The effect of different preconditioning protocols on repeatability of bovine ACL stress-relaxation response in tension

Ebrahimi, M

Elsevier BV
The effect of different preconditioning protocols on repeatability of bovine ACL stress-relaxation response in tension

Mohammadhossein Ebrahimi, Ali Mohammadi, Aapo Ristaniemi, Lauri Stenroth, Rami K. Korhonen

PII: S1751-6161(18)30956-1
DOI: https://doi.org/10.1016/j.jmbbm.2018.10.041
Reference: JMBBM3055

To appear in: Journal of the Mechanical Behavior of Biomedical Materials

Received date: 26 June 2018
Revised date: 26 October 2018
Accepted date: 31 October 2018


This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting galley proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.
The effect of different preconditioning protocols on repeatability of bovine ACL stress-relaxation response in tension

Mohammadhossein Ebrahimi¹, Ali Mohammadi¹, Aapo Ristaniemi, Lauri Stenroth, Rami K. Korhonen

Department of Applied Physics, University of Eastern Finland, POB 1627, FI-70211 Kuopio, Finland

E-mail: ebrahimi.bioengineering@gmail.com
mohammadhossein.ebrahimi@uef.fi
rami.korhonen@uef.fi

¹Corresponding author: Department of Applied Physics, University of Eastern Finland, POB 1627, FI-70211 Kuopio, Finland, Tel. +358-44-9691559

Abstract

Mechanical characterization of soft tissues such as ligaments remains challenging. There is variability in the measured material parameters of ligaments, most of which is related to natural tissue variability, but some of it can be a result of using different testing protocols. Generally preconditioning (cyclic loading-unloading) is performed prior to actual tests to reduce the experimental variability. Commonly, preconditioning protocols for ligaments with a small strain level and 10 sinusoidal loading-unloading cycles are used. The effect of preconditioning and its parameters including strain level, number of cycles and number of

¹ contributed equally to this study.
preconditioning repetitions on the repeatability of tensile stress-relaxation tests are poorly known for knee ligaments. In the present study, forty-eight dumbbell-shaped bovine anterior cruciate ligament (ACL) samples were used to evaluate the repeatability of stress-relaxation response. Different preconditioning protocols with 2% and 6% strain levels and 1, 5 or 10 preconditioning repetitions were applied. After preconditioning, one-step stress-relaxation test was carried out twice with an hour resting period in between the tests. The equilibrium stress showed no systematic bias when only one preconditioning repetition was applied (2.0±3.1% difference and p>0.05 between repeated tests). Systematic bias in the peak-to-equilibrium stress ratio was not observed when higher strain level and number of repetitions were used (0.5±1.6% difference and p>0.05 between repeated tests). In conclusion, the commonly used preconditioning protocol is capable of producing repeatable equilibrium stress levels of bovine ACLs from stress-relaxation tests in tension. However, if repeatable peak-to-equilibrium stress ratio is desirable, higher strain and number of preconditioning repetitions are recommended.

Keywords: Preconditioning; Anterior Cruciate Ligament; Tensile test; Stress-relaxation; Repeatability

1. Introduction

Ligaments like other soft tissues show a time- and history-dependent mechanical response. Time-dependent response is related to visco- and poroelastic behavior, whereas variability in the repeated tests of soft tissues may originate from the history-dependent behavior, which can be overcome via preconditioning (Fung, 2013). Preconditioning is typically performed in vitro by cyclically loading the tissue prior to the actual test in order to get a stable state, and subsequently consistent and reproducible material level properties by overcoming the history
dependency (Carew et al., 2000; Cheng et al., 2009; Fung, 2013; Quinn and Winkelstein, 2011). This is different from an in vivo state (Beynnon and Fleming, 1998), which may vary. Often, in vivo setting also characterizes structural level properties of tissues. Depending on the preconditioning protocol, alterations in recovery patterns of soft tissues may occur (Sverdlik and Lanir, 2001). Collagen fiber reorganization produced by preconditioning, particularly in tensile tests, is thought to be the main mechanism contributing to the soft tissue response following preconditioning. As the crimped collagen fibers become straightened and employed, the tensile response stabilizes (Carew et al., 2004; Quinn and Winkelstein, 2011).

