Mechanical stretching changes cross-linking and glycation levels in the collagen of mouse tail tendon

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Running title: *Changes in collagen crosslinks and glycation under strain*

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Abstract

Collagen I is a major tendon protein whose polypeptide chains are linked by covalent cross-links. It is unknown how the cross-linking contributes to the mechanical properties of tendon or whether cross-linking changes in response to stretching or relaxation. Since their discovery, imine bonds within collagen have been recognized as being important in both cross-link formation and collagen structure. They are often described as acidic or thermally labile, but no evidence is available from direct measurements of cross-link levels whether these bonds contribute to the mechanical properties of collagen. Here, we used MS to analyze these imine bonds after reduction with sodium borohydride while under tension and found that their levels are altered in stretched tendon. We studied the changes in cross-link bonding in tail tendon from 11-week-old C57Bl/6 mice at 4% physical strain, at 10% strain, and at breaking point. The cross-links hydroxy-lysino-norleucine (HLNL), dihydroxy-lysino-norleucine (DHLNL), and lysino-norleucine (LNL) increased or decreased depending on the specific cross-link and amount of mechanical strain. We also noted a decrease in glycated lysine residues in collagen, indicating that the imine formed between circulating glucose and lysine is also stress-labile. We also carried out mechanical testing, including cyclic testing at 4% strain, stress relaxation tests, and stress-strain profiles taken at breaking point, both with and without sodium borohydride reduction. The results from both the MS studies and mechanical testing provide insights into the chemical changes during tendon stretching and directly link these chemical changes to functional collagen properties.

Main

In the field of collagen research there remains a lack of understanding of how the different crosslinks found in collagen contribute to function. The classes of collagen crosslinking are described as immature, mature and Advanced Glycation End products (AGEs)[1]. The immature crosslinks are formed through the action of lysyl oxidase on lysine and hydroxylysine residues to generate aldehydes which then react with nearby lysine or hydroxylysine side chain amines to form imine crosslinks[2]. These can then react further with another oxidised lysine to form mature crosslinks (the pyridinolines)[2]. AGE crosslinks are formed through the spontaneous reaction of circulating sugars with lysine, hydroxylysine and

arginine through chemical steps which have yet to be identified[1].

While both the mature and AGE crosslinks are chemically stable, the situation for the immature imine crosslinks is uncertain. They are often described as acid labile or hydrothermally labile, but whether they hydrolyse under stress is less clear. It has previously been concluded through physical testing that the imines in tendon must be hydrolysable under stress[3,4], although the importance of this in the mechanical properties displayed by tendon or whether they are really stress labile has subsequently been questioned[5,6]. We aim to shed light on the status and reactivity of these bonds in the work presented here through the direct measurement of these crosslinks at different strains in 11 week C57Bl/6 mouse tail tendon.

Increases in AGE crosslinks and mature crosslinks with age are generally considered to cause increases in tendon stiffness[1]. We have previously shown that the glycation of lysine residues within the collagen structure are also likely to contribute to collagen stiffening[7]. Glycated lysine is not considered a covalent crosslink. The chemistry of glycated lysine is similar to that of the immature crosslinks in originating from the chemical reaction of a sugar aldehyde with an amine to form an imine. In the light of this similarity, we included analysis of glycated lysine in this study.

To understand the role of reversibility of the imine bonds in the normal functioning of tendon, we use sodium borohydride reduction to convert the imines into bonds which cannot be broken. This preserves the number of crosslinks and glycated lysine residues as well as their position in the collagen structure. We then compare the results of mechanical testing of reduced samples where the chemical bonds cannot break under stress with the results from unreduced tendon where the crosslinks and glycation could break under stress to look for differences in the stress-strain profiles.

For clarity in this paper we describe the immature bonds in terms of the reduced products that we actually measure by HPLC mass spectroscopy, dihydroxy-lysino-norleucine (DHLNL), hydroxy-lysino-norleucine (HLNL) and lysino-norleucine (LNL) (structures shown in figure S1).

Results

Changes in tendon stress-strain profile by chemical treatment

Imines in an aqueous system are expected to be in an equilibrium as shown in figure 1. The addition of sodium borohydride will reduce the imine and the aldehyde in this equilibrium to a secondary amine and alcohol respectively, irreversibly fixing the crosslink equilibrium at that point. These reductions are the only reactions that will occur in tendon under the conditions used here. If collagen chemistry is changing under tension, then adding sodium borohydride to tendons should change the stress-strain profile seen because the crosslinking can no longer change. While the reduction of an imine to a secondary amine might in itself cause a change in the profile due to increased rotation about a single bond verses a double bond, this might be expected to decrease tendon stiffness. Increases in tendon stiffness and increases in permanent deformation on stretching would be signs of change corresponding to the conversion of a reversible bond into an irreversible one.

Demonstration of change in tendon stress-strain profile when cycling to 4% strain with and without reduction. Fresh tendon was stretched to 4% strain and back to zero in a tensile stress stage through four cycles with 15 minute rests between each cycle. The process was then repeated with tendon which had been reduced with sodium borohydride for 1 hour prior to testing. The unreduced tendon showed a large change in stress-strain profile between the first cycle and second cycle but then subsequent cycles showed very similar stress-strain plots,

figure 2a. We found that this difference between the first test cycle and subsequent cycles is consistently observed. The toe and heel regions seen in cycles 2 onwards are commonly referred to in the literature[8], however, in our experience they are not seen clearly unless a pre-test stretch is carried out first, a procedure which is commonly described to "condition" tendon before experimentation.

Reduced tendon behaves quite differently on repeated testing to 4% strain, figure 2b. Repeated cycles show a continual plastic deformation between each testing cycle (a plastic deformation is a non-reversible change). This is shown by the recorded stress starting to increase at higher strains with each test cycle, indicating that the tendon length has increased slightly on each cycle due to deformation. The reduced tendon does not recover on each cycle repeat, whereas the tendon that had not been reduced shows an ability to largely recover from the second cycle onwards.

