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Muscle loading is necessary for the formation of a functional tendon enthesis

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Abstract

Muscle forces are essential for skeletal patterning during development. Eliminating muscle forces, e.g., through paralysis, leads to bone and joint deformities. Botulinum toxin (BtxA)-induced paralysis of mouse rotator cuffs throughout postnatal development closely mimics neonatal brachial plexus palsy, a significant clinical condition in infants. In these mice, the tendon-to-bone attachment (i.e., the tendon enthesis) presents defects in mineral accumulation and fibrocartilage formation, presumably impairing the function of the tissue. The objective of the current study was to investigate the functional consequences of muscle unloading using BtxA on the developing supraspinatus tendon enthesis. We found that the maximum endurable load and stiffness of the supraspinatus tendon attachment decreased after four and eight weeks of post-natal BtxA-muscle unloading relative to controls. Tendon cross-sectional area was significantly reduced by BtxA-unloading, suggesting that the reduction of mechanical function resulted in part from geometric changes. However, strength, modulus, and toughness were also decreased in the BtxA-unloaded group compared to controls, indicating a decrease in tissue quality. Polarized-light microscopy and Raman microprobe analysis were used to determine collagen fiber alignment and mineral characteristics, respectively, in the tendon enthesis that might contribute to the reduced biomechanical performance in BtxA-unloaded shoulders. Collagen fiber alignment was significantly reduced in BtxA-unloaded shoulders. The mineral-to-matrix ratio in mineralized fibrocartilage was not affected by loading. However, the crystallographic atomic order of the hydroxylapatite phase (a measure of crystallinity) was reduced and the amount of carbonate (substituting for phosphate) in the hydroxylapatite crystals was increased. Taken together, these micrometer-scale structural and compositional changes partly explain the observed decreases in the mechanical functionality of the tendon enthesis in the absence of muscle loading.

Keywords

postnatal development; Botulinum toxin A; biomechanics; tendon-to-bone insertion; biomineralization; mechanobiology

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1. INTRODUCTION

Musculoskeletal tissues are highly sensitive to their mechanical environments. Mechanical forces not only maintain skeletal tissue homeostasis, they also guide tissue development. Disruptions in the loading environment during development often lead to pathological conditions. For example, in neonatal brachial plexus palsy (NBPP), damage to the brachial plexus during birth leads to shoulder paralysis and results in a number of shoulder pathologies in children, including bony defects, a decreased range of motion, and ultimately a loss of limb functionality in the most severe cases [1-3]. This condition affects approximately 1 in 100 births, making it one of the most common pediatric shoulder disorders [4].

We recently developed an animal model of this condition. Botulinum toxin A (BtxA) injections into the supraspinatus muscles of neonatal mice led to reversible paralysis that closely mimicked the developmental pathologies characteristic of NBPP [5-7]. This model was validated by comparison to mice that underwent neurotomy of the upper trunk of the brachial plexus at birth [8]. Mouse shoulders that were paralyzed at birth exhibited internal rotation contractures due to the muscle imbalance across the shoulder, as seen in the clinical condition [1, 4]. This muscle imbalance ultimately led to bone loss and humeral head deformities. Previous studies using this animal model also showed that unloading disrupted the development of the tendon enthesis, a tissue critical for transferring loads from muscle to bone [6, 8]. Notably, unloading led to a loss of fibrocartilage transitional tissue and a loss of underlying bone at the tendon-to-bone attachment [6].

The gross morphological and bone architectural consequences of upper limb paralysis throughout postnatal development have been previously described [6-8]. However, the structural and functional consequences of reduced loading on the tendon enthesis have not yet been investigated. We hypothesized that a loss of bone and fibrocartilage would have a detrimental effect on the ability of the enthesis to transfer forces from tendon to bone. We propose that this loss of mechanical function would be due to disruption of collagen fiber structural organization and due to alterations in the composition of the mineralized interface. In this study, we investigated the functional consequences of muscle unloading throughout post-natal development of the tendon-to-bone attachment. Furthermore, we characterized micrometer-scale structural and compositional changes that might contribute to changes in mechanical behavior.

