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Recapitulation of the Achilles tendon mechanical properties during neonatal development: a study of differential healing during two stages of development in a mouse model

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Abstract

During neonatal development, tendons undergo a well orchestrated process whereby extensive structural and compositional changes occur in synchrony to produce a normal tissue. Conversely, during the repair response to injury, structural and compositional changes occur, but a mechanically inferior tendon is produced. As a result, developmental processes have been postulated as a potential paradigm through which improved adult tissue healing may occur. By examining injury at distinctly different stages of development, vital information can be obtained into the structure-function relationships in tendon. The mouse is an intriguing developmental model due to the availability of assays and genetically altered animals. However, it has not previously been used for mechanical analysis of healing tendon due to the small size and fragile nature of neonatal tendons. The objective of this study was to evaluate the differential healing response in tendon at two distinct stages of development through mechanical, compositional, and structural properties. To accomplish this, a new *in vivo* surgical model and mechanical analysis method for the neonatal mouse Achilles tendons were developed. We demonstrated that injury during early development has an accelerated healing response when compared to injury during late development. This accelerated healing model can be used in future mechanistic studies to elucidate the method for improved adult tendon healing.

Keywords

Development; tendon; mechanics; healing; injury

INTRODUCTION

Tendon injuries are a particular concern because of scar formation. Scar is created when tendons undergo a reparative process [1] after injury that includes inflammation, extracellular matrix production, and tissue remodeling. Prior to the production of the extracellular matrix, fibroblasts become rounded and proliferate. Subsequent to fibroblast proliferation, increased extracellular matrix production then alters the composition of the tendon from its uninjured state. For example, biglycan levels increase and decorin levels decrease [17, 18]. At this stage, not only is the composition altered but also the structure.

For instance, the fibril diameter size distribution is narrowed and consists mainly of small diameter fibrils [16]. Extracellular matrix is also laid down in a disorganized fashion, altering the parallel alignment of the fibers [28]. During remodeling, the final stage, collagen fibrils begin to coalesce laterally and fibers reorganize along the long axis of the tendon. While scar can return some function to the tendon over time, the tendon rarely regains its original functionality or mechanical properties [2].

Unlike adult reparative healing, fetal tendons heal through a regenerative process [3] which is characterized by an absence of scar [4]. It is believed that the regenerative response is a property of the tissue and not the environment [4]. Defining and utilizing the mechanisms involved in fetal healing could lead to therapies for improved adult healing; however, there are fundamental differences between adult and fetal healing which cannot easily be overcome. A new model system needs to be developed that not only parallels adult healing but also demonstrates improved healing parameters.

It has been hypothesized that healing parameters change from the fetal stage, through development, and into adulthood [5]. For example, neonatal developing tendons may heal through scar formation but regain normal composition, structure, and function faster than healing adult tendons. If this hypothesis is true, tendon healing during neonatal development may be a beneficial model system to study improved adult healing. Few studies have been able to develop neonatal injury models due to the small size and fragile nature of developing tendons [6, 7]. A greater understanding of healing at different stages of neonatal development will provide guidance to develop new therapeutic strategies to improve the outcome of adult tendon healing.

The objective of this study is to develop and quantitatively characterize a mouse model of neonatal tendon healing at two stages of development in the Achilles tendon. The following parameters will be quantified at two time points post injury: collagen content, biglycan and decorin mRNA expression, collagen fibril parameters, collagen organization, cell shape, cell number, and elastic and viscoelastic mechanical properties. We hypothesized that there will be a differential healing response between early and late neonatal development. Specifically, we hypothesized that maximum stress, modulus, and decorin levels will be significantly higher in early developmental injury when compared to late developmental injury (injured tendon normalized by contralateral uninjured tendon). We also hypothesized that collagen content, fibril diameter spread, and percent relaxation will be significantly higher at late stages of healing in early developmental injury when compared to late developmental injury (injured tendon normalized by contralateral uninjured tendon).

