Nonlinear Viscoelasticity in Rabbit Medial Collateral Ligament

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Abstract—The goal of this study was to characterize the viscoelastic behavior of the rabbit medial collateral ligament (MCL) at multiple levels of strain (between 0% and ∼5%) and their corresponding stresses (between 0 and ∼55 MPa) for stress relaxation and creep, respectively. We hypothesized that in the rabbit MCL the rate of stress relaxation would be strain dependent and the rate of creep would be stress dependent. Thirty MCLs from 15 rabbits were tested *ex vivo* for this study. Results show that within the physiologically relevant region of ligament behavior, the rate of stress relaxation is strain dependent in the rabbit MCL, with the rate of relaxation decreasing with increasing tissue strain. The rate of creep is stress dependent in the rabbit MCL, with the rate of creep decreasing with increasing stress. These results support our hypothesis, with the greatest nonlinearities in a physiologically relevant region of loading. As such, these nonlinearities should be considered when quantifying ligament viscoelastic behavior with a rabbit model.

Keywords—Stress relaxation, Creep, Nonlinear superposition, Quasilinear viscoelasticity (QLV).

INTRODUCTION

Ligaments display biomechanical properties that are time and load history dependent i.e. they are viscoelastic. The mechanical properties of ligaments play an important role in normal joint mechanics. Alterations in ligament properties can lead to abnormal joint mechanics, which may lead to further deterioration of diseased joint tissue. Therefore, it is important to understand the properties of normal ligaments at varying levels of load and elongation in order to distinguish between healthy and diseased tissue. Surgical reconstructions can then strive to mimic normal behavior using natural or prosthetic grafts.

A number of studies have explored viscoelastic behavior in soft tissues such as ligament and tendon.[1,10,11,17,21,25,26] Early reports of ligament and tendon behavior by Haut and Little,[10,11] Viidik and coworkers,[6,7,24] Woo,[25] and Woo *et al.*[26] reveal ligaments and tendons display nonlinear stress–strain behavior, hysteresis, rate dependent stress-strain behavior, and undergo stress relaxation during constant or cyclic loading. The model most commonly applied to describe this behavior is the quasi-linear viscoelasticity (QLV) formulation of Fung.[8] At a single load or elongation level this model has been shown to describe tendon and ligament very well.[11,25] However, it was observed that QLV could not interrelate creep and stress relaxation[21] nor robustly describe the nonlinear rate behavior observed in rat ligaments.[17] For instance, stress relaxation proceeds faster than creep, a phenomenon shown by Thornton *et al.* in rabbit ligament[21] and later also observed in rat ligament.[17] Using the single integral form of nonlinear superposition, this behavior can be modeled with a relaxation function that is not separable into strain-dependent and time-dependent parts as discussed below.[14] The difference in the rate of relaxation and creep may also be understood by continuum concepts[14] or by microstructurally incorporating collagen fiber recruitment when predicting creep from stress relaxation as shown by Thornton *et al.*[20] In addition to differences between the rate of creep and relaxation at a specific load, it has been observed that rat ligaments display stress relaxation behavior that is strain dependent and creep that is stress dependent in nature.[17] The rabbit MCL, however, is much more commonly used as a model to explore biomechanical effects for various treatments and conditions. As such, recognizing and characterizing mechanical differences in the rabbit MCL could help distinguish between normal and pathological conditions and treatment outcomes. The goal of this study was therefore to study potential stress- and/or strain-dependent creep or stress relaxation behavior, respectively, in the rabbit MCL model. We hypothesize that the relaxation rate is strain dependent and creep rate is stress dependent in rabbit MCL.

METHODS AND MATERIALS

White New Zealand rabbits ($n = 15$) age 6–8 months weighing 3.2–3.6 kg were used as an animal model in this

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study. Animals were acquired from separate unrelated studies, which did not affect the musculoskeletal system or connective tissue of the specimen. All animal procedures comply with our institutions regulations for animal welfare. The animals were euthanized using a 1 ml dose of pentabarbitol. The hindlimbs were disarticulated at the hip joint and stored at $-73°C$. In 10 sets of paired contralateral MCLs, stress relaxation and creep at various levels of strain or stress, respectively, were performed. In five sets of paired contralateral MCLs, repeated stress relaxation and creep tests at one strain or stress level were performed.