2. METHODS

2.1 Animal Model

The use of animals for this study was approved by the Animal Studies Committee at Washington University. 70 CD1 mice of mixed genders, obtained from Charles River Labs (Wilmington, MA), were divided into two groups, 1) experimental and 2) normal age-matched control animals, consistent with previous studies [5-7]. Experimental mice received injections of BtxA (BOTOX; Allergan, Irvine, CA) in the left supraspinatus muscle and injections of saline in the right supraspinatus muscle beginning at birth. Specifically, 0.2U of BtxA dissolved in 10 μ L of saline solution was injected twice per week into the left shoulders for the first two weeks after birth (the frequency of injection was necessary to maintain paralysis). Injections of 0.15 U in 10 μ L of saline were then administered twice per week until the mice were 4 weeks old, and then once per week until euthanasia. 10 μ L of saline solution was injected concurrently with BtxA into the right supraspinatus muscles as an internal control. Previous experiments comparing paired normal and saline-injected shoulders indicated no differences in shoulder development that could be attributed to the saline injections [6]. However, previous studies using this model have noted differences

between saline-injected shoulders of BtxA animals and age-matched controls [6, 7]. Mice were euthanized by carbon dioxide narcosis at two timepoints: postnatal days 28 (P28) and 56 (P56). One shoulder was used from each normal animal at each time point and both shoulders were used from each BtxA experimental animal, resulting in three groups of samples from each of two time points. Five shoulders per group were allocated for analysis of collagen fiber alignment and processed immediately as described below. Five animals per time point from the experimental group were allocated to Raman microprobe analysis and stored at -20°C until processing, as described below. Data from Schwartz et al. [9] were used for normal age-matched comparisons for the Raman microprobe analyses. The remaining 40 animals (20 normal and 20 experimental) were stored at -20°C until processing for biomechanical analysis, as described below.

2.2 Tendon cross-sectional area

Eight shoulders from each of the six groups were used to determine tendon cross-sectional area. Mice were thawed and dissected to isolate the humerus, supraspinatus tendon, and muscle. Micro-computed tomography (μ CT40; Scanco Medical AG, Switzerland) was used to determine tendon cross-sectional area. Samples were stored in saline-soaked gauze until scanning. Immediately prior to scanning, the distal humerus was potted in agarose in a plastic tube and the tube was inverted within a sample holder. In order to limit background signal in the measurement, the tendon and muscle were suspended in air, aligned with the long axis of the humerus. Scans were performed at X-ray tube settings of 55 kV and 145 μ A with a 99 ms integration time. These parameters resulted in 30 μ m isometric resolution. The measurement time was <7 minutes and the total time in the scanner was <20 minutes. No appreciable dehydration was seen in any specimen after scanning. The scanned region was approximately 2 mm in the axial direction and included a small amount of the humeral head and supraspinatus muscle. The cross-sectional areas of sagittal slices through the tendon were estimated using thresholding and the minimum cross-sectional area was recorded.

2.3 Biomechanical testing

Uniaxial tensile testing was used to evaluate the biomechanical properties of the tendon entheses. After micro-CT analysis, the humerus was potted in epoxy up to the humeral head to stabilize the bone. A paperclip was embedded in additional epoxy over the articular surface of the humeral head away from the tendon insertion to prevent fracture of the growth plate. Tendons were wrapped in saline-soaked gauze throughout potting to maintain hydration. Tendons were sprayed with Verhoeff's tissue stain to generate a random pattern of speckles that was used to optically determine strain. Each tendon was secured between two layers of thin paper with a drop of cyanoacrylate adhesive before the sample was mounted in custom aluminum grips. Samples were tested in a 37°C saline bath on an Instron Electropuls E1000 (Instron Corp., Canton, MA) fitted with a 30 N load cell. The tendon was imaged continuously throughout the testing protocol using either an Olympus DP70 (1360 $\times$ 1024 pixels) or an Illunis VMV-8M (3296 $\times$ 2472 pixels) CCD camera at ~3fps. The tendon gauge length was determined optically by comparison to an internal standard on the top grip and was approximately 2 mm for all groups.