Stress relaxation tests show continued change after first cycle. Tendons were pre-conditioned by stretching to 3% strain four times before experimentation. The tendon was then repeatedly extended to 3% strain and held for 120s while the decrease in stress (stress relaxation) monitored before returning to the initial length. Figures 3a and 3c demonstrate the effect when sodium borohydride was added during the rest period after the first test cycle. It can be seen that the stress profile in the cycle after treatment is different to that before treatment. The stress drops during the 120s period (due to structural relaxation processes) in both cases, but after reduction the stress does not drop as far during this period as that seen before treatment, although the initial stress on extension is similar. This shows that fixing the crosslinks by reduction so that they cannot break decreases the ability of tendon to relax when stretched.

In the experiment shown in figures 3b and 3d the sodium borohydride was added 60s into the 120s stress relaxation period. In this case it can again be seen that the profile in subsequent cycles is changed by the reduction step. The initial stress achieved after reduction is lower than that seen before reduction while the stress measured in the plateau phase is similar. This shows that the network of crosslinks have been fixed while the tendon is in an extended state and cannot recover the original structure.

These two experiments show that the crosslink network is changing and can be chemically fixed by reduction at different points in the stress relaxation profile. This implies that chemical change is occurring even after pre-conditioning stretches.

The plastic phase is lost on reduction. Tendon, particularly from young animals, exhibits a plastic phase after a strain of 5%, figure 4a. Figure 4b shows a comparison of the break test profiles of unreduced and reduced tendons (without pre-conditioning). A loss of the plastic phase in the profile of the reduced tendon when compared to unreduced tendon is seen. It is also clear that a higher final stress is reached in the reduced tendon. This shows that the plastic deformation phase is lost when the crosslinks cannot break and also that higher stresses can be achieved when the crosslinks cannot break.

Crosslinking and glycation change under stress. To demonstrate that bonds break and form under stress, the change in crosslink levels at defined strains were measured. We chose to make measurements at 4% strain, 10% strain and at breaking point (BP). These were chosen as representative points on a typical mouse tail tendon stress-strain profile as illustrated in figure 4a. The tendon was held at the defined strain for 10 minutes to maximise any chemical changes occurring.

The initial experiment is shown in figure 5a where tendon was taken to a 4% strain, cut out of the equipment and then reduced with sodium borohydride. In each case a sample of tendon was removed before stretching to act as the control with which to compare the crosslink level in the stretched tendon. This experiment

did not show any convincing change in the mean for immature crosslinks, although the glycation of lysine was seen to decrease.

The spread of data points around the mean was greater than we would have expected which led us to question whether the rate of change was fast and whether we needed to trap the reaction in the stretched state. Further experiments were carried out where the tendon was reduced in-situ while still under strain to fix the tendon chemistry present at that point. The previous experiment was repeated with this modification to the reduction procedure and the result can be seen in figure 5b. Clear changes can now be seen in all the analytes shown. The data in figure 5 is displayed as a percentage change, however, the DHLNL and LNL are found in tendon at very low levels. Figure 6 shows the same data as in figure 5b but normalised to the average mol/mol collagen level for each analyte on the same scale so that the relative contributions of each crosslink type can be assessed. This figure is to emphasise that a seemingly large and significant percentage change in analyte level can represent quite a small absolute change relative to other analytes.

Figures 7a-e summarise the data from the different stretching experiments grouped by analyte with the average starting level found in unstretched tendon shown. Each data point represents tendon from different animals. At 4% strain HLNL levels increase, though this increase only just reaches statistical significance. At 10% strain and BP the HLNL levels show a clear decrease. While the increases in DHLNL at 4% and at BP are small they occur consistently. This is also true for LNL at all test strains. Lysine glycation drops at 4% strain, and does not appear to decrease further with increasing strain.

Figure 7e shows the result for the crosslink histidine-hydroxymerodesmosine (HHMD), which is formed through the reaction of two aldehydes, one hydroxylysine and a histidine. Although there is debate[9,10,11] about what

HHMD actually represents in native collagen, it does reflect a reversible chemical change occurring within the tissue and is a structure which also contains an imine. A standard for this compound which would allow quantification by mass spectroscopy is not available and so the data has been expressed as a percentage change. Levels of HHMD have been reported to be as high as 0.89 mol/mol collagen by others[12] determined using tritium labelling.

The results described above collectively led us to a hypothesis that there might be free aldehyde derived from lysine oxidation (allysine) in resting tendon and that the levels of allysine increase on stretching. To explore this idea, a sample of tendon was taken to 10% strain, reduced under tension and compared with the unstretched control. The level of reduced allysine in this sample before stretching was found to be 89 ng/mg tendon and after stretching 235 ng/mg tendon (dry delipidated tendon weights). The levels of the aldehyde derived from hydroxylysine were too low to be detected. (These results are summarised further in figure S2).

Discussion

Until the work described here, there was no proof that crosslinking within collagen can change in response to mechanical strain. We have demonstrated that crosslinking and glycation within tendon changes under strain. Both increases and decreases in the mean number of individual crosslinks were seen which shows that both bond formation and bond breaking were occurring.

The cyclic stress-strain profiles in figure 2a show that the first testing cycle is quite different to subsequent ones. The loss of glycation is a permanent change which occurs during this stretch phase, figure 7b. In the light of this, it would seem plausible that the loss of glycation contributes to the initial drop seen between the first and second cycle and that glycation has a

role in the stiffening of tendon during the early stretching phase.

In figures 2a and 2b, it can be seen that reduction has a large impact on the profiles when tendon is stretched to 4% strain. The un-treated tendon, after the first cycle, shows a remarkable reproducibility while the reduced tendon shows a marked deformation on each subsequent stretch cycle. This difference indicates that the ability for bonding to change in the un-reduced tendon allows an adaptive change that largely prevents through stress relaxation (or mitigates through bond re-organisation) the plastic deformation seen in reduced tendon.

