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

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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 (Festing, 2006). It was assumed that a litter was a single specimen. 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:

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

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

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

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
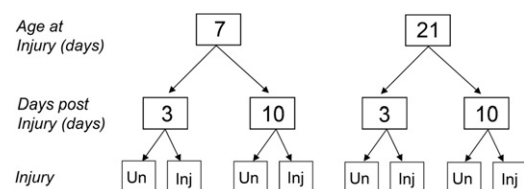

Fig. 1. Schematic demonstrating the 8 groups investigated in the current study. Un=Uninjured; Inj=Injured.

are based on the degrees of freedom in the model. This range for F corresponds to a significance level of 0.1 for a single test. Tolerance is defined as $1-R_k^2$ is the coefficient of multiple determination the X_k is regressed over the other included x variables in the model. The tolerance level was set at 0.01, which means that a variable is excluded if its coefficient of multiple determinations, when it is regressed over the other x-variables, exceeds 0.99. Typical values for tolerance level range between 0.01 and 0.001. This is one measure to prevent entry of variables that are highly correlated with other x-variables. Significance was again set at $p \leq 0.1$, this time due to the small sample size ($n=11$) per regression model and variances within the data

$$Y_i = \beta_0 + \beta_1 Col + \beta_2 Big + \beta_3 Dec + \beta_4 Ave + \beta_5 StDev \qquad (3)$$

Durbin Watson (DW) statistic was calculated to identify the existence of correlations between independent variables. For each equation 3 ($n=11$/group), based on established guidelines for interpretation of DW statistic, a DW value lower than 1.08 indicated correlation between the independent variables (Neter, 1996a, b).

3. Results

3.1. Correlations between the independent parameters

The Pearson correlation coefficients (r) showed some relationships between the independent variables. Correlations were determined between independent variables within each group. Correlations were considered strong for $r < 0.7$, moderate for $0.5 < r < 0.7$ and weak for $r < 0.5$ (Devore, 1991). In the 8 experimental groups examined, fibril diameter average was correlated to fibril diameter standard deviation moderately in 2 groups and strongly in 3 groups. Similarly, decorin and biglycan were moderately correlated in 3 groups and strongly correlated in 3 groups. Finally, collagen content and fibril diameter average were moderately correlated in 3 groups.

3.2. General linear model

A general linear model showed that the interaction terms Days × Age and Days × Age × Injury both had a significant effect on all the mechanical parameters. In addition, injury had a significant effect on stress and modulus. This analysis states that the mechanical parameters are highly dependent on all three categorical parameters (age at injury, days post injury and injury) providing support to analyze this data through regression analysis.

3.3. Whole model analysis—backward stepwise linear regression model

To further understand the relationships present in the entire data set, a set of linear regression models was developed (Eq. (2)). Results of this analysis showed not only predictive effects from the main categorical parameters but also from many of the interactions, including interactions with Age, Days and Injury. Due to the complexity of interpreting these equations and the hypotheses developed prior to initiation of the study, the data was separated into experimental groups (age, days, injury). A clearer understanding of how the structural and compositional parameters affect the mechanics, without the confounding effects of the age, days and injury, will therefore be obtained.

3.4. Grouped analysis—refined backward stepwise linear regression analysis

For this analysis, data was grouped according to age at injury (7, 10 day old), days post injury (3, 10 day post injury) and whether the tendon was injured or uninjured (I, U). This resulted in 8 groups with 3 regression equations for each (percent relaxation, stress and modulus) for a total of 24 equations

Table 1
Multiple regression equations for percent relaxation, maximum stress and modulus with statistically significant predictors. Subscript=age at injury (7 or 21), days post injury (3 or 10), Injured/Uninjured (I/U). Col=Collagen content; Ave=Fibril diameter average; StDev=Fibril diameter standard deviation; Big=Biglycan; Dec=Decorin.