The testing protocol (WaveMatrix software, Instron Corp., Canton, MA) consisted first of a 50 mN (P28) or 100 mN (P56) preload followed by 5 cycles of preconditioning using a triangle waveform with a peak load of 50 mN. A lower preload was used for the P28 samples due to the very low observed maximum load (>0.3 N) in the P28 BtxA unloaded samples. Next, a stress-relaxation test was performed with a strain rate of 100%/s to a maximum strain of 3% followed by 5 minutes of relaxation. Tendons were returned to the baseline state and held isometrically for 5 minutes before loading to failure at 0.5%/s. Strain was determined optically for the linear region by tracking the speckled stain pattern within

two regions, one at the top of the tendon including the grip and one on the bone near the insertion using a custom texture correlation algorithm written in Matlab (Mathworks, Natick, MA). The cross-sectional area used for normalization of the biomechanical properties was estimated by assuming an elliptical cross-section with the width of the tendon observed in the video as the major axis and the average thickness for each group determined from the microCT analysis as the minor axis.

Estimates of the stiffness/modulus, yield strength/stress, yield strain, resilience, and toughness were made from the load-displacement curves of each load-to-failure test. The stiffness and elastic modulus of each sample was determined from the linear portion of each load-displacement curve. The linear portion was identified by adjusting a window of points within the load-deformation or stress-strain so as to maximize the R^2 value for a linear least squares regression of the data in the window. The yield stress was defined as the stress value when the tangent modulus decreased to 50% of the maximum value in the load-to-failure tests. The yield strain was defined as the strain value corresponding to the yield stress. The energy to yield, a measure of resilience, was defined as the total area under the stress-strain plot beginning from the point at which the tendon reached a load of 0.05 N and continuing until the yield stress was attained. The toughness was defined as the total area under the stress-strain curve beginning at 0.05 N and continuing until this baseline value of stress was again attained. The ultimate force and stress were defined as the peak values on the force-displacement and stress-strain curves, respectively. Samples that failed either at the grip or the growth plate were excluded from analysis of failure properties.

Estimates of the fully relaxed modulus of the samples were made from stress-relaxation data. These data, however, were extremely noisy for the low loads sustained by the P28 tendons, so this data was excluded from analysis. For the P56 samples, the fully relaxed state was defined as that achieved after 4.5 minutes of viscoelastic relaxation in isometric conditions. Percent relaxation was defined as the percent change in load from the maximum value of force at the beginning of the stress-relaxation test to the average load measured over the last 30 seconds of the 5 minute isometric test.

2.4 Collagen fiber alignment

Quantitative polarized light microscopy was used to analyze the structure of collagen fibrils within the enthesis fibrocartilage. Details of this technique are described in Thomopoulos et al. [10]. Tendon insertions from each of the six groups previously mentioned were dissected free of all surrounding tissues, leaving only a small amount of muscle and bone, and then processed for histology. Dissected muscle-tendon-bone complexes were immediately fixed in 4% paraformaldehyde with the tendon forming a 180° angle with the humerus (equivalent to 90° of abduction in the shoulder joint), dehydrated, embedded in paraffin, and sectioned at 5µm in the transverse plane. One section per tendon was manually selected to control for orientation and stained in 0.1% Picrosirius Red solution, destained in 1% acetic acid, counterstained with hematoxylin, and differentiated in 1% HCl in 70% ethanol. The distribution of collagen fiber angles was evaluated for a region of each tissue section encompassing both mineralized and non-mineralized fibrocartilage within the tendon enthesis. Each section was imaged through crossed polarizers at 10 different polarization angles using a 40x objective lens with and without a compensator ($\lambda/4$ wave plate oriented NW-SE). The extinction angle was calculated for groups of pixels (5 µm × 5 µm) within a user defined region of interest in the insertion fibrocartilage using custom MATLAB (Mathworks, Natick, MA) scripts [10, 11]. The angular deviation of the distribution of angles was calculated for each sample using circular statistics [10].