MATERIALS AND METHODS

Animals

This study was an IACUC approved study. Neonatal mice in a C57BL/6 background were bred in-house. All litters were weaned at birth to 6 pups to reduce variance from litter size [8]. Care was taken to ensure that mice from the same litter were not used in the same assay.

Surgical Model

288 neonatal mice at 7 (7d) and 21 (21d) days old (144 mice /age) were anesthetized. Mice were placed ventral side down, a skin incision was made, then a plastic coated blade was placed underneath the Achilles tendon and gripped by a hemostat for support (Figure 1). A 0.3mm diameter biopsy punch (Shoney Scientific, Waukesha, WI) was used to create a central, full thickness, partial width (~50%), excisional injury in the Achilles tendon, approximately 2mm proximal from the calcaneus. While no suture repair was performed on the Achilles tendon, a single suture closed the skin. The contralateral Achilles tendon

remained uninjured to serve as an internal control of developmental changes. Mice resumed normal cage activity until euthanized at 3 or 10 days post injury (Total of 4 groups: 7d-3days post, 7d-10 days post, 21d-3 days post, 21d-10 days post).

A pilot study was conducted to determine if the uninjured contralateral tendon was altered due to compensation. To conduct this study, eight 7d mice underwent unilateral injury (contralateral uninjured) while four 7d had no surgery performed on either tendon. All mice were sacrificed at 17 days of age (10 days post injury). All uninjured tendons were then mechanically tested (contralateral uninjured and no surgery uninjured). Mechanical parameters from uninjured contralateral tendons in injured mice were compared to both AT in uninjured mice through a t-test. No differences were seen between the two groups in any of the mechanical parameters (data not shown). Therefore, the uninjured contralateral tendon in this model can be considered normal for the parameters measured.

Mechanical Analysis

To determine the mechanical properties of the neonatal mouse Achilles tendon, both Achilles tendons (injured and uninjured) from each of 48 mice (12/group) were carefully dissected out under a dissection scope. The tendons were cleaned of excess tissue leaving only the calcaneus. Tendon cross-sectional area (mm^2) was measured using a laser-based device [9]. Tendons were dumbbell stamped to 0.3mm width with a custom device to remove the uninjured fibers on either side of the central injury. The cross-sectional area was measured again for material property calculations. Stain lines were placed to measure strain optically, 1.5 mm or 2 mm apart for Achilles tendons injured at 7d or 21d respectively. Sandpaper was glued to the calcaneus and myotendinous end of the Achilles tendon, 3 mm or 4 mm apart for Achilles tendons injured at 7d or 21d respectively. Due to the fragile nature of neonatal Achilles tendon, custom fixtures were designed to ensure the Achilles tendons remained unloaded during handling[7] (Instron 5543, Instron Corp., Canton, MA). Each Achilles tendon underwent the following protocol while immersed in a 37°C PBS bath (Figure 2)—preloaded, preconditioned for 10 cycles (0.01N-0.02N or 0.02N-0.04N for Achilles tendons injured at 7d or 21d respectively) at a rate of 0.1%/s, and held for 300s. Immediately following, stress relaxation was performed to a strain of 5% at a rate of 5%/s, followed by a relaxation for 600s, returned to pre-stress relaxation elongation for 60s and finally, a ramp to failure at 0.1%/s. A 10N Instron load cell was used for all tests and the preloads were well within the resolution of the load cell which was 0.0002N. Local tissue strain was measured optically. Mechanical parameters (maximum stress (MPa), modulus (MPa), percent relaxation (%)) were then normalized within an individual mouse by dividing the value from the injured Achilles tendon by the value from the uninjured Achilles tendon. This normalization was performed to isolate changes in healing from changes in development.

Hydroxyproline Analysis

For analysis of total collagen content, both Achilles tendons from 48 mice (12/group) were dissected and the length of tendon corresponding to the grip-to-grip gauge length was isolated. Standard procedure was followed for analysis of *o*-Hydroxy-proline (OHP), a measure of collagen content (ug collagen/mg dry tissue) [10]. Collagen content was normalized to the dry weight of the individual samples and then for developmental changes within each mouse as described.

