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NON-ENZYMATIC GLYCATION STRENGTHENS ANNULUS FIBROSUS THROUGH CROSSLINKS ALIGNED WITH PRIMARY COLLAGEN FIBERS

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INTRODUCTION: The annulus fibrosus (AF) in the intervertebral disc is a fiber-reinforced tissue consisting of collagen fibers embedded in a proteoglycan-rich extracellular matrix, resulting in great load-bearing and energy absorption capacities. However, due to the disc's avascularity, the AF is susceptible to catabolic remodeling with disease and degeneration, causing debilitating back pain and disability.[1-2] Diabetes mellitus, a prevalent metabolic disorder that has been shown to cause or aggravate irreversible disc tissue damage, is a significant risk factor low back pain.[3-6] A well-documented diabetes-induced AF structural change is the accumulation of advanced glycation end-products (AGEs), which can form irreversible covalent crosslinks with AF collagens due to their slow biological turnover and long half-life.[7-8] Understanding AF structure-function relationships with diabetes-induced non-enzymatic glycation (i.e., AGEs) can provide insight into tissue failure mechanisms, which is pivotal for developing injury prevention measures and effective therapeutic interventions.

Previous studies reported an increased AF stiffness and toughness with non-enzymatic glycation.[9-10] Our recent work examined AF uniaxial tensile mechanics at physiologically relevant AGEs levels and observed significantly increased tensile modulus and failure stress in circumferential-axial (circ-ax) samples (**Fig. 1A**).[11] Thus, how diabetes increases or accelerates AF tissue mechanical failure remains unclear.

Crosslinking schematics with AGEs are still unclear due to experimental limitations.[12] Finite element models (FEMs) are a valuable tool for predicting stress-strain distributions in complex tissue structures. We recently developed and validated a robust multiscale structure-based FEM framework for the disc. Tissue-level FEMs were able to accurately predict multiscale AF mechanics under different loading configurations (e.g., uniaxial and biaxial tension, simple shear) and specimen geometries.[13-14] Thus, the objectives of this study were to: (1) examine the effect of AGEs on AF uniaxial tensile mechanics in loading directions with less fiber engagement, and (2) to expand our FEM framework to describe AF mechanics with non-enzymatic glycation.

METHODS: *Experimental:* Bovine caudal discs were dissected from mature coccygeal spines. Circumferential-radial (circ-rad) and radial samples were prepared using a freezing stage microtome to obtain 2 mm-thick rectangular specimens. The lengths of circ-rad and radial specimens were ~10 and ~5 mm; specimen widths were ~5 mm for both orientations. For repeatable midlength failure, a 1-mm full-width notch was created using a custom-made cutting jig in the thickness direction for circ-rad specimens.[15] Radial specimens exhibited limited grip failure during preliminary testing and were thus unnotched (**Fig. 1A**).

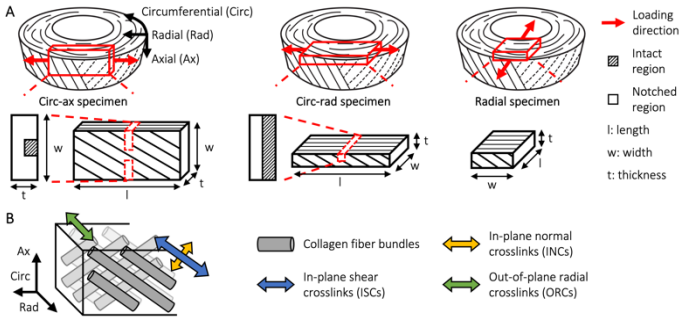

Fig. 1: (A) Specimen orientation, loading direction, and notch geometry. **(B)** Model schematics demonstrating possible crosslinking mechanisms.