On the day of testing, tissues were placed in an environment of approximately $6°C$ for approximately 4 h and then brought to room temperature prior to testing. During dissection, all the surrounding tissues were carefully removed, leaving the MCL with intact tibial and femoral insertions. Bone blocks of about 2.0 cm in length were cut from the femoral condyle and the tibial plateau. The bone blocks were then fixed with polyester resin in plastic molds. No fixative contacted the ligament or the insertion sites of the ligament. Care was taken to keep the bone blocks in an anatomic position. During this procedure, the specimen was kept hydrated with saline. Once potted the width and thickness of the ligaments were measured simultaneously using two vernier calipers, with caliper accuracy of 0.01mm. This measurement was repeated three times and the mean values were used to compute the cross-sectional area. After the fixative cured, the specimen was placed into the testing machine (MTS 858 BIONIX Test System; 200N load cell) containing a hydration bath. Bone blocks were arranged to depict a joint with 70 degrees of flexion. A literature survey shows that this angle of knee flexion provides a natural anatomic position for the rabbit MCL that assists in uniform loading (see [23]) and as such is suitable for biomechanical evaluation.[20,22] Ligaments were marked near their insertions with Verhoff stain, and these markers were used with video analysis to calculate ligament strain. The gage length of the ligament was measured at the onset of consistently measurable load (0.5 N). The bath was then filled so that the ligament was submerged in PBS (phosphate-buffered saline $1\times$). The ligament was then allowed to recover for a period of 10 min in order to reduce the effect of any loading history that it may have acquired during handling of the tissue and to allow it to be consistently hydrated.

In 10 sets of paired contralateral ligaments stress relaxation and creep tests at various levels of strain or stress, respectively, were performed. One knee (left or right selected randomly) was tested for stress relaxation at varying levels of strain between 0 and 5%. A preload of 0.5 N was applied and then an idealized step input elongated the ligament (displacement was applied in 10%/s and resulted in ramp times less than 0.3 s for strains less than 5%). The displacement was maintained for 100 s, after which the tissue grips were returned to their starting position and the ligament recovered for 1000 s while remaining hydrated.

This recovery period was based upon experience in rats, in which below the damage threshold of $\sim 5\%$ strain, a time period 10 times greater than the testing period was sufficient for recovery of load history effects.[17,18] After recovery the above displacement protocol was repeated at a different strain level. In all, five different strain levels were examined per ligament. The order of tests (i.e. the order of the five different levels of applied strain) was random. Creep testing followed the same procedure as described above for stress relaxation, except a load was applied to approximate a fixed step load. Load values corresponding to the peak force of the contralateral stress relaxation test were used in the creep tests. Hence, a force equal to the peak force seen at the start of the relaxation test was applied to the ligament for a period of 100 s followed by a 1000 s recovery period. Five corresponding creep tests were carried out on the ligament at increasing levels of load resulting in engineering stresses (force/initial area) between 0 and ~ 55 MPa. All tests were performed below 5% tissue strain to avoid structural damage to the tissue. In rats, structural damage does not occur below 5% tissue strain.[18] Although this has not been thoroughly evaluated in rabbit tissue, viscoelastic rates measured from testing below 5% strain were reproducible in the rabbit MCL (see Results). In five sets of paired contralateral MCLs we performed repeated stress relaxation tests at a particular strain level in one MCL, while the contralateral ligament was used for repeated creep tests at the same loads corresponding to the loads acquired during the stress relaxation tests. The MCLs from different animals were strained to different magnitudes. The rest of the test protocol was the same as described above.

