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Lysyl oxidase-mediated collagen crosslinks may be assessed as markers of functional properties of tendon tissue formation

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Abstract

Mechanical property elaboration of engineered tissues is often assumed on the basis of gene and protein characterizations, rather than mechanical testing. However, we recently demonstrated mechanical properties are not consistently correlated with matrix content and organization during embryonic tissue development. Based on this, mechanical properties should be assessed independently during natural or engineered tissue formation. Unfortunately, mechanical testing is destructive, and thus alternative means of assessing these properties are desirable. In this study, we examined lysyl oxidase (LOX)-mediated crosslinks as markers for mechanical properties during embryonic tendon formation and the potential to detect them non-destructively. We used tandem mass spectrometry (LC-MS/MS) to quantify changes in hydroxylysyl pyridinoline (HP) and lysyl pyridinoline (LP) crosslink density in embryonic chick tendon as a function of developmental stage. In addition, we assessed a multiphoton imaging approach that exploits the natural fluorescence of HP and LP. With both techniques, we quantified crosslink density in normal and LOX-inhibited tendons, and correlated measurements with mechanical properties. HP and LP crosslink density varied as a function of developmental stage, with HP-to-dry mass ratio correlating highly to elastic modulus, even when enzymatic crosslink formation was inhibited. Multiphoton optical imaging corroborated LC-MS/MS data, identifying significant reductions in crosslink density from LOX inhibition. Taken together, crosslink density may be useful as a marker of tissue mechanical properties that could be assessed with imaging non-destructively and perhaps non-invasively. These outcomes could have significant scientific and clinical implications, enabling continuous and long-term monitoring of mechanical properties of collagen-crosslinked tissues or engineered constructs.

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Keywords

Tendon; Crosslinking; Mechanical properties; Mass spectrometry; Multiphoton microscopy

1. Introduction

Functional properties of engineered tissues are typically assessed at late time-points, often due to limited sample numbers and difficulty handling fragile tissue constructs prior to significant extracellular matrix (ECM) protein deposition. Thus, assessments of tissue formation, especially at earlier stages, are frequently based on protein production and organization. However, proper elaboration of mechanical properties is critical for tissues with mechanically demanding roles in the body. Tendons transfer load from muscle to bone to enable movement and locomotion, and thus their mechanical properties are essential for normal function. Unfortunately, adult tendon fails to heal when injured, or heals with scar tissue that possesses aberrant mechanical properties even after surgical repair, compromising physical function and leading to secondary disorders such as arthritis [1, 2]. Aberrant mechanical properties of scar tissue are associated with abnormal matrix content and organization. Consequently, efforts to enhance healing or regeneration have focused on controlling protein expression and tissue microstructure. Similarly, during engineered or native tissue formation, the elaboration of mechanical properties and structural integrity are commonly assumed if increased ECM content and organization are observed. Thus, in multiple contexts, researchers often forgo mechanical property characterization and instead rely on tissue morphology and biochemical content to assess functional tissue formation.

Collagen crosslinking has been reported to contribute significantly to the mechanical properties of adult homeostatic tendon, as well as to those of dysfunctional tendon that develop with aberrant, disorganized matrix during healing, disease progression (e.g., tendinopathy) and aging [1-3]. We recently showed that inhibition of lysyl oxidase (LOX) activity to reduce collagen crosslinking during embryonic chick tendon development resulted in dramatic decreases in nanoscale elastic modulus [4]. Unexpectedly, we also found that these changes in mechanical properties with enzymatic crosslinking inhibition did not affect total collagen or glycosaminoglycan (GAG) content, or apparent matrix organization [4]. The lack of correlation between mechanical property changes and ECM morphology and content was a significant finding, demonstrating the need to also characterize functional properties of newly forming tissues. Unfortunately, mechanical testing is time-consuming and destructive, requiring high sample numbers and precluding the ability to continuously monitor a single sample over time. Therefore, non-destructive means to monitor functional tissue formation would be desirable. Based on our previous study, we proposed that enzymatic crosslink density correlates directly with mechanical properties during development, and that LOX-mediated crosslinks may be useful as functional markers for tissue formation. The objectives of this study were to characterize LOX-mediated crosslink densities of developing embryonic tendon, to assess the relationship between enzymatic crosslink density and tendon mechanical properties during tissue formation, and to determine whether these crosslinks may be detected non-destructively with imaging.

Collagen crosslinks in tendon include GAGs, non-enzymatic and enzymatic (LOX-mediated) crosslinks. GAGs appear to physically bridge adjacent collagen fibrils in tendon. However, mechanical testing of proteoglycan-deficient tendons or those digested to remove GAGs found decreases in elastic modulus in some tendons [5] and increases or no change in others [6]. In embryonic tendons, we previously demonstrated no correlation between total GAGs and elastic modulus [4]. Other crosslinks implicated in tendon mechanical properties include non-enzymatic collagen crosslinks seen in aging, such as pentosidine [2], though incubation of tendons with glucose to generate pentosidine produced minimal mechanical effects [7]. Taken together, non-enzymatic crosslinks may not be significant contributors to developing tendon mechanical properties. Therefore, we focused on LOX-mediated crosslinks.