2.5 Raman microprobe analysis

Raman microprobe analysis is a useful technique for investigating mineralization at the tendon enthesis because it can be used on unfixed, hydrated tissues with minimal processing that might disrupt the interface. Five shoulders per group were thawed and dissected as described above for the polarized light analysis. Shoulders were directly embedded in Optimal cutting temperature (OCT) medium and sectioned to 20 μm on a cryostat. Immediately before analysis, one tissue section per sample was thawed and washed twice with phosphate buffered saline.

The Raman microprobe apparatus (HoloLab Series 5000 fiber-optically coupled Raman Microscope, Kaiser Optical Systems, Inc.) has been described previously [12]. Spectra were acquired using 10 mW of a 532 nm laser focused using an 80x objective (N.A. = 0.85) to a $\sim 1 \mu\text{m}$ beam spot on the surface of the sample. The scattered light was collected for 32, 4 second acquisitions in a backscattered configuration through the objective lens to a 2048-channel CCD detector. This allowed for concurrent detection of the spectral range 100-4000 Δcm^{-1} . Raman spectra were collected from at least three ($\sim 1 \mu\text{m}$ diameter) regions within fully mineralized regions of enthesis fibrocartilage for each tissue section. The Raman signal was optimized at each spot via manual focusing and care was taken to avoid cells and vascular tissue that might affect the spectral signal.

Spectra were analyzed as previously described [9, 12, 13]. Briefly, spectra were background corrected to eliminate the effects of fluorescence, and peaks within the spectral range 700-1200 Δcm^{-1} were deconvolved with a mixed Gaussian-Lorentzian peak fitting algorithm in the Grams32 software package (Galactic, Salem, NH). The relative mineral concentration of the mineralized matrix within the enthesis fibrocartilage was inferred from the ratio of the peak intensities for the ν_1 P-O stretching band of hydroxylapatite (960 Δcm^{-1}) and the aromatic ring stretching band of phenylalanine residues in collagen (1003 Δcm^{-1}). This measure does not indicate the absolute amount of either mineral or matrix present, but provides useful information about the relative concentration, which can be compared between groups.

Two other spectral signatures were used to characterize the carbonated hydroxylapatite within the mineralized fibrocartilage. First, the crystallographic atomic order of the hydroxylapatite crystals (i.e., one aspect of crystallinity) was measured by the full width at half maximum (FWHM) of the 960 Δcm^{-1} peak. A narrower peak is indicative of increased crystallographic order, or less variation in bond angles and distances. Second, biological hydroxylapatite is characterized by carbonate substitutions for phosphate [14]. Therefore, the carbonate content of the mineral within the enthesis fibrocartilage was evaluated by taking the ratio of the heights of the 1070 Δcm^{-1} peak, indicative of carbonate substitution for phosphate, and the 960 Δcm^{-1} peak. Our past studies have shown more reproducible results from ratios of peak heights, in this particular case, compared to the standard ratio of peak areas [12]. Following Raman microprobe analysis, sections were fixed in 10% neutral buffered formalin and stained using von Kossa's method (for mineral) and toluidine blue (for cells and organic matrix) so that the Raman-analyzed regions could be interpreted histologically.

2.6 Statistical analysis

Normal, saline, and BtxA groups at P28 and P56 were compared using a two-way analysis of variance (ANOVA) to determine effects of and interactions between treatment groups and animal age. When the effect of experimental group reached significance ($p < 0.05$), Fisher's least significant difference tests with a Bonferroni correction were used to evaluate post hoc differences for six predetermined comparisons ($\alpha = 0.05/6 = 0.0083$). The comparisons used

were P28 normal vs. P28 BtxA, P28 saline vs P28. BtxA, P28 normal vs. P28 saline, P56 normal vs. P56 BtxA, P56 saline vs. P56 BtxA, and P56 normal vs. P56 saline. All data are reported as the mean $\pm$ standard deviation.

3. RESULTS

3.1 Effects of paralysis on tendon morphology and mechanics

Local muscle paralysis was evaluated by observing shoulder motion during cage activity. BtxA animals had decreased abduction and external rotation, consistent with previous studies [5-7]. Micro-computed tomography measurements indicated that paralysis did not significantly reduce supraspinatus tendon cross-sectional areas relative to normal age-matched or saline contralateral controls at both P28 and P56 (Table 1). Tendons significantly increased in size from P28 to P56.