Testing was recorded using a SONY CCD-IRIS camera, a VCR, and a viewing monitor which displayed synchronized displacement data with force data acquired using Labtech Notebook data acquisition software (Laboratory Technologies Corp., Wilmington, MA). Video images of the tests were digitized and evaluated to calculate strain for a particular load and time in the tissue with N.I.H. Image software. Using a custom N.I.H. image macro, the x-y coordinate center (centroid coordinates) of each marker was used to calculate the distance between the Verhoff markers, thus, ligament displacement after loading. Data were plotted on log–log scales and fit with a power law. As shown previously,[14,17,19] a power law in time describes ligament data well and can be attained through nonlinear (modified) superposition,

$$\sigma(\varepsilon, t) = \int_0^t E(t - \tau, \varepsilon(\tau)) \frac{d\varepsilon(\tau)}{d\tau} \, d\tau, \qquad (1)$$

which allows the relaxation function, E, to depend on strain level, ε, to describe stress relaxation, σ, as a function of time, t. The form of the resulting nonseparable relaxation function is[19]

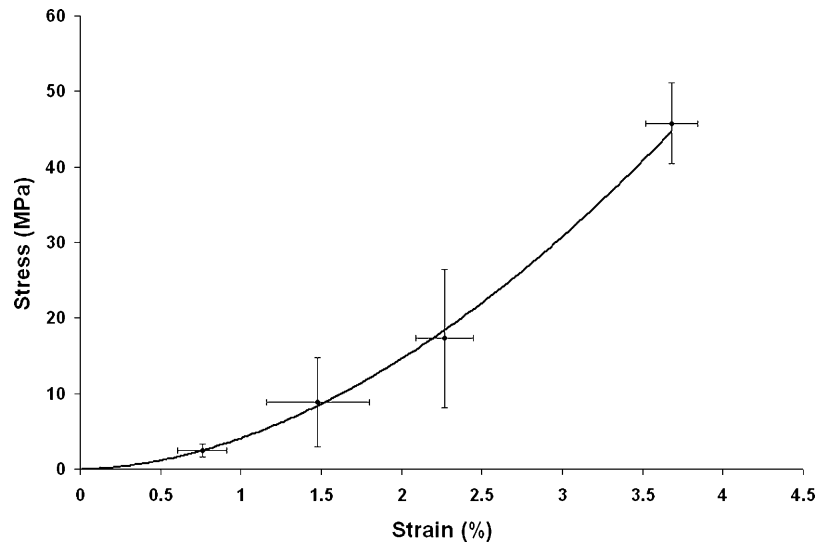$$E(\varepsilon, t) = A(\varepsilon) t^{n(\varepsilon)}, \qquad (2)$$

FIGURE 1. Isochronal curve at time 2.4 s obtained from stress relaxation tests performed on 15 MCLs: $\sigma = 4.095\ \epsilon^{1.835}$, $R^2 = 0.9984$. The graph shows the nonlinear strain-stiffening behavior found in ligaments. Error bars represent standard deviation.

where A is a function representing the initial elastic modulus that provides a description of the stress response,

$$\sigma(\varepsilon, t) = \sigma_0 t^{n(\varepsilon)}. \qquad (3)$$

With this representation σ_0 is the initial stress that depends upon strain and the strain-dependent rate of stress relaxation is defined as n. An analogous formulation exists for creep in which the creep compliance function is dependent upon time and stress level.

Statistical analyses were performed on the data in order to determine if the rate of stress relaxation and the rate of creep are strain- and stress-dependent, respectively. To account for the subsampling within individual specimens, data were analyzed with repeated measures analysis of covariance. The null hypothesis being tested is that there is no association between rate and stress or strain. This hypothesis is rejected if the p value is less than 0.05. All analyses were performed with SAS PROC MIXED (SAS Institute, Inc., Cary, NC).

