PREGNANCY AND LACTATION IMPAIR SUBCHONDRAL BONE LEADING TO REDUCED RAT SUPRASPINATUS TENDON FAILURE PROPERTIES

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INTRODUCTION
During pregnancy and lactation, women experience hormonal and physiological changes that increase the risk of musculoskeletal joint disorders such as shoulder, lower back, wrist, and knee pain [1]. Estrogen levels rise dramatically during pregnancy followed by a rapid decline during lactation before returning to normal levels after weaning. Increased joint laxity from hormonal fluctuations [2] and substantial maternal bone loss due to increased calcium demands for fetal and infant growth [3] contribute to impaired joint function. We previously showed that rats with a history of reproduction exhibited inferior supraspinatus tendon and humeral trabecular bone properties despite a lengthy post-weaning recovery period [4]. However, the transient effects of pregnancy and lactation on the supraspinatus tendon and proximal humerus remain unknown. Therefore, the objective of this study was to evaluate changes in supraspinatus tendon mechanical properties and in the microstructure of the bony insertion site during pregnancy, lactation, and post-weaning recovery in female rats. We hypothesized that substantial bone loss during lactation would compromise the subchondral bone microstructure leading to reduced supraspinatus tendon mechanical properties.

METHODS
30 Sprague-Dawley female rats (IACUC approved) were used: virgin (n=7), end of pregnancy (n=9), 2-week lactation (n=7), and 2-weeks post-weaning (n=7). Rats in the pregnancy group were sacrificed at parturition, while the lactation group underwent pregnancy and 2 weeks of lactation. Rats in the weaning group underwent pregnancy, 2-weeks lactation, and 2-weeks post-weaning recovery. All rats were sacrificed at 7 months of age, and shoulders were harvested for supraspinatus tendon mechanical testing and subchondral and trabecular bone analysis. Mechanics: Supraspinatus tendons were marked with stain lines, cross-sectional area was measured using a custom laser device, and humeri were secured in PMMA. Right supraspinatus tendons underwent viscoelastic tensile testing, consisting of pre-conditioning, stress relaxation at a 5% strain hold for 600s, a dynamic frequency sweep at 5% strain (0.1-10Hz), and a quasi-static ramp to failure at 0.3%/s. Left supraspinatus tendons underwent fatigue cyclic loading until failure at 2Hz between loads corresponding to 7% and 40% maximum stress, determined from quasi-static results. Peak cyclic strain, secant modulus, tangent modulus, hysteresis, and laxity, were recorded at two breakpoints (ends of the primary (BP1) and secondary (BP2) phases of a triphasic fatigue life curve). Subchondral and trabecular bone analysis: Prior to mechanical testing, left proximal humeri were μCT scanned (6μm, μCT35, Scanco). Trabecular bone proximal to the humeral growth plate was analyzed. Additionally, average thickness and the mineralization gradient was calculated (Amira 6.7) across the subchondral plate, defined as the mineralized fibrocartilage of the supraspinatus tendon enthesis and subchondral bone. Briefly, a 100x120x230 voxel volume was identified in the greater tuberosity at the supraspinatus tendon insertion site. After global thresholding, the innermost layer of the subchondral bone was defined to exclude trabecular bone. Individual layers were subsequently defined outwards towards the mineralized fibrocartilage boundary. Layer intensity values were averaged to construct a mineralization gradient, normalized to the total subchondral plate thickness. Bone mineral density (BMD) was compared at 0, 0.5, and 1.0, marking the boundaries between trabecular bone, subchondral bone, mineralized fibrocartilage, and tendon. Statistics: Comparisons across groups were made using one-way ANOVAs with Bonferroni post-hoc corrections. Significance was set at p≤0.05 and trends at p≤0.1. All data is presented as mean±SD.
RESULTS

Supraspinatus Tendon Properties. Cross-sectional area was significantly increased (Fig 1A) and midsubstance modulus trended higher (Fig 1B) during pregnancy compared to virgin. After weaning, however, modulus was significantly lower compared to both pregnancy and lactation groups and trended lower compared to virgin (Fig 1B). Percent relaxation was also significantly greater post-weaning compared to pregnancy and lactation (Fig 1C). For fatigue, tangent stiffness at BP1 for both lactation and weaned trended lower compared to virgin and was significantly lower compared to pregnancy (Fig 1D). However, there were no differences in cycles to failure, peak cyclic strain, hysteresis, or laxity (data not shown).