We recently demonstrated LOX crosslinks contribute to mechanical properties of embryonic tendon [4], but did not quantitatively characterize their relationship with mechanical properties of developing tissue. The few published studies focused on LOX-mediated collagen crosslinking in developing tendon are limited to a single embryonic timepoint [8]. In contrast, LOX-mediated crosslinking has been studied extensively in adult tendon. LOX oxidatively deaminates specific telopeptidyl lysine and hydroxylysine residues of the collagen molecule [9] to facilitate the formation of a covalent bond between three laterally aligned collagen molecules [10]. The most prevalent enzymatic crosslink in adult tendon appears to be the trivalent hydroxylysyl pyridinoline (HP) crosslink, and to a lesser degree the trivalent lysyl pyridinoline (LP) crosslink [10, 11]. Interestingly, these crosslinks seem to play distinct roles in events primarily outside of homeostasis. In normal tissues, HP-to-collagen ratio was positively correlated to elastic modulus of tendons in goat [12], but not in human [13] or equine [14]. In human, normal tendons maintained constant levels of HP- and LP-to-collagen ratios in the adult [2], whereas aging tendons showed increased HP-to-collagen ratio, higher elastic modulus, and significantly lower collagen content than younger tendons [3]. Chronically injured adult human tendons showed significantly higher HP- and LP-to-collagen ratios compared to non-degenerated tendons [2]. Additionally, acutely injured rabbit ligaments sharply reduced the HP-to-collagen ratio, which progressively increased over a 40-week healing period along with bulk elastic modulus [1]. Taken together, the lack of correlation between modulus and enzymatic crosslinks during homeostasis, but apparent correlation with changes with ageing and injury, suggest roles for LOX-mediated crosslinking in events that affect mechanical properties, such as tendon formation, degeneration, injury and healing. Based on this, we proposed that enzymatic crosslink density changes during embryonic development, prior to formation of additional non-enzymatic (e.g., pentosidine) crosslinks or introduction of confounding environmental factors, may correlate with mechanical property elaboration.

Traditional methods to measure crosslink density include high performance liquid chromatography (HPLC) and mass spectrometry techniques. Both methods require tissue hydrolysis to solubilize crosslinks for analysis. Drawbacks to hydrolysis are that the sample is destroyed during measurement and it is not possible to resolve spatial differences in crosslink density at small scales. In contrast, non-destructive and potentially non-invasive techniques could enable repeated measurements of crosslink density in a single sample over a length of time, or allow for additional assays subsequent to crosslink measurements. This

would be advantageous for *in vitro* and *in vivo* applications where samples are limited (e.g., small, rare, expensive) or too important to be destroyed. Infrared microspectroscopy has been utilized to assess divalent crosslinks in bone [15], though a recent report concluded that this method is actually sensitive to the secondary structure of collagen instead of collagen crosslinking [16]. Therefore, there is a need for a specific and non-destructive method to quantify collagen crosslink density in tendon and other collagenous tissues.

In this study, we aimed to provide a quantitative profile of LOX-mediated crosslinks during tendon development and to examine the relationship between elastic modulus and enzymatic collagen crosslink density in embryonic tendon. We hypothesized that LOX-mediated crosslink density increases during embryonic development, and that crosslink density is directly correlated with nanoscale elastic modulus. We employed tandem mass spectrometry (LC-MS/MS) to characterize changes in HP and LP density and collagen content in embryonic tendon as a function of developmental stage and LOX activity inhibition. To assess a non-destructive method to characterize enzymatic crosslink density, we also utilized multiphoton microscopy to optically measure crosslink density and collagen content of normal and LOX inhibitor-treated embryonic tendons, by exploiting the natural fluorescence of HP and LP crosslinks and second harmonic generation (SHG) signal from fibrillar collagen. With these analyses, we profiled HP and LP crosslink density during tendon development and examined potential relationships between HP and LP crosslinks with elastic moduli to characterize whether collagen crosslink density may be a functional marker of developing tendon tissue. Characterization of these crosslinks and understanding their relationship with mechanical properties could provide functional markers of tendon formation, which would be useful for assessing quality of tissue regeneration during healing or engineered tissue development.

2. Materials and Methods

2.1. In ovo culture and tendon harvest

All animal procedures received prior approval from the university institutional animal care and use committee board. All reagents were from Sigma-Aldrich Co. (St. Louis, MO) unless otherwise specified. White leghorn chick embryos (University of Connecticut Poultry Farm, Storrs, CT) were cultured in a humidified rocking incubator at 37.5° C. Embryos were sacrificed and staged according to Hamburger and Hamilton (HH) [17] at HH 28, 35, 40 and 43, equivalent to approximately days 5.5, 9, 14, and 18 out of a 20-day gestation period, respectively. At 24 h before each timepoint, embryos were injected with 200 µL of (β-aminopropionitrile (BAPN; inhibitor of LOX activity) in saline equivalent to either 0, 5 or 15 mg/g of dry embryo mass [18] into the chorioallantoic membrane [19]. The shell hole was sealed with liquid paraffin and embryos were cultured *in ovo* for an additional 24 h. For the stages and BAPN doses tested, the viability rate 24 h after injection was 97.9%.

After sacrifice, the calcaneus tendon was dissected from skin and muscle tissues. At HH 28, since tendon is not visible in the gross, lower limbs were removed at the hip, the feet were removed, and the lower two-thirds of the remaining limb were used for analysis based on tenascin-positive immunohistochemical staining in this region [20] and our previous

histological analysis [4]. Tissues were then prepared for either mass spectrometry or imaging analysis.

2.2. Sample preparation for tandem mass spectrometry (LC-MS/MS)

Samples were prepared following the method of Gineyts et al. [21]. Briefly, freshly excised chick tendon tissues, including an adult porcine Achilles tendon control group, were minced, washed in dH₂O, and lyophilized for 1 week to obtain dry mass. Lyophilized tendons were then suspended in DPBS and reduced by addition of 10 mg/mL NaBH₄ in 1 mM NaOH to yield a 1:30 reagent-to-tendon dry mass ratio. The reaction was allowed to proceed for 2 h and was terminated by addition of acetic acid to pH 3. Samples were then washed in dH₂O and lyophilized for 1 week. Weighed, dried samples were added to 6 M HCl at 10 mg/mL and hydrolyzed for 20 h at 110° C. Acid was evaporated and hydrolysate were resuspended at 10 mg/mL in LC-MS grade H₂O and subsequently passed through 0.2 µm filters. Samples were stored at -20° C until use. Degradation of HP and LP during storage or freeze thaw was not expected due to an estimated stability of 99.9% after 25 years at -20° C and an absence of degradation after 10 freeze-thaw cycles [22].

