Nanoscale Behavior of Collagen is Reflected in Whole-Disc Biomechanical Behavior and is Impaired by Diabetes

C. Acevedo[1,2], S. Sampson[1], E. Schaible[2], J.L. Graham[3], P.J. Havel[3], R.O. Ritchie[2], T.N. Alliston[1], J.C. Lotz[1], A.J. Fields[1]
[1]University of California, San Francisco, CA, [2]Lawrence Berkeley National Laboratory, Berkeley, CA, [3]University of California, Davis, CA

Disclosures: Acevedo (N); Sampson (N); Graham (N); Havel (N); Schaible (N); Ritchie (N); Alliston (N); Lotz (N); Fields (N)

Introduction: Knowledge of intervertebral disc biomechanical behavior can provide insight into disease mechanisms and inform tissue engineering strategies. However, the hierarchical structure of the disc is complex and spans multiple length scales, which makes it difficult to measure and relate the deformations of the constituents at the nanoscale to the biomechanical behavior of the whole disc. Therefore, a common strategy involves *ex situ* testing of the disc's sub-structures, fitting the stress-strain data to a constitutive model that predicts the nanoscale deformation mechanisms, and then extrapolating the predictions to an intact disc. The goal of this study was to demonstrate a new method for directly measuring the deformations of the nanoscale constituents of the disc *in situ*. In doing so, we sought to answer two questions: **1) How is the nanoscale behavior of collagen reflected in whole-disc biomechanical behavior?** and **2) How do alterations in collagen behavior caused by disease impair whole-disc biomechanical behavior?** To understand how alterations in collagen impair whole-disc behavior, we focused on type 2 diabetes (T2D), which is a global epidemic that affects several collagenous tissues.

Methods: *Animals*: Coccygeal motion segments (CC7-CC8) were harvested from 6-month-old lean Sprague Dawley rats and diabetic UCD-T2DM rats (n = 3 rats/group). The UCD-T2DM rats display adult-onset obesity, insulin resistance, and hyperglycemia [1]; this model of T2D compromises disc GAG and water contents, matrix homeostasis, and whole-disc creep properties [2]. *Small Angle X-ray Scattering (SAXS)*: Motion segments were speckle-coated and then compressed at a rate of 0.25% strain/sec during intermittent exposure to X-rays at beamline 7.3.3 at the Advanced Light Source (LBNL). The custom loading device permits investigation of collagen reorganization and stretching during *in situ* whole-disc compression (**Fig. 1**). During testing, the hydrated discs were exposed to 10 keV X-rays for 0.5 sec at ~3 sec intervals. Collagen diffraction data were recorded by a detector (Pilatus 1M), transformed to polar coordinates, and background-subtracted [3]. We analyzed the azimuthal angle of the first-order peak intensity versus the scattering vector q ($=2\pi/d$), where d is the d-periodic overlap, or "d-spacing" (**Fig. 1**). First, peaks were fitted with exponential Gaussian functions to locate the angles of orientation of the collagen fibrils belonging to the two groups of lamellae in the annulus. The degree of alignment of fibrils belonging to each group was characterized using Herman's orientation factor, *e.g.* a higher degree of alignment indicates more uniformly oriented fibrils. Next, scattering curves for the fibrils were made by integrating a 10°-wide sector centered on the peak position of the fibril scattering arc. This yielded two curves of intensity versus q, which were fitted with modified Gaussian functions and analyzed for peak location and full-width-at-half-maximum (FWHM). *Whole-Disc Behavior*: Load-displacement data collected during the SAXS tests were converted to tissue stress-strain curves. Stress was calculated from disc cross-sectional area using pre-test radiographs, and tissue strain was calculated from the speckle pattern. *Statistics*: SAXS and stress-strain data were compared between discs from lean and diabetic rats.

Results: Overall, discs from diabetic rats were significantly stiffer than discs from the lean rats. Typical results from each group are shown. Discs from lean rats exhibited the three characteristic toe-, heel-, and linear-shaped regions (stages I-III; **Fig. 2A**) seen in other collagenous tissues. In stage I (toe stage), the dominant mechanism of deformation at the nanoscale was fibril rotation and straightening. In fact, applied strains of almost 4% were accommodated by primarily by a 10° reorientation of collagen fibrils (**Fig. 2B**), and, to a lesser extent, by straightening of the collagen fibrils as reflected by their increasing degree of alignment (**Fig. 2C**). Notably, there was almost no collagen stretching during stage I, as indicated by the constant d-spacing (**Fig. 2D**). The decrease in the width of the SAXS peak (FWHM) also suggests less variability in the fibril d-spacing, and hence, more uniform collagen stretching. In stage II (heel stage), the main deformation mechanism at the nanoscale transitioned from fibril rotation to fibril straightening and stretching. The amount of fibril rotation began to plateau, while fibril straightening persisted. There was also a sharp decrease in the width of the SAXS peak as fibrils began to engage and resist the applied load. Yet, fibril stretching was small compared to the applied strain, just ~0.5 nm. In stage III (linear stage), fibril alignment and stretching predominated. Collagen stretching continued to occur; however, the small elastic fibril strain could not accommodate the applied strain, and the sudden broadening of the SAXS peak suggests that inter- and intra-fibrillar sliding and delamination occurred.