RESULTS

An isochronal curve, which is generated from a set of points at the same time, but at different strain levels within a set of relaxation curves, was produced to describe rabbit stress–strain behavior. The isochronal serves to decouple time-dependent (viscoelastic) and strain-dependent (nonlinear elastic) behavior so that the nonlinearity can be properly visualized in a stress–strain plot independent of the viscoelasticity. Herein, an isochronal curve was generated from stress relaxation tests at time $t = 2.4$ s and represents the range of the stress–strain curve in which our tests were performed (Fig. 1). This curve indicates that our viscoelastic data are obtained from tests in which the elastic

behavior is consistent with the typical strain-stiffening behavior commonly reported for rabbit MCLs. For their respective tests, all tissues show a definite nonlinear trend in both creep and stress relaxation rates with stress or strain, respectively (Figs. 2 and 3). Both stress relaxation and creep rates changed by approximately an order of magnitude throughout the loading range examined herein (Figs. 2 and 3). Statistical analyses reveal that the rate of stress relaxation is dependent upon strain magnitude ($p = 0.0001$),
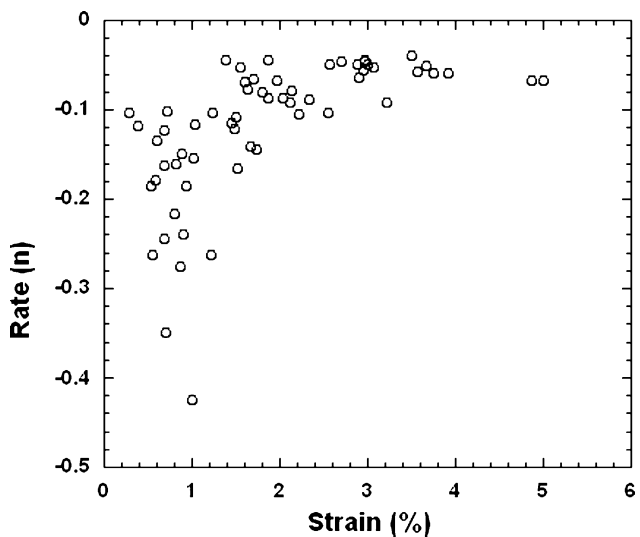

FIGURE 2. The rate of stress relaxation is seen to be nonlinear with respect to strain. This figure represents the data pooled together from both testing protocols (multiple strain level and repeated strain level) for stress relaxation tests. The graph shows that the rate of stress relaxation (*n*) changes by an order of magnitude throughout the low load region.
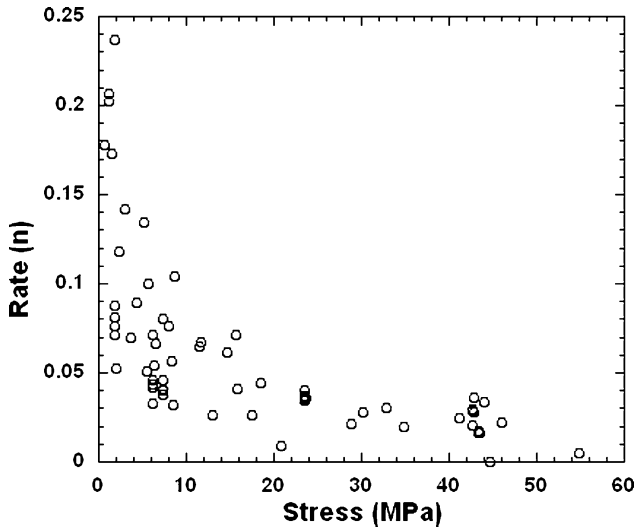
FIGURE 3. The rate of creep is seen to be nonlinear with respect to stress. This figure represents the data pooled together from both testing protocols (multiple stress level and repeated stress level) for creep tests. The graph shows that the rate of creep (n) changes by an order of magnitude through the low load region.