Bovine cortical diaphyseal femur bone samples from 24 month-old calves were processed for LC-MS/MS as a control tissue. Femur samples were obtained from a local abattoir (Research 87, Boylston, MA) the same day as slaughter and frozen at -80° C. Diaphyseal femur sections were excised and split longitudinally with a diamond rotary saw. Marrow and soft tissue were removed, and samples were immersed in liquid nitrogen for 10 min and pulverized. Bone fragments were then demineralized in 0.5 M EDTA and 50 mM Tris (pH 7.4) for 21 days at 4° C with daily solution changes [21]. Demineralized bone samples were subsequently processed in an identical manner as tendon samples for LC-MS/MS analysis.

2.3. Sample preparation for multiphoton microscopy

Freshly harvested tendon and demineralized bone samples were washed and immersed in OCT cryosectioning media (Sakura Finetek, Torrance, CA) for multiphoton microscopy analysis. Tissues were oriented for longitudinal sectioning and frozen at -80° C until use. On the same day as imaging, tissues were placed in a -19° C cryostat, sectioned longitudinally at 50 µm, and immersed in saline to remove OCT. Sectioned samples were washed with saline and stored at 4° C until imaged. This sectioning protocol was designed to minimize differences in optical properties resulting from tissue thickness.

2.4. LC-MS/MS analysis and analyte quantification

A triple quadrupole linear ion trap mass spectrometer (3200 QTRAP, AB SCIEX, Foster City, CA) coupled to a binary pump HPLC (1200 Series, Agilent, Santa Clara, CA) was used for the identification and quantification of HP, LP, pyridoxine and hydroxyproline following the method of Gineyts et al. [21] with minor modifications. Prior to sample analysis, MS-specific parameters were optimized for each target analyte to identify the multiple reaction monitoring transition (precursor/product fragment ion pair) with the highest intensity under positive mode. For each compound, the following precursor-product fragment transitions were monitored: 429.2 to 82.0 m/z for HP, 413.2 to 84.0 m/z for LP, 170.1 to 152.1 m/z for pyridoxine, and 132.05 to 68.0 m/z for hydroxyproline [23].

Chromatographic separation was achieved using a Waters Corp. (Milford, MA) Atlantis T3 4.6×100 mm reversed phase column with $3 \mu\text{m}$ particle size, in series with a Waters 4.6×20 mm guard cartridge at 22°C . The LC solvents used were Solvent A: 0.12% heptafluorobutyric acid (HFBA) in LC-MS grade H_2O ; Solvent B: 50% acetonitrile. The LC gradient used was as follows: $t=0$, 10% B; $t=60$ min, 70% B, $t = 61$ min, 10% B, $t = 70$ min, 10% B at a flow rate of $400 \mu\text{L}/\text{min}$. Tendon hydrolysates were mixed with $10 \mu\text{M}$ pyridoxine as an internal standard and 1% HFBA to yield, in a $50 \mu\text{L}$ injection, 100 pmol of pyridoxine and an appropriate dry tissue mass ($50\text{--}500 \mu\text{g}$) to maintain signals within the linear range of the standards. The elution times of HP and LP were confirmed by spiking hydrolysates with an HP and LP standard (Quidel Corp., San Diego, CA).

Peak areas from chromatograms were quantified using the Applied Biosystems Analyst software v1.5.1 employing automatic peak integration. Standard curves were developed for HP, LP and hydroxyproline analytes by injecting varying molar amounts of these compounds in triplicate with 100 pmol of pyridoxine and calculating the analyte-to-pyridoxine peak area ratio. The number of collagen moles in the sample was calculated assuming 300 moles of hydroxyproline per mole collagen [24, 25]. HP- and LP-to-collagen ratios were calculated as the molar ratio of HP- or LP-to-collagen. HP- and LP-to-dry mass ratios were calculated as the ratio of HP and LP moles measured normalized to the amount of hydrolyzed dry tissue mass injected onto the LC-MS/MS. Collagen content was calculated as the mass ratio of measured hydroxyproline mass to hydrolyzed mass injected on the LC-MS/MS [23, 24].

2.5. Multiphoton microscopy and data analysis

The imaging system was composed of a Leica (Wetzlar, Germany) TCS SP2 laser scanning confocal microscope equipped with a 100 femtosecond pulsed Ti:sapphire laser (Mai Tai, Spectra Physics, Irvine CA) delivering either 720 or 800 nm light onto the specimen. A two-photon excited fluorescence (TPEF) image was acquired at 720 nm excitation in epi-detection mode with a $63\times$, 1.2 NA water immersion objective using a descanned photomultiplier tube (PMT), collecting light between 380 and 425 nm. SHG images were acquired in the forward direction (F-SHG) with a 400 ± 10 nm bandpass filter using linearly polarized illumination at 800 nm. Incident laser power and photomultiplier tube (PMT) gain were adjusted to optimize fluorescence signal collection (PMT offset was kept constant at zero). To compare images acquired with different laser power and PMT gain settings, image pixel intensities were calibrated using a fluorescein solution, as described previously [26]. The incident power varied between 20 mW to 23.5 mW for 720 nm excitation, and from 7.6 to 31.8 mW at 800 nm. Z-stacks of images were acquired through the full depth ($50 \mu\text{m}$) of the tissue at $7 \mu\text{m}$ increments from 4-5 locations (stacks) over each cryosectioned tissue specimen. Background noise was assessed at each tissue imaging location by taking images $20 \mu\text{m}$ below the tissue section, focused in the saline medium. The mean intensities of these images were subtracted from the mean tissue image intensities at each respective location to remove background contributions.

