Repeated Subrupture Overload Causes Progression of Nanoscaled Discrete Plasticity Damage in Tendon Collagen Fibrils

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ABSTRACT: A critical feature of tendons and ligaments is their ability to resist rupture when overloaded, resulting in strains or sprains instead of ruptures. To treat these injuries more effectively, it is necessary to understand how overload affects the primary load-bearing elements of these tissues: collagen fibrils. We have investigated how repeated subrupture overload alters the collagen of tendons at the nanoscale. Using scanning electron microscopy to examine fibril morphology and hydrothermal isometric tension testing to look at molecular stability, we demonstrated that tendon collagen undergoes a progressive cascade of discrete plasticity damage when repeatedly overloaded. With successive overload cycles, fibrils develop an increasing number of kinks along their length. These kinks—discrete zones of plastic deformation known to contain denatured collagen molecules—are accompanied by a progressive and eventual total loss of *D*-banding along the surface of fibrils, indicating a loss of native molecular packing and further molecular denaturation. Thermal analysis of molecular stability showed that the destabilization of collagen molecules within fibrils is strongly related to the amount of strain energy dissipated by the tendon after yielding during tensile overload. These novel findings raise new questions about load transmission within tendons and their fibrils and about the interplay between crosslinking, strain-energy dissipation ability, and molecular denaturation within these structures. © 2012 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 31:731–737, 2013

Keywords: collagen fibril; mechanical overload; discrete plasticity; tendon strain; molecular denaturation

A critical mechanical feature of tendons and ligaments is their resistance to catastrophic failure when overloaded, resulting in a strain or sprain rather than a rupture. Strains and sprains are common. In 2006 and 2010, nearly 40% of all occupational injuries in the United States resulting in time off work were strains or sprains.[1,2] The resulting economic impact of these injuries is sizable. In 2009, workplace injuries cost $170 billion U.S. dollars, half of which was attributed to lost wages and productivity.[3] With such a high rate of incidence, strains and sprains not only accounted for more lost days of work than any other occupational injury, but more than the next three leading causes combined.[1]

To medically treat sprains and strain more effectively, it is necessary to know how collagen fibrils, the primary load bearing elements of tendons and ligaments, respond to mechanical overload. Given the economic impact of these injuries, it is surprising how little is known. Among the few ultrastructural studies of overloaded collagen fibrils that have been conducted, nearly all have used the same tissue model, rat tail tendons, and the same investigative technique, transmission electron microscopy.[4–7] These studies all document the same overload-induced phenomena: the dissociation of fibrils into their subfibrillar components.

Recently, using a bovine tail tendon model and scanning electron microscopy (SEM) combined with enzymatic probes to assess both fibril structure and molecular conformation simultaneously, we documented a mode of strain-induced collagen fibril damage that had not been previously reported.[8] We found that ruptured tendons contained fibrils with repeating kinks partially made up of unwound (denatured) collagen molecules. In a subsequent study,[9] we showed that the same mode of fibril disruption, which we have termed "discrete plasticity," also occurs at subrupture levels of overload, that is, after the tissue's yield point but prior to macroscopic tearing.

In the present study we investigated how repeated application of subrupture tensile overload affects the phenomenon of discrete plasticity in collagen fibrils. We showed that with successive overload cycles, fibrils undergo a progressive cascade of discrete plasticity damage. The sequence of events that we documented reveals that the zones of plastic deformation created within fibrils as a result of overload do not progress to failure, and consequently that discrete plasticity does not diminish the load bearing capacity of fibrils. These novel findings raise new questions about load transmission within tendons and their fibrils and about the interplay between crosslinking, strain-energy dissipation ability, and molecular denaturation within these structures.

METHODS

Tails were collected from 24 to 36 months old steers, freshly killed for food at a local abattoir. Tendons were dissected from the dorsal, proximal region of each tail, laid flat between sheets of gauze moistened with phosphate buffered saline (PBS), double bagged, and stored at −86°C. Forty-two

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tendons dissected from five tails were used. Before testing, frozen tendons were allowed to thaw in their sealed bags. The tendons were evenly distributed between two mechanical overload groups: 5 and 15 cycles. Each tendon was cut to a length of 40 mm. The cross-sectional area of each tendon was measured using a previously employed technique.[8] Each tendon was first vertically suspended above a scale marker and photographed four times using a 12.0 megapixel digital camera. Between each photo the tendon was rotated 90° axially. Using ImageJ software (version 1.44o; NIH), the diameter of each tendon was measured in each photo at three locations equally spaced over the tendon's length. The measured diameters were then used to calculate each tendon's mean elliptical cross-sectional area.