Anterior Cruciate Ligament (ACL) is one of the main knee joint ligaments and is prone to daily and sport injuries. Knowledge of the mechanical behavior of ACL is highly important in order to understand its significance in the knee, when planning rehabilitation protocols and ACL reconstructions, and in computational models of knee joint mechanics. Material parameters that characterize the mechanical response of this tissue are mostly obtained from tensile tests, and there is a considerable variability in these parameters (Woo et al., 1991). Although this experimental variability is mostly attributed to natural tissue variability, part of it can be also a result of different test protocols used between studies.

Various studies have been conducted to evaluate the effect of preconditioning in different soft tissues on their mechanical properties (Carew et al., 2000; Cheng et al., 2009; Gefen et al., 2003; Pinto and Patitucci, 1980; Sverdlik and Lanir, 2001). Depending on the material being tested and the type of the mechanical test performed, the preconditioning protocols might vary. For instance, for aortic valve, the number of reported preconditioning cycles has ranged from a few (three to five) (Carew et al., 1999) to several cycles (>10) (Carew et al., 2000; Lee et al., 1994). Carew et al. (Carew et al., 2004) showed that aortic valves subjected to at least
five cycles of repeated load preconditioning and stress-relaxation can generate repeatable stress-relaxation responses (Carew et al., 2004). Cheng et al. (Cheng et al., 2009) studied rats’ spinal cord and proposed to perform preconditioning to the highest strain designated in the study. These studies would motivate applying a repeated preconditioning protocol and high strain also for ligaments.

Different preconditioning of quadriceps and patellar tendons was shown to alter their stiffness via progressive recruitment of collagen fibers (Schatzmann et al., 1998). Schatzmann et al. (Schatzmann et al., 1998) showed that tendons reached a stable dynamic stiffness and constant energy loss in the preconditioning protocol after performing 150 cycles ranging from 2.2 to 23 MPa. However, they did not assess the effect of preconditioning on a subsequent tensile test. Sverdlik et al. (Sverdlik and Lanir, 2001) investigated tendons and showed that after a few preconditioning cycles, the sample did not reach a stable stress-strain response. Moreover, they delineated that preconditioning is accompanied by elongation of the reference length, while repeating the whole preconditioning protocol can reduce the amount of elongation in consecutive loading steps (Sverdlik and Lanir, 2001). This study would further motivate repeating the preconditioning loading protocol rather than increasing the number of cycles.

Studies investigating ligaments and tendons have typically used various preconditioning protocols with 5 (Haraldsson et al., 2005; Miller et al., 2012a), 10 (Funk et al., 2000; Moon et al., 2006) and up to 240 (Dommelen et al., 2005) number of cycles. Strain levels of 1% (Miller et al., 2012b), 2% (Teramoto and Luo, 2008), 4% (Ciarletta et al., 2006), 8% (Dommelen et al., 2005) and even up to 20% (Funk et al., 2000) have been used in tests. A preconditioning protocol including 10 cycles with strain levels typically from 2 to 3% is found to be the most commonly used (Bigliani et al., 1992; Criscenti et al., 2016, 2015;
Yamamoto et al., 1999). However, the effect of different preconditioning protocols on repeatability of the time-dependent response of ACL in tension has not been well characterized.

Commonly used and published preconditioning protocols are generally utilized regardless of the specific knowledge of which protocol might produce the most repeatable results for a certain type of tissue and testing protocol or geometry. Furthermore, in some studies the mechanical test has been conducted without any preconditioning of the sample (Bigliani et al., 1992; Provenzano et al., 2001).

Some studies have investigated differences of the mechanical properties between preconditioned and unconditioned tendons and ligaments. Preconditioned tendons and ligaments have shown higher reference length, elastic modulus (Staubli et al., 1999) and ultimate strength (Schatzmann et al., 1998; Teramoto and Luo, 2008) as compared with unconditioned samples. These studies suggest that re-alignment of collagen fibers may be an underlying mechanism of preconditioning leading to lengthening and altered tensile properties. If one wants to avoid the initial tissue tension loss and lengthening, preconditioning is recommended. However, those studies did not evaluate whether the tensile behavior of ligaments and tendons in tests following preconditioning become stable and reproducible.