AF specimens were prepared at three physiologically relevant glycation levels (18 hr soak). Control samples were soaked at 25 °C in a SPEG5 solution to minimize excessive swelling (CTRL, $n_{circ\text{-}rad} = 15$, $n_{radial} = 7$).[16] Glycated samples were soaked in 0.3 M methylglyoxal pH-balanced to 7.4 at 25 °C (GLY25, $n_{circ\text{-}rad} = 15$) or 50 °C (GLY50, $n_{circ\text{-}rad} = 15$, $n_{radial} = 7$). Samples were gripped with custom-made clamps and tested in SPEG5 to maintain hydration. Quasi-static monotonic uniaxial tension was applied at 0.1 mm/min until failure. Linear-region modulus, failure stress, and failure strain were evaluated for specimens that failed at midlength post-testing. AGE content was also measured.

Computational: The multiscale structure-based FEM framework previously developed and validated for the AF was adapted to describe crosslinks within the extrafibrillar matrix with model geometries determined using experimental samples.[11] Non-enzymatic crosslinks, whose orientations are still unknown, were described in three possible directions, including crosslinks parallel to collagen fibers (in-plane shear crosslinks, ISCs), crosslinks perpendicular to collagen fibers (in-plane normal crosslinks, INCs), and out-of-plane radial crosslinks (ORCs, **Fig. 1B**). All crosslink components were modeled as directional homogeneous interfibrillar reinforcements with identical mechanical properties. A higher AGE content was assumed to generate denser AGEs compounds per unit volume, leading to a larger overall crosslink stiffness. Crosslink modulus was calibrated by comparing model-predicted bulk AF tensile modulus to experimental data; other model parameters were directly linked to reported AF physical or biochemical properties.[13] Simulated boundary and loading conditions replicated those of experiments. Multivariate linear regression was conducted based on a parametric group of FEMs with varying crosslink modulus to estimate the relative contribution of crosslink modulus to the increase in bulk AF tensile modulus with non-enzymatic glycation.

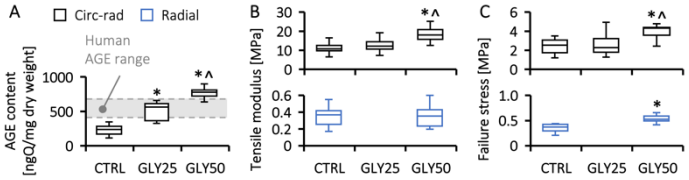

Fig. 3: (A) AGE content, **(B)** modulus, and **(C)** failure stress at each glycation level. *: $p < 0.05$ vs CTRL; ^: $p < 0.05$ vs GLY25.

RESULTS: *Experimental:* 51/59 specimens experienced midlength failure and exhibited a nonlinear stress-strain response. AGEs content normalized by dry weight increased by 95% in GLY25 and 206% in GLY50 samples ($p < 0.001$ vs CTRL; **Fig. 3A**). For circ-rad specimens, non-enzymatic glycation increased linear-region modulus and failure stress by 62% (18.13 vs 11.22 MPa; $p < 0.001$) and 60% (4.09 vs 2.55 MPa; $p < 0.01$) in GLY50 samples. For radial specimens, AGEs did not increase tissue modulus but increased failure stress by 42% (0.54 vs 0.38 MPa; $p = 0.04$; **Fig. 3B-C**). Failure strain was not affected by the non-enzymatic glycation treatment in both directions.

Computational: Preliminary FEMs predicted that ORCs could result in a 10 to 100× increase in AF radial tensile modulus. However, experimental work showed that AF radial modulus did not increase with glycation (**Fig. 3B**). Thus, ORCs were excluded from the model.

Model predictions agreed well with experiments when the crosslink modulus was 12.5% and 25% of collagen fibers in GLY25 and GLY50 specimens, respectively (7.5 and 15 vs 60 MPa). For circ-ax specimens, model-predicted linear-region modulus were 25.2, 38.4, and 48.7 MPa for CTRL, GLY25, and GLY50 samples, respectively (< 0.35× standard deviation (std) from experimental means; **Fig. 4A**).[11] Model-predicted linear-region modulus were 8.7, 13.0, and 17.3 MPa for CTRL, GLY25, and GLY50 circ-rad samples, respectively. Model predictions were within 0.8× std from experimental means except for Werbner et al. (8.7 vs 11.3 ± 1.8 MPa; **Fig. 4A**).[17] Model-predicted tensile modulus was 0.38 MPa for both CTRL and GLY50 radial specimens (< 0.23× std from the experimental means; **Fig. 4A**).