Using a servo-hydraulic materials testing system run by custom software programmed using LabVIEW (version 6.1, National Instruments), each tendon was subjected to a maximum of 5 or 15 cycles of repeated tensile overload (Supplementary Fig. S1). The mechanical overload procedure, designed to induce plastic deformation during each cycle while preventing tendon rupture, was conducted as follows. After mounting each tendon to an inter-grip gauge length of 15 ± 1 mm, the tendons were stretched at a strain-rate of 0.005 s^{-1} and kept moist via a regular application of PBS droplets. During stretching, the load-deformation response of each tendon was monitored in real time at a rate of 20 Hz. When a linear trend line fit to the last 40 recorded data points reached a slope of zero, the actuator reversed direction and moved back to the zero position at the same rate. This stretching process was then immediately repeated until either the prescribed number of overload cycles had been completed, or the tendon failed to support a load greater than 25 N. The gripped ends of each tendon were then cut away, isolating the gauge region for subsequent assessment using SEM or hydrothermal isometric tension (HIT) testing.

Mechanical overload data were analyzed using Microsoft Excel (Office 2011 for Mac). Based on the results of a previous study,[9] the primary response of interest was post-yield dissipated energy (*PYDE*): the amount of strain energy that a tendon dissipated after yielding during a loading cycle (Fig. 1). The *PYDE* was calculated for each overload cycle a tendon experienced. A tendon's cumulative *PYDE* was calculated by summing its *PYDE* values from cycle 2 onward.

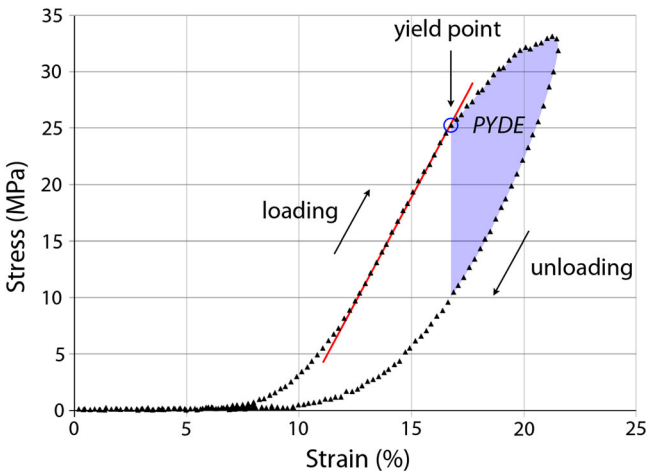

Figure 1. For each overload cycle, the area enclosed within the loading–unloading loop is equal to the amount of input strain energy per unit of tendon volume that is not elastically recovered during unloading. The portion of this "dissipated" strain energy that occurs after a tendon's yield point is termed the post-yield dissipated energy (*PYDE*).

Following the mechanical overload procedure, two tendons from the five-cycle-group and two from the 15-cycle-group were prepared for SEM. Each tendon was fixed immediately after the mechanical damage procedure using 2.5% SEM-grade glutaraldehyde. The tendons were then rinsed in distilled water and cut in half longitudinally to expose their interior. One half of each sample was then dehydrated in graded ethanol, critical-point dried, coated using gold-palladium, and examined at magnifications up to 90,000× using a Hitachi S-4700 SEM operating at 3 kV and 15 μA. Using the resulting micrographs of damaged fibrils, separation distances between fibril kinks were measured using ImageJ software (version 1.44o). Using all measurements made, relative frequency histograms for each sample group (5 and 15 cycles) were constructed using Microsoft Excel.