![Figure 1: Cross-sectional area and midsubstance modulus are significantly greater after pregnancy. After weaning, modulus is reduced while percent relaxation is increased compared to pregnancy and lactation. In contrast, tangent stiffness at BP1 also decreased during lactation and after weaning. Solid lines denote significance for p<0.05 and dashed lines for trends p<0.1.](image)

Trabecular and Subchondral Bone Properties. Bone volume fraction (BV/TV) (Fig 2A) and trabecular number (Tb.N) (Fig 2B) was significantly lower while trabecular separation (Tb.Sp) (Fig 2C) was significantly greater during lactation compared to virgin and pregnancy groups. After weaning, BV/TV recovered with no significant difference from virgin. However, Tb.N and Tb.Sp trended lower and higher, respectively, post-weaning compared to virgin.

![Figure 2: Lactation resulted in (A) decreased bone volume fraction, (B) increased trabecular number, and (C) increased trabecular separation compared to all other groups. Solid lines denote significance for p<0.05 and dashed lines for trends p<0.1.](image)

DISCUSSION

Pregnancy and lactation induced substantial changes in supraspinatus tendon and proximal humerus bone properties. Lactation impaired subchondral and trabecular bone, but properties recovered post-weaning. In contrast, supraspinatus tendon mechanical properties improved during pregnancy and these changes persisted during lactation before returning to pre-pregnancy levels post-weaning. Estrogen has been linked to increased collagen synthesis and tissue elasticity [5], providing a mechanism by which tendon properties increase by the end of pregnancy, while the time at which estrogen levels peak. In contrast to bone tissue, the rapid decline of estrogen levels during lactation had no adverse effects on tendon properties, suggesting additional protective mechanisms associated with lactation.

Furthermore, bony avulsion failures and decreased maximum stress during lactation support further study into the subchondral plate microstructure. Reduced overall thickness and bone mineral density during lactation suggest that independent mechanisms modulated by osteoblast-osteoclast coupling regulation and osteocyte perilacunar-canalicular remodeling, respectively, may be at play.

Supraspinatus tendon tears most commonly occur at the insertion site, and reduced bone mineral density of the humeral head is a known risk factor [6]. Our results provide insight into the mechanisms that may govern this increased risk due to compromised bone, which may arise with menopause, aging, and other metabolic diseases. To further elucidate the onset of rotator cuff disease and hormonal regulations on tendon health, future studies will investigate the biological mechanisms underlying transient changes in tendon and bone properties during reproduction and lactation.

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DECORIN, ALONG AND IN TANDEM WITH BIGLYCAN, ALTERS VISCOELASTICITY IN AGED TENDONS

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INTRODUCTION

Risk of tendon injury increases with age, yet the age-associated changes to tendon tissue that lead to increased injury risk have yet to be elucidated [1]. Decorin (Dcn) and biglycan (Bgn) are small, leucine-rich proteoglycans (SLRPs) present in the tendon extracellular matrix that regulate collagen fibrillogenesis [2,3]. Both SLRPs are highly expressed during development, and conventional knockouts of either molecule impact tendon mechanical properties [4]. While knockout of decorin protects against age-associated reductions in tendon mechanical properties, acute deletion of biglycan or both SLRPs in mature tendons reduces mechanical and structural properties [5,6,7]. These results demonstrate that these SLRPs play a role in tendon homeostasis in addition to their role in development. However, the differential roles of these molecules in aged tendon homeostasis is unknown. Therefore, the objective of this study was to determine the effect of acute deletion of decorin, biglycan, or both SLRPs on aged tendon mechanics. Due to the negative impact of decorin during tendon aging and the positive impact of biglycan in tendon homeostasis, we hypothesized that acute deletion of biglycan would reduce tendon mechanical properties more than acute deletion of decorin, and that acute deletion of both SLRPs would result in similar mechanical properties to biglycan-deficient tendons.

