Tendon tissue microdamage and the limits of intrinsic repair

Stauber, Tino ; Blache, Ulrich ; Snedeker, Jess G

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Tendon tissue microdamage and the limits of intrinsic repair

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Abstract

The transmission of mechanical muscle force to bone for musculoskeletal stability and movement is one of the most important functions of tendon. The load-bearing tendon core is composed of highly aligned collagen-rich fascicles interspersed with stromal cells (tenocytes). Despite being built to bear very high mechanical stresses, supra-physiological/repetitive mechanical overloading leads to tendon microdamage in fascicles, and potentially to tendon disease and rupture. To date, it is unclear to what extent intrinsic healing mechanisms of the tendon core compartment can repair microdamage. In the present study, we investigated the healing capacity of the tendon core compartment in an ex vivo tissue explant model. To do so, we isolated rat tail tendon fascicles, damaged them by applying a single stretch to various degrees of sub-rupture damage and longitudinally assessed downstream functional and structural changes over a period of several days. Functional damage was assessed by changes in the elastic modulus of the material stress-strain curves, and biological viability of the resident tenocytes. Structural damage was quantified using a fluorescent collagen hybridizing peptide (CHP) to label mechanically disrupted collagen structures. While we observed functional mechanical damage for strains above 2% of the initial fascicle length, structural collagen damage was only detectable for 6% strain and beyond. Minimally loaded/damaged fascicles (2–4% strain) progressively lost elastic modulus over the course of tissue culture, despite their collagen structures remaining intact with high degree of maintained cell viability. In contrast, more severely overloaded fascicles (6–8% strain) with damage at the molecular/collagen level showed no further loss of the elastic modulus but markedly decreased cell viability. Surprisingly, in these heavily damaged fascicles the elastic modulus partially recovered, an effect also seen in further experiments on devitalized fascicles, implying the possibility of a non-cellular but matrix-driven mechanism of molecular repair. Overall, our findings indicate that the tendon core has very little capacity for self-repair of microdamage. We conclude that stromal tenocytes likely do not play a major role in anabolic repair of tendon matrix microdamage, but rather mediate catabolic matrix breakdown and communication with extrinsic cells that are able to effect tissue repair.

Introduction

A third of all musculoskeletal clinical visits relates to tendon and ligament disorders, with tendinopathy being the most common of these complaints [1,2]. Tendons are exposed to some of the most extreme mechanical stresses in the body, adapting to variations in mechanical load and appearing to be capable of self-repair to a limited extent [2,3]. Although tendinopathy is a complex disease with multiple factors leading to its onset, repetitive tendon overloading is suspected to play a central role [3]. Structurally (Fig. 1A), tendons are dense connective tissue bands that almost exclusively consist of extracellular matrix (ECM) produced by fibroblast-like stromal cells (tenocytes). The tendon ECM is mainly composed of type-1 collagen molecules that self-assemble into fibrils [4]. Tenocytes arrange these