The remaining 19 tendons from each group were prepared for HIT analysis immediately following the mechanical overload procedure. Each tendon was first longitudinally bisected, producing two samples. One sample from each tendon was reserved for a separate study, while the other sample underwent HIT analysis. As documented previously, longitudinally bisecting tendons does not alter their response during HIT analysis.[10]

HIT analysis was conducted using a custom-built, six-sample apparatus, described previously.[11] Samples were vertically mounted between the system's load cells and rigid rods using small clamps, then submerged in a beaker of distilled, room temperature water. A 100 g pre-load was applied to each of the specimens, which were then left to relax at fixed extension for 30 min. Using a hotplate, the water was heated at a rate of 1.5 ± 0.1°C/min to 75°C. The rate was then decreased to 0.7 ± 0.2°C/min until the temperature reached 90°C, marking the end of the test. During HIT testing, time, temperature, and load data were recorded at 0.2 Hz.

HIT data were analyzed using Microsoft Excel. For each sample, two temperatures relating to its denaturation event were defined: the initial denaturation temperature, $T\mathrm{d}_\mathrm{i}$, marking the start of a sharp increase in load, and the final denaturation temperature, $T\mathrm{d}_\mathrm{f}$, marking the point (after $T\mathrm{d}_\mathrm{i}$) when load began to increase linearly with temperature (Fig. 2). The temperature at which each sample generated its maximum load, T_{Fmax}, was also recorded.

All data are presented as mean ± SD. Statistical analyses were conducted using JMP software (version 9.0.2). Values of $p \leq 0.05$ were considered significant. Responses for each overload group (5 and 15 cycles) were compared using *t*-tests. Relationships between the mechanical overload and HIT responses were investigated by fitting linear regressions, which were tested for significance using ANOVAs.

RESULTS

All of the overloaded tendons examined via SEM contained severely damaged collagen fibrils, easily recognizable by their highly kinked appearance (Fig. 3). While quasi-periodic repeating kinks of this nature are known to be strain-induced,[8,9] the kinked fibrils seen here were significantly more damaged than those previously found in tendons simply pulled to rupture:[8] that is, they were more highly kinked, and many had undergone a complete loss of *D*-banding along their

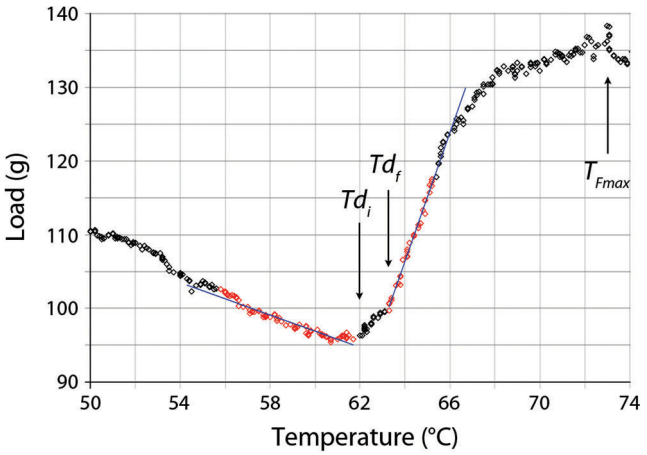

Figure 2. Three temperatures were recorded from each tendon's HIT response: (i) the initial denaturation temperature, $T\mathrm{d}_\mathrm{i}$, (ii) the final denaturation temperature, $T\mathrm{d}_\mathrm{f}$, and (iii) the temperature of maximal isometric force generation, T_{Fmax}. Linear trend lines fit to the data were used to help identify $T\mathrm{d}_\mathrm{i}$ and $T\mathrm{d}_\mathrm{f}$.

