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Detection and Characterization of Molecular-Level Collagen Damage in Overstretched Cerebral Arteries

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Abstract

It is well established that overstretch of arteries alters their mechanics and compromises their function. However, the underlying structural mechanisms behind these changes are poorly understood. Utilizing a recently developed collagen hybridizing peptide (CHP), we demonstrate that a single mechanical overstretch of an artery produces molecular-level unfolding of collagen. In addition, imaging and quantification of CHP binding revealed that overstretch produces damage (unfolding) among fibers aligned with the direction of loading, that damage increases with overstretch severity, and that the onset of this damage is closely associated with tissue yielding. These findings held true for both axial and circumferential loading directions. Our results are the first to identify stretch-induced molecular damage to collagen in blood vessels. Furthermore, our approach is advantageous over existing methods of collagen damage detection as it is non-destructive, readily visualized, and objectively quantified. This work opens the door to revealing additional structure-function relationships in arteries. We anticipate that this approach can be used to better understand arterial damage in clinically relevant settings such as angioplasty and vascular trauma. Furthermore, CHP can be a tool for the development of microstructurally-based constitutive models and experimentally validated computational models of arterial damage and damage propagation across physical scales.

Graphical abstract

Arteries play a critical role by carrying oxygen and essential nutrients throughout the body. However, trauma to the head and neck, as well as surgical interventions, can overstretch arteries

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and alter their mechanics. In order to better understand the cause of these changes, we employ a novel collagen hybridizing peptide (CHP) to study collagen damage in overstretched arteries. Our approach is unique in that we go beyond the fiber- and fibril-level and characterize molecular-level disruption. In addition, we image and quantify fluorescently-labeled CHP to reveal a new structure-property relationship in arterial damage. We anticipate that our approach can be used to better understand arterial damage in clinically relevant settings such as angioplasty and vascular trauma.

Keywords

artery; overstretch; molecular damage; collagen triple helix; unfolded collagen

1 Introduction

Arteries play a critical role in carrying essential nutrients and oxygen throughout the body. However, both trauma and surgical interventions such as angioplasty, can distend arteries beyond their physiological range. Even in the absence of hemorrhage, such ‘subfailure’ deformations can cause cellular damage [1]; [2]; [3] and permanent deformation of the tissue [3]; [4], leading to compromised vessel function [5]. Furthermore, experimental and computational work has demonstrated that overstretch alters arterial passive mechanics in cases of both axial [6]; [7]; [8]; [9] and circumferential; [9]; [10]; [11] loading. This alteration is characterized by a softening of the tissue stress-stretch behavior, or, an increase in wall compliance.

A key vascular constituent in all of these processes is collagen. Not only do collagen fibers dominate the passive mechanical behavior of arteries at high strains [12]; [13], but they also transfer exogenous loads to cells [14]. Therefore, damage to collagen may alter cellular signaling and, similarly, endogenous loading originating in cells may not transfer properly to the extracellular matrix.

Despite the key role of collagen in arterial mechanics, there is limited understanding of collagen damage following overstretch. The majority of findings come from research on the effects of angioplasty, with investigators using electron microscopy to observe dehiscence [15] and tearing [16]; [17] of collagen fibers. Even less is known about collagen damage following axial (longitudinal) loading, with one report of macroscale tears in the tissue following repeated tensile strains yet with little investigation of the damaged structures [18]. The limited insight on structural damage in arteries is largely due to the lack of an objective assay to detect and localize collagen damage in a quantitative fashion.

We recently demonstrated a new method of identifying molecular-level damage in collagen matrix that employs collagen hybridizing peptide (CHP) specifically binding to denatured collagen strands [19]; [20]; [21]. CHP is a synthetic peptide with a collagen-mimicking Glycine(G)-Proline(P)-Hydroxyproline(O) repeating sequence, and has a strong propensity to form the triple helical structure of collagen. As a result, a CHP strand can effectively bind to unfolded collagen chains through triple helix formation, whereas such binding sites are not present in intact collagen, making CHP a promising probe for detecting mechanically-induced unfolding of collagen. Previously, we reported that single-strand CHP with the sequence (GPO)₉ can hybridize to unfolded collagen strands that have been denatured by heat, protease activity [19]; [20], or most recently, large deformations [21]. We also demonstrated that CHP has negligible affinity to intact collagen and virtually no non-specific binding to non-collagenous proteins in tissues [22]; [23] due to its neutral and hydrophilic sequence [19]; [20]. Given that the presence of unfolded collagen molecules has been suggested in connective tissues (e.g., tendon) subject to heavy loading [21]; [24]; [25]; [26]; [27], we hypothesized that individual collagen molecules can be unfolded in overstretched arterial tissue and that such collagens can be probed by CHP.

Here we present the quantitative staining of overstretched arteries with a solution of fluorescently-labeled CHP, testing both the presence of unfolded collagen and the efficacy of the CHP probe. Furthermore, we leverage this novel marker to characterize the location, orientation, concentration, and stretch thresholds of damaged (unfolded) collagen within arteries following various levels of axial and circumferential loading. Finally, we use these results to demonstrate a close association between changes in tissue-level material properties and molecular-level collagen disruption. Studies are carried out in cerebral arteries for the potential translation of findings to cerebral angioplasty and brain trauma. Results for each objective are reported in detail and, as successful preliminary results in arteries prompted a parallel study in rat tail tendon fascicles [21], comparable findings are noted. Finally, the advantages of this technique over existing technology, its potential as a tool for better understanding vascular damage in both trauma and clinical interventions, and the role of molecular-level damage in arterial softening are discussed.