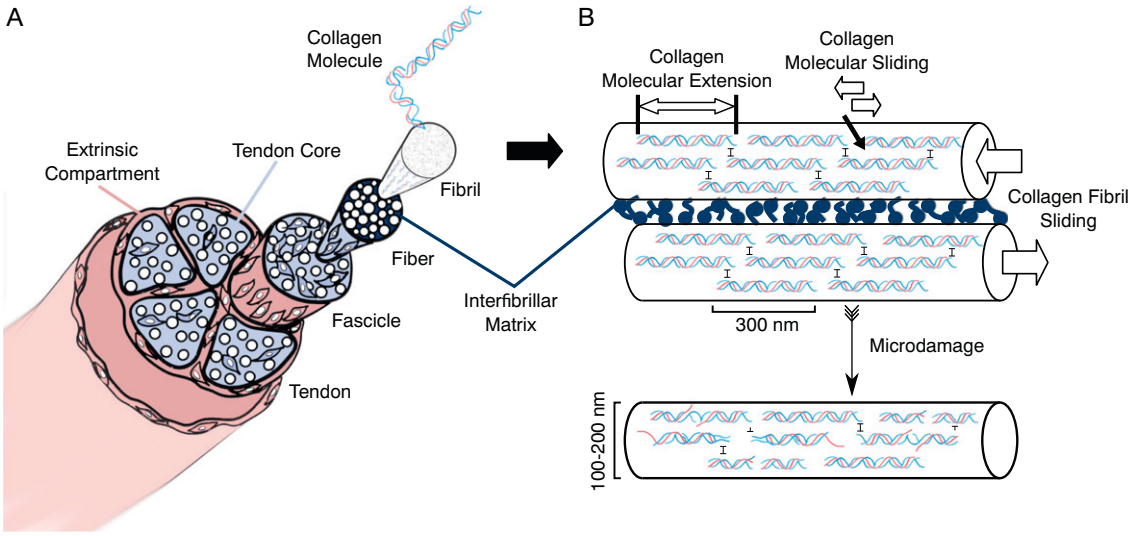

Fig. 1. Tendon hierarchical structure and microdamage concept. A) In tendon, the vascular, nervous and immune system reside in the synovial-like, extrinsic compartment. These systems are vital for the regeneration of whole tissue macrodamage, which often develops from accumulating microdamage in the load-bearing tendon core [2,9–13]. Fibroblastic cells/tenocytes present in the tendon core organize collagen-1 fibrils into fibers and then maintain the resulting, densely packed matrix [3,5]. Collagen fibrils form from collagen molecules through self-assembly. B) At low strains, collagen fiber/fibril sliding and load-transfer by the interfibrillar matrix are the dominating mechanisms creating microdamage [18–22]. Once the interfibrillar matrix fails at higher strains, collagen molecular sliding and extension become increasingly important [23]. At a certain (unknown) threshold, the intermolecular crosslinks fail and allow collagen molecular damage to accumulate [25–29].

fibrils into highly ordered and tightly packed collagen fibers [3–5]. Multiple fibers together with resident tenocytes form a fascicle, which is the fundamental functional unit of the tendon and therefore often considered to represent the so-called tendon core [2]. In their role as the main load-bearing tendon component, fascicles are subjected to very high mechanical stress and accumulate mechanical damage in the ECM [6–8]. It has been widely discussed that "microdamage" can accumulate until it exceeds a certain threshold, culminating in whole tissue damage with an activation of extrinsic repair mechanisms; e.g. recruitment of the vascular, nervous and immune system from the synovial-like tendon compartment surrounding the tendon core [2,9–13].

Our conceptual understanding of microdamage in the tendon core is still incomplete. In particular, only little is known about the intrinsic healing capacity and the mechanisms of tendon microdamage [2,11,14]. Moreover, it remains unclear how increasing strain levels actually impair the different load-transferring substructures of tendon fascicles and where the critical threshold of irreversible microdamage lies. In the present work, we study so-called positional tendons, which in contrast to energy-storing tendons transmit forces without storing energy for later release [15,16]. To fulfill this function, positional tendons are relatively inextensible under physiological loads and consequently experience low strains [17]. Here, tensile loads are transferred through both tensile and shear forces that respectively result in collagen fibril extension and sliding (Fig. 1B) [18–22]. The structures that enable and support collagen fibril sliding remain unknown but are probably located in the interfibrillar space. However, it has recently been shown that once a certain strain threshold is reached (2% strain), collagen fibril sliding becomes non-recoverable and intrafibrillar load-transferring mechanisms gain importance [23]. At the collagen intrafibrillar level, the onset of both recoverable and non-recoverable microdamage is still debated. Collagen fibrils gain their strength from intermolecular crosslinks. In the fibrils of energy-storing tendons, three collagen tropocollagen molecules are linked together forming a mature, trivalent crosslink. In contrast, only two tropocollagen molecules are linked in the fibrils of positional tendons. With increasing strain, these immature, divalent crosslinks are relatively unable to prevent collagen molecular sliding and molecular extension [24–27]. This sliding contributes to the mechanical disruption of triple-helical collagen molecules and α-chain pullout [28].

In this study we characterize the impact of increasing strain levels on the functional and structural properties of the tendon core over time. To do this, we combine biomechanical measurements of tendon properties with the visualization of collagen disruption at the molecular level using a collagen hybridizing peptide (CHP), which binds to exposed collagen α-chains [29–31]. Further, we investigate the long-term reaction

of the tendon core and its resident tenocytes to different levels of microdamage. We used rat tail tendon fascicles as a uniquely tractable explant model system that is amenable to high throughput ex vivo studies [32–34]. Within constraints of the model as a rodent positional tendon, it is ideally suited for the controlled study of tendon core biology and matrix structure-function interactions [35–39], as it lacks the vascular, nerve, and immune compartments and therefore extrinsic wound healing responses. Here, we exploited tendon fascicles to characterize the ability of the intrinsic tendon stromal compartment for self-repair after varying degrees of mechanically imposed matrix damage.

Results

Relation of strain and microdamage in (ex vivo) tendon fascicles

First, we imposed a single dose of stretch-induced matrix damage to explanted rat tail tendon fascicles (Fig. 2A) mounted to a custom-made stretching device (Fig. 2B) and assessed changes in the stress-strain curve and the collagen structure post-loading. Representative stress-strain curves of undamaged and damaged (8% strain) tendon fascicles are depicted in Fig. 3A. The recorded stress-strain curve of an intact