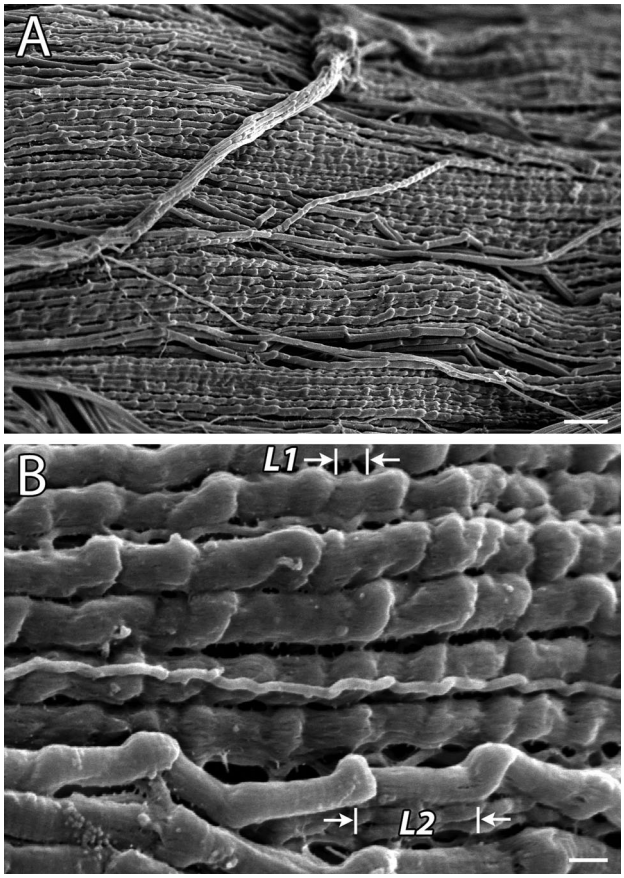

Figure 3. (A) Kinked fibrils were usually found in large arrays, meaning that a damaged fibril was usually bordered by many similarly damaged fibrils, as shown here. Bar 1 μm. (B) Several severely damaged fibrils with short distances between successive kinks and a complete loss of *D*-banding are shown ($L_1 = 165$ nm). A less damaged fibril with a greater separation distance between successive kinks and slightly obscured *D*-banding is also shown ($L_2 = 635$ nm). Bar 200 nm. The sample shown in these micrographs had undergone five overload cycles.

surface (Fig. 3B). Using micrographs, the distance between consecutive kinks on individual fibrils was measured. For the two tendons that had been subjected to five overload cycles, 190 measurements made along 39 kinked fibrils showed that kinks were separated by 287 ± 113 nm. For the two tendons that had been subjected to 15 overload cycles, 275 measurements made along 59 kinked fibrils showed that kinks were separated by 217 ± 111 nm, a significantly shorter distance ($p < 0.0001$). Plotting all measurements on a relative frequency histogram revealed that fibril kinks were most commonly separated by 175–200 nm in both the 5- and 15-cycle groups (Fig. 4).

Among the four tendons examined, one that had undergone 15 cycles of overload contained two unique features. First, after having undergone severe kinking, some fibrils had begun to break apart laterally into subfibrils (Fig. 5). Second, seen under low magnification, fibrils in one region of that tendon formed a sharp, triangular waveform crimp (Fig. 6A) with a mean wavelength of 6.6 ± 1.5 μm (eight measurements). We have not previously seen crimp of this nature in unloaded, control tendons. When examined at higher magnification, the fibrils forming this crimp were found to be highly kinked (Fig. 6B).

All tendons in the 5-cycle-group completed all five cycles. Tendons in the 15-cycle-group completed an average of 12 overload cycles (range 9–15 cycles). The cross-sectional areas of the tendons in the 5- and 15-cycle groups were 4.1 ± 1.3 and 4.5 ± 1.4 mm^2, respectively. The cumulative *PYDE*s were not significantly different between samples in the 5- and 15-cycle groups (660 ± 374 $\mathrm{kJ/m}^3$ vs. 871 ± 520 $\mathrm{kJ/m}^3$, respectively). For the 15-cycle-group, 72.2 ± 9.8% of the samples' cumulative *PYDE* was accumulated prior to the sixth loading cycle (Supplementary Fig. S2). Similarly, none of the three thermal responses measured via HIT differed between the two groups. While no differences were found between the groups, regression analysis

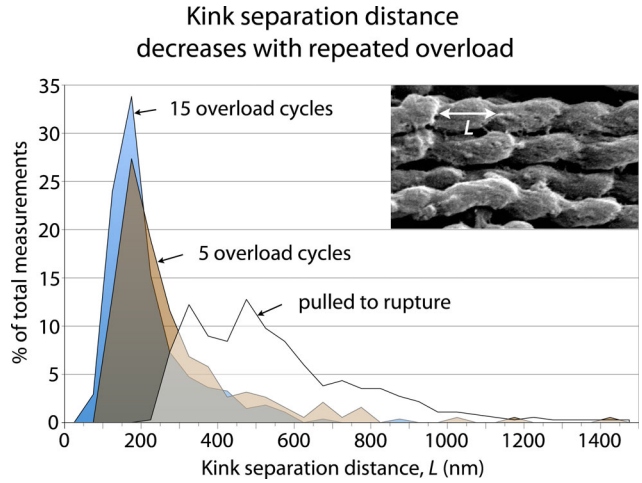

Figure 4. Comparing data for the current samples to those we previously pulled to rupture[8] shows that repeated overload decreases the distance between consecutive fibrillar kinks.