2 Materials and Methods

2.1 Mechanical testing of axially overstretched samples

Sheep heads (n=5) were obtained from a local slaughterhouse immediately after death and transported to the lab on ice. Within five hours of death, the brains were removed and the middle cerebral arteries (MCAs) were dissected from the brain, taking care to remove surrounding connective tissue. Straight lengths of each MCA were cut into 3-5 mm segments (n=21), and branches of these segments were ligated with unwound 6-0 suture. Reference cross-sectional area (*A*) was measured from unloaded vessel cross-sections cut from the end of each segment. Segments were bathed in PBS throughout the dissection and testing process.

Mechanical testing was performed with a custom-made pressure myograph system described previously [28]. Briefly, each segment was cannulated with hypodermic needles and secured with 6-0 suture and cyanoacrylate glue. Microspheres were applied to the adventitial surface

to allow determination of local strains. Vessel segments were perfused with room temperature PBS.

Following mounting, segments were preconditioned by oscillating the luminal pressure (0-20 kPa; 0-150 mmHg) for five cycles while length was held constant. Preconditioning cycles were repeated at gradually increasing lengths while actively monitoring variations in axial force with pressure. The axial *in vivo* length (L_{IV}) was identified as the length at which pressure cycles had a negligible effect on the tensile load [29], and the appropriateness of this estimate was evaluated as additional inflation cycles were done at stretches up to $1.05 \cdot L_{IV}$ (roughly $1.03 \cdot L_{IV}$ in local axial strain). Variations in tensile loads were only evaluated during the fifth and final inflation cycle at each length so that preconditioning at each length (cycles 1-4) was complete prior to evaluating force. Samples were then returned to the *in vivo* length and subjected to a single, triangular wave, quasi-static axial overstretch while maintained at an internal pressure of 100 mmHg (13.3 kPa) and a rate of 0.1 mm/s (strain rate $\approx 0.025 \text{ s}^{-1}$ (Fig. 1). Ten samples were stretched to a variety of subfailure levels and four were pulled to failure. In order to distinguish remodeling-induced unfolding from stretch-induced unfolding (in subsequent CHP staining), six segments (at least one per brain) served as mechanical controls. These segments underwent the same protocol (dissection, preconditioning) but were not subjected to the triangular wave axial overstretch.

2.2 Mechanical testing of circumferentially overstretched samples

A sheep brain was acquired from the Lamb Intensive Care Unit (LICU) at the University of Utah after being euthanized with an overdose of Beuthanasia (MWI Veterinary Supply). All procedures met requirements established by the Institutional Animal Care and Use Committee at the University of Utah. Segments of MCA ($n=5$, 5-9 mm long) were similarly dissected and preconditioned using the same procedures as that described above. Proximal and distal cross-sections of each MCA were imaged, and wall thicknesses were measured and averaged to define segment reference wall thickness (H). Following preconditioning, segments were removed from the needles and cut into rings approximately 1 mm long (axial dimension, $n=31$, Fig. 2). Rings were then individually cannulated with two adjacent 28 gauge hypodermic needles and, in a configuration similar to wire myography, distended circumferentially by a triangular wave displacement of one needle at a rate of 0.1 mm/s (strain rate $\approx 0.06 \text{ s}^{-1}$). Ten rings were stretched to a variety of subfailure levels and eleven were pulled to failure. Similar to that done in the axial overstretch experiments, a total of seven rings (at least one per preconditioned segment) underwent the same protocol (dissection, preconditioning) but were not subsequently cannulated with the 28 gauge needles as they were not overstretched (mechanical controls). Unloaded reference (W) and current (w) ring length were measured from images captured at 3 Hz with a digital video camera (PL-A641, Pixelink).

2.3 CHP staining for collagen damage

CHP labeled with the fluorophore 5-FAM (F-CHP) was obtained from 3Helix (catalog number: FLU300, Salt Lake City). Following mechanical testing, all vessel segments were stained with F-CHP to detect the unfolded collagen molecules [19]. Stock F-CHP was diluted with PBS to a concentration of 20 μM . Because the CHP slowly self-assembles into

its own triple helix over time, losing its driving force to hybridize with collagen, its solutions must be heated to dissociate the trimers into single CHP strands before use. Therefore, CHP solutions were heated for 6 min using a hot plate set to 70 °C and subsequently cooled on ice for 2 min to room temperature and immediately used for staining. Vessel segments were incubated in 100 µL of the activated CHP solution for at least 1 hr at 4 °C. Samples were then rinsed (3 times in 300 µL of PBS for 10 min each at 4 °C), cut open longitudinally, laid flat on glass slides, and prepared for imaging with a mounting media (Fluoromount G; Southern Biotech) and cover slip.

2.4 Confocal imaging

Following staining, samples were imaged at 10× (512 × 512 pixels) using a laser scanning confocal microscope (Fluoview 1000, Olympus). Serial slices were taken in 2 µm increments, producing a z-stack through the entire wall. The CHP signal was excited with the 488 nm Argon gas laser. Using a custom MATLAB script, adjacent images were stitched together to form a single montage covering the full circumference of the vessel.

2.5 Quantification of tissue stretch

Stretch (λ) was used to characterize tissue deformations with stretch being calculated as the ratio of current and reference dimensions. The *in vivo*, rather than unloaded, configuration was used as the stretch reference as previous work in our lab has shown that doing so reduces the sample-to-sample variability in mechanical response [30]. The reader is referred to Nye et al [31] for approximate *in vivo* stretches of this tissue. Stretches in axially loaded samples were determined from needle displacements and calculated relative to the estimated *in vivo* length of each specimen using Eq. 1

$$\lambda_z = \frac{l}{L_{IV}} \quad \text{Eq. 1}$$

where l is the current length of the sample. As mentioned earlier, the *in vivo* length (L_{IV}) was determined during preconditioning. In samples pulled to failure, maximum tissue stretch was measured just prior to loss of internal fluid pressure as, beyond this point, additional deformation was localized to the eventual failure site which was disregarded in the subsequent analysis. Stretches in circumferentially loaded ring samples were similarly calculated from needle displacements using Eq. 2