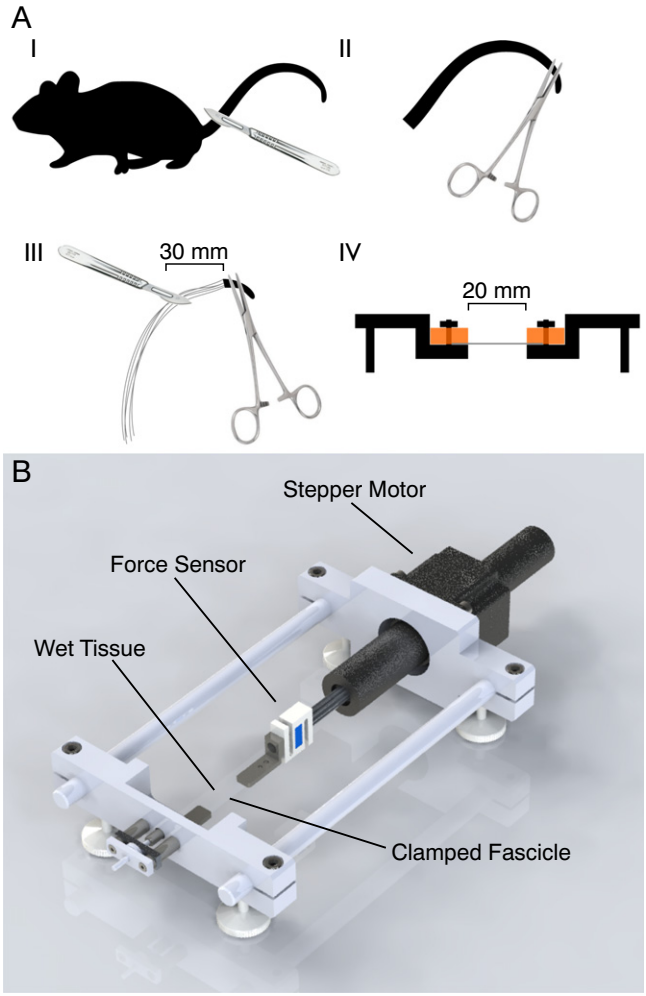

Fig. 2. Tendon fascicle extraction and ex vivo mechanoculture/testing. A) The rat tail tendon was dissected after euthanasia (I). Fascicles were extracted from the tail by gently pulling (II), cut into 30 mm long pieces (III) and mounted using metal clamps with a clamp-to-clamp distance of 20 mm (IV). B) Uniaxial stretching device used to conduct mechanical tests and expose fascicles to different strains. The clamped fascicles were mounted onto the metal holders and fixated with screws. Strain was applied using the stepper motor while the force sensor measured the resulting force. By combining the recorded force-displacement curve with the diameter measurements, we could generate the stress-strain curves.

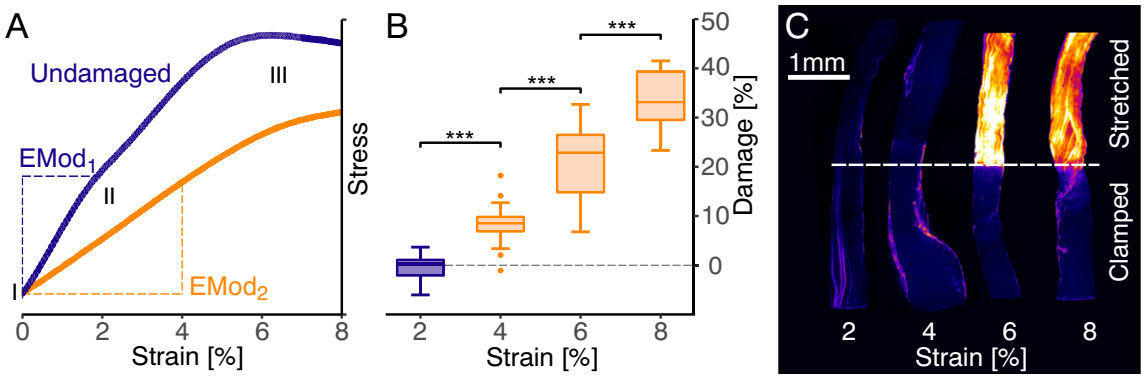

Fig. 3. Strain-damage relation of ex vivo tendon fascicles. A) Representative stress-strain curves of undamaged and damaged (8% L_0 strain) rat tail fascicles highlighting manifestations of microdamage: An elongated toe region (I), a flattening deflection point separating the stress-stiffening from the strain-softening region (II), and a decreased ultimate strain (III) [3,23]. To counteract the elongated toe region and achieve reproducible strain levels, we set the preload to the end of the toe region. In consequence, this region was not measured and is therefore not depicted. B) Boxplot showing the mechanical dose-dependency of functional damage in alive fascicles stretched from 2% to 8% L_0 ($n = 24$) after one loading cycle (day 0). The upper and lower hinge correspond to the first and third quartile (25th and 75th percentiles) and the middle one to the median. Whiskers extend from the upper/lower hinge to the largest/smallest value no further than 1.5 times the interquartile range. Data beyond the whiskers are treated as outliers. For the statistical analysis we performed a one-way ANOVA followed by Tukey post hoc tests (***$p < 0.001$). C) Representative fluorescence microscopy images of fascicles stained with collagen hybridizing peptide (CHP) after being subjected to different strain magnitudes. Regions with detectable collagen fibril disruption are indicated by the CHP signal (orange), regions without collagen fibril disruptions appear blue due to the autofluorescence of collagen.

