Healing of subfailure ligament injury: comparison between immature and mature ligaments in a rat model

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Abstract

This study evaluated biomechanical properties of healing ligament following subfailure (grade II) injury by comparing young and mature animals in a rat lateral collateral ligament (LCL) model. One randomly selected LCL was stretched in situ using a custom designed device in eighteen young (21 days) and eighteen skeletally mature (8 months) male rats. Animals were euthanized at 0, 7, and 14 days post-surgery, and ligament ultimate stress, strain at failure and laxity were determined (n = 6 pairs per group). At time 0 after introduction of stretch injury, ligament laxity was present in both groups. The mature rats had 54 ± 9% strength of the control while the immature rats had 58 ± 11% of the strength of the control, representing a consistent and significant injury. The immature and mature ligaments showed similar patterns of cellular damage post-injury and had similar modes of mechanical failure. Ligament laxity decreased in each group as healing time increased, however ligament laxity did not completely recover in either group after 2 weeks of healing. After 7 and 14 days of healing, the mature rats, respectively, had only 63 ± 14% and 80 ± 8% strengths of the controls while the immature rats had 94 ± 6% and 94 ± 10%. Hence, mechanical data showed that immature animals recovered their strength after a grade II sprain at a faster rate than mature animals. However, ligament laxity was still present in both groups two weeks after the injury and was not completely removed by growth in the immature group. These findings are clinically relevant since joint laxity after injury is common, and these results may explain the presence of continued instability in a joint injured at a young age. Hence, this study, with a new injury model, showed differences in ligament healing associated with maturity and quantified the clinically observed persistance of ligament laxity.

Introduction

Ligament sprains commonly occur and can be quite debilitating, frequently causing persistent joint instability, prolonged pain, and progressive degeneration. A sprain is defined [3,26] as an acute injury to a ligament or joint capsule without dislocation. Sprains are classified by severity based upon clinical examination or imaging. Grade I sprains are mild stretches with no discontinuity of the ligament and no clinically detectable increase in joint laxity. Grade II sprains are moderate stretches in which some fibers are torn. Enough fibers remain intact so that the damaged ligament has not failed. These grade II sprains produce detectable laxity at the joint. Grade III sprains are severe and consist of a complete or nearly complete ligament disruption and result in significant joint laxity.

Numerous studies investigated the mechanical, biochemical, and morphologic properties of healing ligament in a grade III, mechanically injured medial collateral ligament (MCL) [8,9,34,36]. Results demonstrated that ligaments healed with scar tissue that is mechanically inferior and biochemically abnormal in its composition and architecture, concluding that these properties do not return to normal [8,36]. Although collagen content returned to normal fairly quickly after injury, the mechanical properties of healing tissue were inferior to normal ligament [11,34,36] and the diameter and orientation of collagen fibrils did not return to normal, even after 2 years [8]. Even with a greater amount of collagen in the healing ligaments, ultimate loads of healing MCLs returned to only 73% of their...
controls [36]. Indelicato [15] performed a clinical study to compare the results of operative (16 patients) and nonoperative (20 patients) treatments of isolated grade III MCL injuries over 3.1 and 2.4 years, respectively. Results indicated that no patient in either group re-established absolutely normal stability of the MCL when compared to the contralateral knee. The above studies examined a clinical injury or utilized a ligament injury model with complete tear or sharp incision/excision of the ligament tissue. None studied subfailure [20,22,24,30] ligament injuries.