Proteoglycan Gene Expression

For analysis of biglycan and decorin mRNA expression, both Achilles tendons from 48 mice (12/group) were dissected and the length of tendon corresponding to the grip-to-grip gauge length was isolated. Individual Achilles tendons were mechanically homogenized with a

mortar and pestle in RNase-free conditions. RNA was extracted using the TRIZOL (Invitrogen, Carlsbad, CA) and RNeasy Mini Kit (Qiagen Inc., Valencia, CA). cDNA was produced by reverse transcription-polymerase chain reaction (RT-PCR) and quantitative polymerase chain reaction (Q-PCR) with a SYBR Green PCR Master Mix (Applied Biosystems) was conducted with primers specific for GAPDH, biglycan and decorin [11]. Each sample was assayed in triplicate. The relative quantity of mRNA for each gene of interest was computed using a six-fold dilution standard curve [12]. Biglycan and decorin were normalized by the GAPDH value within the same tendon (Biglycan/GAPDH and Decorin/GAPDH). All parameters were then normalized for development within each mouse.

Histological Analysis

Hind limbs from both Achilles tendons from each of 48 mice (12/group) were evaluated histologically. Standard histological procedures were employed including paraffin embedding, sectioning, and staining with hematoxylin-eosin. Sections were then graded for cell density, cell shape, and collagen fiber organization on a nonparametric scale [4] of 1-4 where 1 represented low cell density, spindle cell shape and organized fibers and 4 represented high cell density, rounded cell shape, and disorganized collagen fibers. The histologic analysis was performed independently by 3 masked graders. The overall median and interquartile range for both the injured and uninjured Achilles tendons within each group were then determined.

Fibril Diameter Parameters

Both Achilles tendons from 48 mice (12/group) were analyzed by transmission electron microscopy [13]. Briefly, the Achilles tendons were dissected out with the calcaneus and muscle intact, fixed, dehydrated, and infiltrated. Thin sections (80 nm) were cut and post-stained. The Achilles tendons were examined at 80 kV using a JEOL 1400 transmission electron microscope equipped with an Orius wide field side mount CCD camera. The injury was visualized and micrographs were taken at 60,000x from non-overlapping regions in a predetermined grid. Six unique regions on each micrograph were sampled for injured Achilles tendons and four for uninjured Achilles tendons. Fibril diameters were measured using a RM Biometrics-Bioquant Image Analysis System (Nashville, TN). The fibril diameter mean (nm) and standard deviation (nm) were determined and then normalized for development within each mouse.

Statistical Analysis

Each quantitative parameter was measured and normalized within each mouse by the contralateral Achilles tendon (injured/uninjured) as described. The mean +/- the standard deviation was reported unless otherwise stated. A two-way ANOVA with Fisher's LSD post hoc was performed across age at injury and days post injury for each quantitative parameter. For all statistical tests, significance was set at less than or equal to 0.05 ($p \leq 0.05$) and a trend was set at less than or equal to 0.1 ($p \leq 0.1$).

RESULTS

Mechanical Properties

All comparisons were made between normalized values unless otherwise stated. In Achilles tendons injured at 7d, the normalized cross-sectional area at 10 days post injury was significantly smaller ($p \leq 0.001$) than at 3 days post injury (Figure 3). In contrast, Achilles tendons injured at 21d showed a trend ($p=0.1$) towards increased cross-sectional area at 10 days post injury when compared to 3 days post injury. When comparing across 3 days post