murine tail tendon fascicle consists of a toe region followed by a strain-stiffening, linear elastic region at the transition point (Fig. 3A, I) [3,23,40]. The strain-stiffening linear elastic region ends at the deflection point (Fig. 3A, II), and the strain-softening linear elastic region begins. The strain-softening region continues until the ultimate tensile stress is reached (Fig. 3A, III). There, the curve plateaus until the fascicle fails macroscopically and tears apart. The curve slope changes, such as after the toe region (I), at the deflection point (II), and when reaching the ultimate tensile stress (III), indicate shifts in the matrix load-transferring mechanisms. Alterations in these slope changes are indicative of changes in the underlying load-transferring mechanism and therefore of functional damage. It has been shown that stretch-induced microdamage results in a drawn-out toe region, for which we controlled for with a specific preload to consistently reach comparable strains post-damage (Fig. 3A, I) [28]. Secondly, the inflection point separating the strain-stiffening from the strain-softening linear elastic region became less pronounced post-damage (see Fig. 3A, II). This alteration originated from the loss of the strain-stiffening linear elastic region in fascicles loaded above 2% of the initial clamp-to-clamp distance (L_0). We therefore recorded functional impairment at D0 starting between 2% and 4% L_0 strain (Fig. 3B), a strain region coinciding with the inflection point. At 4% L_0 strain we measured a decrease in the linear elastic modulus of around 10%. The functional impairment then increased in a mechanically dose-dependent manner, reaching around 20% at 6% L_0 strain and over 30% at 8% L_0 before the fascicle failed macroscopically for strains above 10% L_0. All differences in the imposed functional impairment that accompanied the various strain dosing levels were statistically significant.

Next, we examined how functional damage corresponds to the onset of collagen fibril disruption. Therefore, we incubated fascicles stretched to different strains with the collagen hybridizing peptide (CHP). No collagen molecular damage was detected for fascicles stretched to 2% and 4% L_0 (Fig. 3C). However, in fascicles stretched to 6% and 8% L_0, we observed a strong hybridization of CHP and therefore structural microdamage at the molecular level. No CHP hybridization/collagen damage was seen in the clamped (non-stretched) regions, as the compressive forces resulting from the clamping do not cause collagen fibril unfolding. Interestingly, the start of collagen disruption coincides with the end of the strain-softening, second linear elastic region in the stress-strain curve, located between 4 and 6% L_0 strain (Fig. 3A & C).

Taken together, these data show how different strains translate to structural and functional tendon tissue microdamage phenotypes.

Longitudinal tracking of microdamage in tendon fascicles

We next investigated if and how the fascicles are able to recover from the different levels of induced

microdamage. Undamaged (clamped) fascicles fully maintained their mechanical functionality over 7 days (Fig. 4A). In contrast, already the small strains necessary for the daily measurement of the mechanical properties in the 2 and 4% L_0 strain groups caused a significant decrease in the elastic modulus in addition to the initial drop in the 4% L_0 strain condition when comparing pre-damage to D7 (see Supplementary Table 1 for the statistical analysis). Interestingly, this trend of decreasing mechanical functionality did not occur in devitalized fascicles stretched to 2 and 4% L_0 (Fig. 4A), which had elastic moduli similar to the undamaged fascicles after seven days (non-significant difference), indicating that cellular-driven processes are contributing to the progressive decrease of mechanical tendon properties. We expected this effect to be even more pronounced in fascicles exposed to a higher degree of functional microdamage. However, the mechanical competence of fascicles stretched to 6% and 8% L_0 did not further decrease with time but on the contrary even increased until D3 (statistically significant compared to D0 post-damage in the 8% L_0 strain group only). As we observed a similar trend in devitalized fascicles, is seems that this effect depends on passive, matrix-based mechanisms, occurring in strongly damaged tendon fascicles.

We showed that collagen fibril unfolding through α-chain pullout occurred only above 4% L_0 strain. Meanwhile, the newly discovered matrix-based recovery mechanisms also only occurred in fascicles stretched above 4% L_0. Thus, we hypothesized that it could be the matrix-components damaged at strain levels above 4% L_0 which refold and thus recover mechanical integrity. Therefore, we repeated the CHP-staining protocol in fascicles three days after they were stretched to 8% L_0, which corresponds to the plateau-phase of the functional recovery process (Fig. 4A). However, the obtained fluorescence images did not support the hypothesis, as no difference in the CHP-staining was observed (Fig. 4B).

In summary, the recovery capacity of the tendon core matrix is minimal under the herein used conditions. Resident tenocytes conversely seem to cause further matrix degeneration at low strain levels, at least when stretched daily. At high strain levels, a passive recovery mechanism was observed.