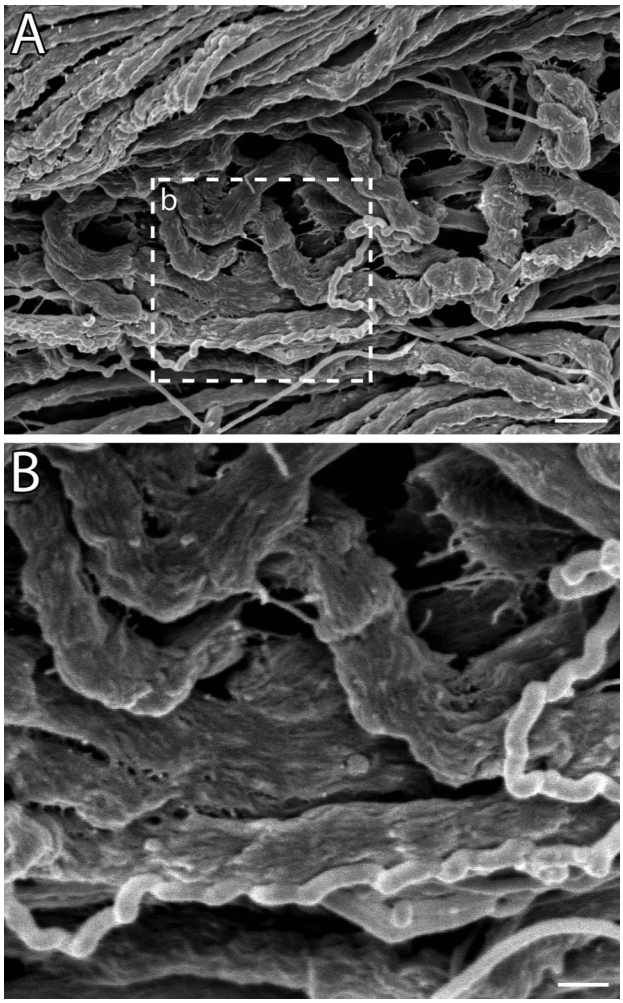

Figure 5. (A) The most severely damaged fibrils were found in a tendon that had undergone 15 overload cycles. Bar 600 nm. The dashed area "b" is shown below. (B) As well as having suffered the typical kinking and loss of *D*-banding resulting from excessive strain, these fibrils had also begun to break up into their subfibrillar components. Fibrils such as this extended over a relatively large area of >10,000 μm^2. Bar 200 nm.

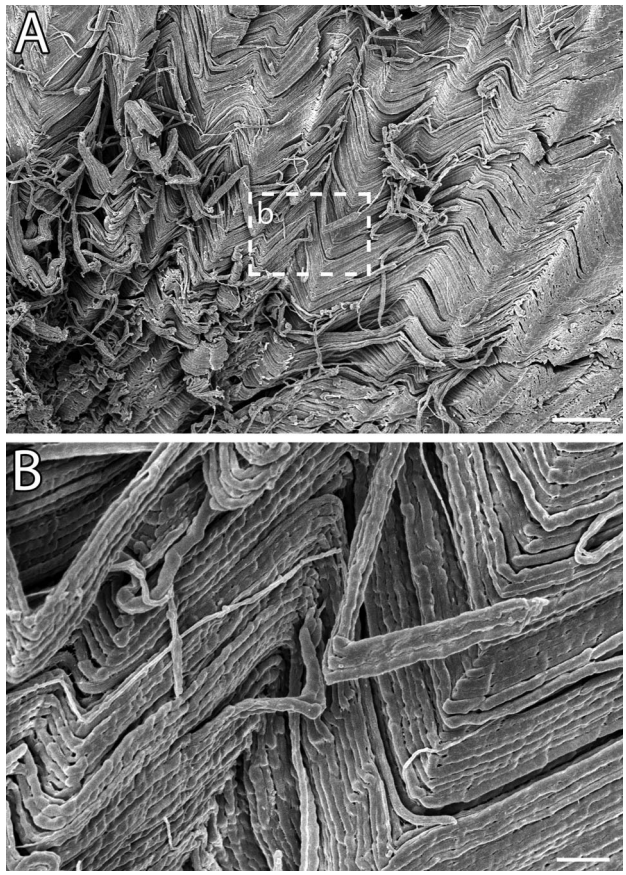

Figure 6. (A) A sharp triangular waveform crimp was observed in one tendon that had been subjected to 15 overload cycles. Bar 6 μm. The dashed area "b" is shown below. (B) At higher magnification, this crimp was found to be superimposed on kinked fibrils. Bar 1 μm.