The metrics of mechanical performance estimated for tendon entheses were all significantly lower in the BtxA group compared to controls. In the absence of gripping failures, the tissue consistently failed at the tendon-to-bone interface in all groups, and all groups exhibited force-displacement that were qualitatively similar at low strains (Figure 1A). Maximum force was reduced at P28 and P56 in the BtxA group compared to normal controls and saline controls at P56 (Figure 1B). Significant differences were also detected between saline and normal controls at P28 and P56. Tendon stiffness was decreased relative to saline and normal controls after 56 days of unloading (Figure 1C). At P28, both saline control and BtxA groups had lower stiffness than normal controls. Maximum force and stiffness both increased with increasing animal age.

At P56, ultimate stress was reduced in the BtxA group compared to controls (Figure 2A). At P28, strength was also reduced in both the saline and BtxA groups compared to the normal group. The elastic modulus was significantly decreased in the BtxA group relative to normal and saline controls after 28 days of unloading but no significant differences were detected at P56 among the three groups (Figure 2B). Yield stress was significantly reduced after both 28 and 56 days of unloading compared to saline and normal controls (Table 1). The resilience (energy to yield) was also decreased at P28 and P56 in the BtxA group relative to saline and normal controls.. The toughness was decreased relative to normal controls at P28. At P28, yield stress, energy to yield, and toughness were also reduced in saline controls compared to normal age-matched animals. The percent relaxation associated with viscoelastic relaxation at P56 was not significantly affected in BtxA tendons.

3.2 Effect of paralysis on collagen fiber alignment

Representative analysis regions for polarized light analysis of collagen fiber alignment are shown in Figures 3E-G. There was a statistically significant increase in the angular deviation of the distribution of collagen fiber angles (i.e., the fiber distribution was less organized) within the enthesis fibrocartilage in BtxA samples compared to normal aged-matched and saline contralateral controls at P28 and P56 (Figure 3A-D).

3.3 Effect of paralysis on mineralized fibrocartilage

Representative mineralized tissue sections from normal, saline and BtxA entheses at P56 are shown in Figures 4A-C respectively. The Raman microprobe measured mineral-to-matrix ratio averaged from three $\sim 1 \mu\text{m}$ analysis spots per sample was not significantly changed by BtxA unloading (Figure 4D). Carbonate substitution for phosphate, determined by comparing the 960 cm^{-1} peak for phosphate to the 1070 cm^{-1} peak for B-type carbonate substitution for phosphate [14, 15], was increased in entheses unloaded using BtxA for 56 days compared to normal controls (Figure 4E).. The crystallinity of the mineral within

mineralized fibrocartilage, averaged over the sampling volume, was significantly reduced in BtxA-unloaded entheses at P56 compared to normal and saline controls (i.e., FWHM was increased, Figure 4F).

4. DISCUSSION

In this study, we investigated the effect of muscle unloading on the postnatal development of the tendon enthesis, and found diminished biomechanical function in BtxA-unloaded tissues. An important distinction must be made between the concepts of material and structure when interpreting biomechanical results. We define material effects as those due to local changes to tissue, including its composition and hierarchical structure, at a specific point along the tendon-to-bone insertion site. We define structural effects as those due to changes in the spatial distribution of material (e.g., cross-sectional area, the spatial distribution of material with different mechanical properties). Clear effects of BtxA-induced unloading were observed in the biomechanical tests performed, and these likely involved both material and structural effects.

The simplest gross structural metric studied was tendon size, and this was maintained relative to saline contralateral controls but not relative to normal age-matched controls following BtxA treatment. The constant size of the BtxA tendons relative to contralateral saline controls is consistent with the results of Eliasson et al., who reported that tendons continue to increase in size normally in the absence of loading during development [16]. Relative to normal controls however, we observed decreases in tendon size due to BtxA-unloading, similar to studies using other unloading models in mature animals [17-19]. These results suggest that developing tendons may respond differently to unloading compared to mature tendons.