Effects of microdamage on cell viability

We investigated the functional and structural aspects of microdamage and whether resident tenocytes contribute to microdamage and/or recovery. To examine the converse impact of different microdamage levels on tenocytes, we measured cell viability. Representative images show the "mechanotoxic" effect of high strains after three days in minimal culture conditions (Fig. 5A). To assess whether this effect results directly from acute tearing of the cell membrane during stretching, we repeated the staining immediately post-stretching and after one day in minimal culture conditions. We found that tendon cell viability was not affected immediately after the initial stretch in all conditions, as we detected no statistically significant differences compared to the undamaged control (see Supplementary Table 2 for the detailed statistical analysis). Furthermore, the viability remained stable in the undamaged and clamped controls over three days (Fig. 5B). However, we could record a significantly reduced viability in the unclamped regions of fascicles stretched to 4, 6 and 8% L_0 compared to the undamaged controls at D1. At D3, the drops in cell viability across all stretched conditions are more pronounced and the differences to the undamaged control statistically significant. The 8% L_0 strain group is statistically no longer distinguishable from the methanol control group.

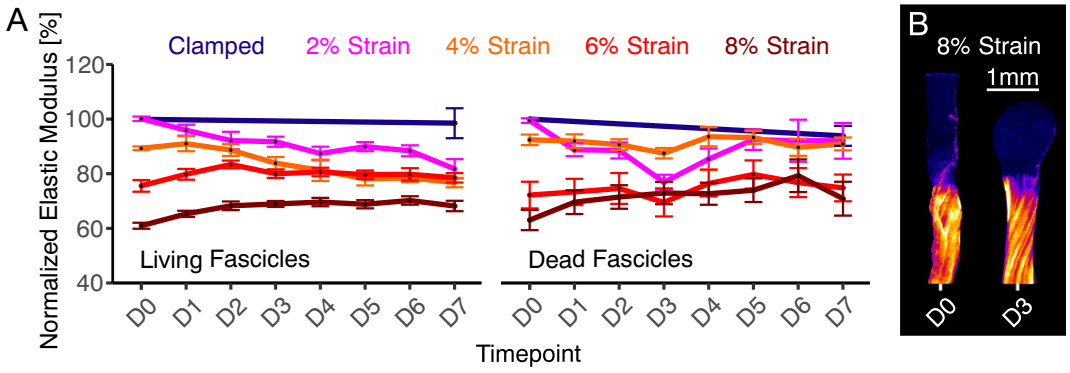

Fig. 4. Functional and structural recovery of damaged tendon fascicles over time. A) The graphs show the linear elastic modulus of living and dead fascicles over a 7-day time course (D0-D7) after experiencing different strains once at D0, normalized to the initial (pre-damage) linear elastic modulus [%]. The data (n = 7–8) are displayed as mean (± sem) except for D0 of the clamped-only fascicles (=100%). B) Fluorescence images of dead fascicles stretched to 8% L_0 stained with CHP immediately after damage and after a 3-day recovery period.

