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Mechanical property changes during neonatal development and healing using a multiple regression model

Heather L. Ansorge [a], Sheila Adams [b], Abbas F. Jawad [c], David E. Birk [b], Louis J. Soslowsky [a,*]

[a] McKay Orthopaedic Research Laboratory, University of Pennsylvania, 424 Stemmler Hall, 3450 Hamilton Walk, Philadelphia, Pennsylvania 19104-6081, United States
[b] Department of Pathology and Cell Biology, College of Medicine, University of South Florida, Tampa, Florida 33612, United States
[c] Division of Biostatistics, Department of Pediatrics, University of Pennsylvania, Philadelphia, Pennsylvania 19104, United States

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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 for elucidation of mechanistic insight required to develop treatment modalities to improve adult tissue healing. The objective of this study was to compare and contrast normal development with injury during early and late developmental healing. Using backwards multiple linear regressions, quantitative and objective information was obtained into the structure–function relationships in tendon. Specifically, proteoglycans were shown to be significant predictors of modulus during early developmental healing but not during late developmental healing or normal development. Multiple independent parameters predicted percent relaxation during normal development, however, only biglycan and fibril diameter parameters predicted percent relaxation during early developmental healing. Lastly, multiple differential predictors were observed between early development and early developmental healing; however, no differential predictors were observed between late development and late developmental healing. This study presents a model through which objective analysis of how compositional and structural parameters that affect the development of mechanical parameters can be quantitatively measured. In addition, information from this study can be used to develop new treatment and therapies through which improved adult tendon healing can be obtained.

1. Introduction

Tendons are complex tissues whose mechanical behavior depends on their structure, composition, and location within the body. It has long been hypothesized that the structure and composition of tendons are a result of the specific function tendons perform (Parry et al., 1978). Tendon is composed mainly of type I collagen organized into aligned fibrils and fibers. It is believed that these aligned fibers bear the tensile loads tendons experience in vivo. Proteoglycans are minor constituents in tendon and it has been hypothesized that they also play a significant role in mechanics (Danielson et al., 1997; Robinson et al., 2005). Specifically, proteoglycans play a role in resisting compression (Arokoski et al., 2000) but they may also bridge the gap between neighboring fibers, providing shear resistance within the tendon. However, the removal of dermatan sulfate did not have any effect on shear or tensile mechanics (Lujan et al., 2007). Based on these facts and assumptions, previous studies have used numerous methods to measure compositional and structural parameters of tendon to correlate them to mechanical behavior.

Previous studies have provided insight into the complex interplay of a tendon's constituents, which is critical to formalizing structure–function relationships (Andarawis-Puri et al., 2010; Derwin et al., 1999; Goh et al., 2008; Robinson et al., 2004). For example, collagen fibril cross-sectional area fraction explained more than half the changes in mechanical properties of mature tendons through linear regression (Goh et al., 2008). Interestingly, the addition of data from younger tendons improved the regression model, thereby supporting the need for further structure–function analyses of developing tendons. While these studies have made significant contributions to understanding the structure–function relationships in tendon, there are still areas that require further investigation. For example, one previous study found significant correlations between compositional and structural parameters with mechanics; however, the models accounted for only 50–70% of the variance (Robinson et al., 2004).

* Corresponding author. Tel.: +1 215 898 8653; fax: +1 215 573 2133.
E-mail address: soslowsk@upenn.edu (L.J. Soslowsky).

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Therefore, studies using new experimental techniques or different independent parameters may better predict mechanics.

While many studies have demonstrated that fibril diameter size is correlated with tendon mechanics in normal tendons, similar relationships have not been shown in healing tendons. A study in the healing MCL showed that the mean fibril diameter did not change up to 104 weeks post injury; however, there was an increase in the spread of the fibril diameters (Frank et al., 1997). This finding indicates fibril diameter mean and standard deviation are important parameters to investigate during tendon healing.