The stress relaxation studies show that adaptive changes occur under tension to allow relaxation and recovery processes to occur. When tendon is reduced the adaptive changes are stopped and the tendon is chemically fixed with the bonding network at the point of reduction. The only known chemistry that is fixed in collagen using sodium borohydride is that around the imine bond equilibrium illustrated in figure 1 indicating that it is this reversible chemistry that is responsible for the adaptive change.

The figure 5b shows that a 4% strain can actually lead to an increase in the number of reversible crosslinks present in tendon under tension. This suggests a more ordered structure where crosslink forming residues have been brought into proximity with each other allowing crosslink formation to occur. This is in keeping with synchrotron X-Ray scattering evidence where it was suggested that strain created a more ordered structure and that the relaxation process was entropically driven[13].

These imine bond breaking and formation reactions are relatively fast as shown by the experiment carried out by reduction of tendon in the relaxed state after stretching in figure 5a, however, the reaction rates in the equilibria are unknown. It is possible that the rate of bond hydrolysis is limiting which may explain why higher stresses and an increased stiffness can be observed with faster rates of extension (an example is shown in figure S3).

In the plastic phase, the collagen fibres are believed to slip against each other. This was described previously by others in studies based on X-ray diffraction studies[14,15]. During this phase we find that there is a net decrease in HLNL bonds and it would seem possible that this could be to allow an extension process to occur, for example in growth, or to reduce stress when over extension occurs. When the reversible bonds are fixed by reduction the plastic phase can no longer occur and is lost from the profile as seen in figure 4b. It can also be seen that higher stresses are then achieved because adaption to the stress cannot occur through bond breaking.

One consistent feature seen in the 4% strain experiments is an increase in DHLNL of between 0.006 and 0.01 mol/mol collagen when compared with unstretched tendon. This might seem small, but a 0.6% to 1% increase in crosslinking on stretching might have an important functional role, particularly if combined with the changes seen in LNL and HLNL. The result requires a pool of the aldehyde derived from hydroxylysine to be present. To prove this by detection of this aldehyde in these experiments would require the development of more sensitive detection methods not currently available, but it is reasonable to think that it is present, otherwise the increase in DHLNL seen would not be possible.

The increases seen in LNL on stretching at all strains tested may be a result of increased levels of aldehyde present in the tissue when stretched and the statistical chance of crosslink reforming with a lysine rather than a hydroxylysine. This increase in LNL could also be linked to structural changes involved in stretching during which the proximity of lysine or hydroxylysine to aldehyde changes.

The data shown in figures 2a and 7b indicate that tendon is stiffer when lysine residues in the

collagen structure are glycated. A mechanism by which stiffening from glycation occurs is most likely through the interactions of the sugar component of the glycated lysine residues with adjacent collagen molecules, for example through hydrogen bonding of the sugar hydroxyls with nearby groups. If this did not occur then there would be no reason for the glycated lysine to hydrolyse when the tendon is stretched. There has to be a mechanism by which the transfer of strain can take place to lengthen the sugar-lysine imine bond, thereby lowering the activation energy of the bond towards hydrolysis. This transfer of strain requires at least two fixed points either side of the imine bond. The effect of glycated lysine appears to be important in intra-fibrillar interactions rather than inter-fibrillar interactions because hydrolysis occurs during the initial 4% stretch phase with no further loss in the plastic phase when inter-fibrillar interactions become important.

The largest cross-link mol/mol collagen change is that seen for HLNL, ranging from an average increase of 0.1 mol/mol collagen under 4% strain conditions, to a decrease of 0.2 mol/mol collagen under 10% strain conditions. A schematic model is presented in figure 8 which shows five parallel cross-linked strands of six collagen molecules, each strand overlapping in a staggered formation. For clarity it is presented as a planar 2D structure, initially with a typical cross-link density of 45 cross-links to 30 molecules of collagen (1.5mol / mol collagen). It can be seen in figure 8a that at this level of crosslinking there is a cross-link gap in the matrix. Figure 8b shows the potential impact of a decrease in cross-linking of 0.1mol /mol collagen (3 cross-links). It can be seen that this could greatly weaken the structure creating a fracture plane indicated by the yellow line. Figure 8c shows that an increase of 3 bonds could clearly strengthen the structure by filling the cross-link gap and potentially forming crosslinks with adjacent collagen planes. While the reality of the native collagen structure is far more complex and subtle than this 2D model, it illustrates the impact that changes on the scale that we have observed could have.

The evidence presented here shows that tendon collagen undergoes chemical changes in crosslinking and glycation under strain which are important for correct function. By using reduction with sodium borohydride which fixes the crosslinks, it was demonstrated that the reversibility of crosslink and glycation chemistry is essential for the proper mechanical properties of tendon. The picture that has emerged is one of highly dynamic crosslinks, breaking and re-forming with every stretch. Changes in the levels of crosslinking and glycation would be expected to impact tendon function, with implications for ageing, disease and sport sciences.

Experimental

Animal procedures:

Ethics. Animal experiments were performed according to the UK Animals (Scientific Procedures) Act 1986, licence PPL 70/8303 and approved by the Babraham Institute Animal Welfare and Ethics Review Body.

Sample analysis:

Tissue processing. After removal of the skin from isolated tails, tendon was drawn out of the tail under PBS pH 7.4 by grasping the tip and the base of the tail with forceps. Twisting the forceps holding the smaller vertebrae at the tail tip caused the ligaments holding the vertebral bones together to break, allowing the vertebral bones with attached tendons to be gently pulled out. For larger vertebral joints they were first weakened by inserting the tip of a scalpel between the joints taking care not to cut the tendons. Tendon was isolated sequentially one vertebral bone at a time, working up the tail from the tip to the base. Tendon fibres were detached from the tail vertebrae with a scalpel.

Sample analysis. Acid hydrolysis and sample analysis by HPLC-mass spectrometry was carried out as described previously[6].

Allysine analysis. For this analysis, sodium borodeuteride was used because the background noise and isobaric background was much lower with the increase in mass.