It seems that there is lack of studies evaluating the effect of different preconditioning parameters, such as strain level and repetition number, on repeatability of the subsequently performed mechanical testing of ACL. Therefore, the aims of this study were i) to investigate the repeatability of bovine ACL mechanical response to the commonly used preconditioning protocols and ii) to propose a new protocol to reach reproducible results. For this purpose, we defined several preconditioning protocols based on different number of repetitions and strain
levels. For the repeatability of the time-dependent behavior of ACL following preconditioning, we chose a stress-relaxation test which has been previously used to investigate the time-dependent tensile behavior of ligaments (Oehman et al., 2009; Provenzano et al., 2001). Based on the reported results (Carew et al., 2004; Cheng et al., 2009; Sverdlik and Lanir, 2001) and our pilot measurements, we hypothesized that a preconditioning protocol with higher strain levels and number of repetitions would result in better repeatability of ACL stress-relaxation response.

2. Materials and Methods

2.1. Specimen preparation

Eight ACLs were harvested for testing from bovine stifle joints. To preserve the mechanical and biological properties of ACLs, the specimens were immersed in Phosphate Buffered Saline (PBS, pH 7.4) in plastic containers. They were then fresh frozen at -20°C in PBS using a slow-freezing procedure (Moon et al., 2006). Prior to the mechanical testing, the specimens were thawed at room temperature. The tensile test specimens were cut from the middle part of the ACLs along the direction of collagen fibers using a parallel double razor tool and then ten millimeter long dumbbell shaped specimens were cut using a custom-made tool (n = 48; Fig. 1A). Cross-sectional area of the tissue samples was determined by measuring the width and thickness from the center of the samples using a microscope with a 4.6x magnification. Based on previous studies which used histological sections, the cross-sectional area was assumed to be elliptical and it was calculated accordingly (Duenwald et al., 2009; Pioletti et al., 1999; Provenzano et al., 2001). This assumption was also verified by cutting one sample in the middle and measuring the cross-sectional area directly by light
microscopy. This analysis confirmed that the assumption of elliptical shape is valid as the difference in the cross-section area between these two methods was 0.15% (elliptical assumption vs. direct measurement). In all steps from the sample preparation, geometry measurement and fixation to testing, the specimens were kept moist in PBS.
Figure 1: (A): A representative example of a bovine ACL specimen used in this study. (B) A representative sample between the clamps in a tensile test device (C): The experimental tensile test set-up. (D): The strain time-history protocol for investigating the repeatability.
2.2. Biomechanical testing

The ends of the specimens were glued on double-sided sandpapers (Mirox P80, Mirka Oy, Uusikaarlepyy, Finland), in order to avoid slipping, and clamped between custom-made tensile testing clamps (Chokhandre et al., 2015; Danso et al., 2014; Lynch et al., 2003; Fig. 1B). Preliminary tests showed that gluing sandpapers on the samples rather than on the clamps produced the best grip. The clamp screws were tightened to 4 Nm with a torque wrench. Tests were performed using a linear servo-motorized material testing device (Newport PM500-C Precision Motion Controller, Newport PM1A1798 Actuator and 25 lb load cell, Honeywell Model 31/AL311BL) equipped with a custom-made transparent chamber filled with PBS. Force and actuator displacement (clamp-to-clamp displacement (Hansen et al., 2002; Rigozzi et al., 2009; Sverdlik and Lanir, 2001)) were recorded during the tests at 100 Hz.