BtxA-unloading resulted in tendon-to-bone entheses with inferior mechanical properties. This suggests that the material that comprises the developing tendon enthesis tissue must be altered by BtxA to produce an attachment with diminished mechanical function. To explore potential tissue changes that might contribute to the observed biomechanical changes, we measured collagen fiber alignment and characteristics of the mineralized fibrocartilage matrix within the enthesis. We observed a small, but significant, increase in the angular deviation of collagen fibrils (6° - 8°), i.e., the absence of muscle loading resulted in a less organized extracellular matrix. This is consistent with observations *in vitro*, in which cells within mechanically stimulated tissue constructs deposit matrix proteins predominantly in the direction of loading [20]. Clear structure-function relationships between fiber alignment and tensile properties in collagenous tissues are long-established in the literature [21, 22], and models for this relationship allow quantitative estimation of the degree to which the disorganization of collagen contributes to the lower moduli observed in the entheses of the BtxA-treated group. The essence of this calculation is averaging an estimate of the homogenized moduli of cross-linked, aligned fibers over the measured fiber orientation distribution. Using a simple formulation and that we published elsewhere [10, 13, 23, 24] and the unit cell properties that we used to model adult tendon [13], the decrease in collagen organization caused by BtxA-unloading could account for an approximately 10% reduction in elastic modulus. This is far less than the observed 75% and 42% decreases in elastic modulus with BtxA at P28 and P56, respectively, compared to normal controls. Thus, although alignment is known to correlate positively with tensile strength and stiffness (in the direction of fiber alignment) and with tissue anisotropy [20, 25], the reduction in collagen fiber alignment observed here accounts for only a small fraction of the reduced elastic modulus of the tissue. Other compositional factors must therefore be involved. This conclusion is consistent with observations of Juncosa-Melvin *et.al.* who demonstrated *in vitro* that mechanical stimulation up-regulates production of extracellular matrix genes,

which also correlate with increased stiffness [26]. Factors such as this may affect the tissue as a material and therefore affect its mechanical response.

Failure loads are determined by the interplay between structural effects such as the gross shape and distribution of tissue types within the enthesis, and material effects such as local failure loads. In nearly all of the samples, failure occurred at a location within the tendon-bone interface and not in the tendon midsubstance. The mechanics of the tissue within the enthesis is dominated by collagen for low mineral concentrations, and the mineral phase for higher mineral concentrations [13]; as with many composite materials and tissues, the transition point is the mineral volume fraction associated with percolation [27, 28]. This critical mineral concentration is a strong function of the shape and organization of the mineral phase (e.g., [29, 30]). We assayed the relative mineral concentration of the mineralized matrix within the enthesis using Raman microprobe analysis. We observed no statistically significant differences in the intensity ratio of the $960\text{ }\Delta\text{cm}^{-1}$ peak for mineral and the $1003\text{ }\Delta\text{cm}^{-1}$ peak for collagen. Raman measurements cannot accurately describe the *absolute* volume or mass of material present. Rather, this measure describes changes in the *concentration* of mineral, assuming that the entire tissue volume consists of only mineral and collagen. Previous analyses using X-ray micro-computed tomography have demonstrated decreases in bone volume when muscle forces are removed using BtxA [31-33]. This is also true of the developing murine tendon enthesis [6, 7]. Our results suggest that the local mineral-to-collagen ratio within the extra-cellular matrix is not affected strongly by BtxA, in contrast to the total amount of mineralized tissue, which is decreased due to BtxA-unloading.