Tail tendon (wet weight 10 to 20mg) was prepared and reduced as for the other experiments described here but with $NaBD_4$ used as the reducing agent. Base hydrolysis was then carried out in 200uL 2M NaOH at 100°C for 24hours. The sample was then allowed to cool and 600uL of 1M HCl added and the sample made up to 8ml with 50% acetonitrile in water. The sample was then split in half and 6-hydroxy-norleucine (0.5eq based on dry wt) added to one half. Both samples were then loaded onto disposable 500mg SCX ion exchange columns (Sigma Cat. No 57018). Each column was then washed with 2ml 50% acetonitrile, then the analytes eluted with a mixture of 1.5ml 50mM ammonium carbonate pH7.5 added to 50% acetonitrile in water. The samples were then freeze dried, re-dissolved in 400uL 50% acetonitrile, filtered through a 0.22um nylon filtration membrane and freeze dried again. The samples were then taken up in 30uL 50% acetonitrile in water and 5 uL analysed using the HLPC-MS method and parameters described previously[1]. The increase in non-deuterated signal area in the standard sample was used to work out the amount of deuterated analogue in the sample to which no standard had been added for each sample pair.

The samples were then expressed as ng/mg dry delipidated tendon and an approximate mol/mol collagen value calculated based on the dry delipidated tendon mass assuming 100 % collagen content. The resulting mol/mol collagen values will therefore be expected to be slightly lower than the actual values.

Physical testing:

Stress-strain stage set up procedure. Physical testing was undertaken using a Microtest 200N tensile stress stage (Deben UK Ltd) fitted with a 20N loadcell and a petri-dish bath to allow immersion of the sample. With a distance of 14mm between the jaws, individual processed fibres were clamped submerged in PBS pH 7.4 at room temperature, and pre-loaded to a force of 0.01N. The diameter of each fibre was assessed along its length and the smallest dimension used to calculate stress.

Cyclic strain testing. After the tendon had been loaded to 0.01N and the diameter and length measured, the distance between the jaws was decreased by 0.05mm. At this point the crimp pattern could be seen. After a 15min rest, the tendon was taken to a 4% strain at a rate of 0.5mm/min, held for 0.1s and the relaxed back to the cycle initial strain. The force was recorded every 0.5s during the cycle. After a rest of 15min the cycle was repeated. Four cycles were carried out on each tendon tested. Where tendon was reduced first, this was done by dissolving 8mg $NaBH_4$ in 10uL 1mM sodium hydroxide which was then added to 1ml of PBS. This 1ml solution was then added to the tendon in 4ml PBS and left for 1 hour with gentle agitation every 15 minutes. The reduced tendon was then washed twice in fresh PBS before testing.

Stress relaxation testing. A single tendon fibre after pre-loading to 0.01N, was pre-conditioned by holding at an extension of 3% for 60s and then taken back to its original length to rest for 60s, and repeated a further 3 times. During the stress relaxation experiment the fibre was repeatedly extended to 3% for 120s and then back to its original length to rest for 120s, recording the force every 0.5s. $NaBH_4$ (4mg in 40ul 1mM NaOH added to 1ml PBS) was added by exchanging with 1ml of PBS from the bath (18ml total) and mixed by pipetting.

Break test profile measurements. Tendon was prepared as described above and taken to breaking strain at a rate of 1mm/min. The force was recorded every 0.5s.

Extension crosslink measurements. Tail tendon was removed from male mice as described in the processing method, except the tendons were left attached to the tail vertebrae for ease of handling. With the jaws of the tensile stress stage set 18mm apart, 5-8 vertebral sections were mounted into the jaws, totalling 20-28 tendons. These were clamped in place across the tendon fibres removing the vertebral bones once clamped and submerged in PBS at room temperature. Excess was cut away and used as a control. At a rate of 0.2mm/min the fibres were taken to breaking point, after which the tendon was excised from the jaws and both the control and the broken tendon were reduced as above in 200ul PBS. This process was repeated twice for each mouse tail in order to produce enough material for analysis. The tendon was subjected to acid hydrolysis and analysis by mass spectroscopy as detailed above.

For analysis at 4% and 10% strain, tail tendon was collected from mice as described above and clamped into the jaws of the tensile stress stage, submerged in PBS at room temperature, with the jaws of the tensile stress stage approximately 18mm apart and at an initial load of 0.01N. Control material was collected by cutting away excess not held in the jaws. The jaws were extended to the defined strain at a rate of 0.1mm/min and held at that strain for 10 mins. The samples were reduced in situ under strain by exchange of 1ml of PBS from the bath with 1ml PBS containing $NaBH_4$ (4mg in 40ul 1mM NaOH added to 1ml PBS) for 5mins in the bath, then excised from the jaws. Reduction was allowed to continue for a further 2h in a tube. Samples collected were subject to acid hydrolysis for analysis as described above.

Statistical analysis

For all stretching experiments, ratio paired Student *t*-tests were used. For the other experiments, one-way ANOVAs were used when more than 2 groups were compared followed by Tukey's multiple comparisons tests. Significance was defined as $p < 0.05$ and p-values are reported on figures. Data analysis was performed using GraphPad Prism 8.

Definitions

Stress = Force/cross sectional area

Strain = Extension / initial length

Author contributions

JC, MS, IN designed and performed experiments and analysed data. AS-P carried out statistical analysis. JC wrote the paper and directed the research.

Competing interests

The authors declare no competing financial interests.

Data availability

All data are available from the corresponding author upon reasonable request.