Many in vivo injury models have been used to induce mechanical insult into ligament. Prominent models for grade III injury attempt to simulate ligament rupture and damage the length of the tissue. For instance, Frank et al. [7] pulled a 3-0 braided steel suture transversely through the tissue midsubstance to rupture the medial collateral ligament. This wire rupture technique was extensively compared to the scalpel cut technique in [29]. To create midsubstance “mop-end” tears with simultaneous damage to insertion sites, rabbit MCLs were ruptured by pulling a 2.5 mm diameter rod placed beneath the MCL [14,31]. In addition to grade III injury models, several subfailure injury models have been used. Amiel et al. [1] compared partial laceration (simple side cut with repair, center straight cut) to complete laceration (straight cut with repair, Z step cut with repair). Amiel et al. [2] and Schreck et al. [25] used a surgical injury model with a partial laceration in the midsubstance (approximately 60% of the width) of the rabbit MCL and ACL leaving fibers on both sides intact [2,25]. Hefi et al. [13] transected the rabbit ACLs leaving approximately 25% of the tissue intact. Lastly, Laws and Walton [16] induced a subfailure MCL injury by manually flexing the knee joint until a “characteristic sound caused by soft tissue tearing was heard”. Although these injuries were subfailure, they were all induced by laceration or poorly controlled and consequently do not consistently re-create the cellular and matrix damage done by a grade I or II sprain. The injury model applied in this study attempted to more closely resemble a grade II sprain by damaging the length of the tissue as done in the prominent grade III injury models described above.

In contrast to grade III injuries, subfailure ligament injuries (grades I and II) that represent more than 85% of ligament sprains [3] have not been extensively studied in either a biological and mechanical sense, particularly with in vivo models. In the years from 1985 to 1988, the annual incidence of sprains, strains, and dislocations was 14.6 million, the highest incidence of any musculoskeletal injuries [21]. Frensgaard and Johannsen [10] studied 41 patients with partial ACL tears for 17 months. Twenty-one patients were found to have clinically unstable knees, a condition which may result in progressive deterioration of the joint. Because this was a clinical study, the level and extent of injury to the joint was not controlled. Hence the extent of healing is harder to judge. Laws and Walton [16] examined histological and mechanical changes with grade II MCL injury in a sheep model. The grade II injured ligaments appeared to heal by an intrinsic fibroblastic mechanism, although the subfailure injury was poorly defined. Panjabi et al. [20] investigated the mechanical compromise associated with an 80% subfailure injury in an ex vivo study of rabbit anterior cruciate ligaments (ACLs). The authors reported a significant change in the shape of the load-displacement curve, especially at low loads, which results in a stretching of the toe region to larger deformations. In addition, decreased ultimate stress and elastic modulus and elongated toe region have been reported in rat MCLs after a subfailure stretch [22]. The above studies elucidate some issues, but there clearly is much to learn about the biochemical and mechanical in vivo healing response following subfailure ligament injury.

Previous studies suggest that the ageing process impairs wound healing [4,5,32]. Faster ACL healing of younger rabbits after a partial transection injury has been reported [13], which is consistent with clinical observations in pediatric athletes [6]. Woo et al. [33] examined the mechanical properties of the rabbit MCL as a function of age and reported a rapid rise in strength in the early growth period that leveled off at 6–7 months of age. This behavior may be related to the increased molecular stability, entropy and density of collagen cross-linking with age [28] or differences in collagen biochemistry between immature and mature animals. For instance, type I collagen in embryonic and immature animals contains relatively few covalent cross-links and only $\alpha1(I)$ chains, while mature type I collagen contains $\alpha2(I)$ chain subunit. This is an important fact since embryonic type I collagen is very sensitive to degradation by proteases which is essential in remodeling the extracellular matrix in growing animals [17]. The above studies report differences in normal ligament mechanical and biochemical properties with age and increased healing rate in ACLs in younger animals, however, the effect of age on healing subfailure collateral ligament injuries has not been addressed.

In this study we hypothesized that young animals differ in their response to subfailure (grade II) ligament injury and heal faster and more completely compared to ligaments in mature animals. We use a new in vivo rat model to produce controlled subfailure (grade II) ligament damage and then compare biomechanical properties of healing ligaments of adult and young animals.

Materials and methods

This study was approved by the Institutional Animal Use and Care Committee. Twenty-four skeletally mature (8 months; 284 ± 13 g) and
Fig. 1. Ligament stretching device which laterally restrains the insertions.