Relative crosslink density was estimated from multiphoton images by taking the ratio of power-normalized background-subtracted mean TPEF intensity over the corresponding

power-normalized mean F-SHG intensity at each imaging location. The latter was used as an optical measure of collagen density since we found the intensity of these images to be well correlated to tendon collagen content of corresponding tissues measured by LC-MS/MS ($r^2=0.89$; Fig. S1). Therefore, while the F-SHG intensity is not expected to depend on the collagen content via a simple linear relationship in general [27, 28], this was a reasonable assumption for this set of samples.

To analyze the potential fluorescence efficiency of HP and LP when assessed with imaging, a high performance liquid chromatography (HPLC) approach was employed with fluorescence detection at 295 nm ex./400 $\pm$ 10 nm em. to examine the fluorescent peak area of HP and LP as a function of moles injected on the column [25]. Here an isocratic methanol-based elution buffer was employed as described previously with fluorescence detection of HP and LP [25].

2.6. Statistical analysis

The number of biological replicates measured with each method is summarized (Table 1). For tandem mass spectrometry, each biological replicate consisted of pools of chick tendons from multiple embryos to acquire sufficient material for measurement. For multiphoton microscopy, each biological replicate was from a single chick tendon. Statistical significance testing for LC-MS/MS data was performed using a two-way ANOVA between drug treatment and developmental stage with Bonferroni's post-hoc test. For multiphoton microscopy data a one-way ANOVA with Tukey's post-hoc test was performed between drug treatments for each stage. All statistical calculations including linear regression analyses were performed with Graphpad (La Jolla, CA) Prism v.5.03.

3. Results

3.1. LC-MS/MS chromatograms and standard curves

We first developed standard curves to define linear ranges of quantification for HP, LP and hydroxyproline. Injection of standards for HP, LP, pyridoxine (Pyr) and hydroxyproline (Hyp) produced large separation between analytes and sharply resolved peaks (Fig. 1). We used Pyr as an LC-MS/MS internal standard and Hyp as a measure of collagen content. HP consistently eluted approximately one minute before LP. Standard curves of HP, LP and Hyp were defined using linear regression. The linear range of HP was from 0.28 – 63 pmol ($r^2=0.999$), LP from 0.26 – 29.6 pmol ($r^2=0.997$), and Hyp from 0.5 – 63 nmol ($r^2=0.992$). For embryonic tendon samples, identities of peaks were confirmed by spiking tendon samples with standards.

3.2. Validation of LC-MS/MS method using adult tissue controls

To validate the accuracy of the mass spectrometry method, we used adult porcine Achilles tendon and bovine diaphyseal femur as control tissues. The chromatograms of these two tissues showed clear differences in their HP-to-LP peak area ratios, where this ratio was on average larger in adult tendon (19.1; Fig. 2) than adult bone (4.1; Fig. 2), which was consistent with prior reports [24, 25]. Specifically, the magnitudes of HP and LP density of adult tendon were 0.47 ± 0.05 mol/mol for the HP-to-collagen ratio and 0.02 ± 0.009

mol/mol for the LP-to-collagen ratio (Fig. 2), which were similar to previously reported values ranging from 0.16-0.95 mol/mol for HP-to-collagen and 0.01-0.1 mol/mol for LP-to-collagen depending on age, species and tendon location [2, 3, 13, 14]. The measured bovine femur values were 0.37 ± 0.04 mol/mol for the HP-to-collagen ratio and 0.09 ± 0.02 mol/mol for the LP-to-collagen ratio (Fig. 2). These values were similar to reported values in human and bovine femurs of various ages, which ranged from 0.1-0.4 mol/mol for HP-to-collagen and 0.03-0.08 mol/mol for LP-to-collagen [29-31], and were not significantly different from previously reported values for the same bovine femur tissue of the same age used in this study ($p > 0.05$; [21]). Taken together, these results demonstrate that the LC-MS/MS method in our study was sensitive to HP and LP crosslinks.

3.3. Characterization of crosslink density in embryonic tendon as a function of developmental stage and BAPN dose using LC-MS/MS

Using LC-MS/MS, we first analyzed how collagen content represented as the Hyp-to-dry mass ratio varied as a function of developmental stage (Fig. 3). We found that Hyp-to-dry mass ratio increased nearly exponentially with developmental stage, increasing by 83% from HH 28 to 35, by nearly 6-fold from HH 35 to 40, and by 2-fold from HH 40 to 43 ($p < 0.001$ between stages). When treated with BAPN at concentrations previously used [4], there were no statistically significant differences in Hyp-to-dry mass ratio of BAPN-treated tendons compared to saline-treated tendons ($p > 0.05$). These results were similar to our previous measurements of collagen content using biochemical absorbance assays [4].

