Decreasing Cyclic Load, as Collagen Organization Increases, Does Not Improve Maturation in Engineered Ligaments

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INTRODUCTION: Hierarchical collagen fibers are the primary source of strength in tendons and ligaments. Injuries to these tissues disrupt the collagen organization resulting in loss of function, pain, and decreased mobility.[1] These collagen fibers do not regenerate after injury nor with repair, resulting in limited repair options.[1,2] Engineered replacements are promising; however it remains a challenge to form the large hierarchically organized collagen fibers essential to long-term mechanical success. Mechanical cues, including cyclic muscle activity, are critical for tissue development *in vivo* and have been shown to improve maturation of engineered tissues;[2,3,4] however the effect on hierarchical fiber formation is largely unknown. Previously, we developed a novel culture system which guides Anterior Cruciate Ligament (ACL) fibroblasts in high density collagen gels to form native-size hierarchical fibers,[1] and we have demonstrated that intermittent cyclic stretch further improves their maturation.[5] These constructs are promising ACL replacements, however further maturation is needed to be clinically relevant. Interestingly, cyclic loading of these constructs affected cells differentially depending on the degree of organization, with 10% cyclic strain driving early improvements in mechanics and composition when cells were in unorganized gels, and 5% load being more beneficial later in culture once cells were anchored on aligned collagen fibers, suggesting a shift in mechanotransduction.[5] The objective of this study was to explore whether an adaptive cyclic load that changes in intensity from 10 to 5% strain, as collagen fibers develop, further improves maturation. We hypothesize that progressively decreasing cyclic strain as organization increases will better dive cells to produce more mature hierarchical fibers, resulting in significantly stronger replacements.

METHODS: To form constructs, rat tail type I collagen and neonatal bovine ACL fibroblasts were mixed and cast into 1.5 mm thick sheet gels at 20 mg/mL collagen and $5x10^6$ cells/mL as previously described.[1] Rectangles (8 x 30 mm) were cut from gels, divided between groups, and cultured for up to 6 weeks. All constructs were clamped into a modified CellScale tensile bioreactor (Fig. 1A). Constructs were loaded with an established loading regime[4] of 1 Hz for 1 hr, twice daily, every other day to mimic rapid muscle movement (Fig 1B), with control constructs being loaded at 0, 5, or 10% strain throughout culture and adaptive load constructs loaded with a decreasing percent strain as constructs matured (Fig 1C). Specifically, adaptive constructs were loaded with 10% strain from weeks 0-2, 7% strain weeks 2-4, and 5% strain weeks 4-6 (Fig. 1C). Time points were taken at 0, 2, 4 and 6 weeks, with 0 week constructs collected 24 hours after one loading cycle. Post culture, confocal reflectance was performed to analyze collagen organization. DNA, Glycosaminoglycans (GAGs), collagen content, and LOX activity were measured via Picogreen, DMMB, hydroxyproline (Hypro), and LOX activity assays. Mechanics were analyzed by tensile tests at 0.75% strain/sec to failure. All data are mean ± SD. Significance determined by 1- and 2-way ANOVA with Tukey's post-hoc (p<0.05).

RESULTS: All constructs contracted similarly over 6 weeks of culture (Fig. 1D) and formed aligned collagen fibrils by 2 weeks (data not shown), which matured into larger fibers by 4 and 6 weeks (Fig. 2). Steady cyclic loading at 5 and 10% drove collagen crimp formation by 6 weeks. Interestingly, adaptive load constructs appeared to have less crimp and reduced fiber organization compared to steady loaded constructs. Early in culture at 0 and 2 weeks, when cells were in unorganized gels, 10% and adaptive load produced increased collagen, GAG, and LOX activity (Fig 3A); however later in culture, once cells were on aligned fibers, steady 5% load significantly increased collagen content compared to all other groups. Interestingly, adaptive load did not have the same increase in collagen content as 5% load at 6 weeks, despite being loaded at 5% strain between 4-6 weeks (Fig 3A, hypro/WW). Instead, adaptive load resulted in a significant increase in GAG by 6 weeks compared to steady 5 and 10% load constructs. Further, adaptive load did not improve LOX activity by 6 weeks, despite steady 5 and 10% load having increased LOX activity at 6 weeks. Collectively, this suggests the adaptive decreasing strain may cause a pathological injurious response.[6] All groups had significantly improved tensile properties by 6 weeks compared to 0 weeks (Fig 3B); however, adaptive load was not better than steady load. 5% strain had the highest elastic modulus and ultimate tensile strain (UTS) compared to all other groups, mirroring collagen concentration, and both steady 5 and 10% load groups had significantly improved toe moduli compared to other groups, mirroring increased crimp formation.