$$\lambda_\theta = \frac{c}{C_{IV}} \quad \text{Eq. 2}$$

where c is the current ring circumference and C_{IV} is the estimated *in vivo* circumference. Mid-wall ring circumferences were calculated according to Eq. 3

$$c = 2x_n + \pi(D_n + H) \quad \text{Eq. 3}$$

where x_n is the distance between the central axes of the two parallel needles and D_n is the needle diameter. As the thickness of the wall could not be measured during loading, the reference wall thickness, H , was used. Computational modelling showed this assumption to produce no more than 3% error in circumference measurements [32]. The *in vivo* circumference was taken to be the ring circumference at the configuration during overstretch that produced equivalent wall stress (Eq. (4)) as that experienced when held at *in vivo* length and pressure (13.3 kPa) during preconditioning (Eq. (5)).

$$T_{\theta} = \frac{F_{\theta}}{2wH} \quad \text{Eq. 4}$$

$$T_{\theta} = p_i \frac{d_i}{d_e - d_i} \quad \text{Eq. 5}$$

In Eq. (4), T_{θ} refers to the mean circumferential Cauchy stress, F_{θ} is the experimental force measured by the load cell, and w is the current ring length (axial dimension). In Eq. (5), p_i refers to the internal pressure and d_i and d_e to the vessel current inner and outer diameters, respectively. Controls were slightly overstretched during preconditioning as internal pressures were cycled above the *in vivo* pressure (Appendix A). However, as controls were never cannulated with the parallel hypodermic needles, their maximum overstretch was simply calculated from the change in diameter as captured by video during preconditioning.

2.6 Quantification of collagen damage

The CHP signal was qualitatively evaluated through the entire z-stack (approximately 20 slices) and a single slice within each stack was objectively quantified. The slice quantified was taken from within the media for circumferentially loaded samples and within the adventitia for axially loaded samples. As cerebral arteries have no external elastic lamina, the precise boundary between the media and adventitia was not known. Therefore, the medial and adventitial images used for quantification were selected from the z-stack based on morphological features, roughly 40% and 80% of the way through the wall (values consistent with previously reported wall thicknesses of cerebral arteries [33]). Collagen damage was quantified by calculating the percentage of pixels having an intensity above a specified threshold. This threshold was defined to be 2.0 times the average brightness of a slice within the corresponding control sample taken at the same wall depth (12-bit scale). The threshold was applied to minimize contributions from background signal inherent to CHP binding of remodeling collagen. Other thresholds (1.5 and 2.5) were qualitatively evaluated; however, these were not ultimately utilized as they appeared to increase false classification of damaged and remodeling fibers. The effect that varying this threshold has on the study results is addressed in Appendix B.

Digital masks were applied to the images to eliminate contributions from branches and associated sutures, as well as from tissue in contact with the needles during distension. Regions of enhanced signal near failed ends were also masked out as the motivation behind

the study was to characterize collagen damage within regions of intact (subfailure) arterial tissue. Furthermore, in cases where tears occurred between the needles ($n=2$), the staining associated with the tear was also masked out. In a few of the circumferentially distended rings ($n=3$), tears between the needles were so significant that the samples were excluded from the analysis, as all staining was associated with the torn tissue.

2.7 Quantification of damaged fiber orientation

OrientationJ, an ImageJ plug-in, was used to quantify the orientation of the damaged collagen fibers. Described in detail by Rezakhaniha et al [34], this image analysis tool evaluates the local orientation and isotropic properties of every pixel in an image by analyzing pixel gradients in both the vertical and horizontal directions. While pixel orientation is the primary output of the plug-in, coherency and energy are additional measures of how aligned the structures are. Coherency is bounded between 0 and 1, with 1 indicating highly oriented structures and 0 indicating isotropic areas. The reader is referred to [34] for further details on these parameters.

Prior to processing, pixels having an intensity lower than the control-specific threshold were set to zero. The built-in function ‘Dominant Direction’ was used to quantify the dominant orientation of the damaged fibers. The ‘Distribution’ function was used to provide a hue-saturation-brightness (HSB) color-coded map and histogram of fiber orientations for each image. In order to discriminate between significantly and not-significantly oriented areas, the minimum normalized energy was set to 2%. Finally, a cubic B-spline interpolation was used with a Gaussian window of one pixel.

2.8 Regression analysis

Collagen damage versus tissue stretch data were fit with a piecewise linear regression model to estimate the average stretch at which the onset of damage occurred. Using a method similar to that employed by Provenzano et al [35], the percentage of collagen damage (D) was modeled as a function of stretch (λ) as

$$D=m(\lambda - \lambda_c)I[\lambda] \quad \text{Eq. 6}$$

with

$$I[\lambda]=0 \text{ for } ; \lambda < \lambda_c$$

$$I[\lambda]=1 \text{ for } ; \lambda \geq \lambda_c$$

where λ_c is the critical stretch threshold below which collagen damage is zero and above which damage increases linearly with stretch according to slope m . The indicator function $I[\lambda]$ equals 0 when $\lambda < \lambda_c$ and equals 1 otherwise – defining the onset of damage at λ_c . The piecewise model was fit to the experimental data with a Levenberg-Marquardt algorithm using MATLAB’s ‘lsqcurvefit’ function having tolerances set to 1e-8.

2.9 Statistical Methods

Confidence intervals were calculated for the linear regression model according to standard approaches outlined by Navidi [36] and using the 'regstats' function in MATLAB.