Models with only ISCs or INCs greatly underestimated AF linear-region modulus for circ-ax and circ-rad GLY50 specimens (**Fig. 4B**). Thus, ISCs and INCs were both required to accurately describe the effect of non-enzymatic glycation. Multivariate linear regression suggested that the increase in AF circumferential tensile modulus with glycation was more sensitive to ISC modulus than INC modulus. Particularly, ISC and INC modulus contributed ~90% and ~10% to the increase in tensile modulus for both circ-ax and circ-rad specimens.

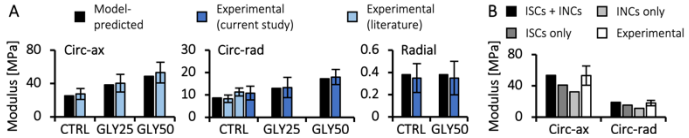

Fig. 4: Model-predicted **(A)** tensile modulus in three specimen orientations and **(B)** GLY50 sample tensile modulus with different crosslinking schematics vs experimental data (mean ± std).[11, 15, 17]

DISCUSSION: Non-enzymatic glycation modifies AF collagen structure via crosslinking. The current study found that glycation increased AF tensile modulus and failure stress without affecting failure strain. These findings agreed with previous studies for circ-ax samples,[9-11] indicating that AGEs accumulation is unlikely to cause premature tissue failure by adversely affecting AF quasi-static monotonic uniaxial tensile mechanical properties. However, tendon studies have reported a significantly reduced tissue viscoelasticity (i.e., energy dissipation capabilities) with AGEs,[18-19] suggesting that non-enzymatic glycation with diabetes could cause premature disc failure by compromising tissue performance under dynamic, especially fatigue loading.

Observations from the current study indicated that non-enzymatic crosslinks had a larger effect along the primary collagen fiber direction. Particularly, glycation did not stiffen the AF in the radial direction. Model predictions also showed that including out-of-plane radial crosslinks led to unrealistically high radial tensile modulus, suggesting that interlamellar crosslinks, most likely through elastic fibers,[20] were not probable. Furthermore, glycation had a greater effect in directions with more fiber engagement. With GLY50 treatment, AF tensile modulus increased by ~100% in circ-ax specimens but by only ~60% in circ-rad samples ($p < 0.001$ for both orientations).[11] Multivariate linear regression suggested that the increase in bulk AF tensile modulus was more sensitive to the mechanics of in-plane shear crosslinks aligned with collagen fibers. These findings agreed with previous tendon studies, where AGEs treatment increased tendon uniaxial tensile modulus along the fiber direction by ~100-400%.[12, 21] Our findings also agreed with a previous quantitative imaging study, which showed that collagens were more responsive to AGEs formation than interlamellar elastic fibers, which are mainly oriented in the radial direction.[20, 22]

FEMs in the current study accurately and robustly predicted AF uniaxial tensile mechanics with AGEs. Since collagen mechanics were not altered in the glycated FEMs, model predictions indicated that glycation could strengthen bulk AF without stiffening individual collagen fibers or fibrils, which remains a debate in the field.[12]

Determining the effect of non-enzymatic glycation on AF mechanics plays a pivotal role in understanding diabetes as a risk factor for tissue injury, which is essential for developing preventive measures and therapeutic interventions for low back pain. Ading to previous literature, the current study showed that AGEs did not compromise AF monotonic quasi-static uniaxial tensile mechanical properties. The proposed FEM framework also provided a powerful tool for examining multiscale AF mechanics with disease and degeneration. Future work aims to examine the effect of glycation on AF damage accumulation.

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