METHODS

Animals: Female wild-type (WT) (n=6) and transgenic Dcn<sup>fl</sup>/<sup>fl</sup> (n=11), Bgn<sup>fl</sup>/<sup>fl</sup> (n=9), and Dcn<sup>fl</sup>/<sup>fl</sup> Bgn<sup>fl</sup>/<sup>fl</sup> (n=15) mice with a tamoxifen (TM) inducible Cre were used in this study (IACUC approved). At 485 days old, mice received three consecutive daily TM injections (4mg/40g body weight) for Cre-mediated excision of targeted genes. Mice were sacrificed 30 days later (515 days of age). Patella-patellar tendon-tibia complexes were harvested from the left hind limb and prepared for mechanical testing as described [5].

Mechanical Testing: Uniaxial, viscoelastic testing was performed with an Instron 5848. The testing protocol consisted of 10 cycles of preconditioning, followed by stress relaxations at 3%, 4%, and 5% strain for 10 minutes. Following each stress relaxation, frequency sweeps of 10 cycles at 0.1, 1, 5, and 10 Hz were performed. A ramp-to-failure followed the final stress relaxation. Percent relaxation, dynamic modulus (E'), and phase shift (δ) were computed for each stress relaxation and frequency sweep. Stiffness, modulus, maximum load, and maximum stress were quantified from the ramp-to-failure data.

Statistics: For all mechanical properties, one-way ANOVAs with Bonferroni post-hoc tests were used to compare across genotypes. Significance was set at p<0.05, and trends were set at p<0.10.

RESULTS

Quasi-Static Mechanics: No differences in CSA were observed between genotypes (data not shown). No differences in stiffness or maximum load were observed between genotypes (Fig 1). No differences in insertion or midsubstance modulus were observed between genotypes (Fig 2). Dcn<sup>−/−</sup>Bgn<sup>−/−</sup> tendons had larger maximum stresses than Dcn<sup>−/−</sup> tendons (data not shown).

Stress Relaxation: Dcn<sup>−/−</sup>Bgn<sup>−/−</sup> tendons exhibited larger stress relaxations than WT and Bgn<sup>−/−</sup> tendons at 3% and 4% strain (Fig 3). Dcn<sup>−/−</sup> tendons exhibited a larger stress relaxation than WT tendons at 4% strain, and Dcn<sup>−/−</sup>Bgn<sup>−/−</sup> tendons trended towards more stress relaxation than Bgn<sup>−/−</sup> tendons at 5% strain.

Dynamic Mechanics: No differences in dynamic modulus were observed between genotypes at any strain level or frequency (data not shown). At 3% strain, Dcn<sup>−/−</sup>Bgn<sup>−/−</sup> tendons had larger tan(δ) values than WT tendons at all frequencies (Fig 4). Dcn<sup>−/−</sup>/Bgn<sup>−/−</sup> tendons had larger tan(δ) values than Bgn<sup>−/−</sup> tendons at 0.1Hz and 1Hz and trended towards larger tan(δ) values at 5Hz. Dcn<sup>−/−</sup> tendons trended towards larger tan(δ) values than WT tendons at 5Hz and 10Hz. At 4% strain, Dcn<sup>−/−</sup>/Bgn<sup>−/−</sup> tendons had larger tan(δ) values than WT tendons at 0.1Hz, 5Hz, and 10Hz (Fig 5). Dcn<sup>−/−</sup>/Bgn<sup>−/−</sup> tendons had larger tan(δ) values than Bgn<sup>−/−</sup> tendons at 0.1Hz and 5Hz and trended towards larger tan(δ) values at 0Hz. Dcn<sup>−/−</sup> tendons had larger tan(δ) values than WT tendons at 1Hz and 10Hz, had larger tan(δ) values than Bgn<sup>−/−</sup> tendons at 1Hz, and trended towards larger tan(δ) values than WT and Bgn<sup>−/−</sup> tendons at 5Hz. At 5% strain, Dcn<sup>−/−</sup>/Bgn<sup>−/−</sup> tendons had larger tan(δ) values than Bgn<sup>−/−</sup> tendons at 0.1Hz and 1Hz (data not shown). Dcn<sup>−/−</sup> tendons trended towards larger tan(δ) values than Bgn<sup>−/−</sup> tendons at 0.1Hz and 1Hz.