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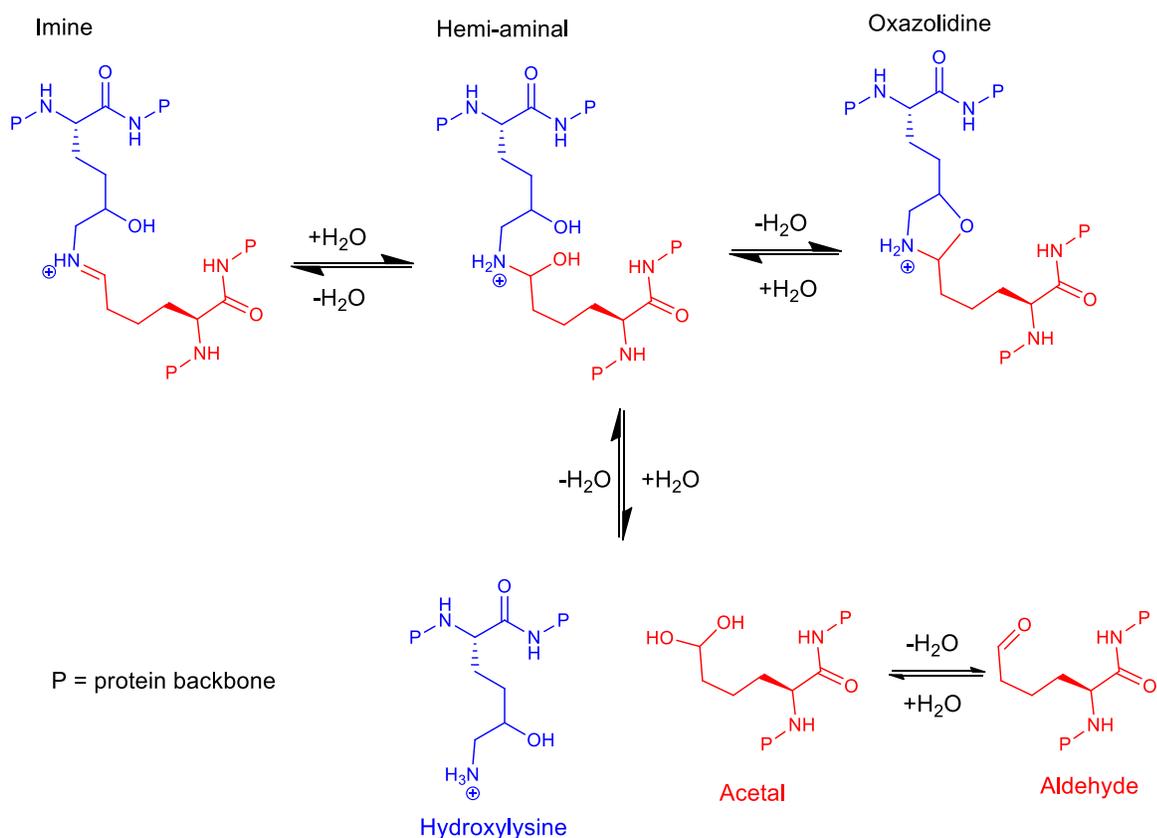
Figure 1 Scheme showing possible structures in the equilibrium between aldehyde (red) and hydroxylysine (blue) residues in collagen. The imine, hemi-aminal and oxazolidine are possible crosslinks that may exist in equilibrium with each other. None of these crosslinks have been observed in native collagen to date possibly due to their low abundance and that current NMR spectrometers do not yet have the required sensitivity.
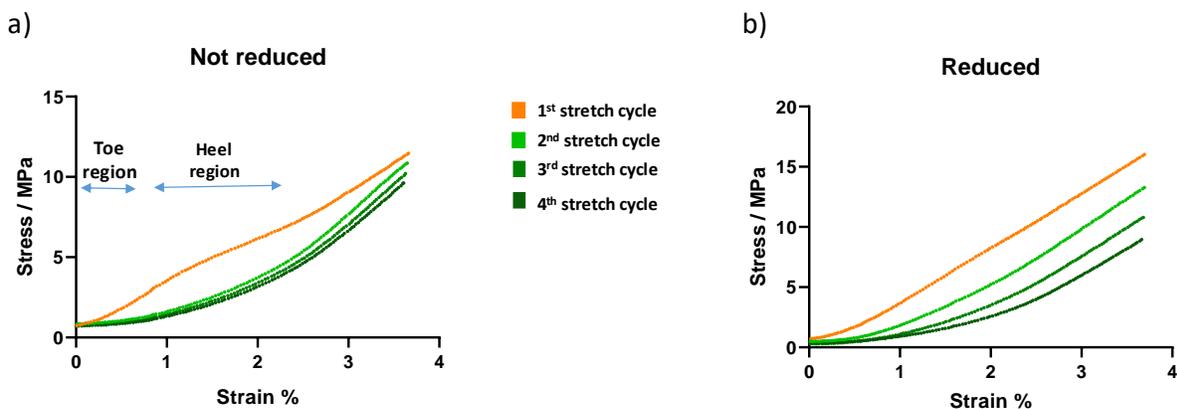
Figure 2 a) Typical stress-strain profile of unreduced tendon, this was repeated 27 times. Further examples where no toe-heel region are observed on stretching of unconditioned tendon can be seen in

