Glycosaminoglycans accumulate during proliferation but minimally affect tensile mechanics during mouse Achilles tendon healing

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DISCLOSURES: None.

INTRODUCTION: Injuries are common in repetitive and high load-bearing tendons such as the Achilles tendon.[1] Glycosaminoglycans (GAGs) are long, negatively charged polysaccharides found in many musculoskeletal tissues, including tendon. Following injury, tendons become swollen, hypercellular, less stiff, and GAG-enriched.[2] Our work has shown that regions of high GAG content in young Achilles tendons demonstrate increased elasticity following GAG-depletion.[3] However, whether GAGs serve a similar role in injured tendon elasticity remains unknown. Therefore, the objective of this study was to investigate the compositional and mechanical role of GAGs in injured mouse Achilles tendons. We hypothesized that GAG content would increase following injury and then decrease with recovery, and that GAG-depleted tendons would exhibit an increase in elastic modulus in the injury region.

METHODS: *Injury model:* Eighty (n=40 male, n=40 female) C57BL/6 mice were assigned randomly to uninjured and injured groups. Injury mice received bilateral excisional injuries (0.3 mm biopsy) to the Achilles tendon midsubstance at 150 days of age (IACUC approved).[4] Mice were euthanized 1, 3, and 6 weeks following the procedure. *Treatment:* Achilles tendons were equilibrated in a tris buffer (pH=8) for 1 hour and then treated using buffer supplemented with protease inhibitors (control) or a solution of protease inhibitor buffer and 0.5 U/mL chondroitinase ABC (cABC, treatment to digest sulfated GAGs) for 18 hours under gentle agitation at 37°C. Assay order was randomized and investigators were blinded to all injury timepoints and treatment groups. *Histology (n=3-6/group):* Left limbs were fixed in 10% neutral-buffered formalin for 1 day, incubated in 0.5M sodium EDTA for 7 days, soaked overnight in 30% sucrose, embedded in OCT, and sectioned transversely at 10 µm. Tape-stabilized sections were stained using 1% alcian blue (GAG quantity, nuclear fast red counterstain) or 0.025% toluidine blue (positive GAG staining). Brightfield images were taken at 20X using a slide scanner. *Biochemistry (n=3-6/group):* Left limb tendons were digested in a 5 mg/ml proteinase K-ammonium acetate solution overnight at 55°C prior to quantifying GAG content using the 1,9-dimethylmethylene blue assay (pH=1.5, CS as standard).[5] The remaining digest was hydrolyzed and collagen content (hydroxyproline) was measured spectrophotometrically.[6] *Biomechanics (n=7-10/group):* Right limb tendons were stamped into a dog-bone shape, the cross-sectional area (CSA) measured using a custom laser device, and Verhoeff stain lines applied for optical strain analysis. The calcaneus was secured in rubber and clamped in a grip, and the proximal tendon was held between sandpaper grips in a 37°C 1X PBS bath. The testing protocol consisted of 10 cycles of preconditioning, holds at 2% and 4% axial strain (toe region and linear region) for 10-minute followed by frequency sweeps at 0.1, 1, 5, and 10 Hz, a 5-minute unloaded rest period, and a ramp-to-failure at 0.1%/s. Elastic (stiffness, modulus[7]) and viscoelastic (dynamic modulus and phase shift (tan δ)) properties were measured. Fiber alignment (angle of polarization, AoP) in the injury was measured using reflectance polarized light imaging. *Statistics:* All data were assessed for statistical outliers (2.2*IQR). Comparisons across groups were conducted using two-way ANOVAs followed by Bonferroni post-hoc tests. Significance was set at α=0.05.

RESULTS: GAGs increased following injury and localized in regions of scar formation during healing ($p<0.0001$, Fig. 1A,C). cABC treatment depleted nearly all GAGs in tendons uniformly at every timepoint ($p<0.0001$, Fig. 1B,D,E). GAGs increased 3 weeks after injury ($p<0.0001$, Fig. 1E), where there was also an increase in water content by 6% ($p=0.0065$, data not shown) and no change in collagen content (Fig. 1F). Achilles tendon CSA increased with injury ($p<0.0001$, Fig. 1G) and was lower in the cABC treatment group ($p=0.016$). While stiffness, modulus, and dynamic modulus decreased following injury ($p<0.0001$, $p=0.0045$, and $p<0.0001$), all were unaffected by cABC treatment. However, phase shift (1 Hz, 4% strain) increased with cABC treatment 3 weeks following injury ($p=0.0076$, Fig. 1K), along with increased fiber disorganization relative to control ($p = 0.0317$, Fig. 1L).