Representing our linear estimate of collagen damage vs stretch (y vs. x) with the form

$\hat{y} = \hat{\beta}_0 + \hat{\beta}_1 x$, the 95% confidence intervals were given by Eq. 7

$$\hat{\beta}_0 + \hat{\beta}_1 x \pm t_{n-2, \alpha/2} * s \sqrt{\frac{1}{n} + \frac{(x - \bar{x})^2}{\sum_{i=1}^n (x_i - \bar{x})^2}} \quad \text{Eq. 7}$$

where t is the t-statistic with $\alpha = 0.05$, n is the number of samples, s is the standard error, and the superscript bar denotes a mean value.

2.10 Quantification of the maximum stiffness and yield point

In order to correlate collagen damage with changes in tissue-level mechanical properties, the yield point and point of maximum stiffness were calculated for each sample. The First Piola-Kirchhoff stresses were calculated according to Eqs. 8 and 9

$$P_z = \frac{F_z}{A} \quad \text{Eq. 8}$$

$$P_\theta = \frac{F_\theta}{2WH} \quad \text{Eq. 9}$$

and plotted against stretch. As done previously [6]; [30]; [31]; [37]; [38], baseline load cell noise was reduced using the digital filter specified in SAE J211 [39] and filtered traces were compared to original data to ensure fidelity. The stiffness at each data point of the loading curve was calculated as the slope of a regression line centered over 1% strain (25 data points for circumferential samples, 48 data points for axial samples). The yield point was defined as the earliest point in the loading curve where the stiffness dropped below 73.4% of the greatest stiffness achieved prior to that point. This threshold was determined in a previous study from the average noise amplitude in stiffness plots of juvenile sheep MCAs [38]. The maximum stiffness was then calculated as the greatest slope prior to the yield point. This sequence was important as in a few samples, stiffnesses higher than that observed in the linear region occurred beyond what we deemed as obvious yielding (see for example Fig. A1, bottom right graph, middle trace). Only stiffnesses beyond $\lambda_\theta = 1.1$ and $\lambda_z = 1.2$ were evaluated, as data below these levels occasionally showed significant changes in slope prior to the primary linear region (possibly attributable to fiber reorientation). Furthermore, care was taken to rule out slope decreases at the end of a subfailure loading which coincided with deceleration of the linear actuator.

3 Results

3.1 Location of molecular-level damage within the arterial wall

Confocal microscopy revealed the presence of unfolded collagen strands in overstretched arteries as manifest by the heightened CHP signal (circumferential loading, Fig. 3, and axial loading, Fig. 4). Interestingly, we found that circumferential loading primarily damages collagen molecules within the media (Fig. 3b) while axial loading primarily disrupts collagen within the adventitia (Fig. 4c). While some CHP signal was observed in the other layers (Figs. 3c and 4b), it was predominantly an artifact from strong fluorescence in a neighboring layer. In general, CHP staining was found to be spatially inhomogeneous with concentrated regions of fluorescence indicating focal collagen damage. Furthermore, adventitial damage patterns (Fig. 4c) typically appeared more fibrous than medial ones (Fig. 3b). As expected, minimal signal was observed in the controls (Fig. 3d-f and Fig. 4d-f) as these were not overstretched.

3.2 Orientation of damaged fibers

The orientations of the damaged fibers revealed via CHP staining (and measured in their unloaded state) aligned greatly with the direction of loading (Fig. 5). Considering only samples having a greater percent damage than control samples, the fiber orientation following circumferential loading was -3.1 ± 6.0 degrees (relative to circumferential axis) with a coherency of 0.27 ± 0.06 (mean $\pm$ SD, $n=16$). Following axial loading, the damaged fiber orientation was 86.9 ± 9.6 degrees with a coherency of 0.20 ± 0.05 ($n=8$). All orientation and coherency values used in this analysis are compiled in Appendix A.

3.3 Correlation between CHP intensity and mechanical overload

We also found that the CHP signal increased with overstretch severity for both loading directions. This was true both qualitatively (Fig. 6) and quantitatively (Fig. 7). Collagen damage was quantified for a single layer within the wall, the media for circumferentially loaded samples and the adventitia for axially loaded samples. The CHP signal following relatively mild stretches (Figs. 6b and 6f) was comparable to that of controls (Figs. 6a and 6e), then increased approximately linearly beyond a critical stretch threshold, λ_C (Fig. 7). Note that controls were overstretched slightly beyond their *in vivo* configuration during preconditioning: approximately 5% in the axial direction when determining the *in vivo* length and roughly 1% in the circumferential direction as pressures were cycled up to 150 mmHg, but 100 mmHg was deemed the *in vivo* pressure. As the amount of ‘damage’ was also quantified in controls, data from them are included in Fig. 7 near the origin.

In order to better define the thresholds and accumulation of collagen damage, the data were fit with a piecewise linear regression model. Regression lines consisted of an initial horizontal line through the origin followed by a second line having a constant positive slope. The intersection of these two lines (λ_C) is indicative of the collagen damage threshold, while the slope (m) of the second line is indicative of the rate of damage accumulation with strain beyond λ_C . The resulting regression lines and 95% confidence intervals are overlaid on the data (Fig. 7) with output parameters presented in Table 1. Calculated values for λ_C appear appropriate given that relatively minimal CHP signal was detected below either threshold. In

a few samples, small amounts of collagen damage could be observed following milder stretches (see thin fibers in the center of Fig. 6f); however, our algorithm could not distinguish these from the background signal inherent to controls. More sophisticated edge detection algorithms were also implemented; however, these did not improve sensitivity without reducing specificity.

3.4 Relationship between collagen molecular damage and tissue yielding

Analysis of the stress-stretch data revealed that the stretch thresholds (λ_C) as determined by CHP staining approximately coincided with the arterial yield stretches (Table 1). For both loading directions, λ_C (red dashed lines in Fig. 7) fell within 3% strain of the average yield point (solid blue circles in Fig. 7). As our measure of yielding is somewhat subjective, it is also instructive to compare the stretch threshold relative to the average point of maximum stiffnesses (solid blue squares in Fig. 7), a measure that some use to define yielding. For the case of circumferential loading, the threshold of collagen damage, λ_C , fell midway between the average stretch at maximum stiffness and the average yield stretch. In contrast, the collagen damage threshold in axial loading fell slightly above the yield stretch. A discussion on the strength of this finding is addressed in Section 4.3. Also, a complete list of circumferential (n=11) and axial (n=10) yield stretches is included in Appendix A.