figure 4b (orange traces). b) Typical stress-strain profile of reduced tendon, this was repeated 8 times. Each plot shows 4 stretch cycles taken to a strain of 4%, the tendon was pre-loaded to 0.01N at the start of testing. Tendon in both examples taken from 11 week old C57BL/6 mice. The orange trace is the first cycle in each plot, subsequent cycles shown in green.
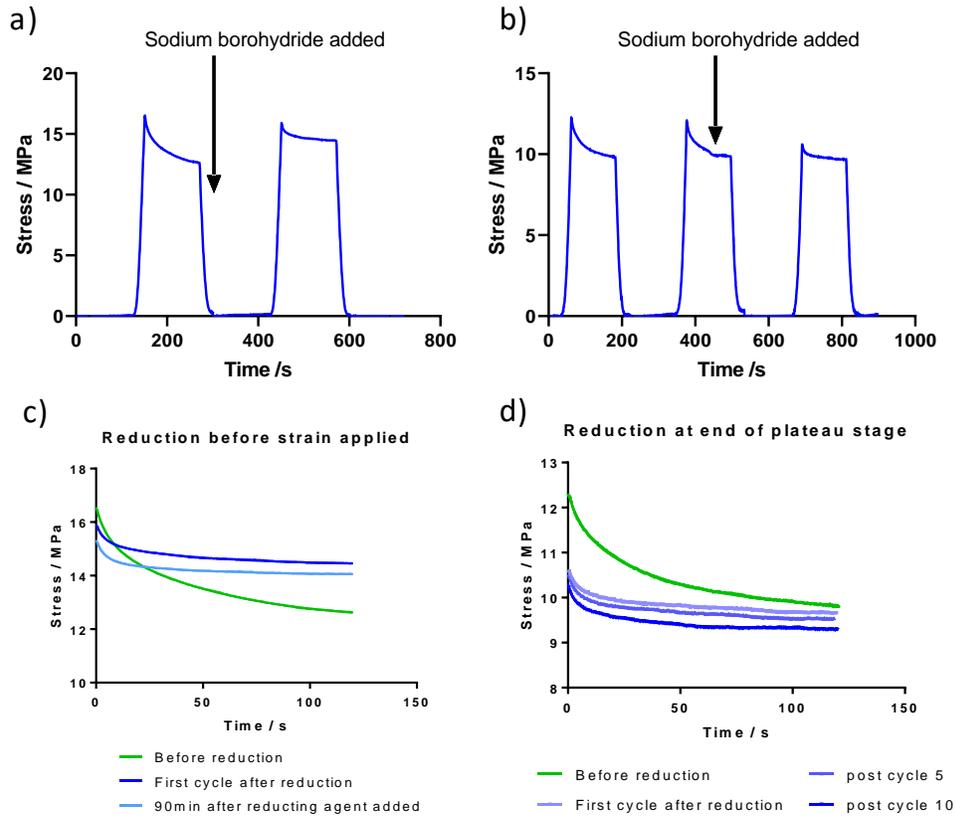
Figure 3 a,b,c,d shows the stress relaxation testing from tendon fibres from 11 week old C57BL/6 mice prior to, and after exposure to, a reducing agent (sodium borohydride) that fixes the reversible bonds. This was repeated 6 times. The reducing agent addition is shown in a & b, overlap plots of the relaxation curves are shown in the lower traces c & d. Tendon was conditioned by 4 pre-stretching cycles before use.
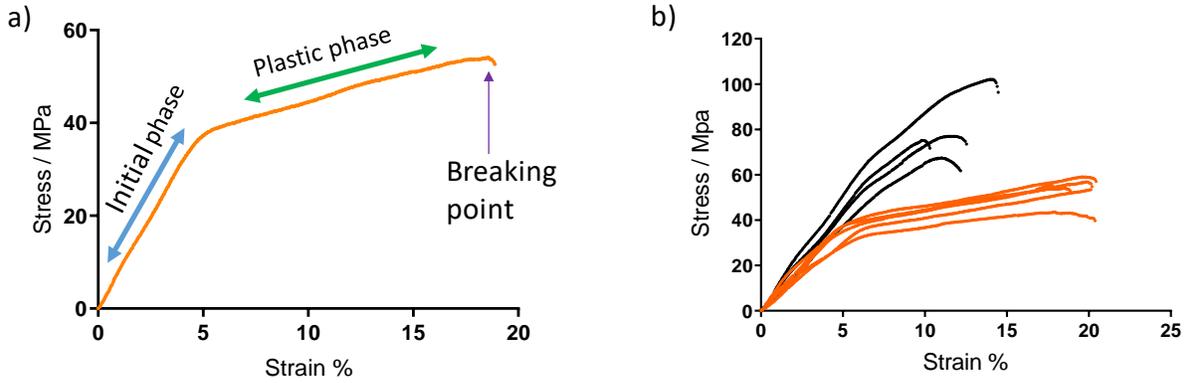
Figure 4 a) A typical stress-strain profile for 11 week old C57BL/6 mouse tail tendon. b) Profile showing overlay of reduced (black) and unreduced (orange) 11 week C57BL/6 tendon showing the loss of the plastic region on reduction. Tendon used was un-conditioned.
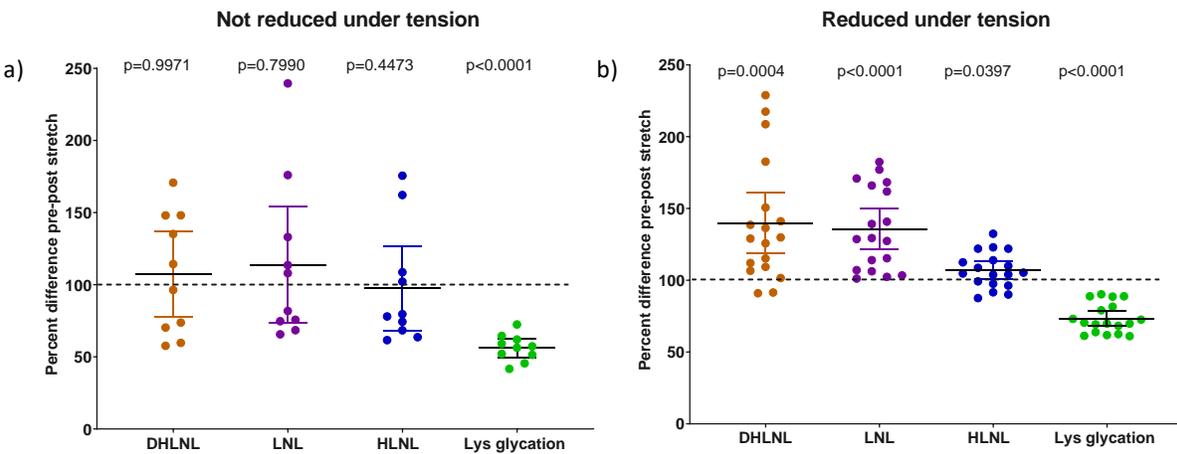