while creep is dependent on stress magnitude ($p = 0.0001$). Multiple tests at varying strain levels demonstrate the rate of stress relaxation decreases in magnitude with increasing strain (Fig. 4). Creep data at multiple levels of stress reveal the rate of creep decreases with increasing stress (Fig. 5).
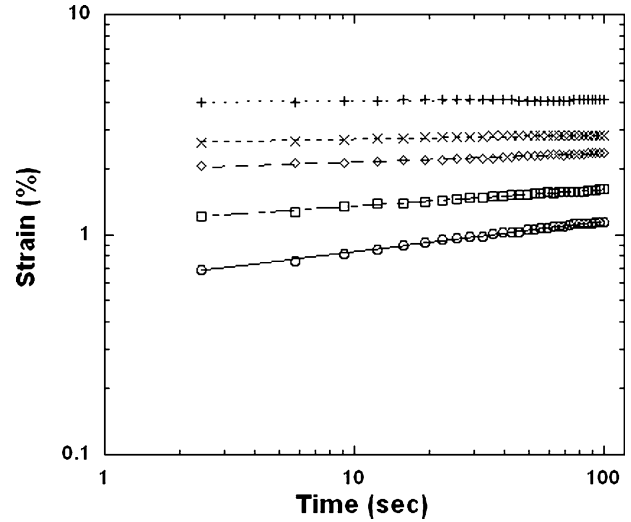

FIGURE 5. Creep at multiple levels of stress (Log–Log scale). The rate of creep decreases as the stress level is increased. The strain (in %) and rate of creep (n) for each of these five tests is represented by the following equations:

○ $\sigma = 2.98\,\mathrm{MPa}$; $\epsilon = 0.60681 \times t^{0.13927}$; $R^2 = 0.9977$,
□ $\sigma = 8.08\,\mathrm{MPa}$; $\epsilon = 1.1323 \times t^{0.075519}$; $R^2 = 0.99578$,
◇ $\sigma = 15.85\,\mathrm{MPa}$; $\epsilon = 1.9495 \times t^{0.039312}$; $R^2 = 0.98648$,
× $\sigma = 34.89\,\mathrm{MPa}$; $\epsilon = 2.5907 \times t^{0.019652}$; $R^2 = 0.95894$,
+ $\sigma = 54.80\,\mathrm{MPa}$; $\epsilon = 3.9825 \times t^{0.006287}$; $R^2 = 0.70028$.

Figures 6 and 7 are representative of the data acquired from specimens tested for repeated strain or stresses, respectively (note the difference in vertical scale). Both stress relaxation tests and creep tests were quite reproducible,
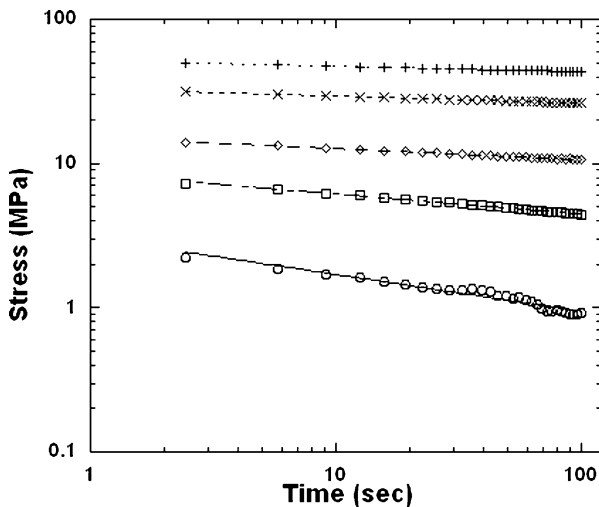

FIGURE 4. Stress relaxation at multiple levels of strain (Log–Log scale). The rate of relaxation decreases as the strain (ϵ) level is increased. The stress (in MPa) and rate of relaxation (n) for each of these five tests is represented by the following equations:

○ $\epsilon = 0.81\%$; $\sigma = 2.4488 \times t^{-0.2619}$; $R^2 = 0.9769$,
□ $\epsilon = 0.87\%$; $\sigma = 6.6249 \times t^{-0.1602}$; $R^2 = 0.9869$,
◇ $\epsilon = 1.24\%$; $\sigma = 13.357 \times t^{-0.1058}$; $R^2 = 0.9919$,
× $\epsilon = 1.96\%$; $\sigma = 27.999 \times t^{-0.0698}$; $R^2 = 0.9966$,
+ $\epsilon = 3.57\%$; $\sigma = 41.978 \times t^{-0.0582}$; $R^2 = 0.9993$.
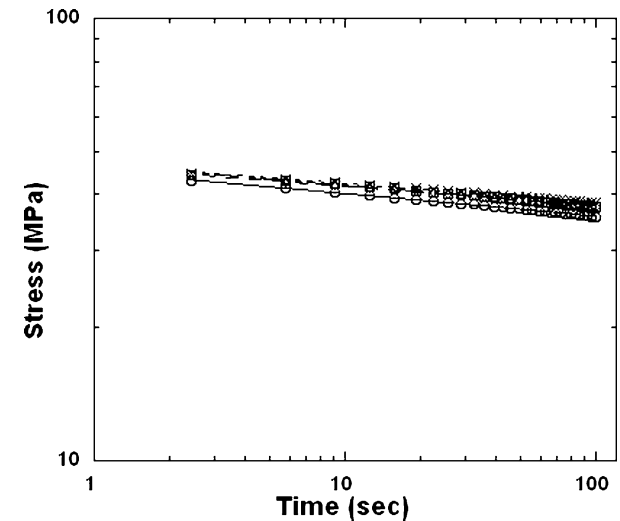
FIGURE 6. Stress relaxation behavior of MCLs subjected to repeated tests at the same strain, separated by 1000 s of recovery (Log–Log scale). Dispersion in the data was mild, providing confidence that specimens had effectively recovered in repeated testing. The stress (in MPa) and rate of relaxation (n) for each of these tests is represented by the following equations:

○ $\epsilon = 1.60\%$; $\sigma = 45.191 \times t^{-0.050937}$; $R^2 = 0.99841$,
□ $\epsilon = 1.55\%$; $\sigma = 46.750 \times t^{-0.047055}$; $R^2 = 0.99736$,
◇ $\epsilon = 1.39\%$; $\sigma = 46.208 \times t^{-0.044441}$; $R^2 = 0.99800$,
× $\epsilon = 1.86\%$; $\sigma = 46.767 \times t^{-0.043266}$; $R^2 = 0.99885$
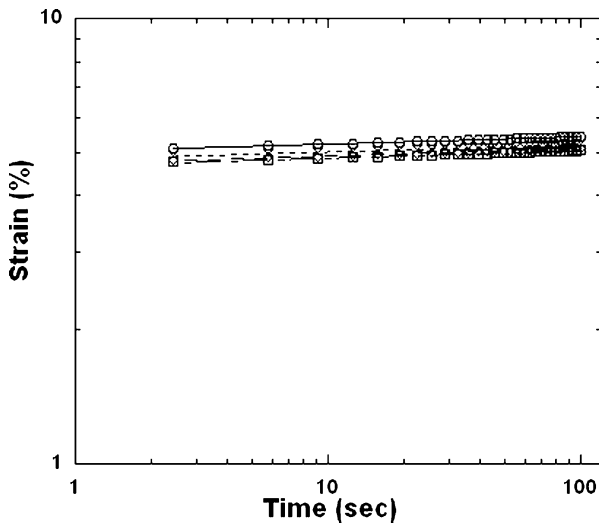
FIGURE 7. Creep behavior of MCLs subjected to repeated tests at the same load, separated by 1000 s of recovery (Log–Log scale). Dispersion in the data was mild, providing confidence that specimens had effectively recovered in repeated testing. The test was performed on the contralateral MCL of the ligament tested in Fig. 5 at the load value acquired from test shown in Fig. 5. The strain (in %) and rate of creep (n) for each of these tests is represented by the following equations:

○ $\sigma = 43.41$ MPa; $\epsilon = 5.0328 \times t^{0.016280}$; $R^2 = 0.99588$,
□ $\sigma = 43.37$ MPa; $\epsilon = 4.6723 \times t^{0.016852}$; $R^2 = 0.99695$,
◇ $\sigma = 43.35$ MPa; $\epsilon = 4.7219 \times t^{0.016769}$; $R^2 = 0.99829$,
✕ $\sigma = 43.36$ MPa; $\epsilon = 4.8319 \times t^{0.015981}$; $R^2 = 0.9982$.

although small variations in specimen strain (since machine input for displacement does correlate perfectly with tissue strain) or load occurred which contributed to noise in the data. Consistent with previous reports[14,17] the rate of relaxation was approximately two-fold greater than the rate of creep.