To characterize how collagen crosslinking changes during tendon development, we quantified the HP- and LP-to-collagen molar ratio in embryonic tendon as a function of developmental stage and BAPN dose (Fig. 4). The HP-to-collagen ratio with saline treatment initially increased by 66% from HH 28 to 35, then decreased by 76% from HH 35 to 40, and finally rapidly increased by 2.8-fold from HH 40 to 43 (Fig. 4A; $p < 0.01$ between stages). However, BAPN treatment significantly altered these changes with developmental stage and reduced the HP-to-collagen ratio at each stage tested ($p < 0.05$). The reduction in crosslink density due to BAPN treatment was stage-dependent; the largest reduction in the HP-to-collagen ratio was 78% at HH 35 ($p < 0.001$), while the smallest reduction was 48% at HH 40 ($p < 0.05$). There were no statistically significant differences between HP-to-collagen ratios with 5 and 15 mg/g BAPN treatments ($p > 0.05$), although those treated with 15 mg/g trended consistently lower than with 5 mg/g. The LP-to-collagen ratio with saline treatment was similar between HH 28 and 35 ($p > 0.05$), but then decreased sharply by 96% from HH 35 to 40 ($p < 0.001$), and remained relatively constant between HH 40 and 43 ($p > 0.05$; Fig. 4B). BAPN treatment significantly decreased the LP-to-collagen ratio at HH 28 and 35 ($p < 0.05$). There were no statistically significant differences due to BAPN treatment at HH 40 or 43, and no statistically significant differences in LP density between 5 and 15 mg/g doses of BAPN ($p > 0.05$). Interestingly, total HP+LP-to-collagen ratio (Fig. 4C) showed a similar trend with saline treatment as the HP-to-collagen ratio (Fig. 4A). BAPN treatment significantly reduced the total HP+LP density at all stages tested ($p < 0.05$). There were no statistically significant differences in total HP+LP-to-collagen ratio between 5 and 15 mg/g doses of BAPN ($p > 0.05$). The HP-to-LP ratio (Fig. 4D) increased by nearly 30-

fold from HH 28 to 43. BAPN treatment did not significantly affect this ratio at any stage ($p > 0.05$).

Subsequently, we normalized crosslinks to dry mass instead of collagen, and found that trends in crosslink density changed substantially (Fig. 5). We attributed this in part to the nearly 30-fold increase in collagen content from HH 28 to 43 (Fig. 3). The HP-to-dry mass ratio with saline treatment increased dramatically during development, first by 6-fold from HH 28 to 35 ($p < 0.05$), then by 50% from HH 35 to 40 ($p > 0.05$) and again by 6-fold from HH 40 to 43 ($p < 0.001$), for a total increase of nearly 55-fold ($p < 0.001$; Fig. 5A). BAPN treatment significantly reduced the HP-to-dry mass ratio of HH 35 and older tendons ($p < 0.05$; Fig. 5A). In contrast, the LP-to-dry mass ratio increased by 5-fold from HH 28 to 35 ($p < 0.01$), but then decreased sharply by 72% from HH 35 to 40 ($p < 0.05$) and was similar between HH 40 to 43 ($p > 0.05$; Fig. 5B). BAPN treatment reduced the LP-to-dry mass ratio at HH 28 ($p < 0.05$; Fig. 5B). Finally, the total HP+LP-to-dry mass ratio (Fig. 5C) showed a similar trend with saline treatment as the HP-to-dry mass ratio (Fig. 5A). The total HP+LP-to-dry mass ratio increased substantially with development, by 6-fold from HH 28 to 35 ($p < 0.05$), remained relatively constant between HH 35 to 40 ($p > 0.05$), and then increased by 5-fold from HH 40 to 43 ($p < 0.001$), for a total increase of nearly 30-fold ($p < 0.001$; Fig. 5C). BAPN treatment significantly reduced the total HP+LP-to-dry mass ratio from HH 35 to 43 ($p < 0.05$; Fig. 5C). There were no significant differences in the HP-, LP- or total HP +LP-to-dry mass ratios between the 5 and 15 mg/g doses of BAPN ($p > 0.05$).

3.4. Contribution of collagen crosslinks to developing tendon mechanical properties

In addition to characterizing changes in crosslink density during development, we were also interested in their relationship to the elaboration of tendon mechanical properties, which increase progressively during development [4]. We compared changes in HP and LP crosslinks during normal development and after BAPN treatment (Table 2; Fig. 6) to previously measured elastic modulus values [4]. Increases in tendon modulus during development showed the highest correlation to the HP-to-dry mass ratio ($r^2 = 0.78$; Fig. 6A) and total HP+LP-to-dry mass ratio ($r^2 = 0.80$; Fig. 6B), and both were statistically significant ($p < 0.0001$; Table 2). In these plots (Fig. 6A-B), when the three highest crosslinking density points were removed, the correlations remained significant ($p < 0.01$) and retained a similar correlation coefficient ($r^2 = 0.68$ for Fig. 6A; $r^2 = 0.73$ for Fig. 6B), supporting the relationships between tendon modulus and HP-to-dry mass (Fig. 6A) and HP +LP-to-dry mass (Fig. 6B) ratios. The correlation between modulus and collagen content showed a lower correlation coefficient ($r^2 = 0.47$), though this was also statistically significant ($p < 0.05$). All other comparisons to modulus (Table 2) including HP-to-collagen ratio (Fig. 6C) and total HP+LP-to-collagen ratio (Fig. 6D) showed relatively low correlation coefficients ($r^2 < 0.1$) and were not statistically significant ($p > 0.05$).

3.5. Analysis of collagen crosslink density via multiphoton imaging

To assess the potential of multiphoton imaging as a non-invasive means to assess collagen crosslink density, we acquired F-SHG and TPEF images of 50 μm thick tendon tissue sections (Fig. 7). In HH 35, 40 and 43 tendons, the spatial distribution of the TPEF signal showed in most cases similar features as the corresponding F-SHG images, suggesting that

the fluorescence was originating from collagen fibers. To obtain a multiphoton-based crosslink density measure (crosslinks/collagen), the mean fluorescence of a tissue area was normalized to the corresponding mean F-SHG signal, since for our studies the F-SHG signal intensity correlated strongly with collagen content (Fig. S1). Comparison of the multiphoton-based crosslink density estimates of normal vs. BAPN-treated tissue showed significant changes due to LOX activity inhibition in a manner similar to that found with LC-MS/MS. Specifically, optical crosslink density was significantly reduced with BAPN treatment at HH 35 and 43 ($p < 0.01$; Fig. 7A,C), but not at HH 40 ($p > 0.05$; Fig. 7B). This was the same pattern found with LC-MS/MS values of HP-, LP-, and total HP+LP-to-collagen ratios, where the largest reductions from BAPN treatment were at HH 35, and the smallest reductions were at HH 40 (Fig. 4A-C).

