycan molecules and the rearrangement of these matrix constituents in order to fine-tune the local physical environment that the chondrocytes respond to. Nanoindentation enables explorations of such local structure-property relationships that are the key to understanding mechanisms of cartilage injury and repair, as well as developing promising rehabilitation and tissue engineering solutions.

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