injury, the Achilles tendons injured at 7d were significantly larger ($p \leq 0.001$) compared to those injured at 21d. For viscoelasticity, the only difference observed was a trend ($p = 0.1$) at 3 days post injury towards increased percent relaxation in the Achilles tendons injured at 21d when compared to those injured at 7d (Figure 3). The maximum stress and modulus were significantly greater ($p = 0.05$ and $p = 0.002$ respectively) in Achilles tendons injured at 21d when compared to 7d at 3 days post injury. However, maximum stress and modulus were significantly lower ($p \leq 0.001$ for both) in Achilles tendons injured at 21d when compared to 7d at 10 days post injury. In the Achilles tendons injured at 7d, the maximum stress and modulus were significantly increased ($p \leq 0.001$ and $p \leq 0.001$) at 10 days post injury compared to 3 days post injury. In Achilles tendons injured at 21d, the maximum stress was trended toward a decrease ($p = 0.09$) and modulus was significant decreased ($p = 0.04$) at 10 days post injury compared to 3 days post injury.

Collagen Content

Collagen content, as measured by hydroxyproline content, showed no significant differences across age at injury or days post injury (Figure 4).

Proteoglycan Expression

Normalized biglycan and decorin expression were significantly increased ($p \leq 0.001$ and $p = 0.002$ respectively) in Achilles tendons injured at 21d between 3 and 10 days post injury. There was a significant increase in normalized biglycan ($p \leq 0.001$) and decorin ($p \leq 0.001$) expression between Achilles tendons injured at 7d and 21d at 10 days post injury (Figure 5).

Histological Analysis

There was a marked increase in disorganization, rounded cell shape, and number from 3 to 10 days post injury in Achilles tendons injured at 21d (Figure 6). No appreciable differences were observed at 3 days post injury or in tendons injured at 7d.

Fibril Diameter Parameters

When examining injured Achilles tendons compared to uninjured, a shift towards smaller fibrils was observed at 10 days post injury (Table 1; Figure 7). Both fibril diameter mean and standard deviation were significantly decreased ($p \leq 0.001$ and $p \leq 0.001$ respectively) at 10 days post injury when compared to 3 days post injury in Achilles tendons injured at 7d. In Achilles tendons injured at 21d, fibril diameter mean was significantly decreased ($p = 0.03$) between 3 and 10 days post injury whereas the standard deviation trended ($p = 0.1$) towards a decrease. When comparing across age at injury, the mean trended ($p = 0.08$) towards a decrease at 3 days post injury between Achilles tendons injured at 7d and 21d. In contrast, both mean and standard deviation were significantly increased ($p \leq 0.001$ and $p \leq 0.001$ respectively) at 10 days post injury in Achilles tendons injured at 21d when compared to those at 7d.

DISCUSSION

The goal of this study was to characterize healing during two distinct stages of neonatal tendon development in a mouse Achilles tendon. Overall, it was demonstrated that Achilles tendons injured during early neonatal development (7d) had an accelerated healing response when compared to those injured during late neonatal development (21d). When injured during early development (7d) there was an initial decrease (3 days post injury) in mechanical properties followed by a quick return to function after only 10 days. These changes were accompanied with only a few compositional and structural changes, specifically cross-sectional area, and fibril diameter mean and standard deviation decreased.

In addition, during early developmental injury cells were numerous and rounded, a characteristic which has often been observed during both development and healing when fibroblasts produce extracellular matrix. These results are counter to previous studies in adult tendons that show little to no mechanical improvement, particularly not by 10 days post injury [14].

In the current study, when Achilles tendons were injured during late development (21d) there was initially (3 days post injury) little to no change from uninjured tendons. However, when examined at 10 days post injury, almost all measured parameters were altered. The changes observed during late developmental healing are indicative of a normal adult reparative healing response which includes an initial delay (3 days post injury) in cellular proliferation and extracellular matrix production [15]. Taken together, these data indicate that Achilles tendons injured during early development may follow an accelerated healing process over those injured during late development.