Commonly the ligaments have been preconditioned using 10 cycles and 2% or 3% strain levels (Bigliani et al., 1992; Criscenti et al., 2016, 2015; Moon et al., 2006; Oehman et al., 2009; S. L. Woo et al., 1986). In this study, six preconditioning protocols with 8 samples in each group were defined (Table 1, Fig. 1C). These protocols included two different strain levels (2% and 6%) and three different numbers of repetitions (1, 5, 10 times). Each repetition consisted of 10 sinusoidal cycles. We chose 6% as the maximum strain level because strain levels more than 6% have been suggested to cause single collagen bundle failure, which might result in permanent changes in the tensile behavior of ligaments (Noyes et al., 1974). Moreover, pilot measurements showed that bovine ligament failure occurs at much higher strain than 6%. In Table 1, the protocols with their abbreviations have been listed.
Table 1. Preconditioning protocols and their abbreviations.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1E2</td>
<td>1 repetition at 2% strain</td>
</tr>
<tr>
<td>R1E6</td>
<td>1 repetition at 6% strain</td>
</tr>
<tr>
<td>R5E2</td>
<td>5 repetitions at 2% strain</td>
</tr>
<tr>
<td>R5E6</td>
<td>5 repetitions at 6% strain</td>
</tr>
<tr>
<td>R10E2</td>
<td>10 repetitions at 2% strain</td>
</tr>
<tr>
<td>R10E6</td>
<td>10 repetitions at 6% strain</td>
</tr>
</tbody>
</table>

1 repetition = establishing zero load length followed by 10 loading cycles.

In each repetition, the zero-load length was first determined by applying a 0.05 MPa tensile stress (Criscenti et al., 2016; Henninger et al., 2013). When a stable force at each zero-load length (each repetition) was observed, the specimen was then elongated with 10 sinusoidal cycles to the predefined protocol strain using a 0.125 Hz frequency (Ristaniemi et al., 2018). After two minutes of recovery following each repetition, which was observed to be enough to stabilize the force, the zero-load length was re-established in such a way that the stable pre-stress of 0.05 MPa was refined. This was again followed by cyclic preconditioning loading. After the entire preconditioning protocol (1, 5 or 10 repetitions), the zero-load length was established again and the specimen was allowed to recover for two minutes before the stress-relaxation tests.

In the stress-relaxation test, the samples were elongated by 6% of their length with a 1%/s velocity, followed by a 30 minute relaxation period. Most of the ligament relaxation has been reported to occur during the first 20 minutes (Criscenti et al., 2015). The chosen strain level was below the yield point of ligaments, assumed to be in the linear stress-strain region. After one hour recovery (Moon et al., 2006) at the same zero load length, the stress relaxation test was repeated to test the repeatability of these tests (Fig. 1C). In our study, the stress at the zero-load length was observed to recover to the pre-stress value during the one hour recovery time.
Engineering strain and stress as a function of time were calculated by a custom MATLAB code (MATLAB R2016b, The MathWorks, Inc., Natick, MA, USA) according to the final zero-load length and the cross-sectional area. The stress was calculated by dividing the force with the initial area. The magnitudes of stress at the peak and at the end of relaxation (from this point onwards termed as equilibrium) were extracted from the data, and the peak-to-equilibrium stress ratio was calculated. The peak-to-equilibrium stress ratio was chosen as a variable to evaluate the level of relaxation following the load application. Tissue tangent modulus was calculated from the loading phase of the stress-relaxation test, as a linear fit between 5% and 6% strain in the stress-strain curve. For illustrative purposes, mean stress-relaxation curves of each protocol were normalized to the peak stress of the first relaxation test.

2.3. Statistical Analysis

Normality of the data were tested using Shapiro-Wilk test. Repeated measures t-test was used to examine if mean values of each variable between the first and second stress-relaxation tests were different from each other. To examine changes in the zero-load length after each preconditioning repetition, a mixed-design analysis of variance was performed (only protocols R10E2 and R10E6). Due to significant interaction effect (strain level × number of repetitions), repeated measures one-way analysis of variance was used to test the effect of repetition number and t-test to test the effect of strain level on the zero-load length. To examine effects of the preconditioning strain and number of repetitions on repeatability of the stress-relaxation response, percentage differences between the first and second stress-relaxation tests were first calculated for each variable. Then, effects of the preconditioning strain and number of repetitions were tested using a two-way analysis of variance. Pairwise post hoc comparisons were Bonferroni corrected. The critical level for statistical significance
was set at p<0.05. Statistical analyses were performed using IBM SPSS Statistics (version 21, IBM Corporation, Armonk, NY, USA).