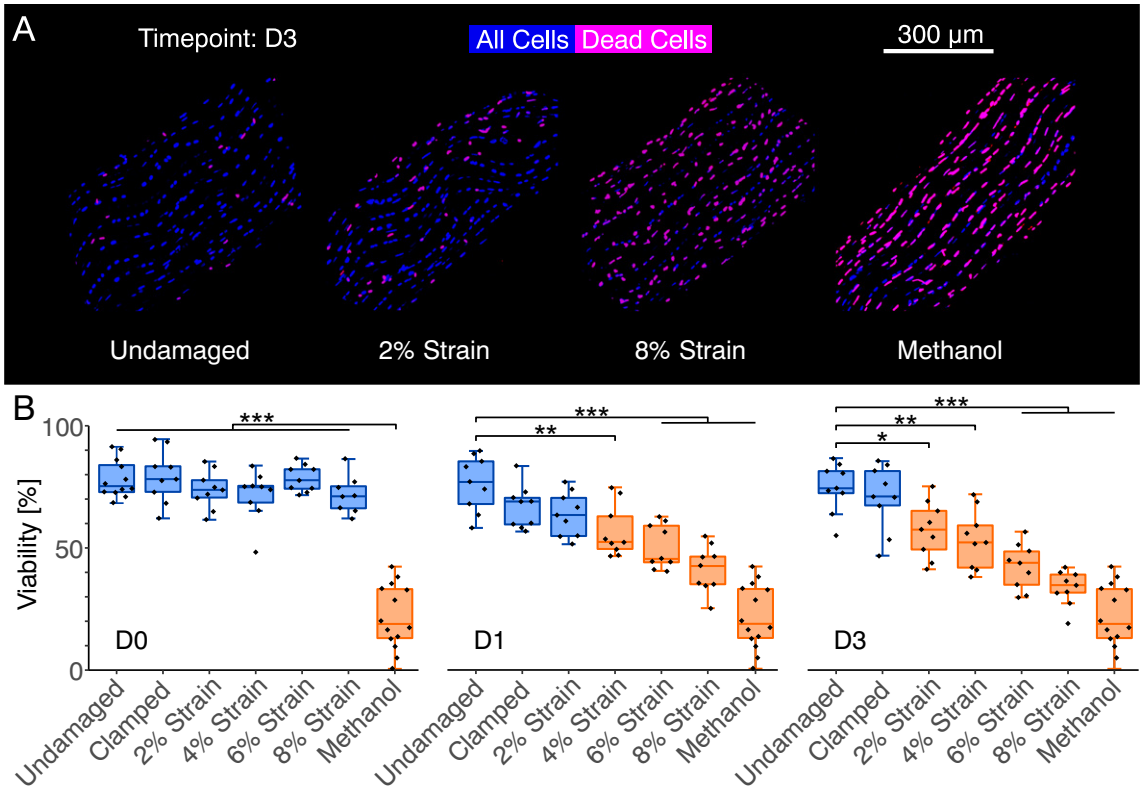

Fig. 5. Cell viability in damaged tendon fascicles over time. A) Representative fluorescence images of undamaged fascicles, fascicles stretched to 2% or 8% L_0 after three days in culture and methanol devitalized tendon fascicles. NucBlue was used to stain all cells (blue) and dead cells were labeled with EthD-1 (violet). B) Boxplots showing the quantified viability of fascicles at different timepoints after they experienced different levels of microdamage (n = 9) or immediately after methanol treatment (n = 14). The upper and lower hinge correspond to the first and third quartile (25th and 75th percentiles) and the middle one to the median. Whiskers extend from the upper/lower hinge to the largest/smallest value no further than 1.5 times the interquartile range. Data beyond the whiskers are treated as outliers. The individual measurements are added as single points to increase transparency. Statistical analysis included a one-way ANOVA and Tukey post hoc tests (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). For the full statistical analysis, see Supplementary Table 2.

In summary, we found a critical loading/strain threshold between 4% and 6% L_0, above which we observed impaired fascicle function and structural collagen disruption. This evident matrix damage then exerts adverse effects on the tissue-resident cell population, resulting in increasing cell death in a time- and strain-dependent manner.

Discussion

The concept of tendon microdamage has been proposed to summarize pathophysiological changes within the tissue hierarchical level of the tendon core. Several studies have assessed the immediate reaction of tenocytes or the tendon matrix to mechanically imposed microdamage [8,23,33,41–48]. However, the long-term structural and functional recovery potential of the tendon core has so far remained obscure, as well-controlled in situ damage and repair studies are lacking. In this study, we aimed at determining whether there is a critical microdamage threshold that might exceed the long-term regenerative capacity of the tendon core, beyond which extrinsic healing and repair mechanisms would have to take over. The involvement of the extrinsic compartment implies vascularization and inflammation, both hallmarks of degenerative tendon pathologies. To identify a critical microdamage threshold, we exposed tendon fascicles to different strain magnitudes to create different levels of microdamage.

The normal physiological loading range of tendon fascicles is considered to include mechanical tissue strains up to the deflection point of the stress-strain curve [3,23]. In the beginning of mechanical loading, the most relevant extension and load absorbing mechanisms involve increasing fiber recruitment through lateral force transfer as well as fiber realignment and initially recoverable collagen fiber and fibril sliding [40,49]. At strains above the deflection point, fiber and fibril sliding becomes increasingly non-recoverable, reducing the linear elastic modulus while

increasing deflection point strain [23]. As a result, the individual collagen fibers and fibrils increase their contribution to the load-transfer, as enabled by molecular sliding and molecular extension [50,51]. The molecular sliding creates shear-forces which cause gradual collagen disruption through α-chain pull-out [29]. Once these mechanisms are exhausted as well, the fascicle ruptures [3]. Our results confirm the onset of functional microdamage above the deflection point, which then further increases in a mechanically dose-dependent manner. For lower strains, the damage seems to be restricted to the matrix structures responsible for the lateral force transfer between fibrils, as we did not detect collagen fibril disruption. In contrast to previous studies [3] however, our results show a lower threshold for the onset of collagen fibril microdamage, as disrupted collagen molecules were detected already at 6% L_0 strain. Similar collagen disruption processes have been reported for individual fibril extensions, following single-peak loading of rat tail tendon fascicles and even after complete tendon failure in human patients, confirming their (patho-) physiological relevance [29,44,52–54].

Further, the results revealed that fascicle-resident tenocytes in the minimally crosslinked collagen matrix of tail tendon are already overwhelmed by the lowest levels of microdamage created in this study, which questions tenocyte ability for tissue repair. Nevertheless, interesting effects of passive, matrix-based recovery were observed in both alive and dead fascicles subjected to strains above 4% L_0. At these higher strains, the dominating damage-creating mechanism is collagen molecular sliding as the divalent aldimine crosslinks fail. It therefore seems plausible that the observed functional recovery involves the re-establishment of those crosslinks, especially since a similar "molecular restoration" mechanism has been reported in bone [55,56]. The viscoelastic damage and recovery mechanisms we expose here extend findings described in other studies that have characterized substantially shorter timescales, on the order of hours [23,57,58].