To confirm the two-photon microscopy data was representative of total HP+LP crosslinks, we evaluated the relative fluorescence intensities of HP vs. LP crosslinks using HPLC. Fluorescence HPLC analysis found that the fluorescent peak areas of HP were 27% larger than LP on a molar basis (data not shown), indicating that the TPEF imaging method is sensitive to both HP and LP crosslinks, rather than being specific to either HP or LP.

4. Discussion

The results of this study demonstrate that enzymatic crosslink densities have the potential to be used as markers of mechanical properties of newly forming tissues, and that these markers can be detected non-destructively via imaging. Using LC-MS/MS, we found that LOX crosslink densities follow distinct trends during tissue formation, and that the HP- and HP+LP-to-dry mass ratios are well correlated to elastic modulus of normal and LOX inhibitor (BAPN)-treated tendon throughout embryonic development. We also demonstrated that multiphoton microscopy has the capability to detect relative changes in collagen crosslinking similarly as LC-MS/MS, showing promise as a non-destructive and potentially non-invasive method to characterize tissue mechanical properties. Imaging of crosslinks could enable long-term monitoring of mechanical properties over multiple time-points, and visualization of their spatial variations throughout the tissue. The latter is currently possible with atomic force microscopy but is time-consuming and destructive. Outcomes of this study could have significant implications for clinical and basic science research where mechanical testing is normally impractical or unfeasible, enabling functional property characterization during engineered or natural tissue formation, healing, aging, and disease progression.

We observed large changes in crosslink density during normal embryonic tendon development, and found that trends in crosslink density during development differed substantially, depending on whether the data were normalized to collagen molecules (Fig. 4) or tissue dry mass (Fig. 5). When normalized to total collagen content, the total HP+LP crosslinks demonstrated a large decrease from HH 35 to 40, followed by a subsequent increase (Fig. 4C), whereas the total HP+LP-to-dry mass ratio (Fig. 5C) progressively increased with development, similar in trend to that previously reported for elastic modulus during embryonic chick tendon development [4]. Different crosslink density trends when normalized to collagen compared to dry mass were unexpected as collagen is considered the

major dry mass component of adult tendon. However, unlike adult tissue, embryonic tendon is highly cellular and composed of significant amounts of various collagens, proteoglycans and glycoproteins that vary in content during development [4, 32, 33], and thus collagen may not be the most appropriate normalizing factor for crosslinks during tendon formation. Another consideration that has been minimally investigated is the kinetics of LOX activity vs. collagen synthesis. We found large and rapid increases in total collagen content during tendon development (Fig. 3), while others have reported that LOX protein density is relatively constant during development in embryonic chick skin [34] and aorta [35]. Given this, perhaps the rate of collagen synthesis was outcompeting that of crosslink formation during HH 35 to 40, resulting in a decrease in the crosslink-to-collagen ratio. However, this would require further study as LOX protein synthesis rate and activation were not characterized in this work.

Another notable finding was that trends in HP and LP density during development were different regardless of whether the data were normalized to collagen (Fig. 4) or dry mass (Fig. 5), evident by a 31-fold increase in HP-to-LP ratio from HH 28 to 43 (Fig. 4D). Because the HP-to-LP ratio was not significantly affected by BAPN treatment, it appeared that LOX did not have a substrate preference for HP compared to LP. Lysyl hydroxylase converts lysine into hydroxylysine, and has been found in embryonic tendon [36]. As HP links three hydroxylysines, and LP links two hydroxylysines and one lysine [11], the formation of HP vs. LP is likely influenced by lysyl hydroxylase activity. Taken together, it is possible that the large reduction in LP-to-collagen ratio from HH 35 to 40 and continued decrease from HH 40 to 43 (Fig. 4B) were due to increases in collagen content and lysyl hydroxylase activity during development. While specific roles of HP and LP crosslinks in musculoskeletal tissues are not well understood, our finding that HP-to-LP ratio increases during tendon development is corroborated by a high HP-to-LP ratio in adult tendon, as LP is thought to contribute towards calcification in bone [10]. Based on this, the HP-to-LP ratio may be useful as a marker of tendon development.

To examine the relationship between collagen crosslinks and mechanical properties of developing embryonic tendon, we compared crosslink density and mechanical property trends of normal and BAPN-treated tendons. In our previous study [4], we found that BAPN treatment of embryonic tendon reduced nanoscale elastic modulus, and hypothesized that this was a result of reduced crosslink density. One objective here was to quantify crosslink density changes due to BAPN treatment, and demonstrate these changes correlate with elastic modulus reductions [4]. We found that BAPN treatment significantly reduced HP-, LP- and total HP+LP-to-collagen ratios (Fig. 4A-C) in embryonic chick tendon. Interestingly, we also found that crosslinks-to-collagen ratios in normal tendon generally decreased as a function of developmental stage (Fig. 4A-C), in contrast to elastic modulus, which increases during development [4]. However, when crosslink density was normalized to tissue dry mass instead of collagen, both HP-to-dry mass ratio (Fig. 5A) and total HP+LP-to-dry mass ratio (Fig. 5C) increased with developmental stage, similar to previous observations of elastic modulus [4]. Correspondingly, higher correlation coefficients to elastic modulus of normal and BAPN-treated tendons were obtained for HP-to-dry mass ($r^2 = 0.78$; $p < 0.0001$) and total HP+LP-to-dry mass ratios ($r^2 = 0.80$; $p < 0.0001$) (Table 2 and