3. Results

Mean changes of the zero-load length of ACLs in the different protocols are presented in Figure 2. There was a significant interaction effect of the repetition number and strain on the zero-load length (p=0.009). The zero-load length increased more with 6% compared to 2% preconditioning protocols (p<0.05 for all repetition numbers). In the preconditioning protocol with 6% strain, the zero-load length stabilized at the 9th repetition, where the significant difference compared to the 10th repetition disappeared (p=0.094). Similarly, as the samples were preconditioned with 2% strain, the zero-load length stabilized after the 6th repetition (p=0.248, compared to the 7th repetition) and after the 9th repetition (p=0.351, compared to the 10th repetition). All other comparisons between subsequent zero-load lengths were significantly different from each other (Fig. 2, p<0.05).
Figure 2: Zero-load length changes during preconditioning (with positive SD indicated by dotted line). Dagger (†) indicates no significant difference between consecutive repetitions (P>0.05). See text for more details.

Mean stress-relaxation responses of each protocol are presented in Figure 3. The absolute values of peak and equilibrium stresses (mean ± SD) for each protocol in each stress-relaxation step as well as the level of ligament relaxation (peak-to-equilibrium stress ratio) and ligament tangent modulus are presented in Table 2. The peak stress in the second relaxation test was significantly lower than that in the first test in all protocols (p<0.05). The equilibrium stresses were reproducible (the first and second measurement were not significantly different) with only one repetition and 2% or 6% strain levels (p>0.05). The level of relaxation showed a repeatable response using a protocol of ten repetitions and 6% strain (p>0.05 between repeated tests). Similarly to the peak stress results, the tangent modulus of ligaments was not reproducible in any of the investigated preconditioning protocols, and in 4 out of 6 protocols the second test produced higher tangent modulus.
Figure 3: The mean (±SD) stress-relaxation responses for each protocol (Table 1). Inset shows detailed view of the instantaneous response to the step-loading phase. Dashed lines represent standard deviation.
Table 2: The absolute values of variables from the first and second stress-relaxation tests.

<table>
<thead>
<tr>
<th>Protocols</th>
<th>Peak Stress (MPa)</th>
<th>Equilibrium Stress (MPa)</th>
<th>Peak to Equilibrium Stress Ratio</th>
<th>Tangent modulus (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st</td>
<td>2nd</td>
<td>1st</td>
<td>2nd</td>
</tr>
<tr>
<td>R1E2</td>
<td>1.51±0.74</td>
<td>1.23±0.55</td>
<td>0.90±0.49</td>
<td>0.88±0.47</td>
</tr>
<tr>
<td>R1E6</td>
<td>2.58±1.56</td>
<td>2.23±1.35</td>
<td>1.62±1.12</td>
<td>1.58±1.07</td>
</tr>
<tr>
<td>R5E2</td>
<td>2.38±1.52</td>
<td>1.94±1.31</td>
<td>1.42±1.12</td>
<td>1.35±1.05</td>
</tr>
<tr>
<td>R5E6</td>
<td>2.56±0.96</td>
<td>2.39±0.93</td>
<td>1.86±0.75</td>
<td>1.81±0.74</td>
</tr>
<tr>
<td>R10E2</td>
<td>2.16±1.21</td>
<td>1.84±1.10</td>
<td>1.38±0.89</td>
<td>1.31±0.88</td>
</tr>
<tr>
<td>R10E6</td>
<td>3.88±1.76</td>
<td>3.80±1.78</td>
<td>2.95±1.42</td>
<td>2.88±1.42</td>
</tr>
</tbody>
</table>

For clarity, parameters that were not significantly different from each other have been shown. There were significant differences between the 1st and 2nd relaxation tests in all parameters except the ones marked with dagger. †: P >0.05