Regression models provide a statistical relationship between a dependent (response) variable and independent (predictor) variables. By regressing independent compositional and structural parameters against dependent mechanical parameters, the level of contribution of each of the parameters on mechanical properties can be determined while simultaneously incorporating the effect of the presence of all the independent parameters. Therefore, the objective of this study is to use rigorous statistical models to objectively and quantitatively compare and contrast not only normal development but also healing during early and late development. This study will determine which parameters are beneficial or detrimental to mechanical properties. Regression will be conducted on the independent parameters collagen content, biglycan and decorin mRNA content and fibril diameter average and standard deviation against the dependent mechanical parameters percent relaxation, maximum stress and elastic modulus.

2. Methods and materials

2.1. Data collection

The experimental methods used for this study have been previously published Ansorge et al., 2012). Briefly, Achilles tendon in neonatal mice were unilaterally injured at both 7 and 21 day old and allowed to heal for 3 or 10 day post injury. The following parameters were quantified: collagen content by hydroxyproline analysis, biglycan and decorin mRNA content by quantitative real time PCR, fibril diameter average and standard deviation by transmission electron microscopy and mechanical parameters percent relaxation, maximum stress and elastic modulus by tensile testing with local tissue strain measured optically. These measurements were taken for both the injured and contralateral uninjured Achilles tendon for each mouse. Imputation was conducted using the regression method (Zantop et al., 2006) where missing values were observed in a specimens. Lastly, the data was examined for outliers and each group was found to have one specimens as an outlier. Outliers were defined as being at least two standard deviations from the mean and having corroborating experimental evidence of some experimental difficulty (for instance, an observation during experimentation that there may have been a dissection/specimen preparation error). Therefore, each group had a total of 11 specimens.

To determine the sample size for multiple regression analysis, an *a priori* power analysis was performed based on 5 predictors (total collagen content, biglycan and decorin mRNA content, fibril diameter average and standard deviation), an alpha set at 0.05, an anticipated *R*-squared of 0.8 and a desired statistical power of 0.8. The R^2 was chosen assuming the current study would predict mechanical parameters better than a previous study done in our laboratory with an R^2 between 0.5 and 0.7. Calculating the sample size based on these expectations (Soper, 2008), an *n* of eleven is needed for each group. An *n* of 12 was used to account for experimental error due to the small size and fragile nature of developmental tendons.

Multiple regression analysis assumes that each dependent and independent parameter is obtained from a single specimen. It was assumed that a litter was a single specimen (Festing, 2006). All litters were weaned to 6 pups to account for experimental error associated with variations in litter size. For each specimen, four distinct and reproducible injuries (along with uninjured contralateral tendons) are needed, one for each of the following assays: biomechanics (percent relaxation, maximum stress, and elastic modulus), transmission electron microscopy (fibril diameter average and standard deviation), biochemistry (collagen content) and QPCR (biglycan and decorin mRNA expression).

2.1.1. General linear model (GLM)

Summary statistics of all variables were examined and described by mean, median, standard deviation, minimum and maximum to ensure that assumptions for linear analysis were met. Pearson's correlations were conducted between all independent parameters to determine correlations. A general linear model (GLM) was run to determine if the dependent variables (percent relaxation, maximum stress and elastic modulus) were significantly related to the independent parameters: collagen content (Col), biglycan (Big) and decorin (Dec) mRNA content, fibril diameter average (Ave) and standard deviation (StDev) and categorical parameters and interactions (age at injury (Age), days post injury (Days), injured or uninjured (Injury)). For all specimens, equations for percent relaxation, stress and modulus were calculated for the injured and uninjured tendon. This resulted in a total of 96 *y*-values inputted in the GLM for Eq. (1), where Y_1–Y_3 were percent relaxation, stress and modulus:

$$E\{Y_i\} = \beta_0 + \beta_1 Col + \beta_2 Big + \beta_3 Dec + \beta_4 Ave + \beta_5 StDev + \beta_6 Age + \beta_7\ Days + \beta_8\ Injury + \beta_9(Age \times Days) + \beta_{10}(Age \times Injury) + \beta_{11}(Days \times Injury) + \beta_{12}(Age \times Days \times Injury) \quad (1)$$

Upon determining that there are significant effects from the independent parameters, an analysis of how the dependent parameters vary with the independent parameters can be conducted.