Fig. 6A-B), than for collagen content alone ($r^2 = 0.47$; $p < 0.05$) or crosslinks-to-collagen ratios ($r^2 = 0.1$; $p > 0.05$) (Table 2). Taken together, these data indicate that collagen crosslinks play a significant role in the elaboration of tendon mechanical properties during embryonic development, and suggest that the HP-to-dry mass ratio may be useful as a functional marker of tendon development. This finding is similar to prior studies of fetal bovine articular cartilage, where the pyridinoline-to-wet mass ratio was significantly correlated to bulk equilibrium and dynamic modulus [37], although crosslinking was not inhibited or manipulated in that report. While we found the other crosslinking metrics to be less correlated with elastic modulus, the observed trends may nonetheless be useful to evaluate if engineered or healing tendons are following temporal patterns of enzymatic crosslinking characteristic of normal development, such as a large decrease in the LP-to-collagen ratio (Fig. 4B) and large increase in the HP-to-LP ratio (Fig. 4D).

After establishing LOX crosslink density as a marker for mechanical properties, we were interested in exploring imaging as a non-destructive method to assess crosslink density. Most collagen crosslinking investigations employ hydrolysis to solubilize crosslinks for analysis, but these techniques are destructive and require many samples, reagents and resources. Imaging capabilities to assess collagen crosslinking would enable faster and repeated measurements of the same sample over multiple time-points, which could be advantageous for research and clinical applications. A previous imaging-based approach to quantify collagen crosslink density employed infrared microspectroscopy with bone tissue [15], however it appears to actually be sensitive to the secondary structure of collagen, rather than crosslinks [16]. In this study we utilized a multiphoton microscopy approach to measure the natural fluorescence of HP and LP crosslinks [38], and normalized these measurements to F-SHG intensity, which is specific to fibrillar collagen [39]. The fluorescence signal showed a similar spatial distribution as collagen fiber SHG images at HH 35, 40 and 43 (Fig. 7), suggesting the fluorescent signal was originating from collagen crosslinks. With BAPN treatment, multiphoton crosslink density (fluorescence-to-SHG ratio) was significantly reduced at HH 35 (Fig. 7A) and 43 (Fig. 7C) but not at HH 40 (Fig. 7B). This pattern was similar to LC-MS/MS results, where the smallest reductions in crosslink-to-collagen ratios were at HH 40 (Fig. 4A-C). BAPN treatment resulted in similar and statistically significant reductions in crosslink density when analyzed with either LC-MS/MS or multiphoton imaging, suggesting that multiphoton imaging could be a useful technique to quantitatively assess HP and LP crosslinks. While we performed multiphoton imaging on tissue sections, this method can also provide high-resolution images of whole tissue, albeit with limitations in penetration depth. In clinical applications, minimally invasive optical probes may circumvent this limitation. In our study, the consistent sample thickness and likely minimal changes in collagen organization within individual stages allowed us to use the F-SHG signal as a reliable metric of collagen content, supported by strong correlation with LC-MS/MS-based quantification of collagen content. In principle, F-SHG imaging intensity is expected to be proportional to the square of the local collagen concentration and to depend on collagen organization and packing [27, 28]. Nevertheless, this linear dependence of SHG signal intensity and area fraction on collagen concentration that we found has also been observed in collagen gels [27]. Similar to collagen gels, embryonic tendon tissue is relatively low density, in contrast to mature collagenous tissues

of much higher density for which this relationship may not hold. For significantly thicker whole tissue samples, the SHG signal detected in the backward direction (B-SHG) could be employed, though the use of B-SHG signal as a metric of collagen content requires more detailed studies focused on the dependence of the signal on fiber diameter and organization [40, 41], and warrants further investigation. For example, inhibition of LOX in dermal fibroblast culture increased B-SHG intensity by nearly twofold compared to control [42]. Additionally, more detailed analysis of the SHG signal, either in terms of its polarization dependence [28, 43] or through algorithms that assess organization at the fiber level [44] could improve understanding of the interdependence of collagen crosslinking, organization and local or bulk mechanical properties. Taken together, these findings suggest that two-photon imaging is an effective non-destructive and potentially non-invasive technique to monitor changes in collagen crosslinking, and hence associated mechanical properties, in collagenous tissues *in vivo* and engineered tissues *in vitro*.

To verify the accuracy of the mass spectrometry technique, we analyzed adult tissues with previously reported crosslink densities. Our mass spectrometry measurements of adult porcine tendon yielded HP- and LP-to-collagen ratios within range of previously reported adult tendon crosslink densities [2, 3, 13, 14] (Fig. 2). Additionally, our measured HP- and LP-to-collagen ratios for adult bone samples were within 25% of previously reported values and not statistically different ($p > 0.05$) [21]. These results suggest that the LC-MS/MS method was sensitive to and accurate for collagen crosslinks in our study. Interestingly, the absolute HP-to-collagen ratios measured here (Fig. 4A) were over 4-fold lower than the adult tendon measurements (Fig. 2), suggesting that significant crosslinking occurs postnatally in tendon.

In conclusion, we characterized changes in collagen crosslinking during embryonic chick tendon development and demonstrated the potential to use enzymatic crosslink density as a marker for mechanical properties of collagenous tissues. We also demonstrated two-photon imaging as a promising method to non-destructively, and perhaps non-invasively, monitor crosslink density during tissue formation. Our findings provide new insights into mechanical property elaboration during tendon development, though future studies will be beneficial to explore additional players and mechanisms of this process in both native and engineered tissues. While this study focused on tendon, these findings may be applicable to other collagenous tissues that are crosslinked via LOX. Further development of the imaging methods to indirectly monitor functional properties of collagenous tissues non-destructively and non-invasively could significantly impact basic science research and advance clinical approaches to monitor native and engineered tissues during development, aging, healing and disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Representative LC-MS/MS chromatogram demonstrating typical peak shapes and elution times of analytes. Chromatogram of standard injection of hydroxylysyl pyridinoline (HP), lysyl pyridinoline (LP), pyridoxine (Pyr), and hydroxyproline (Hyp). Chromatograms are individually normalized to their maximum to illustrate peak shape.