Since we were evaluating the repeatability of the ligaments with two different stress-relaxation steps, the percentage difference of the above-mentioned variables in different preconditioning protocols can be meaningful to assess the repeatability, and can be used in statistical comparison of the protocols. The percentage difference (mean ± SD) between the first and second stress-relaxation responses for the variables including the peak stress, equilibrium stress, level of relaxation (peak stress to equilibrium stress ratio) and tangent modulus are shown in Figure 4. The interaction effect of the strain level and number of repetitions was significant regarding differences in the peak stresses (p=0.036 for interaction effect, Fig. 4A). There was no significant effect of repetitions on differences in the first and second peak stresses at 2% strain (p>0.05). However, at 6% strain, protocols with 5 and 10 repetitions showed significantly lower differences in peak stresses compared to those with one repetition (p<0.05). In the preconditioned ACLs with 5 and 10 repetitions, the mean difference in the first and second peak stresses was significantly lower when 6% strain was applied compared to the applied 2% strain (p<0.05, Fig. 4A).
Figure 4: The percentage differences in the analyzed parameters between the first and second stress-relaxation responses. †: P<0.05

There was no significant effect of the number of repetitions (p=0.104 for main effect) or strain (p=0.150 for main effect) on equilibrium stress differences between the first and second stress-relaxation test (Fig. 4B). There was a significant interaction effect of the strain level and repetition number in the level of relaxation of the ligaments (Fig. 4C). The percentage difference of the first and second relaxation levels showed a significantly lower magnitude using a protocol with 10 repetitions and 6% strain compared to the other protocols (p<0.05), except the protocol with 5 repetitions and 6% strain (p=0.057). The interaction effect of the strain level and number of repetitions was also significant for the differences between the first and second tangent modulus (Fig. 4D). Similarly, with the results of the peak stresses, we did not observe any significant effect of repetitions at 2% strain level (p>0.05 between the
protocols), while at 6% strain level significantly lower differences in the tangent moduli between repeated tests were measured in 5 and 10 repetition protocols compared to the one repetition protocol (p<0.05, Fig. 4D).

4. Discussion

Previous studies have demonstrated that cyclic preconditioning prior to biomechanical testing can change the mechanics and structure of soft tissues, which can lead to different viscoelastic response (Carew et al., 2000; Cheng et al., 2009; Schatzmann et al., 1998; Sverdlik and Lanir, 2001). In order to stabilize the tissue before actual tests, the ACLs have been typically preconditioned with a commonly used protocol (Bigliani et al., 1992; Criscenti et al., 2015; Oehman et al., 2009; S. L. Woo et al., 1986). In this study, we evaluated the repeatability of a commonly used preconditioning protocol and compared it to several preconditioning protocols to assess the repeatability of subsequently measured stress-relaxation responses of bovine ACLs. We specifically showed that, depending on the analyzed parameter and especially ligament relaxation level, the repeatability of test results can be improved by adjusting the preconditioning protocol with higher strain level and number of repetitions. This was consistent with our hypothesis. On the other hand, and in contrast to our hypothesis, the commonly used protocol (smaller strain and one cyclic loading repetition) was shown to be applicable when the equilibrium response is of interest.

Consistent with the present study, a previous study showed an increase in the reference length of tendons during consecutive stretching cycles (Sverdlik and Lanir, 2001). They also showed intensified sample length with increasing strain (Sverdlik and Lanir, 2001). However, the reference length was not shown to be stabilized. In our study, the zero-load length under
constant stress increased as a function of the repetition number until a plateau was reached at the 6th or 9th repetition. It may be that full recovery of the collagen crimp pattern requires active cellular processes (Lavagnino et al., 2017), while re-crimping of the collagen fibers seems to become repeatable with increasing number of cyclic loading repetitions. In addition, it can be assumed that straightening of the collagen crimp was more complete with the higher strain level used during the preconditioning and therefore greater amount of crimp recovery would be needed after higher strains, explaining larger increases in the zero-load length with higher strain levels. Nonetheless, this analysis supports the use of higher number of cyclic loading repetitions in order to obtain repeatable measurements.