4 Discussion

Using CHP, we detected and characterized molecular-level unfolding of collagen in cerebral arteries subjected to a single overstretch. These findings are the first to identify any stretch-induced molecular disruption to collagen in blood vessels, as previous investigations have only explored tissue-, fiber-, or fibril-level disruption. In addition to detecting damage, CHP effectively localized the damage, both revealing the specific layer within which damage occurred and demonstrating that overstretch primarily disrupted fibers aligned with the direction of loading. Finally, comparison of CHP staining with mechanical data showed that arterial yielding was closely associated with the onset of collagen damage, and that, beyond this threshold, collagen damage increased with strain. Each of these findings held true for both axial and circumferential modes of overload. These results suggest that collagen unfolding is likely to occur in trauma and in surgical interventions such as angioplasty, making CHP a potential tool for better understanding vessel damage in clinical settings. Findings also lend insight into the role of molecular-level collagen damage in arterial softening. Observations of elastin damage were also noted; however, these will be addressed in a future work.

4.1 Location and orientation of damaged collagen within the wall

The finding that overstretch primarily damages arterial collagen that is aligned with the direction of loading, with negligible damage to off-axis fibers, is both novel and consistent with the current knowledge of arterial mechanics. While fiber angles were measured in an unloaded state, relatively small changes occur when transforming between this and the *in vivo* state (Appendix C). It is well established that collagen is the primary load bearing constituent in arteries at high levels of strain [40]. Given that collagen fibers are much stiffer in tension than in shear [41], it follows that the fibers bearing the greatest load will be those

aligned with the loading direction. This, coupled with the fact that axially oriented fibers are primarily found in the adventitia [33]; [34]; [35]; [38]; [40]; [41]; [42], is consistent with our finding of an axially oriented CHP signal within the adventitia following axial overstretch. The fact that the arterial media is rich with circumferentially oriented fibers [40] is similarly consistent with our finding of a circumferentially oriented CHP signal within the media following circumferential overstretch. Furthermore, we report that damaged circumferential fibers are more aligned than the damaged axial fibers ($SD=6.0$ compared to 9.6 degrees), paralleling the structure of intact collagen in cerebral arteries [42]. The z-resolution of the confocal objective used was not fine enough to precisely quantify collagen damage among fibers oriented orthogonal to the loading direction, as these images were often influenced by the strong fluorescence from a neighboring layer. Therefore, we cannot conclusively exclude medial damage following axial loading nor adventitial damage following circumferential loading; though, the level of collagen damage in these layers is clearly lower. Helical fibers known to be present in the adventitia (Fig. 7a in [33]), were intact and often observed while imaging and quantifying the adventitia for the axial study (see large diagonal fiber with moderate brightness in Fig. 6g). While such fibers are hypothesized to provide a protective mechanism against overdistension, it is interesting that these were never observed to have much, if any CHP staining. It is possible that such fibers did support axial loading while others more aligned with the loading reached their failure point earlier, leading to crack initiation and propagation across the remaining tissue and subjecting helical fibers to a quicker loading to failure. Preliminary work suggests that the duration of the strain, not just the magnitude, increases the amount of mechanically-induced unfolding.

Despite the correlation between our results and the current knowledge of arterial mechanics, the actual identification of mechanically damaged collagen has only been reported by a handful of investigators, each using either histology [18] or electron microscopy [15]; [16]; [17]. In agreement with the present work, one report showed that circumferential loading with a balloon catheter damaged circumferentially oriented medial fibers. However, the presence/absence of damage within the adventitia was not reported [17].

4.2 Thresholds and accumulation of collagen damage

To our knowledge, this is the first study to define clear thresholds of collagen damage in blood vessels and to characterize the accumulation of damage with strain. While both parameters serve as meaningful inputs for future microstructurally-based constitutive models of arterial damage, neither one appears to have been previously defined for molecular-, fibril-, nor even fibril-level collagen disruption. The work that most closely achieves this for axial loading found the threshold for macroscopic collagen disruption to be between 6-30% beyond the zero strain length [18]. While this range is insightful, it is poorly defined and is limited to the case of 1000 loading cycles in rabbit aorta. For the case of circumferential loading, investigators have reported stretched collagen fibers in human MCAs after subjecting them to 1.5 atm and ruptured fibers after 3.0 atm [17]. However, these two data points fall short of defining a clear threshold or a rate of damage accumulation.