24 weanling (21 days; 48.2 ± 0.94 g) male Wistar rats were used for this study. Wistar rats are commonly used in ageing studies. Of the 24 rats per age group, 18 were used for mechanical testing and six for confocal microscopy. Rats were anesthetized with an isoflurane anesthetic administered by face mask using a Bain nonrebreathing delivery system. The incision area was clipped and aseptically prepared for surgery. A skin incision approximately 10 mm long was made on the lateral side of the stifle, and fascia was incised to expose lateral collateral ligament (LCL). One randomly selected LCL was isolated without violating other joint structures, and stretched using a device that pulled the ligament like a bow string while laterally restraining the origin and insertion to prevent local damage (Fig. 1). The same device design was applied to both the mature and immature animals, but each stretcher was scaled to the respective joint/ligament size for each group. Typical ligament length and thickness for each age group were considered when scaling of the device so that the ‘foot’ of the device, which inserted under the ligament, had the same dimensions (width, thickness, and length) relative to the ligament. The length of the base portions (which sat near the ligament insertion sites) was also scaled relative to typical ligament length in order to be in approximately the same points near the insertion sites for each age group. The amount of stretch was applied in situ in displacement control. The amount of lateral displacement resulting in substantial tissue damage was determined in preliminary studies. Lateral stretch in each age group was scaled relative to the ligament length. The contralateral side was sham-operated to serve as a control. Each incision was closed in a routine manner.

Eighteen young rats (21 days) and 18 skeletally mature rats (8 months) were euthanized at 0, 7, and 14 days post-surgery, therefore, the mechanical portion of the study contained six groups each consisting of 12 ligaments (n = 6 pairs). Dissecting away all extraneous tissue carefully exposed each LCL. The distal femur, the LCL, and proximal femur were dissected with care taken not to disturb the ligament insertion sites. Ligaments were kept moist in Hank’s physiological solution to prevent dehydration, and cross-sectional area was calculated by optically measuring the width and thickness of the ligament and assuming an elliptical cross-section. This elliptical shape is reported in current literature describing the anatomy of the LCL. [18] and seen to be a reasonable assumption in rat based upon histological examination. Ligaments were then placed into a custom designed load frame with special structures to hold the femur and fibula end sections of the sample along the longitudinal axis of the tissue where all fibers appear to load simultaneously. The samples were inserted into the testing machine and a small preload was applied (0.1 N for mature animals, 0.02 N for immature animals) in order to obtain a uniform zero point (i.e. start the tests from the same relative position). The ligaments were preconditioned [12] (1% strain for 10 cycles) and pulled to failure in displacement control at 10%/s.

Strain was measured by placing graphite impregnated silicon grease markers at the ligament insertion sites on the specimen and using video dimensional analysis with 10 μm resolution at this level of magnification) to measure displacement. Force is displayed on the video screen to synchronize with output image based displacements. The resolution of the force data acquisition system was ~0.0025% of the maximum force used to load the ligaments. Using NIH image the video was stored as frames where each frame can be used as a data point in force–displacement, which can then be normalized into stress–strain. The x-coordinate center coordinate (centroid coordinates) of each marker was used to calculate the distance between the silicon markers. Thus, the change in centroid distance and the calibrated force measurement produce force displacement data, which were used to compute the engineering stress (σ) and strain (ε).

The ultimate stress (σu), defined as the largest stress seen by the tissue during the test, of the grade II damaged ligament was normalized by the ultimate stress of the contralateral control knee. The strain was taken as the deformation per unit length where the gage length was taken at preload. Strain at failure (εf) was taken as the strain in the ligament at ultimate stress. These data were also normalized by the contralateral control ligament. In addition, the location of structural failure was identified and recorded. Digital images of the structural failure were analyzed to assess failure location. For the case of potential tibial avulsion the ruptured tibial end of the tissue was examined for bone. Ligament laxity was obtained by optically measuring the difference in ligament insertion to insertion distance at preload in each pair of contralateral ligaments. The difference between control length (Lc) and stretched length (Ls) was normalized by the control length to produce laxity as a percent [%Laxity = 100(Ls - Lc)/Lc]. Parameters that were compared are ligament laxity, ultimate stress, and strain at failure.