The peak to equilibrium stress ratio, which indicates the level of tissue relaxation, showed to be quite repeatable by adjusting the precondition parameters. However, the results for 2% strain levels demonstrated no significant effect of the number of repetitions. In contrast, the repetition effect became more significant using the 6% strain level. At this strain level, increasing the repetition number led to improved ACL relaxation level repeatability. The preconditioning protocols with 10 repetition times at either 2% or 6% strain level showed the smallest differences, 4.4% and 0.5%, respectively, between the levels of relaxation from the first and second measurements, while the difference using the commonly used protocol was 16.3%. Furthermore, the only statistically repeatable protocol (p=0.703 between the first and second measurements) included 6% strain and 10 repetitions, showing that this protocol was best suited for analyzing peak to equilibrium stress ratio among the protocols used in the current study. This further suggests that the repetition number in addition to the strain level of preconditioning might change the ligament structure so that the ligament viscoelastic behavior stabilizes (Cheng et al., 2009; Schatzmann et al., 1998; Sverdlik and Lanir, 2001). On the other hand, the commonly used preconditioning protocol is not suggested to be optimal one for repeatable analysis of this relaxation parameter.
Similar to the results of the relaxation level, at 2% strain level, the number of repetitions did not have a significant effect on the differences between the first and second peak stresses. In the protocols with 6% strain, the second peak stress reached 82% and 97% of the first peak stress when 5 and 10 repetitions were applied, respectively, while that of the commonly used protocol reached 72% of its initial value. In addition, peak stress differences in the protocol with 6% strain and 10 repetitions were significantly different from all other protocols, except that with 6% strain and 5 repetitions. Similarly, difference in the first and second tangent moduli in the protocol with 6% strain and 10 repetitions were different from the other protocols except the one with 6% strain and 5 repetitions. Therefore, consistent with the relaxation level results, the most repeatable peak stresses and tangent moduli were obtained using higher strains and more repetitions. Since the peak stress is mainly controlled by the collagen fiber stiffness and recruitment, it may be that the progressive recruitment of the crimped collagen fibers at higher strain has stabilized the tissue (Provenzano et al., 2001; Viidik, 1972).

The equilibrium stress from repeated stress-relaxation responses was repeatable when the preconditioning protocol of either 2% or 6% strain level and one cyclic loading repetition was used. We would therefore recommend employing the commonly used protocol when a repeatable equilibrium stress level is a desirable parameter. Though, we acknowledge that, in contrast to all other parameters, percentage differences between the first and second repeated equilibrium stresses were not significantly different between any of the protocols. Therefore, firm conclusion regarding superiority of the commonly used protocols for repeatability of equilibrium stresses cannot be drawn.

In tensile tests, the grip between the clamps and the sample is critical and can be considered as a limitation for repeatable measurements (Chokhandre et al., 2015). Typically, sandpaper
is used to avoid slipping. In some studies, sandpaper was not utilized (Henninger et al., 2013; Lujan et al., 2007). In a typical tensile test, sandpaper is glued on the clamps (Chebil et al., 2012; Danso et al., 2014) while here we glued the double-sided sandpaper on the tissue samples because preliminary tests showed it to work better. Good grip was presumably maintained throughout the tests which is supported by repeatable measurements, especially at higher strain and number of repetitions. Actually, the tangent modulus calculated from the rising part of the stress-relaxation test could be even higher at the repeated test (even though not statistically), even further supporting that a good grip was maintained.

Relatively small number of ACLs can also be considered as a limitation of this study. This may explain the lack of significant differences in some of our protocols. However, no systematic differences between two repeated tests were observed (i.e. always higher or lower in the second test) in those protocols where insignificant differences were observed, suggesting that higher number of samples would likely produce the same conclusions.

We measured clamp-to-clamp strain in this study (Rigozzi et al., 2009; Sverdlik and Lanir, 2001). We acknowledge that more local tissue strain analyzed from markers might be slightly different from clamp-to-clamp strain. However, a previous study on rat tail tendon (Hansen et al., 2002) showed that clamp-to-clamp strain corresponds to local tissue strain. Nevertheless, the used strain measure should not affect our conclusions because we always compared the first and second stress-relaxation measurements of each sample.