Fig. 2.

Validation of LC-MS/MS measurements using adult tissue controls. Quantification of HP- and LP-to-collagen ratios produced an HP-to-LP ratio of 19.1 for adult tendon and 4.1 for adult femur bone. HP- and LP-to-collagen ratios were similar to reported literature. The HP- and LP-to-collagen ratios of the 24 mo. Bovine femur were not significantly different from reported values of Gineyts et al. [21] who measured the same tissue using LC-MS.

Fig. 3.

Measurement of collagen content in developing tendon using LC-MS/MS. Quantification of hydroxyproline-to-dry mass ratio (mass-to-mass ratio) as a measure of collagen content in embryonic chick tendon as a function of developmental stage. Collagen content increased nearly exponentially with time. Standard deviation error bars are not shown when error bars are less than the size of the symbol. Saline and BAPN treatment samples were not statistically significantly different at each developmental stage ($p > 0.05$).

Fig. 4.

HP and LP crosslink density normalized to collagen in embryonic chick tendon as a function of developmental stage and BAPN dose measured by LC-MS/MS. **(A)** HP-to-collagen ratio with saline treatment increased from HH 28 to 35, then decreased from HH 35 to 40, and finally increased from HH 40 to 43. BAPN treatment significantly reduced the HP-to-collagen ratio at all stages. **(B)** LP-to-collagen ratio markedly decreased between HH 35 to 40, and BAPN treatment decreased LP density at HH 28 and 35. **(C)** Total HP+LP-to-collagen ratio showed a similar temporal pattern to HP-to-collagen ratio with both saline and BAPN treatment. **(D)** The HP-to-LP molar ratio in embryonic chick tendon increased significantly during development from ~1.5 at HH 28 to ~35 at HH 43 and was not affected by BAPN treatment. X-axes are spaced to be representative of real-time differences. Statistical indicators: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$ compared to saline-treated group.

Fig. 5.

HP and LP crosslink density normalized to dry mass in embryonic chick tendon as a function of developmental stage and BAPN dose measured by LC-MS/MS. **(A)** HP-to-dry mass ratio increased rapidly from HH 28 to 35 and from HH 40 to 43. BAPN treatment significantly reduced this ratio from HH 35 to 43. **(B)** The LP-to-dry mass ratio did not vary considerably during development and BAPN treatment decreased this ratio at HH 28. **(C)** Total HP+LP-to-dry mass ratio showed similar trend to the HP-to-dry mass ratio and was significantly reduced by BAPN treatment from HH 35 to 43. X-axes are spaced to be representative of real-time differences. Statistical indicators: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$ compared to saline-treated group.

Fig. 6.

Correlation plots between collagen crosslinking metrics and nanoscale elastic modulus (AFM data from [4]) of HH 28, 35, 40 and 43 chick tendon for saline, 5 mg/g BAPN and 15 mg/g BAPN treatment groups. Dotted line indicates best linear fit, with corresponding r^2 and p -values for the fit shown. HP (**A,C**) and total HP+LP (**B,D**) crosslinks were significantly correlated to elastic modulus when normalized to dry mass (**A,B**; $p < 0.0001$), but were not significantly correlated to elastic modulus when normalized to collagen molecules (**C,D**; $p > 0.05$).

Fig. 7.

Multiphoton imaging analysis of collagen crosslink density from HH 35 (**A**), 40 (**B**) and 43 (**C**). At all stages tested, the spatial pattern of fluorescence was similar to F-SHG, suggesting the fluorescence originated from collagen fibers. BAPN treatment significantly reduced multiphoton crosslink density at HH 35 (**A**) and 43 (**C**), but not at HH 40 (**B**). This was a similar pattern to that observed by LC-MS/MS (Fig. 4A-C), where the largest reductions in crosslink density due to BAPN treatment were at HH 35, and the smallest reductions were at HH 40. Scale = 50 μ m. Statistical indicators: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$ compared to saline-treated group; “ns” = not significant.

Summary of biological replicates used for LC-MS/MS and multiphoton microscopy measurements of crosslink density in tissues studied. For embryonic tissues, LC-MS/MS measurements were of pooled embryonic tendons from multiple biological replicates, whereas multiphoton microscopy analyzed a single biological specimen per replicate.

Table 1

	No. of biological replicates		Embryonic replicates pooled?	Chick tendons per pool			
	Embryonic	Adult		HH 28	HH 35	HH 40	HH 43
LC-MS/MS	3	3	Yes	10	6	3	3
Multiphoton microscopy	6	3	No				

Table 2

Correlation coefficients between crosslinking densities and elastic modulus in embryonic tendon during development. Correlation includes saline, 5 mg/g and 15 mg/g BAPN treated groups from HH 28, 35, 40 and 43 embryonic chick tendons. Strongest correlations were to the HP-to-dry mass ($r^2=0.78$, $p < 0.0001$) and total HP+LP-to-dry mass ($r^2=0.80$, $p < 0.0001$) ratios.

Correlation to modulus	r^2 value	p -value
HP + LP/dry mass	0.80	<0.0001
HP/dry mass	0.78	<0.0001
Collagen content	0.47	<0.05
HP/collagen	0.1	>0.05
LP/collagen	0.09	>0.05
LP/dry mass	0.04	>0.05
HP + LP/collagen	0.001	>0.05