showed that tendons containing collagen molecules with lower helix stabilities had experienced higher levels of cumulative *PYDE* during the preceding overload procedure (Fig. 7A and B). The same relationship was observed between T_{Fmax}, a measure of the tissue's intermolecular coupling, and cumulative *PYDE* (Fig. 7C).

DISCUSSION

Recently we showed that overloading tendons causes collagen fibrils to develop repeating kinks along their length,[8,9] a damage phenomena that we have termed "discrete plasticity." [9] While individually these kinks appear similar to those that form a tendon's native micro-scale crimp,[12] it is the frequency of their repetition that distinguishes them as being non-native. Fibril kinks that form a tendon's crimp repeat every 20–100 μm.[13–15] In contrast, when tendons are pulled to rupture, the kinks produced along fibrils—discrete zones of plastic deformation—reach a minimum separation distance of >240 nm, with the majority of kinks spaced between 300 and 600 nm apart (mean: 550 ± 225 nm).[8] Here, we showed that this separation distance is substantially reduced when tendons are subjected to repeated, subrupture, tensile overload (Fig. 4). In other words, when a previously strain-damaged fibril is re-overloaded, new zones of plastic deformation must develop between the previously plasticized zones, causing a progressive cascade of fibril disruption (Fig. 8).

Two important ideas arise from our finding that re-overloading a tendon causes new kinks to develop between the previously created kinks present along already damaged fibrils. First, for a fibril to undergo more kinking with additional overloading, the fibril's kinked regions must be sufficiently strong after formation that they remain stable—and do not fail completely—while the intervening regions along the fibril develop new kinks via this discrete plasticity mechanism. If each kinked structure requires significant strain energy input to form, then this mechanism is metaphorically similar to well-understood toughening mechanisms in other materials: for instance, dislocation movement and lock-up in metals.