In order to assess the amount of initial ligament damage in the normal and immature rats after receiving the grade II ligament injury, confocal microscopy was used with a cell viability assay to detect normal and damaged cells. Six mature rats and six immature rats were used to determine the amount of damage in each group. Cellular damage in the tissue was quantified using a technique described by Provenzano et al. [22]. This technique quantifies regions of cellular damage as function of strain. In summary, this process involved preparing the LCLs for confocal microscopy immediately after tissue stretch. The time from stretch to viewing of the tissues was approximately 1 h, during which staining was performed. In situ staining was performed with calcein and ethidium homodimer using a technique similar to that described in [19]. Live cells with intact membranes metabolize calcein and show green fluorescence while cells with damaged membranes are penetrated by ethidium homodimer, which results in red fluorescent staining of the nuclei. The LCLs were imaged with a confocal microscope and image scans through the depth of the tissue were compiled and overlaid. Using NIH image an index was assigned to each color in the micrograph and a constant threshold was set as the boundary between colors. In the micrograph the background index (black in color) was eliminated and yellow regions in the micrograph indicate green and red are overlaid and were counted as damage. Regions of damaged cells were quantified (in pixels) and normalized by ligament area (in pixels). This technique does not count the number or area of damaged cells in the tissue since ratio of the area of fluorescence to cell dimensions is not known and scans are overlaid. In the present study, one of the ligaments was stretched in the manner described above and the other ligament was used as a control. Both ligaments were imaged with confocal microscopy and cellular damage quantified. The difference was taken between the control and stretched ligament (n = 6 pairs) for each of the mature and immature groups for comparison purposes and represented as mean ± standard deviation.

In addition, to confocal microscopy, scanning electron microscopy (SEM) was applied in one additional mature rat (one control LCL and one stretched LCL) to examine the distribution of fiber [27] damage in the stretched ligament. Tissues were processed according to the methods described in [23]. An immature animal was not examined with SEM due to the small ligaments size in immature animals.

Repeated measures analysis of variance (ANOVA) was used to evaluate differences among time groups for each age group. Repeated
measures analysis of variance was also used to assess differences between the sham leg and treated leg ligament length in each animal. When ANOVA revealed significant differences among groups, a post-hoc t-test was performed to analyze these differences. Repeated measures were appropriate to account for correlations between legs within an animal. For display purposes the data were normalized by the sham leg. Differences were considered significant at a probability level of 95% (p < 0.05). A two group t-test (unpaired t-test) was used to determine if differences in ultimate stress, strain at failure, and laxity between mature and immature animals at 0, 7, and 14 days were significantly different.

Results

Scanning electron microscopy revealed initial collagen fiber damage to be distributed along the length of the tissue in mature animals (Fig. 2). At time 0 the mature rats had 54 ± 9% of their original strength, and immature rats had 58 ± 11% of their original strength, representing a consistent and significant injury. Statistically, these two numbers (i.e. the initial decrease in strength for mature and immature animals) are not significantly different (p = 0.624). In addition, confocal microscopy (represented in Fig. 3) revealed the measure of cellular damage (not number of necrotic cells) [22] for mature and immature ligaments at time 0 to be consistent, 30.16 ± 9.57% (mean ± s.d.) for mature rats and 33.57 ± 14.55% (mean ± s.d.) for immature rats. Again, these cellular damage values were not significantly different (p = 0.674). Fig. 4 shows ligament length (mean ± s.d.) for mature and immature rats at all three time groups. The immature rats increased their ligament length from week 0 to 1 and 2. The stretched ligaments were significantly longer than the control ligaments for all groups (p = 0.0001 for all pairs), despite growth of the control in the 1 and 2 week immature groups. Our data show faster recovery of ultimate stress in immature animals than mature animals. After 7 days the immature rats recovered a significant (p < 0.05) portion of their ultimate stress (58 ± 11% at time 0 to 94 ± 6% at 1 week), while mature rats did not display a significant (p < 0.05) recovery until 14 days (54 ± 9% at time 0 to 80 ± 8% at 2 weeks) (Fig. 5). Still, mature rats did not recover as fully as immature animals after 14 days (80 ± 8% for mature compared to 94 ± 10% for immature). Examining the difference in ultimate stresses between mature and immature animals at 7 and 14 days showed significant differences between ultimate stresses for immature and mature groups (p = 0.0006 and 0.0321, respectively). The normalized strain at failure (Fig. 6) was not significantly different. Table 1 reports the location of failure for each group; revealing the majority of failures occurred in the ligament and not the ligament to bone insertion. Ligament laxity significantly decreased (p < 0.05) in immature animals at 7 days (18 ± 7% at time 0 to 8.9 ± 3% at 1 week) while mature animals showed no significant change over the 2 week period (Fig. 7). However ligament laxity at low load did not completely recover in either group after 2 weeks of healing (5 ± 2% for immature and 9 ± 5% for mature).