DISCUSSION

Induced knockout of decorin, biglycan, or both molecules in aged tendons did not impact quasi-static mechanical properties compared to WT tendons. Dynamic mechanical properties were also unaffected by genotype. Viscoelastic properties were most affected by both decorin knockouts, especially at low (3%) and intermediate (4%) strains. Deficiency of both decorin and biglycan led to increased viscoelasticity compared to WT and biglycan-deficient tendons at 3% and 4% strain. Deficiency in decorin alone led to some increases in viscoelasticity at the intermediate strain level compared to WT and biglycan-deficient tendons. The viscoelastic effects of decorin or decorin/biglycan deficiency were largely lost at the high (5%) strain level. These results run contrary to our hypothesis that deletion of biglycan would lead to
larger reductions in mechanical properties than decorin deletion and that double knock-out tendons would have similar mechanical properties to biglycan-deficient tendons. Instead, knockout of both molecules led to large increases in viscoelastic properties, and some of these properties were increased with decorin deficiency alone.

Results demonstrate that decorin plays a larger role than biglycan in aged tendon mechanics. While no differences were observed between WT and Bgn−/− tendons, Den−/− tendons had increased viscoelastic properties compared to control tendons. While biglycan has been shown to impact mechanics in mature tendons, biglycan expression decreases with age [6,2]. This decreased expression may explain the negligible effect of biglycan deletion in aged tendons.

The structure-function role of decorin within the tendon matrix remains controversial. Conventional knockout of decorin impacts both quasi-static and viscoelastic properties of the patellar tendon [4,8]. Depletion of SLRP GAG chains in tail tendon fascicles, however, does not affect quasi-static and viscoelastic properties [9,10]. Results of this study support previous findings that decorin deficiency alters tendon viscoelastic properties at the tissue level.

The viscoelastic changes seen in decorin-deficient tendons were enhanced with the dual deletion of decorin and biglycan. Biglycan expression has been shown to increase in response to induced deletion of decorin [11]. The Dcn−/−/Bgn−/− genotype eliminates this potential compensatory mechanism, which may explain the observed increases in viscoelastic properties in these tendons.

A limitation of this study is the small sample size used across groups, which may lead to certain parameters being underpowered. In addition, the mechanical changes observed lack a mechanistic explanation at this time. Future studies will analyze differential gene expression between these genotypes to better understand how decorin and biglycan together influence aged tendon mechanics.

This study reveals the role of decorin in the viscoelastic properties of aged tendons and demonstrates that biglycan may offset changes in these properties following the loss of decorin. These results provide further understanding of the role of SLRPs in the mechanical properties of aging tendons.

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INTRODUCTION
Rotator cuff tendon tears are a common injury affecting a large portion of the population. Of these rotator cuff tears, 90% involve injury to the supraspinatus tendon, 35% to the infraspinatus, and 25% to the subscapularis [1-6]. Further, advancing age is directly correlated with increased incidence of such tears. However, the age related changes in tendon mechanical properties that may predispose the supraspinatus to injury relative to the other rotator cuff tendons is unclear [7]. Therefore, the objective of this study was to define the age-related alterations in rotator cuff tendon fatigue mechanics to determine whether the supraspinatus is more susceptible to injury due to aging than the infraspinatus and subscapularis. We hypothesized that aging would adversely and preferentially affect supraspinatus tendon fatigue mechanics when compared to the subscapularis and infraspinatus tendons.