We probed the characteristics of the hydroxylapatite crystals within the mineralized fibrocartilage extracellular matrix and found that for the BtxA group the carbonate concentration was increased with respect to self-comparison between P28 and P56, as well as comparison to the normal and saline groups at P56. Crystallinity of the BtxA mineral crystallites was decreased with respect to the normal and saline groups by P56. Although the exact role of carbonate incorporation in matrix remodeling is unclear, several reports have shown decreased carbonate content and/or crystallinity in mineralized tissue from younger animals that has yet to undergo remodeling [9, 34-37]. Of note in the present study, tendon entheses that develop without muscle forces have a dramatically different composition from that of normally loaded controls. The BtxA-unloaded group has a carbonate concentration characteristic of much older mineralized matrix that has likely been remodeled, yet it is less crystalline. The typical effect of aging in bone, however, is the co-varying increase in both carbonate concentration and crystallinity [34, 38, 39]. The present changes in mineral composition and structure may be the result of changes in organization and composition of the organic matrix components. Furthermore, previous results indicated increased osteoclast activity in BtxA unloaded compared to loaded shoulders which resulted in a higher rate of bone resorption [6, 31, 40]. This could amount to disruptions in the balance of mineralized matrix deposition versus resorption, leading to lower net amounts of bone accumulation and a less mature mineralized matrix. In summary, the observed changes in mineral characteristics are likely due to both altered cellular activity and organic matrix composition.

The mechanical consequence of decreased atomic order and increased carbonate content of the mineral crystallites within the enthesis fibrocartilage is unclear. The boundary between mineralized and non-mineralized fibrocartilage is characterized by a narrow zone of increasing mineral content that may dissipate stress concentrations arising at the interface [9, 12, 13, 24]. Carbonate content has been observed to regulate crystal size, with higher carbonate content leading to smaller crystals with elongated aspect ratios [41, 42]. Additionally, changes in the carbonate content and crystallinity have been correlated with inferior bone biomechanical properties [34, 38, 43, 44]. The failure mode of the samples

tested in the current study was invariably the tendon-bone interface if there was not a readily identified gripping problem. However, it is not known whether the failure occurred within the unmineralized fibrocartilage, within the fully mineralized fibrocartilage, or within the mineral gradient between the two. Changes to the mineral aspect ratio are expected to shift the mineral volume fraction needed for percolation, and hence have a strong effect on the mechanics and stress fields within tissue at the enthesis [13]. These material effects could lead at the structural level to macroscopic stress concentrations that reduce strength and toughness of the enthesis.

There were a number of limitations in this study. BtxA injections lead to a small decrease in animal body weight, although this decrease is not statistically significant [6]. The current and prior studies using this animal model have not controlled for animal gender or litter size. These factors contribute to variation in body weights and may also contribute to variations in the functional outcomes measured. Differences in animal sizes may partly explain the observed differences in some outcomes between the normal and saline controls. Additionally, there may be some direct or indirect systemic effects of BtxA, as the injected pups were generally more lethargic and less active. Due to various factors contributing to variation in the animal model, it is possible that some of the outcomes may be underpowered, although statistically significant differences were detected with N=5 for most outcomes.

5. CONCLUSION

In conclusion, the results presented here indicate that muscle forces are necessary for the development of a functional tendon enthesis. Biomechanical behavior was significantly affected after only four weeks of BtxA-unloading during postnatal development. Mechanical property changes were due to a small degree to changes in the organization of collagen fibers. Changes to the mineral composition due to BtxA-unloading likely had deleterious effects on both the material behavior of the tissue and the structural response of the enthesis as a whole. These results have important implications for patients of NBPP. Understanding the developmental time course of loss of enthesis function will lead to a more informed strategy for treatment. Future studies will determine whether these detrimental functional changes to the enthesis are reversible upon return of muscle loading, as this condition spontaneously recovers in a sub-population of patients with NBPP [45]. Understanding the time course of recovery may inform the timing of surgical repair treatments.

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HIGHLIGHTS

- We investigated the functional consequences of muscle unloading on developing murine tendon-to-bone (i.e., enthesis) attachments.
- Biomechanical function was diminished in unloaded entheses compared to controls.
- The reduced biomechanical function may be partially attributed to decreased collagen fiber alignment and changes in mineral content and composition.

Figure 1.

Supraspinatus tendon enthesis structural properties were compromised by BtxA-muscle unloading throughout postnatal development. A) Representative force displacement curves. B) Maximum force was decreased in the BtxA-unloaded group compared to normal age-matched controls at P28 and P56 and saline contralateral controls at P56. Maximum force was increased in P56 animals compared to P28 animals. C) Tendon enthesis stiffness was reduced in the BtxA-unloaded group compared to normal controls at both P28 and P56 and relative to saline contralateral controls at P56. Stiffness increased with increasing animal age. [* $p < 0.05$].