DISCUSSION: Overall, decreasing cyclic load, which changed as cells shift from being in unorganized collagen to anchored on aligned fibers, does not improve hierarchical collagen organization, collagen crimp, or tissue mechanics. Although we previously found that 10% load drove early improvements in composition and mechanics, and 5% load drove later improvements, the adaptive load, with 10% strain early in culture and 5% strain later, did not lead to synergistic improvements in tissue properties. This may be due to cells sensing a decrease in strain and losing tensional homeostasis. Previously, it has been shown that cells respond to decreases in tensile load with initial catabolism of the extracellular matrix, and only shift to a more anabolic state once cells contract their environment enough to sense the new, reduced load.[6] This may indicate that decreasing cyclic load throughout culture may lead to repeated breakdown and remodeling of collagen, which leads to a weaker, less organized construct than steady load, which maintains cellular tensile homeostasis throughout culture.[6,7] Ongoing work is assessing an increasing adaptive load, as well as assessing maturation at the fibril and fascicle length scale.

SIGNIFICANCE: This study provides new insight into how adaptive intermittent cyclic loading affects cell-driven hierarchical fiber. A better understanding of how mechanical cues regulate fiber formation will help to better engineer replacements and develop rehabilitation protocols to drive repair after injury.

REFERENCES:1.Puetzer+*Biomat* 2021; 2.Galloway+*J Bone Surg Am* 2013; 3.Connizzo+*Matrix Bio* 2013; 4. Puetzer+*Tis Eng* 2016; 5.Troop+*ORS Trans* 2023; 6.Lavagnino+JOR 2006; 7.Henshaw+JOR 2005

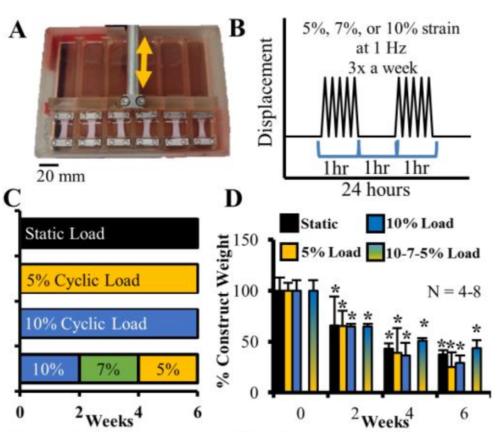

Fig. 1: A) Culture chamber and B) loading regime. C) Experimental setup, D) % original weight of constructs. Significance to *0wk

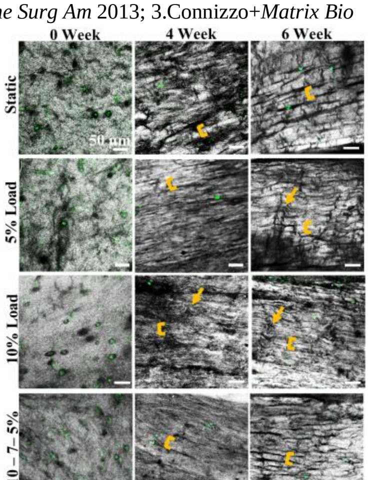

Fig. 2: Confocal of collagen organization, brackets denote fibers, arrows note crimping. grey=collagen, green=auto-fluorescent cells

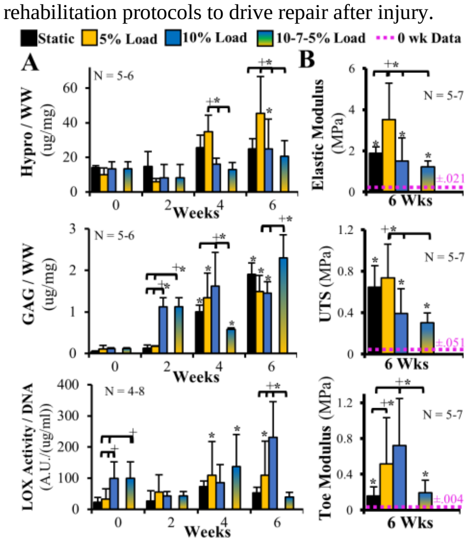

Fig. 3: A) Collagen (hypro) & GAG normalized to wet weight (WW), and LOX activity in constructs. B) Tensile mechanical properties. Significance to *0 wk and +bracket