METHODS
Experimental design and sample preparation: 7-month juvenile (n=10), 18-month adult (n=10), 27-month old (n=10), and 36-month old (n=10) male F344XBN rats were obtained from the National Institute of Aging (IACUC approved), approximating respective human ages of 18, 43, 63, and 90 years old. After 3 weeks of facility acclimation, all animals were sacrificed. Lower and upper subscapularis (LS & US, respectively [7]), supraspinatus (SS), and infraspinatus (IS), muscle-tendon complexes were then each carefully dissected from the scapula of the right shoulder and removed with the proximal humerus for mechanical testing. Muscle, along with extraneous tissue was removed from each tendon and cross-sectional area of each tendon was measured using a custom laser device [8]. Each humerus was potted in a custom acrylic cylinder secured with polymethyl-methacrylate, leaving the proximal humerus exposed. The head of the humerus was secured using a self-tapping screw to prevent failure at the growth plate. Mechanical testing: The LS, US, SS, and IS from each animal were mechanically tested independently on an Instron ElectroPuls E3000. Maximum stresses were calculated from previous ramp to failure testing on contralateral shoulders for each tendon for each group. Mechanical testing consisted of a 0.1N preload, a ramp to 40% maximum load at 1% strain per minute, and cycles to failure (7-40% maximum load at 2Hz). During loading, force and displacement data were acquired and analyzed using MATLAB (Mathworks; Natick, MA). A line was fit to the triphasic peak strain vs. cycles curve, and all fatigue parameters were calculated at breakpoints 1 (BP1) and 2 (BP2) (Figure 1). Cycles to failure, secant modulus and stiffness, peak strain, laxity (resistance of material to elastic deformation), and hysteresis (measure of energy dissipation) were evaluated. A 2-way ANOVA with Bonferroni post-hoc tests was used to compare the different ages for each tendon with significance set to p<0.05.

Figure 1: Example peak strain vs. cycles (% of failure) curve. Fatigue parameters were calculated at breakpoints 1 (BP1) and 2 (BP2).
RESULTS

Significant changes in hysteresis at BP2 were seen with aging in all rat rotator cuff tendons tested in fatigue. Secant modulus at BP2 significantly decreased in the LS, SS, and IS in the geriatric group compared to juvenile animals, and in the LS and IS between geriatric and adult rats (Fig. 2). The SS and IS were similar with significant increases in hysteresis in adult, aged, and geriatric animals compared to juvenile (Fig. 3). Peak strain at BP2 was significantly increased in the LS geriatric tendons compared to juvenile and adult, and in the IS adult tendons from juvenile (Fig. 4). Surprisingly, there were no significant changes in cycles to failure in the LS or SS. The US had increased cycles to failure between juvenile and aged, and the IS had the highest number of cycles to failure in the geriatric group (Fig. 5).

DISCUSSION

Advancing age correlated with significant detrimental changes in the fatigue properties of all rat rotator cuff tendons, supporting that aging, in general, is a factor in the increase of rotator cuff tears seen in humans. Interestingly, supraspinatus fatigue response was not preferentially affected by aging when compared to the other rotator cuff tendons, which is not consistent with tear frequency observed clinically. Significant decreases in secant modulus in the LS, SS, and IS, and changes in hysteresis in all rotator cuff tendons indicate altered load transmission and energy dissipation capacity with aging. Previous literature has shown significant accumulation of advanced glycation end products, cross-linking, and fatty infiltration within the supraspinatus [10]. It has also been shown that the supraspinatus has a decreased fiber alignment response to loading as a result of aging [11]. This altered biochemical and structural environment within the rat rotator cuff tendons may explain the significant changes shown in fatigue mechanical properties in this study. Surprisingly, cycles to failure was highest in the IS in the geriatric group; however, this may be explained by an increase in IS cross sectional area in the geriatric group (not shown). A limitation of this study is that it only analyzes fatigue mechanical properties. It has been shown that significant biochemical compositional, cellular, and structural changes exist with aging in the supraspinatus; however, none of the current studies analyze these changes in reference to the other rotator cuff tendons [10-12]. In summary, this study demonstrates that aging has a significant effect on fatigue properties of the rat rotator cuff, though the supraspinatus was not preferentially affected. Thus, other factors may explain the predominance of supraspinatus tears with age. Future studies will investigate the histological, morphological, and biochemical changes in all rotator cuff tendons in response to aging.

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