DISCUSSION

The aim of this study was to determine whether the rates of stress relaxation and creep are dependent on strain and stress, respectively, in the rabbit MCL within a physiologic range of loading. This type of viscoelastic behavior has previously been observed in rat ligaments,[17] and data from this study confirm similar stress- and strain-dependent nonlinear viscoelastic behavior in rabbit ligament. Provenzano *et al.*[17] show that in the rat MCL the rate of stress relaxation changes by an order of magnitude as the strain level is increased from 0.27% to 5.10%.[17] Results from this current study in rabbit (Fig. 2) are consistent with rat data. Additionally, trends in creep nonlinearity are similar between rat and rabbit MCLs. The nonlinearity seen in the rabbit MCL, however, differs somewhat from that observed in the rat MCLs.[17] Rabbit ligament displays a greater nonlinearity in the rate of creep with changing stress than does the rat. Hence,

within a physiologically relevant region, stress relaxation and creep rate are strain- and stress-dependent, respectively. As such they cannot be robustly described with the QLV formulation, but instead require a more general formulation, such as the modified superposition formulation utilized herein. This dependence does; however, appear to decrease as strain level approaches our assumed damage threshold of 5% strain. Although no definitive statement can be made about the behavior outside of our examined strain range, we speculate that it is very likely that a separate strain- or stress-dependent nonlinearity will present itself at higher strains due to microstructural tissue damage, especially under repeated testing (or joint motion).

As stated in the previous paragraph, nonlinearities in rates of creep and relaxation cannot be robustly modeled by the QLV formulation.[14,17] The QLV formulation is separable with the time-dependent portion of the relaxation modulus (or creep compliance) being strain (or stress) independent, respectively, so the time-dependent behavior cannot change as a function of strain or stress. As previously described[17] and further demonstrated herein, the shape of the relaxation or creep curve is strain- or stress-dependent within the examined range of strains or stresses. This region is particularly relevant since *in vivo* knee studies report 1) goat ACL was never loaded beyond 6% of its ultimate strength,[12] 2) goat patellar tendon was never loaded beyond 29 MPa,[13] and 3) human ACLs rarely exceeded 4% strain (measured without prestress associated with *ex vivo* tests) and were usually strained much lower.[2,4] Hence, the nonlinear viscous behavior shown herein is physiologically relevant and the fact that QLV cannot robustly describe this behavior were largely unrealized, in part, because many experiments were designed under the assumption of QLV and in part because creep and relaxation were determined in a more limited window of loadings. Hence, our conclusions are in contrast to the work of Pioletti and Rakotomanana.[16] They tested human ligament and tendon at successively increasing strain levels and concluded that no correlation between strain and the time for relaxation exists and that variable separation is justified. However, their data were not directly fit with QLV or a more general nonlinear model, in order to interpret the shape of the stress relaxation curve. Instead the difference between force at a particular time and final force normalized by the total difference in force was used, making their data harder to interpret. In addition, Piolletti and Rakotomanana[16] primarily examined anterior cruciate ligament strain well above the physiologic range[2,4] where strain dependence is likely to be more evident. Further, Pioletti and Rakotomanana[16] did not address the effects of damage, which appear likely with ACL strains from 8 to 18%. When considering loads throughout a physiologically relevant range,[2,4,12,13] a nonlinear viscoelastic model would appear more appropriate.