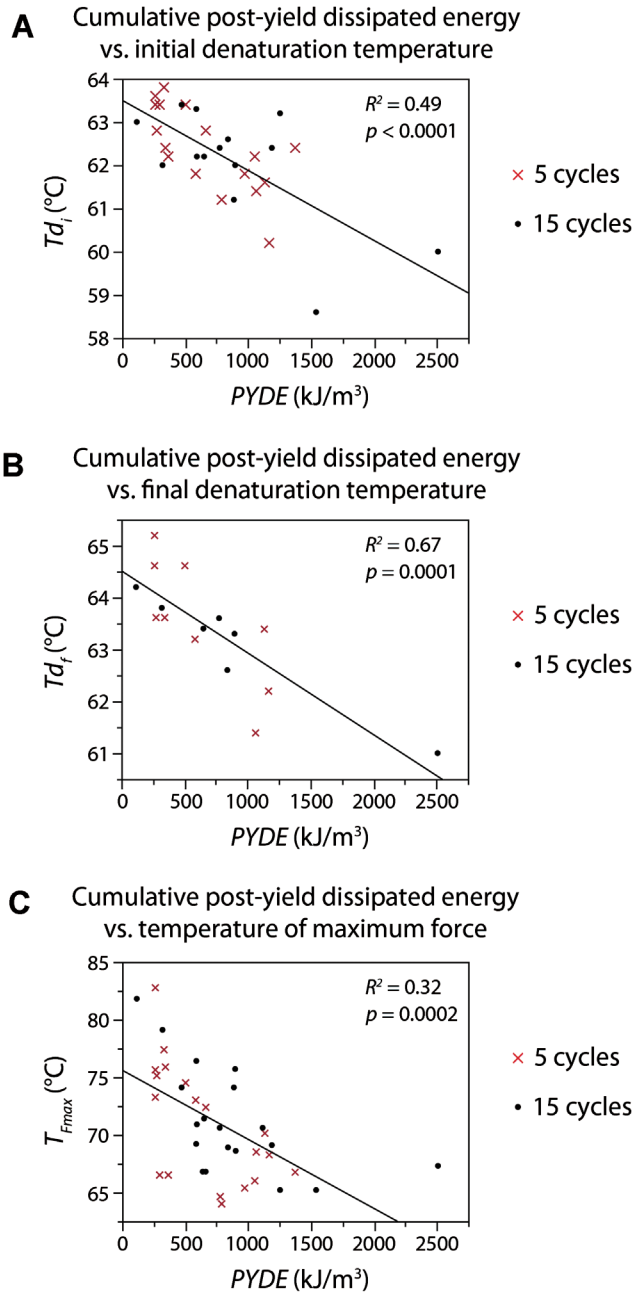

Figure 7. Tendons that dissipated more cumulative energy after yielding during mechanical overload (from cycle 2 onward) had lower temperatures for all three of the HIT responses.

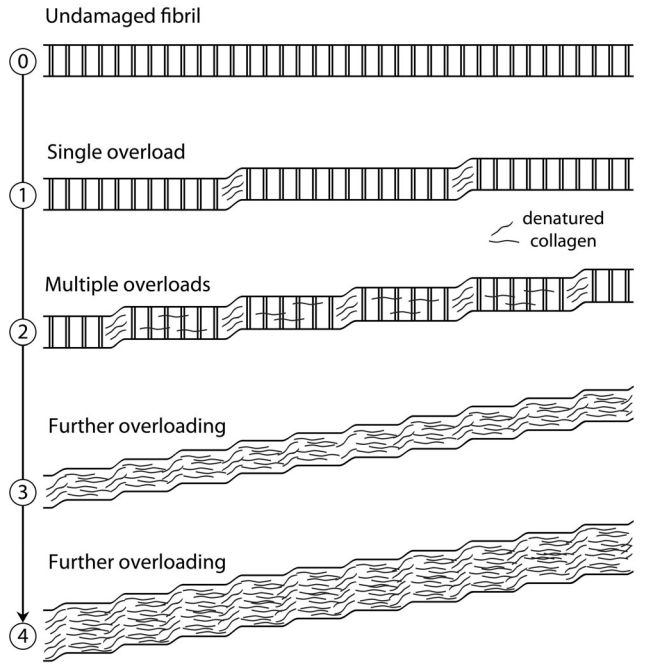

Figure 8. The progression of discrete plasticity damage in collagen fibrils with increasing subrupture overloading: (1) A single tensile overload cycle causes repeating kinks along fibrils. The kinks contain denatured collagen molecules. (2) Multiple overload cycles cause fibrils to develop more kinks between the initial kinks. Denatured collagen molecules are also produced along the surface of fibrils between kinks, partly obscuring the fibril's *D*-banding (bottom of Fig. 3B). (3) With further overloading, more kinks develop and fibrils lose all surface *D*-banding (middle of Fig. 3B). (4) Eventually, fibrils expand and acquire a loose fibrous appearance as the lateral cohesion between subfibrillar components is lost (Fig. 5B).

On the second front, when our tendons were repeatedly overloaded, the resulting damage did not appear to become uniformly distributed. Instead of most fibrils within the tendon reaching an equivalent level of damage, repeated overloading caused those fibrils damaged during the initial cycles to become increasingly damaged. As was the case for tendons simply pulled to failure,[8] it was common in our repeatedly overloaded tendons to find large regions of undamaged fibrils next to damaged, kinked fibrils. Unfortunately, a quantitative measure of the distribution of kinked fibrils within overloaded tendons will not be possible until the nanoscale features of discrete plasticity in fibrils can be mapped over large regions using microscale techniques.

To our knowledge, only one group previously studied the ultrastructure of tendons that had been repeatedly subjected to subrupture overload. Torp et al.[7] found that both repeated subrupture overload and complete tendon rupture had the same effect on rat tail tendons: the dissociation of fibrils into their subfibrillar components. Increasing age, and hence cross-link maturation,[16,17] may increase the resistance of fibrils to this mode of disruption,[6] thereby explaining the differences between these earlier observations and those presented here using our more mature bovine tail tendon model.