Discussion

The purpose of this study was to compare healing of grade II ligament sprains in mature versus immature animals. To our knowledge, this is the first study to describe mechanical properties of healing collateral ligament in immature and mature animals after a controlled grade II injury. In our model the initial mechanical insults for the mature and immature animals was consistent. The initial change in ultimate stress was not significantly different and confocal microscopic analysis for quantifying cellular damage in the ligament revealed consistent measures of cellular damage in the mature and immature animals. These values of cellular damage correlate to the cellular damage produced by ~8% axial tissue strain in [22]. Confocal images showed that not all damage is taking place at the insertion sites or at the location of the stretching device, but is
Fig. 3. Typical confocal microscopy images for control (top) and grade II stretched (bottom) lateral collateral ligaments in (a) a mature animal, (b) an immature animal. Green indicates viable cells with intact membranes while red and yellow indicate regions of cellular damage. Note that damage is not completely localized to the stretcher location or the insertion sites.

Fig. 4. Ligament length for stretched and control mature and immature ligaments at 0, 7, and 14 days (mean ± S.E.M.). The stretched ligaments were significantly ($p < 0.05$) longer than the controls for mature and immature animals at all time points. Actual $p$ values are indicated on the figure. Note the increase in tissue length from the immature rats over time and the larger ligament length in all stretched tissue when compared to their controls.
The fact that cellular and structural damage is distributed supports the use of “wire pull” [7,29] or rod pull [14,31] type models (lateral stretch) for grade II and III injury instead of ligament transection where cellular damage is probably more localized. The observation that tissue damage is distributed is reinforced by Table 1, showing the location of structural failure. Unlike, other studies in the literature involving rabbit MCLs [33,35], which report all immature animals fail by tibial avulsion, our results in a rat LCL model showed failure most commonly occurred in the ligament tissue for both control and stretched tissues. Neither the change in ultimate stress nor cellular damage at time 0 (initial injury) showed any statistically significant difference between mature and immature animals.

A grade II ligament injury increased ligament length and therefore joint laxity, at least temporarily. Although strength recovered almost completely in the immature
animals after one week, ligament laxity was still present two weeks after the injury. The immature animals showed an increase in control ligament length with time (Fig. 4). Even so, the stretched group was still significantly longer than the control, suggesting a stretched ligament continues to grow (in length) similar to a normal ligament and that growth alone will not resolve ligament laxity. There must be healing and remodeling. These findings are clinically relevant since joint laxity after injury is common, and these results may explain the presence of continued instability in a joint injured at a young age.

Subfailure injury initially reduced the tissue ultimate strength, a result consistent with previous reports [13,16,20,22]. Few studies have examined healing after a subfailure injury in collateral ligament. Laws and Walton [16] examined MCL healing in mature ewes up to 6 weeks post-injury. Results of their study indicated a significant increase in ultimate strength after three weeks. Yet, at three weeks the injured ligament strength was only ~39% of the control value. Hence, their value is substantially less than the ~80% reported in this study after two weeks of healing. This difference in the amount of strength recovery is likely due to differences in the amount of initial subfailure damage. The initial post-injury strength reduction reported by [16] was substantially greater than reported here (~13% versus ~54%). Results of this study indicated that immature animals recovered strength more quickly from a ligament injury.