Linear regression equation	Model p	Model R^2
$PercRelax_{7,3,U} = 18.4 - 0.12 \times Col + 0.77 \times Ave$	0.04	0.57
$PercRelax_{7,10,U} = 70.80 - 28.14 \times Dec$	0.07	0.30
$PercRelax_{21,3,U} = 78.00 - 0.06 \times Col$	0.01	0.50
$Stress_{21,3,U} = 47.81 - 0.38 \times Ave$	0.01	0.50
$Modulus_{21,3,U} = 478.26 - 255.52 \times Big - 8.76 \times Ave_4 + 16.62 \times StDev$	0.08	0.60
$PercRelax_{21,10,U} = 136.14 - 0.03 \times Col - 60.84 \times Big - 0.57 \times Ave$	0.05	0.65
$Modulus_{21,10,U} = -39.94 + 3.66 \times Ave_4 - 6.75 \times StDev$	0.03	0.59
$PercRelax_{7,3,I} = 54.14 - 1.05 \times Ave + 2.75 \times StDev$	0.05	0.50
$Modulus_{7,3,I} = 14.65 - 4.62 \times Big + 3.00 \times Dec$	0.06	0.50
$PercRelax_{7,10,I} = 40.54 + 1.25 \times StDev$	0.01	0.57
$Mod_{7,10,I} = 31.7 + 102.2 \times Big - 66.35 \times Dec$	0.00	0.77
$Stress_{21,3,I} = 29.10 - 0.23 \times Ave$	0.06	0.34
$Modulus_{21,3,I} = 370.5 - 3.19 \times Ave$	0.04	0.39

Table 2
Each box demonstrates one of the equations analyzed in the current study for each of 8 groups (Uninjured and Injured, age at injury (7 and 21 day old), days post injury (3 and 10 day)). Independent parameters that are statistically signifcant in each equation are represented by the following abbreviations: C=Collagen content, B=Biglycan, D=Decorin, A=Fibril diameter average, S=Fibril diameter standard deviation.

	Uninjured				Injured			
Age at injury	7 (Early)		21 (Late)		7 (Early)		21 (Late)	
Days post injury	3	10	3	10	3	10	3	10
Perc relax	CA	D	C	CBA	AS	S		
Stress			A				A	
Modulus			BAS	AS	BD	BD	A	

(Table 1). 13 equations were found to be significant. Table 2 is broken into the 8 experimental groups and the three dependent mechanical variables; therefore, each box in the table represents an equation. The independent parameters that were found to be significant within each equation are represented in the corresponding box. Briefly, 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. None of the significant models had a Durban–Watson score lower than 1.08.

4. Discussion

This study employed an Achilles tendon neonatal healing model (Ansorge et al., 2011, 2012) in a mouse to study the structure–function relationships in tendon development and healing. A unilateral injury was created in developing neonatal Achilles tendons at two ages (7 and 21 day old) and two time points post injury (3 and 10 day post injury). The uninjured

and injured Achilles tendons were assayed for mechanical parameters (percent relaxation, maximum stress and elastic modulus), compositional parameters (collagen content, biglycan and decorin expression) and structural parameters (fibril diameter average and standard deviation). The compositional and structural parameters were used as independent variables in multiple regression models to predict the mechanical parameters. Briefly, 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.

Prior to this study, the viscoelastic parameter percent relaxation had not been regressed against compositional and structural parameters in tendon. We demonstrated that collagen content is a predictor of percent relaxation in three of the four uninjured percent relaxation equations. In addition, both proteoglycans are predictors in one uninjured percent relaxation equation while fibril diameter average is a predictor in two. Proteoglycans and water, along with the inherent viscoelasticity of the collagen itself, are thought to be primarily responsible for viscoelastic behavior (Woo et al., 2000, 1997). Uniform fibrils are thought to contribute to viscoelastic parameters due to possible discontinuity allowing sliding between the fibrils (Zhang et al., 2006). Surprisingly, in the current study, only fibril diameter average and standard deviation are significant predictors of percent relaxation during healing. This may be due to increased discontinuity of fibrils in the injured tendon. Future studies will further investigate the differential mechanisms behind viscoelastic parameters during normal development and developmental healing.