Several authors previously studied fatigue damage in tendons at the microscale under cyclic loading.[18–21] These studies all document the same initial structural change: development of a new, sharp crimp pattern with a relatively short wavelength. Consistent with these studies, we also observed a sharp crimp pattern within one of our repeatedly overloaded tendons (Fig. 6). Measurements of this overload-induced crimp using micrographs showed that its wavelength varied between ~5 and 8 μm, similar to the wavelengths seen by Dale and Baer[19] in rat tail tendons that had

been repeatedly loaded beyond their yield point. Unique to the crimp that we documented here is the observation that the microkinked fibrils had experienced discrete plasticity damage at the nanoscale. This finding illustrates that, while these two phenomena both involve fibril kinking, they may be the results of distinctly different processes. Further, discrete plasticity kinking does not inhibit the fatigue-induced kinking that produces sharp crimp.

HIT analysis has been used extensively to assess the thermal stability of collagen molecules within soft tissues.[22–25] During this test, tension is generated within isometrically constrained tissue samples when the α-chains of collagen molecules gain sufficient kinetic energy to rupture their restraining intrahelical hydrogen bonds. Individual α-chains are then entropically driven to coil randomly, but are prevented from doing so by the sample grips, resulting in the generation of a measureable internal tension. As recently explained,[9] two temperatures describing a collagenous sample's denaturation event can be measured from such experiments (Fig. 2). The initial denaturation temperature ($T\mathrm{d_i}$) marks the beginning of the increase in load with temperature, corresponding to the denaturation of a sample's least stable collagen helices. The final denaturation temperature ($T\mathrm{d_f}$) marks the start of the directly proportional relationship between load and temperature. At this point, the total number of dissociated, water-solvated, α-chains has plateaued, and the sample begins to behave according to the theory of rubber elasticity.[26]

Willett et al.[10] used an HIT system closely related to our version[11] to show that a single overload cycle significantly reduced the initial denaturation temperature of young adult bovine tail tendons: the same tendon model that we used. Five cycles of subrupture overload further destabilized the collagen molecules. Our previous ultrastructural observations on ruptured tendons showed that the strain-induced kinks produced along fibrils contain collagen molecules that are at least partially denatured.[8] While it remains to be definitively proven, a logical connection exists between these two sets of observations: the reduced (onset) denaturation temperature of overloaded tendons results from collagen molecules located within the kinks of damaged fibrils. Similarly, the progression of discrete plasticity with repeated overload (Fig. 8) explains why tendons that were overloaded five times had lower denaturation temperatures than those overloaded only once.[10]

Unlike Willett et al.,[10] who reported a significant decrease in denaturation temperature for tendons overloaded five times versus only once, the tendons overloaded 15 times in our study did not have a lower denaturation temperature than those overloaded five times. This may be because the cumulative *PYDE* dissipated by our tendons was largely accumulated within the first five cycles (Supplementary Fig. S2), giving damaged fibrils in the 5- and 15-cycle groups similar kink densities (Fig. 4). While no differences between the 5- and 15-cycle groups were found, the tendon's denaturation temperatures were related to their preceding mechanical damage history. Tendons that had dissipated more cumulative energy after yielding (that is, those which had incurred more plastic deformation) had lower denaturation temperatures (Fig. 7A and B). Increasing overload damage produces more fibril kinks and hence more denatured collagen molecules. Therefore, that the strain energy "dissipated" after yielding is likely absorbed by collagen molecules, providing the energy input required for kinking/denaturation. In this scenario, higher levels of cumulative post-yield dissipated energy during mechanical overload would cause lower denaturation temperatures.

We have only explored discrete plasticity in low-load carrying tendons to date, but it is worth speculating on the functional advantages that the overload-induced progression of discrete plasticity in collagen fibrils may provide to tendons and possibly ligaments. From a mechanical standpoint, the ability of overloaded fibrils to undergo discrete plasticity provides a mechanism for dissipation of excessive strain energy via molecular slip and denaturation without catastrophic failure. If this is a general phenomenon in mammalian collagen, it could have evolutionary survival value. Further, because the denatured collagen molecules created in a fibril during discrete plasticity are restricted to specific subfibrils,[8] enzymatic removal of this damaged collagen could yield a mechanically sound scaffold on which to conduct rebuilding. This repair option seems preferable to joining a fibril's free ends were it to rupture when overloaded.

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