2.1.2. Model refinement

Upon determining which main categorical effects and interactions were significant, a backward regression analysis for the entire model was performed to find the "best" predictors of the dependent parameters ($n=96$) (Eq. (2)). It was initially assumed the categorical parameters would have statistically significant interactions. Based on this assumption, the initial study design separated experimental groups for regression analysis. The following analysis was conducted to confirm this assumption; however, the *a priori* power analysis based on 0.05 for sample size was done for only 5 independent parameters. Since the number of independent parameters in Eq. (2) is larger than 5, the level of significance was set to 0.1 for this analysis. Therefore, a *p*-value of 0.10 and a tolerance of 0.001 was used due to the larger number of independent variables being regressed against the dependent variables, which decreases the power. This analysis gives an overall understanding of how the mechanical parameters vary within the framework of the entire study. If there are significant effects of the categorical parameters, then the data can be broken down into experimental groups for further analysis by multiple regressions

$$\begin{aligned} E\{Y_i\} = {} & \beta_0 + \beta_1 Col + \beta_2 Big + \beta_3 Dec + \beta_4 Ave + \beta_5 StDev + \beta_6 Age + \beta_7 Injury \\ & + \beta_8(Age \times Days) + \beta_9(Days \times Injury) + \beta_{10}(Age \times Days \times Injury) \\ & + \beta_{11}(Col \times Age) + \beta_{12}(Big \times Age) + \beta_{13}(Dec \times Age) + \beta_{14}(Ave \times Age) \\ & + \beta_{15}(StDev \times Age) + \beta_{16}(Col \times Injury) + \beta_{17}(Big \times Injury) \\ & + \beta_{18}(Dec \times Injury) + \beta_{19}(Ave \times Injury) + \beta_{20}(StDev \times Injury) \\ & + \beta_{21}(Col \times Age \times Days) + \beta_{22}(Big \times Age \times Days) + \beta_{23}(Dec \times Age \times Days) \\ & + \beta_{24}(Ave \times Age \times Days) + \beta_{25}(StDev \times Age \times Days) \\ & + \beta_{26}(Col \times Injury \times Days) + \beta_{27}(Big \times Injury \times Days) \\ & + \beta_{28}(Dec \times Injury \times Days) + \beta_{29}(Ave \times Injury \times Days) \\ & + \beta_{30}(StDev \times Injury \times Days) + \beta_{31}(Col \times Age \times Days \times Injury) \\ & + \beta_{32}(Big \times Age \times Days \times Injury) + \beta_{33}(Dec \times Age \times Days \times Injury) \\ & + \beta_{34}(Ave \times Age \times Days \times Injury) + \beta_{35}(StDev \times Age \times Days \times Injury). \quad (2) \end{aligned}$$

2.2. Final multiple regression models

Due to the high number of categorical interaction terms, the data for final analysis was split into experimental groups. Analysis of the equations for the entire model (above) confirmed that this is an appropriate refinement for the data. The final multiple linear regression models quantified the relationship between the dependent variables (percent relaxation, stress and modulus) and the continuous independent variables (collagen content, biglycan and decorin content, fibril diameter average and standard deviation) (Eq. (3)) within each experimental group (Neter, 1996a, b) (Fig. 1). The data was delineated into 8 categories based on age at injury (7 and 21 day old), days post injury (3 and 10 day post injury) and injured or uninjured (*I*, *U*). This resulted in a total of 11 *y*-values inputted in the linear regression for equations 3, where Y_1–Y_3 were percent relaxation, stress and modulus. Backward linear regression was then performed as described in Neter, 1996a, b. Specifically, the *F* to enter was set at 3.3 and *F* to be removed was set at 3.2. The criteria for choosing the appropriate range of *F* to enter and *F* to remove

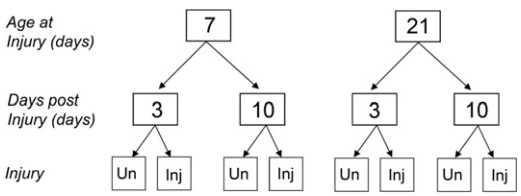

Fig. 1. Schematic demonstrating the 8 groups investigated in the current study. Un=Uninjured; Inj=Injured.