In contrast to the three stages of disc compression observed in discs from lean rats, discs from diabetic rats only exhibited the heel- and linear-shaped regions (stages I-II; **Fig. 3A**). During stage I (heel stage), we observed all three mechanisms of deformation seen with healthy discs. At the nanoscale, fibrils rotated into alignment with the tensile axis (**Fig. 3B**), straightened (**Fig. 3C**), and stretched (**Fig. 3D**). However, fibril rotation played a much smaller role. Specifically, angular changes were just 2° (**Fig. 3B**), compared with 10°–15° in the healthy discs. We also observed fibril stretching in this early stage and an increase in the width of the SAXS peak (**Fig. 3D**). This suggests that fibrils were engaging and resisting the applied load, but not enough to accommodate the applied strain, so they began to delaminate. In stage II (linear stage), fibrils continued to reorient slightly, fibril alignment reached a maximum, and fibril delamination and failure increased, which suggests that fibril rotation, straightening, and stretching were unable to take up the applied strain.

Discussion: Here we show for the first time how the nanoscale behavior of collagen and the unique structural arrangement that causes the fibrils to work in tension confers resistance to disc compression, and we demonstrate how these mechanisms are compromised by T2D, an increasingly common metabolic disease. Resistance to disc compression involved the following mechanisms: in healthy discs, collagen fibril rotation (stages I and II), straightening (all stages), and sliding (stage III); in diabetic discs, fibril straightening (stage I) and sliding (all stages). Fibril rotation, which coincided with the toe stage in healthy discs, was absent in the discs from diabetic rats. The annulus of the discs from diabetic rats had 29% higher concentrations of pentosidine [2], an AGE that is known to cross-link intradiscal collagen [4], and could therefore hinder collagen fibril rotation and sliding. Together these findings show that fibril rotation is important for resisting disc compression, and they suggest that AGE accumulation in T2D may stiffen the disc by preventing fibril rotation.

Significance: Here we quantified for the first time the deformation mechanisms of collagen fibrils during whole-disc compression. Our results demonstrate the important roles of fibril rotation, straightening and sliding, and they show how these mechanisms are impaired by an increasingly common disease.

References: 1. Cummings+, *Am J Physiol Regul Integr Comp Physiol* 2008; 2. Fields+, *J Orthop Res* 2015; 3. Yang+, *Nat Commun* 2015; 4. Sivan+, *Biochem J* 2006.

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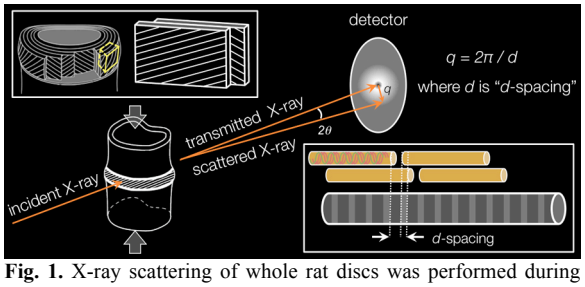

Fig. 1. X-ray scattering of whole rat discs was performed during compression. The lamellae of the annulus fibrosus have alternating collagen fibril orientations of ~30° and ~150° (upper left), giving rise to two distinct scattering peaks. The scattering vector is related to the d-periodic overlap, or "d-spacing", between adjacent collagen molecules (yellow) that form fibrils (grey; lower right).

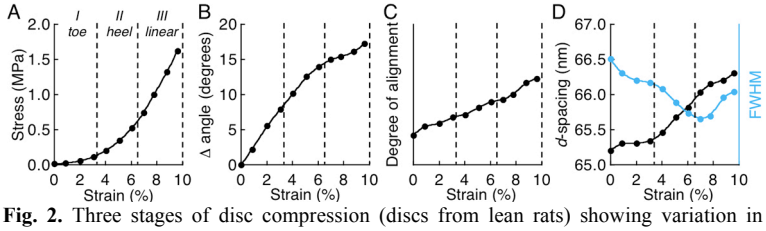

Fig. 2. Three stages of disc compression (discs from lean rats) showing variation in collagen fibril orientation, alignment, d-spacing and full-width-at-half-maximum.

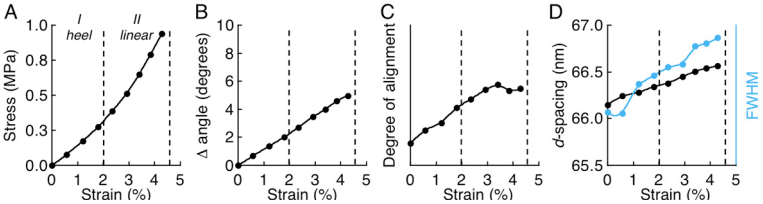

Fig. 3. Two stages of disc compression (discs from diabetic rats) showing variation in collagen fibril orientation, alignment, d-spacing and full-width-at-half-maximum.