Figure 2.

Supraspinatus tendon enthesis mechanical property values were compromised by muscle unloading with BtxA throughout postnatal development. A) Tendon enthesis strength was reduced in the BtxA-unloaded group relative to normal controls at P28 and P56 and relative to saline controls at P56. Strength increased with increasing animal age. B) The elastic modulus was reduced in the BtxA-unloaded group relative to normal and saline controls at P28 and recovered by P56. [* p<0.05].

Figure 3.

Tendon enthesis collagen fiber alignment was affected by muscle unloading throughout postnatal development. A) The angular deviation of collagen fiber angles within the enthesis of BtxA-unloaded shoulders was increased (i.e., the fibers were less organized) at P28 and P56 BtxA relative to loaded controls. Angular deviation decreased with increasing animal age. [* $p < 0.05$]. B-D) Representative histograms of the collagen fiber angles, as determined by polarized light microscopy for P56 entheses (B: normal, C: saline, and D: BtxA-unloaded). E-G) Representative picrosirius red stained sections are shown, as viewed under polarized light. Local collagen fiber mean angles are indicated by the colored dots (E:

normal, F: saline, and G: BtxA-unloaded, corresponding to the respective histograms shown in B, D, and E). [Scale bar = 50 μ m].

Figure 4.

Raman microprobe analysis of mineralized fibrocartilage within the enthesis of BtxA-unloaded shoulders. A) The relative mineral content determined by the ratio of the 960 Δcm^{-1} peak (phosphate ν_1) representing mineral and the 1003 Δcm^{-1} peak (phenyl group) representing collagen was not significantly changed by muscle unloading. B) The amount of carbonate substituted for phosphate in the hydroxylapatite mineral phase was increased at P56 by BtxA unloading relative to normal controls. Carbonate substitution increased with increasing animal age. C) The crystallinity (i.e., relative atomic order) of the hydroxylapatite mineral was decreased (i.e., FWHM was increased) at P56 in BtxA-unloaded mineralized fibrocartilage compared to loaded controls. [* indicates $p < 0.05$]. D-F) Example von Kossa and Toluidine blue stained mineralized sections from D) Normal, E) Saline, and F) BtxA shoulders. [Scale bar = 50 μm].

Cross-sectional area and biomechanical properties of supraspinatus tendon entheses subjected to BtxA muscle unloading at postnatal days 28 and 56.

Table 1

Group	Area (mm ²) ^{&}	Yield Stress (MPa) ^{&}	Energy to Yield (MPa)	Yield Strain	Toughness (MPa)	% Relax
P28 Normal	0.24 ± 0.06	9.27 ± 3.48 [#]	0.84 ± 0.45 [#]	0.18 ± 0.03	4.02 ± 2.80 [#]	
P28 Saline	0.19 ± 0.05	5.25 ± 1.80 [*]	0.49 ± 0.19 [*]	0.22 ± 0.07	2.02 ± 0.69 [*]	
P28 BtxA	0.18 ± 0.05	1.82 ± 1.08 ^{**}	0.15 ± 0.13 ^{**}	0.14 ± 0.06	0.68 ± 0.41 [*]	
P56 Normal	0.28 ± 0.07	9.15 ± 2.79	0.74 ± 0.33	0.21 ± 0.07	3.43 ± 1.81	51.1 ± 7.6
P56 Saline	0.26 ± 0.03	8.56 ± 2.32	0.63 ± 0.21	0.17 ± 0.05	2.02 ± 0.61	51.8 ± 12.9
P56 BtxA	0.24 ± 0.04	4.03 ± 1.16 ^{**}	0.22 ± 0.09 ^{**}	0.13 ± 0.04	1.39 ± 0.80	61.8 ± 8.7

* p<0.05 relative to age-matched normal

[#] p<0.05 relative to age-matched saline

[&] significant effect of age