When considering the results of this study the limitations to this work must be taken into consideration. First, during video analysis the strain obtained from the optical markers is an average strain over the entire length of the ligament between the two ligament insertions. Hence, our strain measure does not reflect regional variations in strain within the tissue. Second, the specimen might lose some of its water content over periods of loading and may not be able to regain it completely during periods of recovery. Such hydration changes would influence stress relaxation[3] or creep[22] behavior. However, the same nonlinear trends were present herein after random, serial, or repeated testing was performed supporting the validity of our testing protocols. No consistent order effect was detected. In addition, the repeated tests (see Figs. 6 and 7) carried out on the MCLs show some dispersion at the same strain or corresponding stress levels (mainly due to testing-system-produced experimental error). This dispersion, however, was mild compared to the differences in behavior associated with multiple levels of testing (Fig. 2.). Hence, the relatively small amounts of dispersion in the repeated tests (as seen in Figs. 6 and 7) provide confidence that specimens had effectively recovered before a repeated test and that the reported stress (or strain)-dependent nonlinearities in creep (or relaxation) were not significantly affected by our experimental protocols. Third, ligament stress relaxation and creep data were gathered over 100 s. Although test times of 100 s may not describe the entire temporal behavior of the tissue, this time scale provides a good indication of rat ligament behavior[15] and facilitates serial testing on a specimen with recovery times (10 times the length of the test) that do not become excessive. Fourth, the structural damage threshold for rabbit MCLs has not been defined. Previous studies have shown that testing under ∼5% strain (40–60% of the failure strain) in rat medial collateral ligaments did not cause any changes in the mechanical properties of the ligament nor did it result in any structural damage in the tissue.[18] In rabbit MCL Thornton et al.[21] suggest that 14MPa stress corresponds to the approximate start of the linear region and a normal rabbit MCL fails at approximately six times this value. Woo et al.[27] show that the strain at failure for the rabbit MCL is greater than 7% tissue strain, while the entry into the linear region is around 40 N tissue load. Other studies[5,28] show similar results. Further work by Thornton and coworkers,[23] although not specifically indicating a damage threshold for rabbit MCLs, does report apparent microdamage in 6 of 12 MCLs tests in static creep followed by cyclic creep at 28 MPa (three damaged during static creep and three damaged in cyclic creep following static creep). Potential lasting effects of this possible damage after recovery, such as changes in tissue length, stress–strain behavior, or viscoelastic behavior, were not examined. In addition, the superposed stress history utilized by Thornton and colleagues[23] makes direct interpretation of the results in terms of a damage threshold extremely difficult. One note is that in the rat MCL, the damage threshold is at a strain level approximately equaling 40–60% of the failure strain. Extending this analogy to rabbit MCLs, the stress–strain curve described as typical in Thornton et al.,[23] would give a damage threshold with a corresponding stress of ∼40–60 MPa, which is the upper range of our creep tests. Hence our tests fall within the predicted ranges of recoverable deformations for rabbit MCL tissue and correspond to strain and stress levels that do not result in structural damage in rat MCLs. In addition, the levels we tested are very likely to be relevant to include normal physiologic levels of ligament loading.[2,4,12,13]

The mechanisms driving viscoelastic behavior in ligament are not yet completely defined. We have previously speculated[17] that "the decrease in relaxation rate with increasing strain could be the result of larger strains causing greater water loss (wringing out effect) which causes the tissue to be more elastic (less viscous) than tissues subjected to lower strains." Studies supporting this hypothesis have reported increased relaxation with increased hydration[3] and a decrease in tissue water content with cyclic loading,[9] which probably drives fluid out of the ligament during loading. Further study of fluid content under varying levels of strain could add insight into the mechanism by which stress relaxation varies with strain. In regards to creep behavior, Thornton et al.[21] speculated that differences in stress relaxation and creep behavior are due to progressive recruitment of collagen fibers during creep and that this microstructural behavior is unlikely to have as significant an effect on stress relaxation as on creep. Subsequent experiments strongly suggest that collagen fiber recruitment does in fact play a role in creep behavior.[20,23] Hence, the progressive recruitment of collagen fibers could also explain the decrease in the rate of creep with increasing load. As larger loads are applied to the ligament more fibers are recruited leaving fewer fibers to be progressively recruited after initial loading and therefore decreasing the creep response. Further work is required to better understand viscoelastic mechanisms in collagenous tissues.

Overall this study expands our understanding of the viscoelastic behavior of the rabbit MCL by examining various levels of strain and stress. These data can assist researchers with the development of more robust characterizations of normal rabbit ligaments that can better distinguish between normal and pathological conditions. The viscoelastic nonlinearities described herein should therefore be considered when describing ligament behavior in a rabbit model.

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