We also briefly considered that the refolding of straightened α-chains might potentially play a role in this passive recovery mechanism, but CHP-staining performed on fascicles three days after damage did not support this hypothesis. As living tenocytes in fascicles stretched to strains between 2% and 4% L_0 led to further losses of mechanical competency over 7 days, one could expect a reduced passive recovery in living fascicles stretched to 6% and 8% L_0 strain for the same reason. However, we did not detect this, which might be due to their decreased cell viability. The observed decrease in cell viability is in agreement with studies assessing tenocyte death under isolated shear loads [59] and changes in tenocyte morphology following overstretching [60]. In our study, the decrease in viability did not occur immediately after overstretching but with a certain lag time, indicating that apoptotic pathways and not immediate/physical disruption caused cell death. It is conceivable that the destruction of the pericellular matrix already occurs at 2% L_0 strain and plays an important role in triggering apoptosis in tenocytes [59,61,62]. These results further substantiate the pathophysiological relevance of matrix microdamage, as it also triggers molecular mechanisms instead of just affecting mechanical competence. This novel finding is in line with a plethora of recent studies highlighting the importance of the extracellular matrix in human disease [63–67].

As we could show that the intra-fascicular tenocytes have only minimal capabilities for driving tissue-recovery, the possibly apoptosis-driven cleansing mechanism combined with the also observed continuously ongoing matrix degradation might be functional and even crucial for the subsequent tissue-recovery [68]. The reason for the matrix degradation could lie in the increased activity of matrix-metalloproteases, aiming to further degrade the extracellular matrix. This degradation would facilitate the invasion of macrophages and more active fibroblast populations residing in the extrinsic tendon compartment including the interfascicular matrix [69], the tendon surface [70–72] or homing from newly formed blood vessels. These more active fibroblasts might be functionally analogous to the satellite cells in skeletal muscles [73–75] or osteoblasts in bone [76,77]. It seems therefore possible that the physiological function of the intra-fascicular tenocytes studied here could be reduced to mechano-sensing, similar to osteocytes in the bone [78–80]. Unearthing more analogous mechanisms shared by the components of the musculoskeletal system like this could be stimulating for the whole field of musculoskeletal matrix biology [67].

Conclusion

The data we present characterizes the dose-dependent response of the tendon matrix and the resident tenocytes to mechanically induced microdamage and supports the premise that extrinsic support is required to cope with microdamage at any level. We speculate that microdamage in the tendon core is likely to accumulate unabated until extrinsic healing mechanisms are triggered, perhaps marking the onset of more advanced and clinically relevant states of tissue degeneration.

Experimental procedures

Fascicle preparation

Rat tail tendon fascicles were harvested from 12 to 18-week-old female Wistar rats. All experiments

were approved by the responsible authorities (Canton Zurich, license number ZH239-17). The fascicles were gently extracted from the tail within 4 h after the sacrifice of the animal and cut into 30 mm long pieces that were then randomly assigned to the different groups (Fig. 2A). The fascicles used as dead ("devitalized") controls for the mechanical testing were kept frozen at –20 °C inside the tails but thawed and cut once starting the experiments. Previous studies have shown that cryopreservation conducted like this does not influence the mechanical properties of rat tail tendon fascicles assessed here in this study [81]. After the isolation and prior to the stretching, both alive and dead fascicles were kept in the same minimal culture conditions (DMEM/F12 GlutaMAX with 1% Penicillin/Streptomycin, 120 µM L-Ascorbic Acid Phosphate Magnesium Salt n-Hydrate from Wako, and 1% N2 supplement containing transferrin, insulin progesterone, putrescin, and selenite from Gibco, at 29 °C, 5% CO_2 and 3% O_2) to preserve native cell phenotype and morphology [37]. Fascicle diameter (d) was measured at three different places using a 10× magnification (EVOS XL, Thermo Fisher Scientific, Waltham, MA) and the mean diameter was calculated to estimate the preload (PL) necessary to normalize the initial stress to 1 MPa in fascicles with different diameters and to ensure reaching identical strains consistently. To do this, we used formula (1) and assumed a round specimen shape:

$$PL\,[N] = \pi * \left(\frac{d}{2}\right)^2 * 1\ MPa \qquad (1)$$

Mechanical testing & analysis

Assessing the strain-damage relation of ex vivo tendon fascicles

To investigate the strain-damage relation, fascicles (n = 24 from 3 different animals) were harvested as described in the methods for fascicle preparation and randomly distributed to the used strain conditions. Applied exclusion criteria for fascicles were diameters outside the range of 200–350 µm and visible kinks. The fascicles were mounted to custom-made clamps (Fig. 2A) at a clamp-to-clamp distance (L_0) of 20 mm using paper pieces soaked, as described in the methods for fascicle preparation, with minimal culture media to keep the fascicles hydrated. The whole construct was transferred to the custom-made uniaxial stretching device (Fig. 2B) operated by our own software and equipped with a 10 N load cell (KD 24 s, Lorenz Messtechnik GmbH, Altdorf, Germany) [50,82]. The preload was set individually for each fascicle as calculated (formula 1, see methods for fascicle preparation) and the fascicles were preconditioned by five stretch cycles to 1% L_0 strain superimposed onto the preload at a speed of 1 mm/s. The fascicles were then stretched 1× to different pre-defined strain magnitudes (2%, 4%, 6%, 8% L_0) also superimposed onto the preload while recording the resulting forces. The generated force-displacement curves were fed into MATLAB (R2017a) and combined with the diameter measurements to first calculate the nominal stress and then the tangent linear elastic modulus ($EMod_1$). The region of the curve used to calculate the $EMod_1$ was defined by the end of the non-linear toe region and the deflection point and determined visually. Next, the fascicles were stretched again to measure the linear elastic modulus post-damage ($EMod_2$) and both measures were used to calculate the damage magnitude using formula (2):