4.3 Coincidence of molecular damage and yielding

Aside from the novelty of detecting molecular-level collagen damage in vascular tissue, perhaps our most significant finding is the approximate coincidence of the yield point and the onset of CHP binding. To the best of our knowledge, no investigations have even compared thresholds of tissue-level yielding with any form of collagen damage in arteries. We hesitate to overstate our finding, though, as our methods for quantifying both yielding and collagen damage could not be purely objective. We did, however, do our best to seek objectivity in all of our methods (see Appendix B for a discussion on quantifying collagen damage). Regarding yielding, there is no standard approach for quantifying the yield point in soft biological tissues; however, two general methods seem to be used. One method defines the yield point as the point of greatest stiffness either directly from the data [43]; [44] or after fitting the data with a polynomial [45]; [46]; [47]. A second method defines the yield point as the point where the stress-strain curve first departs from the linear region based on a user-defined threshold [38]; [48]; [49]. While this latter approach is more sensitive to user inputs, it is better suited to detect yielding at the end of a sustained linear region rather than in the middle of it, which was our goal. We ultimately employed both methods, with the latter defining the yield point and the former defining the point of maximum stiffness. These two numbers provide some sense of the amount of variability in our calculated yield point. For both loading directions, we found the average difference between the yield point and the point of maximum stiffness to be 5% strain (Table 1). In defining the threshold for yielding, we did our best to be objective by taking the threshold from a previous study in similar tissue [38]. Furthermore, post-hoc qualitative analysis showed that this threshold did a reasonable job of identifying the end of the linear region in all axial samples (Fig. A1). However, samples loaded circumferentially often exhibited a less pronounced linear region and less dramatic changes in stiffness, making it harder to qualitatively verify the identified yield points. In light of this, it is interesting to note that we found the axial threshold of collagen damage to best align with our defined yield point while the onset of collagen damage in circumferential samples (where the yield point was less defined) fell closer to the point of maximum stiffness. The fact that changes in stiffness were more dramatic in axial loading may be due to the fact that the adventitia consists of thicker bundles of collagen fibers [13] compared to the media, which, upon rupture, cause more significant changes in stiffness.

4.4 Comparisons between circumferential and axial results

While the primary objective of the present work was to detect the presence of unfolded collagen following circumferential and axial loading separately, we believe there is still value in comparing the qualitative and even quantitative findings from the two datasets. In general, the CHP signal in the media following circumferential loading highlighted finer, more closely packed fibrous damage than that observed in the adventitia following axial tests (demonstrated in both Figs. 5 and 6). This is likely due to the fact that smaller fibers are found in the media while the adventitia consists primarily of thick bundles of collagen fibers [13] having a larger spatial distribution [42]. These large adventitial bundles may explain the brighter CHP signal in axial tests (Figs. 5 and 6), as larger damaged fibers would provide more binding sites for CHP. This brighter adventitial signal may also have contributed to the higher rate of damage accumulation beyond λ_C in axial tests (Table 1), since this would

increase the likelihood of damaged fibers being above the defined pixel intensity threshold. Finally, the stretch threshold, λ_C , and related yield threshold, λ_Y , were found to be lower in circumferential tests (Fig. 7 and Table 1). This may be due to fact that medial fibers are more aligned with the circumferential direction than are adventitial fibers with the axial direction [42], causing them to become taut and subsequently fail at lower stretches. Additional factors such as collagen crimp and cross-linking may also contribute to differences in these damage parameters. However, to our knowledge, these have not been quantified in cerebral arteries. It should also be noted that there was an age difference in axial (juvenile, approx. 3-9 months) and circumferential (adult, approx. 3-7 years) samples. While microstructure is expected to change with age, our recent study on supraphysiological axial loading of sheep MCAs found no significant differences in the maximum stiffness, the ultimate stretch, nor in the ultimate stress between the juvenile and adult groups [31]. As collagen dominates the passive mechanical response at high strains, these results suggest negligible changes in collagen over these same age ranges. In contrast, biaxial tests in the physiological range from the same study found the circumferential stiffness to be roughly five times higher than the axial stiffness for juvenile and adult samples, paralleling the difference in supraphysiological stiffnesses observed in the present work (Fig. 7). While these observations are not conclusive, they suggest that effects from structural differences in the two age groups are likely secondary to the inherent structural differences between the two loading directions.

Though not directly related to collagen damage, we also noted differences in the stress-stretch behavior for the two loading directions. As the curves shown begin from the *in vivo* configuration (Fig. 7), they include a slight initial stress offset, are missing much of the toe-region and are, therefore, more linear than those commonly presented for blood vessels. However, we have provided more complete axial loading curves for this same tissue previously [31]. Furthermore, the average axial and circumferential *in vivo* stretches presented in the same work can be used to convert the stretches reported here to be relative to the unloaded length. The stress offset in the axial samples is lower than expected, likely due to some stress relaxation when determining the *in vivo* length (Section 2.1). In addition, the stress-stretch response over the loading range was generally stiffer in the circumferential direction than the axial, bearing roughly four times the stress at half the stretch. A similar disparity was also observed at the *in vivo* stretch, in agreement with our previous investigation in sheep [31] yet contradictory to our studies in human [30] and rat [37] cerebral arteries. Further investigation is needed to determine whether these discrepancies are simply due to test methodology or actual species-specific differences.

4.5 Comparisons with CHP results in rat tail tendon fascicle

As mentioned earlier, successful preliminary results of CHP binding in mechanically overstretched arteries led to a parallel study in rat tail tendon fascicles [21]. Three similarities can be drawn between the recent findings from that study and the present work. First, the study in tendon showed a nearly linear increase in CHP binding with strain beyond a critical threshold, comparable to that reported here in arteries (Fig. 2 of [21]). Second, the onset of CHP binding in tendon also coincided with a decrease in stiffness (Fig. 2a and Supplementary Fig. 2a of [21]). Although a precise yield point was not defined in the tendon

study, it was qualitatively observed for all samples tested. Finally, the authors noted that CHP did not bind to ends of tissue that were cut with scissors, suggesting that such loading does not unfold collagen strands. We, too, observed this phenomenon in arteries. One key mechanical difference between the two tissues, however, was that both molecular- and tissue-level failure occurred at lower stretches in the tendon fascicle than in arterial samples. This is expected as arteries, in general, have a much higher fraction of elastin [50].