DISCUSSION: GAG content increased 3 weeks following Achilles tendon injury during the proliferative phase of healing and decreased 6 weeks following injury during tissue remodeling (Fig. 1E). Interestingly, this finding corresponds with higher expression of decorin (and thus dermatan or chondroitin sulfate) at 3 weeks following excisional injury.[8] At 3 weeks, we observed an increase in water content that was unaffected by GAG digestion. Yet, GAG removal decreased CSA, suggesting that GAGs may increase ECM area independent of water content. Contrary to our hypothesis, removing nearly all GAGs from the tissue had no effect on midsubstance elastic tensile mechanics during injury, as observed in healthy tissues.[9] However, cABC treatment did affect viscoelasticity at 3 weeks following injury, where GAG content was the highest (7.52 µg/mg, 3.5x control) and tended to localize in regions of scar tissue formation (Fig. 1A,B). While the suspected mechanism for GAGs in tendon tensile mechanics is primarily at the microscale,[10,11] these data may indicate an additional yet minimal role for GAGs in modulating scar tissue viscoelasticity in injured tendon during the proliferative phase of healing.

SIGNIFICANCE: Tendons are among the most injured musculoskeletal tissues, and treatments to promote healing are lacking. Understanding how glycosaminoglycans accumulate during healing and modulate tensile tissue mechanics may help advance future therapies.

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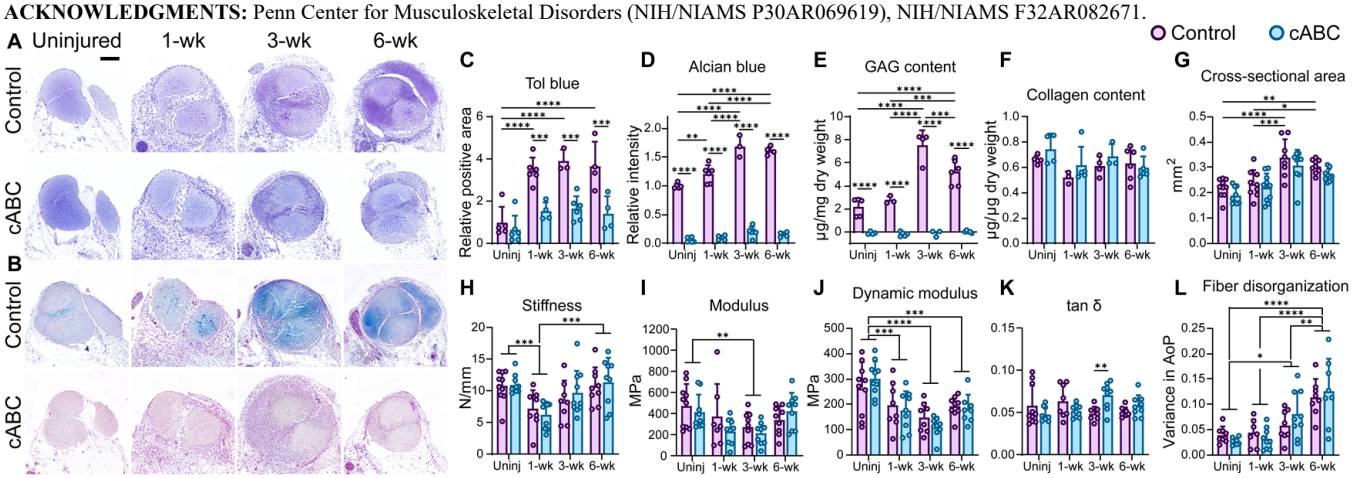

FIGURE 1: Transverse histological sections show progressive scar formation with high GAG localization (A,C) and quantity (B,D) with healing, and that cABC treatment depleted GAGs uniformly from the tissue (scale bar = 200 µm). GAG and water content peaked at the 3-week timepoint (E) without changes to overall collagen content of the tendon ECM (F). Following injury, Achilles tendons had a higher CSA (G) and inferior mechanics (H-J). Phase shift 3 weeks following injury increased following GAG depletion (K) (1 Hz, 4% strain shown). Finally, fibers in the injury region were more disorganized with healing, but this was unaffected by GAG depletion (L) (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).