$$Damage\,[\%] = \frac{EMod_1 - EMod_2}{EMod_1} * 100 \qquad (2)$$

For the 2% L_0 strain experimental group, stretching again to 2% L_0 was sufficient to record the linear elastic modulus, but a stretching regime of 4% L_0 had to be used for all other groups as the linear elastic region shifts following damage from overstretching [23]. We verified the validity of our measurement region in pre-experiments recording the whole force-displacement curve post-damage (f.e. Fig. 3A). For the statistical analysis we performed a one-way ANOVA followed by Tukey post hoc tests using R (version 1.1.463).

Assessing the functional recovery of tendon fascicles over time

Here, we first induced again the different damage phenotypes at day 0 (D0) as described in 5.2.1. The linear elastic modulus post-damage ($EMod_2$) was then measured daily for seven days during the prolonged culture of the fascicles in the minimal culture conditions [37]. These measurements aimed at assessing the functional recovery of the fascicles and included the clamping, the mounting to the mechanical testing device and the stretching to 2% or 4% L_0 respectively. A one-way ANOVA with a Tukey post hoc test was used to analyze differences between timepoints using R (version 1.1.463), which was also used to calculate the means and standard errors of the means (sem). To separate cellular from matrix-based recovery effects, we tested both alive and devitalized fascicles. For additional control, one group of both alive and devitalized fascicles were just measured once at the beginning of the experiment and then again after seven days but clamped and mounted to the mechanical testing device every day to separate clamping artefacts from the effects of stretching (n = 8 fascicles per group, from three animals).

CHP staining

Frozen fascicles ($n = 3$, from 1 animal) were damaged by stretching them to 2%, 4%, 6% or 8% L_0 after preconditioning. Collagen hybridizing peptide labeled with carboxyfluorescein (CHP, Echelon, C – 660F) was diluted in PBS to a 100 μM stock solution, heated to and kept at 80 °C for 10 min for activation before rapidly cooling on ice. The specimens were stained with a final concentration of 9 μM CHP in PBS overnight at 4 °C, then washed three times with PBS for 10 min each time. Imaging was done using an iMic spinning disc confocal microscope (FEI/Till Photonics) equipped with a Hamamatsu Flash 4.0 sCMOS camera and a SOLE-6 Quad laser (Omicron)) at an excitation wavelength of 488 nm. Tiled and stacked images were acquired with a 4× air immersion objective (N.A. 0.16) with a stack height of 180 μm and 25 stacks with an exposure time of 47 ms per stack.

Cell viability assay

Cell viability of undamaged fascicles, fascicles clamped for 10 min and fascicles stretched to different strains (2%, 4%, 6%, 8% L_0) was measured at three different timepoints: Immediately after damage (D0), after 1 day (D1) and after 3 days (D3). Fascicles were cultured separately from each other in 12-well plates filled with 1 ml of minimal culture media ($n = 9$, from 3 different animals). Fascicles treated with methanol for 10 min were used a dead control ($n = 14$, from 3 different animals). Fascicles were washed with PBS and then stained with Ethidium Homodimer (EthD-1, Sigma-Aldrich, 2 mM stock in DMSO) diluted to 4 μM in PBS for 10 min while keeping them at 37 °C. After three subsequent washing steps with 1 ml of PBS for 10 min each, the samples were fixated by 4% formaldehyde (Roti-Histofix, Karlsruhe) for 20 min at room temperature (RT) and then washed again. If not immediately analyzed, the fascicles were then kept in 1 ml PBS and at 4 °C before aspirating the PBS and applying one drop of NucBlue Live ReadyProbes Reagent (Thermofisher, R37605) together with 0.5 ml PBS and left to stain for 20 min at RT following the manufacturer's instructions to mark DNA irrespective of cell viability and obtain the total cell number. Two-channel imaging was performed using an iMic spinning disc confocal microscope (FEI/Till Photonics) equipped with a Hamamatsu Flash 4.0 sCMOS camera and a SOLE-6 Quad laser (Omicron) with 510 ms exposure time at excitation wavelengths of 405 nm for NucBlue and 561 nm for Ethidium Homodimer with a 10× air immersion objective (N.A. 0.40). For each fascicle, three image stacks (81 planes, 120 μm stack height) were recorded and their mean viability used for analysis. Quantification of the viability was performed using MATLAB (R2017a) while images representative for their respective conditions were processed with Fiji (version 1.52 h). Finally, R (RStudio, version 1.1.463) was used to conduct the statistical testing consisting of a one-way ANOVA with Tukey post hoc tests.

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Declaration of Competing Interest

None.

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