Two technical differences between the two studies are also apparent. First, multiple test groups in the tendon study, each having a specific target stretch, define the increase in CHP staining beyond the critical strain threshold (Fig. 2 of [21]). This was not possible to achieve in the present work as our linear trends were primarily defined by samples pulled to failure (Fig. 7 and Appendix A), with each having unpredictable failure stretches. Therefore, we characterized the rise in CHP staining with a regression line and associated confidence intervals rather than with error bars for each strain group. A second difference between the two studies is the additional noise observed in the arterial damage-stretch plots (Fig. 7). This is likely due to both the structural heterogeneity of arteries as well as the increased CHP signal observed in arterial controls (for example, see Fig. 6e). This latter factor led us to develop a more sophisticated algorithm for classifying collagen damage in arteries – defining pixels with intensities greater than two times the average control intensity as being mechanically damaged. It is still unclear why arteries were more prone to background binding compared to rat tail tendons; however, one possible explanation would be a higher native collagen-remodeling rate in arteries.

4.6 Advantages of CHP over existing technology

The reported binding of CHP to mechanically damaged collagen in arteries is a novel discovery for the field of arterial mechanics. This technique improves upon existing methods in four important ways. First, CHP opens the door to understanding molecular-level damage to collagen whereas other common techniques (SEM, TEM, and histology) only reveal fibril-, fiber-, or tissue-level disruption. Therefore, CHP can be used to further elucidate the progression of collagen damage across these physical scales. Furthermore, the previously mentioned study in rat tail tendon fascicles suggests that collagen triple helices may unfold long before disruption or damage of larger scale structures – making CHP a more sensitive metric of collagen damage [21]. In addition, CHP lends insights into inter- and intra-molecular damage progression. For example, the fact that CHP bound along the length of damaged fibers in the present work suggests that the stress/strain mechanisms leading to molecular failure (likely alpha-chain pullout, [21]) are not localized to a single point on a fiber, but that they propagate along the length of a fiber during the overload. Second, CHP conjugated to a fluorophore provides an approach for detecting vascular damage that is non-destructive in both its imaging of subsurface detail and minimal tissue preparation. In contrast, electron microscopy and histology require serial slicing in order to reveal subsurface damage. Furthermore, previously mentioned studies using an SEM [17] required fixation, formic acid digestion in a 45 °C oven, and freeze drying - all of which can alter the very structures being studied. Third, the damage-specific CHP provides a straight-forward method for positive identification of damage in tissue compared to a ‘needle-in-the-haystack’ hunt with an SEM. Fourth, in contrast to classifying the severity of tissue damage

with the help of a blinded observer [18], quantifying the fluorescence intensity of CHP provides a simple, objective measure of damage severity.

4.7 CHP as a tool for probing vascular damage in angioplasty and trauma

In light of the advantages that CHP offers over existing techniques, this marker has the potential to serve as a useful probe of vessel injury in both angioplasty and trauma. While molecular-level collagen damage has yet to be demonstrated in either of these settings, we have reason to believe it occurs. For example, balloon angioplasty has been shown to rupture collagen fibers [17]. Given our recent evidence that collagen unfolding precedes fiber failure [21] it is likely that collagen unfolding occurs in balloon angioplasty. Therefore, CHP could serve as a useful research tool for assessing the severity of balloon or stent loading acutely as well as tracking collagen remodeling over time. More specifically, CHP could be used to answer Humphrey's call for "multiaxial data from which balloon-induced wall damage can be quantified as a function of clinically relevant balloon parameters" [51]. Furthermore, as we have shown collagen unfolding to be correlated with tissue yielding, this molecular-level denaturation may be the key mechanism behind the well-established remnant deformations observed in angioplasty [3]; [52]. Regarding trauma, it is well known that blood vessels are often torn and bleed as an immediate consequence of TBI [53]; [54]. It follows that other vessels undergo subfailure deformations. Therefore, CHP could also serve to increase understanding of vascular strains in TBI by probing for intact yet damaged vessels, potentially elucidating their prevalence, loading magnitude, and even their loading direction. However, as the present work only explored quasi-static loading, the effect of strain rate on CHP binding should first be understood *in vitro*.

Should an investigation require detection of mild collagen damage, efforts should first be made to improve the signal-to-noise ratio of CHP binding in arteries. This could be accomplished by reducing or blocking the background signal inherent to continuously remodeling collagen in arteries with unlabeled CHPs prior to overstretch. Alternatively, development of a more sophisticated algorithm that distinguishes between CHP binding of remodeling and damaged fibers could also facilitate detection of mild damage.

4.8 Role of collagen unfolding in arterial softening

As arterial mechanics are known to soften in angioplasty [8]; [9]; [10] and theorized to do so in trauma [6], another motivation behind this investigation was to shed light on the microstructural mechanisms responsible for arterial softening. Numerous experimental and computational studies have demonstrated that a single overstretch softens the subsequent reloading curve in cases of both axial [6]; [7]; [8]; [9] and circumferential [8]; [9]; [10]; [11] loading. While this softening behavior has been attributed to collagen [55]; [56], the underlying mechanism is not known. Using the same tissue and experimental approach as done here, Bell et al showed that softening occurs following axial stretches as low as 1.2 times the *in vivo* length [6] – lower than the threshold for collagen damage observed in the present study. This comparison suggests that mechanisms other than the unfolding of the collagen triple helix must be at play in arterial softening. While this is likely true, we hesitate to rule out the present mechanism, as small amounts of CHP staining were observed

in samples stretched to 1.2 (Fig. 6f), although, these subtle signals were difficult to quantitatively distinguish from background signals.

The literature on preconditioning provides additional mechanistic insights for arterial softening, as the softening during preconditioning is similar to that observed by Bell et al. Quinn and Winkelstein showed collagen fiber reorientation to be correlated with softening during preconditioning of ligaments [57]. Others have suggested that macromolecule unfolding [13], as well as irreversible proteoglycan deformation [58], could also play an important role in preconditioning. Therefore, these alternative mechanisms may be important following mild overstretch of arteries.