Figure 5 a) Shows the change in analytes on stretching tail tendon fibres of C57BL/6 mice to 4% strain with reduction after removing from testing apparatus (mean $\pm$ 95% c.i, n=10 mice). Statistical analysis: two-sided ratio Student's paired t-tests. b) Shows the change in analytes on stretching tail tendon fibres of C57BL/6 mice to 4% strain with reduction before removing from testing apparatus (mean $\pm$ 95% c.i, n=18 mice). Statistical analysis: two-sided ratio Student's paired t-tests. Tendon used was un-conditioned. *The structures of LNL, HLNL and DHLNL are shown in Figure S1. Abbreviations: HLNL (hydroxy-lysino-norleucine), DHLNL (dihydroxy-lysino-norleucine), LNL (lysino-norleucine, Lys-glycation (the addition of a hexose to the side chain NH2 of lysine).*
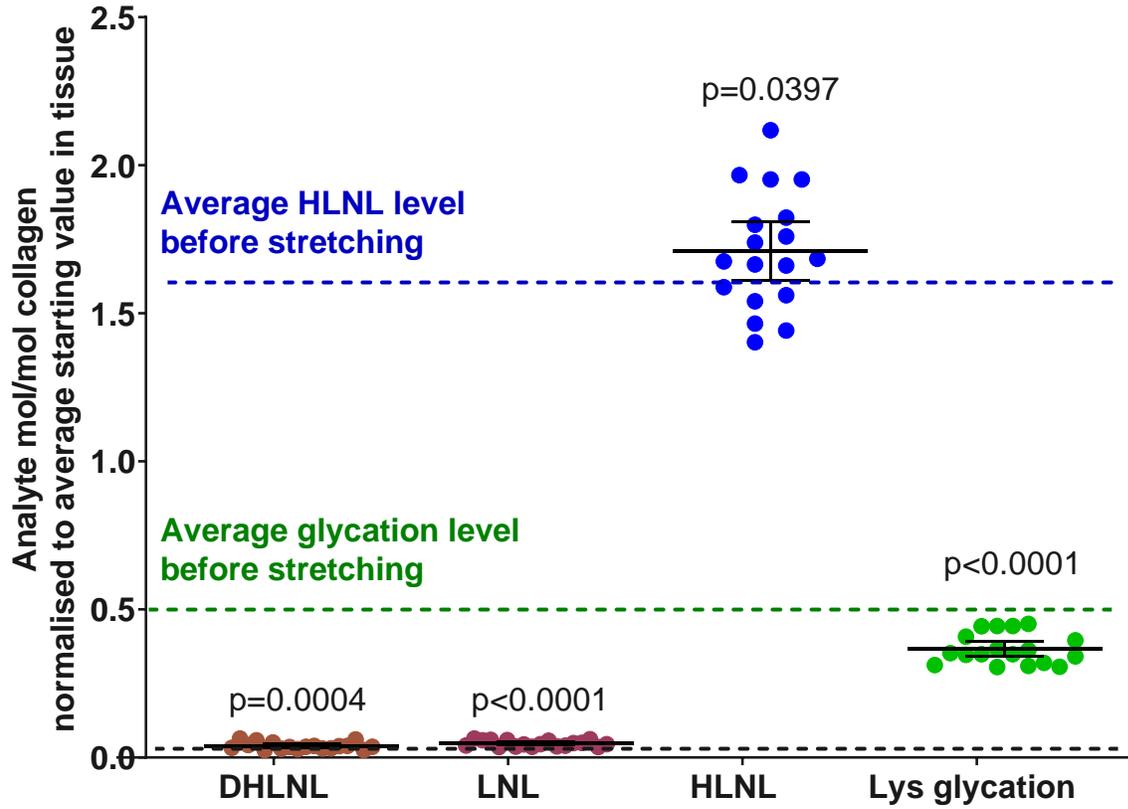
Figure 6 Graph shows the change in analytes on stretching tail tendon fibres of C57BL/6 mice to 4% strain normalised to the average mol/mol collagen value before stretching (mean ± 95% c.i, n=18 mice). Statistical analysis: two-sided ratio Student's paired t-tests. This plot highlights the relative abundance of each analyte.
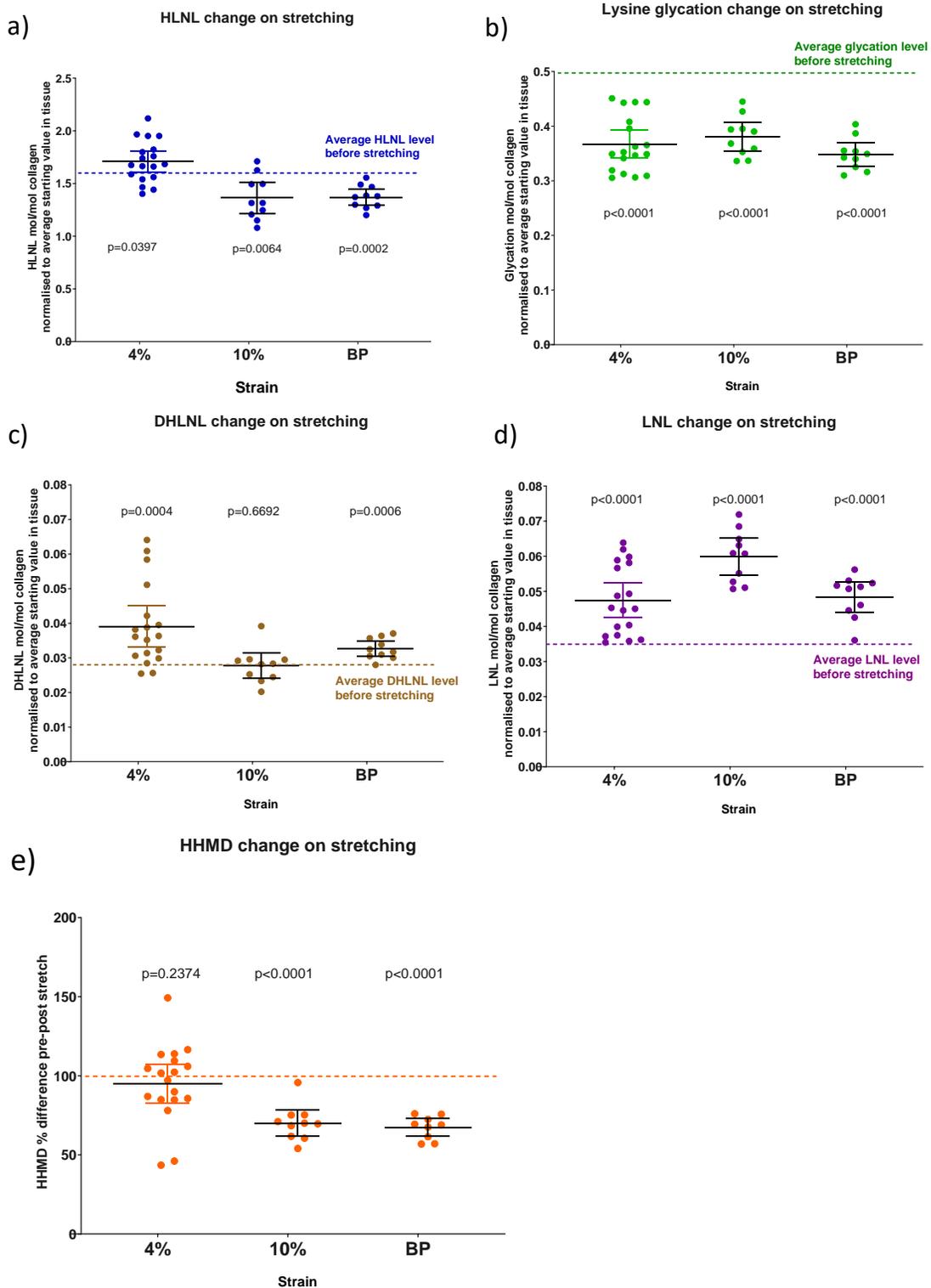
Figure 7 Graphs to show the change in analytes on stretching tail tendon fibres of C57BL/6 mice (mean $\pm$ 95% c.i, n=10 except 3a where n=18 mice). a-d values normalised to the average mol/mol collagen value before stretching. Statistical analysis: two-sided ratio Student's paired t-tests. HHMD (histidine-hydroxymerodesmosine).