Changes in composition, structure, and mechanical parameters during healing in adult tendons have been extensively studied previously. This knowledge is important because one must understand the normal healing process to improve upon it effectively. However, new models of accelerated or improved tendon healing could facilitate the discovery of natural mechanisms that can be implemented to improve adult healing. For instance, the scarless healing model from fetal tendons; however, fetal tendons have inherent differences from adult tendons such as the presence of amniotic fluid and limited mechanical stresses. These inherent differences complicate the use of fetal development as a paradigm through which to study adult healing [4]. Alternatively, neonatal tendons are more similar to adult tendons but also demonstrate changes in composition, structure, and mechanics that are similar to healing [7]. Importantly, the current study demonstrated for the first time a return to normal tendon mechanics after only 10 days post injury, without treatment. This healing neonatal mouse tendon model provides a new paradigm through which to study improved healing. The mouse model has historically presented logistical complications due to the small size and fragile nature of the neonatal tendons; however, this study utilizes consistent, reproducible methods to study tendon neonatal healing. A mouse model can be used further to investigate the differential mechanisms through genetically modified mice and commercially available assays.

The fibril diameter parameters were two of the main differential responses between early and late developmental healing in the current study. Fibrils are formed through fibrillogenesis, a regulated multi-step process. During normal adult tendon healing, fibril diameter mean and spread both decrease and do not return to normal even after an extended time post injury. These changes were accompanied by reduced mechanics [16]. Interestingly, a shift to smaller diameter fibrils in both early and late developmental healing was observed; however, the shift was more pronounced during early development when the mechanics had been restored. The differential effect of small diameter fibrils being associated with both a decrease and an increase in mechanics in this study could be due to mechanisms not examined here.

Proteoglycan expression has been associated with many processes including normal tendon development and fibrosis. During reparative adult healing, biglycan levels are elevated and remain so [17] but decorin expression either does not change [18] or decreases [17]. In the current study, when tendons were injured during early development, proteoglycan expression did not change drastically from normal development. It is possible that despite the presence of an injury, the tendon progresses through normal developmental stages of extracellular matrix deposition. However, during late developmental healing, there was an increased expression of both decorin and biglycan, which is contrary to studies in adults

[11]. Therefore, there may also be mechanisms at play in late developmental healing that are different than those in adult injury. Based on these results, decreasing proteoglycan expression in adult tendon healing may lead to accelerated healing.

The direct role of proteoglycans in tensile and viscoelastic properties is relatively unknown in tendon. It has been hypothesized that proteoglycans have a structure-function role through interconnecting neighboring fibrils and force transfer [19 - 21]. Tendons from a decorin knock out mouse [22] and tendons that had decorin blocked during ligament healing [23], both demonstrated increased mechanical properties. These results are consistent with the current study. Alternatively, flexor digitorum longus biglycan knockout tendons had a significantly lower modulus and maximum stress [22], which is contrary to results presented here. However, the overall quantity of biglycan in tendons older than 7 days old is much less than that of decorin; therefore, the effect of increased decorin may overshadow the effect of biglycan.

Cellular response to injury is an important factor in tendon healing. Reparative healing in adult tendons may be the inability of adult fibroblasts to recapitulate neonatal fibrillogenesis. However, a recent study demonstrated the initiation of developmental fibrillogenesis by adult human tendon fibroblasts when cultured under tension [24]. This suggests that the environment in developing and mature tendon determines mechanisms of fibril synthesis, deposition and alignment [25]. The current study demonstrated, during both normal and healing early development, increased cell number and rounded cell shape, both are which are indicative of extracellular matrix production. Therefore, in conjunction with the current study, this indicates that adult tendon healing may be improved if an early developmental neonatal environment is recreated.

During scarless fetal healing, there is a lack of inflammatory response that many have hypothesized could explain scarless healing [3]. Similarly, the immune system in neonates is not fully mature and different cell subsets are seen in the immune response [26]. A permissive environment may be created in neonates for growth and development and could help explain the results of the current study. In fact, previous work has shown that although healing follows the same trajectory in neonates and adults, wounds in neonates heal faster. Fibroblasts are present in greater numbers, collagen and elastin are more rapidly produced, and granulation tissue forms more quickly [27]. Future work in the presented model will include an investigation into the influx of inflammatory cells and the expression of pro-inflammatory and anti-inflammatory cytokines, which will provide further insight into the mechanisms observed in this study.