4.9 Study limitations

Two limitations of this study are noted. First, despite the application of microspheres on all overstretched samples, the stretches reported in the present work were calculated from a more global measure of needle displacements. While local strain measurements do result in different damage parameters, additional analysis suggests that this factor doesn't undermined the association between tissue yielding and the onset of collagen damage (Appendix D). Second, the method used for axial loading was biaxial (due to the internal fluid pressure) rather than uniaxial (as done for circumferential loading). This was done out of an initial desire to improve translation of findings to an *in vivo* setting. While this does limit comparisons between the two modes of loading, the contribution of circumferential stress during axial loading was rather small, approximately 7% of the axial stress at the onset of collagen damage. Therefore, we would not expect the axial results to be much different had they been conducted in a purely uniaxial manner.

5 Conclusion

For the first time, molecular-level collagen damage was detected, localized, and quantified in overstretched vascular tissue through the use of a collagen hybridizing peptide (CHP). Consistent with established literature on arterial mechanics [33]; [34]; [35]; [38]; [40]; [41]; [42], we showed that overstretch primarily damages (unfolds) collagen fibers that are aligned with the direction of loading. Furthermore, we demonstrated that the onset of collagen damage is closely associated with tissue-level yielding. This was true for both axial and circumferential loading. As samples were only loaded quasi-statically, it remains unknown how results may vary at higher strain rates. Not only does this approach provide the first insights into molecular-level collagen damage in vascular tissue, but it improves upon existing methods of detecting collagen damage as it is non-destructive, readily visualized, and objectively quantified. We anticipate that this approach can be used to better understand arterial damage in clinically relevant settings such as angioplasty and vascular trauma. Furthermore, CHP can be a tool for the development of microstructurally-based constitutive models and experimentally validated computational models of arterial damage and damage propagation across physical scales.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Artery configuration for axial overstretch

Light microscope image of a single sheep MCA mounted to notched hypodermic tubing just prior to (left) and at peak (right) overstretch. Small branches were ligated so that the vessel could maintain 100 mmHg (13.3 kPa) during overstretch. Small black dot are microspheres used to track local strain. Axes and scale bar (0.5 mm) apply to both images.

Fig. 2. Artery configuration for circumferential overstretch

Light microscope image of a single arterial ring from a sheep MCA just prior to (left) and at peak (right) overstretch. These rings, or short lengths of otherwise intact artery, came from longer MCA segments that were first mounted and preconditioned similar to segments loaded axially (Fig. 1). However, following preconditioning, segments were chopped into short lengths so that the two 28 gauge hypodermic needles shown could be passed through the lumen. Small black dots are microspheres used to track local strain. Axes and scale bar (0.5 mm) apply to both images.

Fig. 3. Confocal images from an artery loaded circumferentially relative to its control
The fluorescent CHP signal was most prominent in the media (b), indicating that that layer had the highest concentration of unfolded collagen molecules. Fluorescence in the intima and adventitia (a, c) is primarily an artifact from the medial signal (b). Axes and scale bar (200 μm) apply to all images.

Fig. 4. Confocal images from an artery loaded axially relative to its control

The fluorescent CHP signal for axially loaded samples was most prominent in the adventitia (c). The two horizontal bands in (a) show rupture of the internal elastic lamina. Fibrous fluorescence in intima and media (a, b) is primarily an artifact from the adventitial signal (c). Axes and scale bar (200 μm) apply to all images.

Fig. 5. Representative micrographs showing the orientation of damaged collagen fibers

The damaged fibers primarily exhibit a circumferential orientation (0 degrees) within the circumferentially loaded samples while samples loaded axially show damaged fibers mainly with an axial orientation (± 90 degrees). After setting all pixels below the control-specific intensity threshold to zero, OrientationJ was used to quantify the orientation of each pixel within the image, outputting an HSB color-coded map and an associated histogram (Hue: local orientation; Saturation: coherency; Brightness: original image). Histograms count the total number of pixels with a defined orientation (one degree resolution). Axes and scale bar (200 μm) apply to all images.

Fig. 6. Increase in CHP binding (collagen damage) with overstretch severity

Samples subjected to relatively mild levels of overstretch (b, f) appeared similar to mechanical controls (a, e); however, the increase in CHP binding became more apparent at higher strains (c, d, g, h). Images only show a portion of the entire sample. Black circular dots are microspheres. Axes and scale bar (200 μm) apply to all images. Note that samples serving as mechanical controls were slightly overstretched beyond their *in vivo* configuration during preconditioning: (a) $\lambda_{\Theta_MAX} = 1.01$ and (e) $\lambda_{Z_MAX} = 1.05$ as described in Section 3.3).

Fig. 7. Collagen damage and arterial stress vs. overstretch

Collagen damage was measured following CHP staining as the percentage of pixels above a control-specific intensity threshold. Both directions of loading produced an increase in damage with overstretch severity. The intersection of the horizontal and sloped regression lines defines the critical threshold for collagen damage (λ_C , red dashed line). In both cases, this damage threshold fell within 3% strain of the average yield point (solid blue circle). Two representative stress-stretch curves are overlaid for each loading case. Stretches are measured relative to the *in vivo* configuration.

Collagen damage parameters (calculated from linear regression of CHP data) compared to stretches at maximum stiffness and yield stretches (calculated from stress-stretch curves).

Table 1

Loading Direction	Stretch at Max Stiffness (mean $\pm$ SD)	Yield Stretch (mean $\pm$ SD)	Collagen Damage Parameters		R ²
			Damage Threshold (95% CI)	% Damage Per Unit Stretch beyond λ_C	
Circumferential	$\lambda_{MAX} = 1.15 \pm 0.01$	$\lambda_Y = 1.20 \pm 0.01$	$\lambda_C = 1.17 (1.15-1.19)$	m = 52.8	0.55
Axial	$\lambda_{MAX} = 1.28 \pm 0.07$	$\lambda_Y = 1.33 \pm 0.06$	$\lambda_C = 1.35 (1.29-1.42)$	m = 66.7	0.79