<table>
<thead>
<tr>
<th>Failure location</th>
<th>Ligament midsubstance</th>
<th>Ligament fibular third</th>
<th>Growth plate/avulsion</th>
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<td><strong>Week 0</strong></td>
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<tr>
<td>Mature</td>
<td>Control</td>
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<td><strong>Week 1</strong></td>
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<td><strong>Week 2</strong></td>
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All failures occurred in one of the following locations (1) midsubstance—characterized as the middle third of the ligament, (2) fibular section—characterized as the third of the ligament near the fibula bone and (3) avulsion—characterized by insertion failure at the bone/growth plate of the fibula. The frequency for each failure mode is shown for each group of six ligaments.

![Fig. 7. Ligament laxity represented as a percent is calculated by laxity = 100(L_s - L_c)/L_c, where L_c is the control length and L_s is the stretched length. Standard deviation error bars are not included for clarity. The standard deviations in percent are: immature: 0 days: ±6.7, 7 days: ±3.1, 14 days: ±1.8; mature: 0 days: ±1.9, 7 days: ±2.8, 14 days: ±5.4. Letter difference indicates significant differences among time groups for each age group (p < 0.05). No interaction between the age groups is indicated with the lettering. Comparing age groups at a given time revealed a significant difference in laxity between immature and mature animals after the initial injury (time 0). Laxity was not significantly different between age groups at 7 and 14 days.](image-url)
then mature animals. This result is similar to results reported by [13] where immature animals recovered ACL strength more quickly than mature animals up to three months post-partial ACL transection. However, ligaments in their study did not completely recover in either mature or immature animals after one year. This difference in the amount of strength recovery is most likely due to the poor healing characteristics of the ACL compared to collateral ligaments. However, further study of the mechanisms driving the healing response in collateral and cruciate ligaments is required to elucidate better the difference in collateral and cruciate ligament healing characteristics.

A number of studies suggest that the ageing process impairs wound healing. Ashcroft et al. [4,5] studied acute cutaneous wounds and observed delay in the appearance of growth factors that increased with animal age. Interestingly, TGFβ1,2 isoforms increased at all time points in the wounds of younger animals, whereas TGFβ3 increased in intensity after post-wounding day 7 in the old animals. Ashcroft et al. [4,5] also observed marked differences in healing between young and old animals with a delayed inflammatory response, decreased immunostaining for fibronectin, as well as a decreased and delayed appearance of extracellular matrix components in wounds of the old animals. The above ideas may play a role in the superior healing properties of immature tissue reported in this study. However, further study into the biological mechanisms of remodeling/healing after injury in young and old animals need to be examined before firm statements about causation can be made.

Limitations to this study should be noted. The pull to failure is structural, therefore, we do not know if results in immature and mature are measuring healing of different anatomical regions, i.e. insertion in young versus midsubstance in mature. However, quantified cellular damage did not show any gross differences, and the immature group did not commonly fail at the insertion site. This study does show healing and recovery from a functional structural point of view. Second, the in vivo stretching device does not perfectly simulate a clinical grade II injury, however it does provide a controlled injury that the authors believe will add insight to this common injury. In addition, it is difficult to correlate the amount of lateral stretch to axial strain due to the direction of loading and the nonrigid nature of ligament. Lastly, the study group only went out to two weeks post-surgery. Study at longer times may further elucidate ligament laxity, especially in light of the interesting result that laxity in young animals is not removed by growth in the early post-injury time period.

In summary, results from this study quantified the increased rate at which immature animals recovered from a grade II ligament injury compared to mature animals. Neither of the groups completely recovered its control length after two weeks, demonstrating the persistence of tissue laxity and hence joint laxity, lagging the recovery of strength. This study presented a new model for investigation of grade II injuries and healing differences between immature and mature animals. Further histological, biochemical, and molecular biology studies should be performed to understand the mechanisms of biological healing in both immature and mature animals.

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