When examining the elastic modulus during early develop and healing, decorin and biglycan were the only significant predictors in healing tendon. It has been previously hypothesized that collagen bears the main load in elastic tensile mechanics; however, the results in this study are not surprising as chondroitin sulfate and dermatan sulfate have previously been demonstrated to significantly predict elastic mechanical parameters (Robinson et al., 2004). In addition, we previously demonstrated that the same experimental group had an accelerated healing response with a return of mechanical function (Ansorge et al., 2012). This could indicate that increased proteoglycan expression is beneficial during healing. Proteoglycans are modulators at multiple stages of fibrillogenesis during development (Zhang et al., 2005). The current study points to a potential role of proteoglycans during fibrillogenesis during healing. Due to size constraints of the neonatal tendons, the actual protein levels of proteoglycans were not measured and this is a limitation of this study. However, previous studies have shown that the mRNA message is similar to the protein level expression in C57BL/6 mice at both 30 and 60 day old (Appendix). Therefore, the message levels measured can be useful to get some estimate of proteoglycans for this analysis and can be useful. Future studies should develop more sensitive techniques to measure proteoglycan levels to further understand their role in development and healing. Further investigation into the role of the proteoglycans could lead to new treatments for improved tendon healing.

Interestingly, no significant predictors were seen in any group for maximum stress. During development, tendon fibroblasts must ensure that the tendon is growing and functionally strong enough to accommodate an increasing body size. However, these tendons probably do not experience stresses that would cause rupture. Based on this study, we hypothesize that functional mechanical parameters are more important during development, than failure mechanics. Therefore, it is possible that in the developmental healing tendon fibroblasts first respond to injury by regulating the functional mechanics via matrix production and remodeling.

We previously demonstrated an accelerated healing response during early development that was not seen in late development. Interestingly, 4 out of the 6 possible early developmental healing equations had significant predictors and only 2 out of the 6 had significant predictors during late developmental healing. The predictors in late developmental healing were also present in normal development in comparable groups, indicating that these predictors may be an effect of developmental growth and not healing. One possible explanation is that the healing response during late development is slower and the only significant predictors are those seen during normal development. The information from these comparisons is important because it will help focus new research on possible treatments for improved adult tendon healing. For instance, the addition of proteoglycans to adult healing tendons may help recapitulate mechanical parameters quickly, as seen during early developmental healing.

An analysis of the independent parameters measured in this study revealed some multicollinearity between independent parameters. In particular, the pairs, fibril diameter average and standard deviation, decorin and biglycan, and collagen content and fibril diameter all showed correlations in some of the groups analyzed. These correlations are observationally supported by previous studies that have examined compositional and structural properties of tendon. For instance, as tendons develop, both the fibril diameter average and standard deviation (spread) increase with age (Zhang et al., 2005). In addition, biglycan and decorin are thought to have differential effects on collagen fibrillogenesis and be differentially regulated (Danielson et al., 1997; Robinson et al., 2005; Zhang et al., 2006). Lastly, increased collagen production and increased fibril diameter average occur during similar stages of development (Ansorge et al., 2012; Birk et al., 1997; Bland et al., 1997; Zhang et al., 2005).

The coefficient of determination (R^2) for the significant equations ranged from 0.3 to 0.77. A low R^2 indicates that a small portion of the variability in the mechanical parameter is attributable to these independent parameters. It is likely that the inclusion of factors not considered in these models would result in an improvement in some of the R^2 values. Such additional factors could include compositional parameters such as type XII and XIV collagens, and structural parameters, such as fibril area fraction, or biologic parameters such as the number of inflammatory cells present.

In summary, 8 different experimental groups (based on age at injury, days post injury and injured or uninjured) were analyzed in backward stepwise linear regression to determine how the structural and compositional parameters (collagen, biglycan and decorin content, and fibril diameter average and standard deviation) predicted the mechanical parameters (percent relaxation, maximum stress and elastic modulus). 13 out of the 24 possible equations were demonstrated to have significant predictors. This study provides quantitative, objective information on the development of mechanical strength not only during healing, but also during normal development. This information can be used to focus future research on improving healing in adult tendons. Understanding the exact mechanisms of action behind these predictor variables could provide new therapeutic treatments for the improvement of mechanical properties during adult healing.

Conflict of interest statement

None of the authors of this paper have any conflicts of interest to disclose.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jbiomech.2012.01.030.

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