a) "Hole" in crosslink matrix

b)

Decrease in cross-linking by 0.1 mol / mol collagen

Increase in cross-linking by 0.1mol/mol collagen shown in black

Between layer crosslinking

Hole filled and ease of fracture decreased

Line of potential fracture in structure shown in yellow

c)
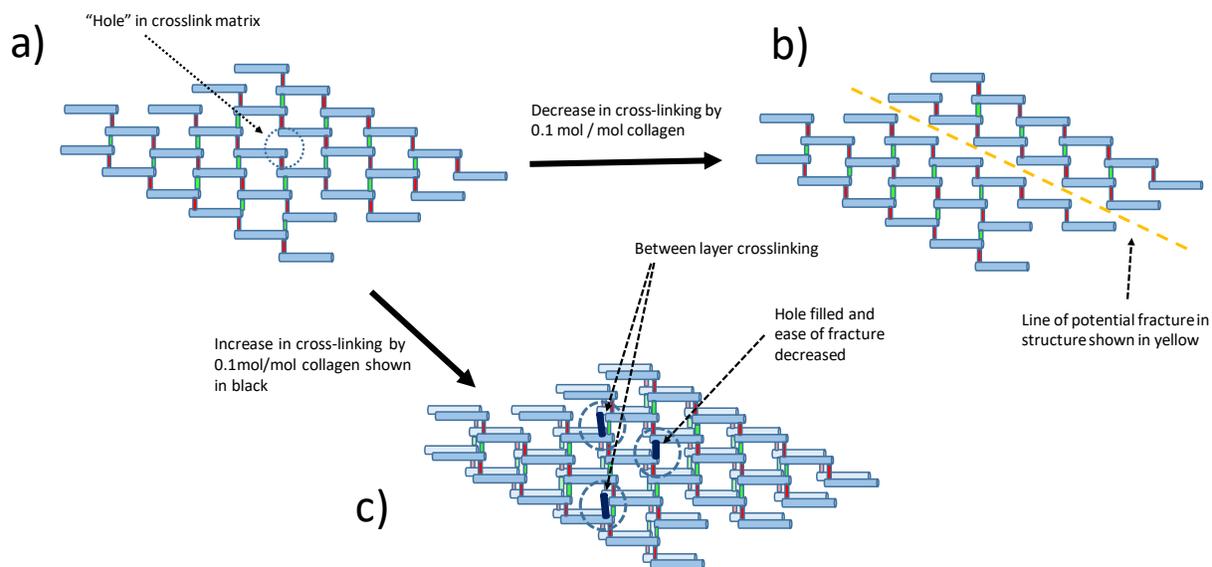
Figure 8 A schematic model of collagen to illustrate the potential impact of a 0.1mol/mol collagen change in cross-linking. This model shows a typical initial level of 1.5 mol cross-linking / mol collagen in each layer. 30 collagen molecules per layer shown in blue with 30 cross-links shown in red and assigned as unchanging with 15 cross-links shown in green and assigned to be sites of bond formation and breakage. In this model, a decrease or increase of 0.1mol/mol is equivalent to a change of 3 bonds per 30 collagen molecules. a) The top left model illustrates that at this level of crosslinking there are holes in the structure, i.e. the level of cross-linking is not sufficient to completely cross-link in a 2D plane. b) The top right model shows that when there is a decrease of 3 cross-links in the model there is the potential for a fracture line to develop through the polymer. c) The bottom structure illustrates that when there is an increase of three cross-links, the hole in this layer can fill and crosslinking then has to occur between layers, which would clearly make the overall structure much stronger.

Mechanical stretching changes cross-linking and glycation levels in the collagen of mouse tail tendon

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