In summary, two different models of healing during neonatal development, early and late, in a mouse model were characterized. Overall, it was demonstrated that Achilles tendons injured during early development (7d) had an accelerated healing response when compared to those injured during late development (21d). During early development, a decrease in mechanical parameters and an increase in cross-sectional area were seen without changes in composition and structure at 3 days post injury. By 10 days post injury, the mechanical properties had returned to almost normal levels and the only other changes were the fibril diameter parameters which had become smaller with a decreased spread. On the other hand, in Achilles tendons injured during late development, few changes were seen in mechanical, compositional and structural parameters at 3 days post injury. At 10 days post injury however, a reduction in mechanical properties was observed along with significant changes in composition and structure. Taken together, these observations indicate that healing during development either has unique mechanisms or an accelerated healing process. Early developmental healing provides a model system where mechanical properties are quickly recapitulated and can be used to investigate new methods for improved adult healing.

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Figure 1.

A) Photograph of standard surgical procedure on a 7 days old mouse Achilles tendon. The plastic backing is placed beneath the neonatal Achilles tendon and gripped with the hemostat. A 0.3mm biopsy punch is then used to create a central full-thickness, partial width injury in the Achilles tendon. B) Graphical representation of the surgical procedure.

Figure 2.

Graphical representation of a typical testing protocol for neonatal mouse AT. The protocol includes a preload, precycle, stress relaxation and constant ramp to failure.

Figure 3.

A) A return to normal cross-sectional area was observed in early developmental injury (7d) but a trend towards increased cross-sectional area was observed in late developmental injury (21). B) Few changes were seen in percent relaxation when comparing both across age at injury and at time post injury. C and D) In early developmental healing (7d), maximum stress and modulus increased between 3 and 10 days post injury, returning to normal by 10 days post injury. However, both mechanical parameters decreased between 3 and 10 days post injury in late developmental injury (21d). The horizontal line at 1 indicates normal development. Error bars represent standard deviation. *: $p \leq 0.05$; #: $p \leq 0.1$

Figure 4. Collagen content showed no significant changes across any group. The horizontal line at 1 indicates normal development. Error bars represent standard deviation.

Figure 5.

A and B) Significantly increased proteoglycan expression was observed between 3 and 10 days post injury in late developmental injury (21d), but not during early developmental injury (7d). Significantly increased proteoglycan expression was also observed between early and late developmental injury at 10 days post injury. Error bars represent standard deviation. *: $p \leq 0.05$; #: $p \leq 0.1$

Figure 6.

Median and interquartile range of histological parameters fiber organization, cell shape and cell number. Achilles tendon injured at 21 days old show marked increase in disorganization, rounded cell shape and cell density whereas Achilles tendon injured at 7 days do not. Inj = Injured; Un = Uninjured.

Figure 7.

Representative transmission electron micrographs for each experimental group, both uninjured and injured. Histograms of the frequency of fibril diameter size of uninjured and injured Achilles tendon during neonatal development. A shift towards small diameter fibrils and decreased spread can be seen in both the micrographs and histograms in Achilles tendon at 10 days post injury for both ages. This shift is more pronounced for Achilles tendons injured at 7d.

Table 1

Fibril diameter mean and standard deviation for each group (mean +/- stdev).

Age at Injury	Days post Injury	Mean (nm/nm)	Standard Deviation (nm/nm)
7	3	1.01 ± 0.14^{ad}	1.04 ± 0.13^a
7	10	0.52 ± 0.13^c	0.41 ± 0.14^c
21	3	0.91 ± 0.09^a	0.97 ± 0.12^b
21	10	0.83 ± 0.10	0.88 ± 0.13

^a significantly different than 10 days post injury

^b trend showing differences from 10 days post injury

^c significantly different than injury at 21 days old (at same time point post injury)

^d trend showing differences from injury at 21 days old (at same time point post injury).