Due to logistic reasons, we froze the samples before thawing them for the tests. The samples were frozen at -20 °C with a slow-freezing procedure. Fast freezing has been shown to reduce the loading capacity of tendons (Oswald et al., 2017) due to structural damages (Park et al., 2009). In contrast, slow freezing at -20 °C has been shown to produce no alterations on the tensile mechanical properties of ligaments (Jung et al., 2011; Moon et al., 2006). The most
recent study on rat Achilles tendon demonstrated that the tensile properties of tendons start to alter when frozen for 9 months or when the sample is subjected to 5 freezing-thawing cycles (Quirk et al., 2018). However, we kept our samples frozen for a short time and thus we expect no drastic alterations of the tensile properties of our samples due to freezing. Furthermore, when comparing fresh and frozen samples, one freezing-thawing cycle has shown to produce no significant alterations on the mechanical properties of ligaments and tendons (Viidik and Lewin, 1966; S. L.-Y. Woo et al., 1986). Nevertheless, we cannot rule out the possibility that freezing the samples might have affected our results.

Our values for the tensile tangent modulus of protocols with 6% strain and 5 or 10 preconditioning repetitions were consistent with an earlier study for bovine ACL using frozen and thawed dumbbell-shaped samples (Pioletti et al., 1999). Our values were also consistent with the literature values of human ACL modulus obtained using frozen and thawed dumbbell-shaped samples (Chokhandre et al., 2015) and using fresh bone-ligament-bone complexes (McLean et al., 2015) as well as frozen and thawed bone-ligament-bone complexes (Chandrashekar et al., 2006; Noyes and Grood, 1976). Our values for the peak and equilibrium stresses were consistent with the values obtained in earlier measurements on frozen and thawed bone-ligament-bone complexes (Dommelen et al., 2005). However, our values for the tensile tangent modulus in other protocols (except protocols with 6% strain and 5 or 10 preconditioning repetitions) were generally lower than those of the abovementioned literature values. The possible reason for this might be that the collagen fibers are not fully employed and straightened using low strain and low number of preconditioning repetitions, leading to lower tensile modulus. On the other hand, there was a lot of sample-specific variability in the stress and tangent modulus values and the mean values could differ substantially from group to another. Considering the design of the experiment, sample preparation and the chosen relaxation time (30 min) may also affect the values of the
analyzed parameters. However, those factors should not affect the results regarding repeatability because always the same sample was tested twice, enabling the comparison between the first and second stress-relaxation tests.

In this study, bovine ACL samples were used due to easy availability, ethical issues and good comparability with respect to human ACL. Animal models are typical in musculoskeletal biomechanics studies (Cone et al., 2017) and among commonly used large animal models, the anatomy of bovine knee ligaments was found to be the most corresponding to human ligaments (Proffen et al., 2012). The collagen content of human knee joint ligaments (Mow and Huiskes, 2005) is also comparable to that of bovine ligaments (Eleswarapu et al., 2011). Moreover, ACL has similar composition and structure (e.g. collagen and water content) compared to other knee joint ligaments (Rumian et al., 2007). However, even though with these similarities, the mechanical properties of different ligaments can be different (Ristaniemi et al., 2018) and dependent on the species (Noyes and Grood, 1976), and the results of the present study may not be directly applicable to ACL of humans or other species, or to other ligaments. This should be clarified in the future.

The findings of the current study reveal the importance of strain levels and number of repetitions of preconditioning protocols on subsequently measured and reproducible stress-relaxation responses. We recommend that our findings should be considered when planning tensile tests of ligaments and aiming for reproducible results. However, care should be taken if applying the preconditioning protocols of this study to other studies with different boundary conditions and tissue types.

**Conflicts of interest statement:**

The authors have no conflicts of interest to declare.
Acknowledgment:

The research leading to these results has received funding from the Academy of Finland (Grant 286526).

References:


Chokhandre, S., Colbrunn, R., Bennetts, C., Erdemir, A., 2015. A comprehensive specimen-specific multiscale data set for anatomical and mechanical characterization of the


Oswald, I., Rickert, M., Brüggemann, G.-P., Niehoff, A., Ulloa, C.A.F., Jahnke, A., 2017. The influence of cryopreservation and quick-freezing on the mechanical properties of


Mechanical Tensile Properties of the Quadriceps Tendon and Patellar Ligament in Young Adults. 27, 1–11.


https://doi.org/10.1177/036354659101900303

