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Effects of tissue fixation and dehydration on tendon collagen nanostructure

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ABSTRACT

Collagen is the most prominent protein in biological tissues. Tissue fixation is often required for preservation or sectioning of the tissue. This may affect collagen nanostructure and potentially provide incorrect information when analyzed after fixation. We aimed to unravel the effect of 1) ethanol and formalin fixation and 2) 24 hour air-dehydration on the organization and structure of collagen fibers at the nano-scale using small and wide angle X-ray scattering. Samples were divided into 4 groups: ethanol fixed, formalin fixed, and two untreated sample groups. Samples were allowed to air-dehydrate in handmade Kapton pockets during the measurements (24 h) except for one untreated group. Ethanol fixation affected the collagen organization and nanostructure substantially and during 24 hours of dehydration dramatic changes were evident. Formalin fixation had minor effects on the collagen organization but after 12 hours of air-dehydration the spatial variation increased substantially, not evident in the untreated samples. Generally, collagen shrinkage and loss of alignment was evident in all samples during 24 hours of dehydration but the changes were subtle in all groups except the ethanol fixed samples. This study shows that tissue fixation needs to be chosen carefully in order to preserve the features of interest in the tissue.

Keywords: X-ray scattering; d-spacing; amino-acid spacing; collagen delamination;

INTRODUCTION

Collagen is found in most biological tissues and its amount, organization, and constituents, *i.e.* collagen cross-links and type of collagen affect tissue function and mechanical properties. Characterizing collagen is key for understanding tissue homeostasis, disease and repair. Although its amount and organization varies between different tissues and sites, its basic form and function are similar, collagen, together with its cross-links, provides tissues with tensile strength and ductility. In total 24 different types of collagen have been identified, from which collagen type I and II are the most prominent in musculoskeletal tissues.

Tendon comprises mainly of water (70%), collagen (collagen type I ~90% of dry weight) and proteoglycans (2-5% of dry weight), with a small amount of elastin and other molecules (Freedman et al., 2014; Parkinson et al., 2011; Sharma and Maffulli, 2005). The collagen fibers are aligned highly parallel along the loading axis in tendons and ligaments. The collagen fibers are formed in a complex hierarchical manner. Fibers are formed from fibrils, which are further formed from microfibrils. Microfibrils are assembled from five ~300 nm long right-handed triple-helical collagen molecules with a characteristic ~67 nm d-spacing, which forms the basic crystallographic unit for collagen (Petruska and Hodge, 1964). The base of the d-spacing is the alternate gap and overlap regions, with lower and higher electron densities, respectively, and cross-links stabilize the structure at the molecule terminals (Orgel et al., 2000). Collagen molecules are formed from three left-handed helical alpha chains that consist of 1056 amino-acid residues with ~0.29 nm spacing. Because of relatively high collagen content and alignment in tendon, it is a suitable tissue for studying the nature of collagen, such as damage and biomechanical behavior under loading (Bianchi et al., 2016; Fessel et al., 2014; Sasaki and Odajima, 1996), and the effect of hydration (Bigi et al., 1987; Fratzl et al., 1993; Wess and Orgel, 2000).

To characterize the collagen protein structure, varying sample preparation steps are

usually needed. The tissue is usually fixed, e.g. in ethanol or formalin, to preserve the tissue from degradation. Fourier transform infrared (FTIR) spectroscopy has shown that ethanol fixation produces shifting of amide I and II peaks to lower wavenumbers, thus substantially affecting the collagen secondary structure (Pleshko et al., 1992). On the other hand, it has been found that formalin has only minor effects on the collagen secondary structure, (Bot et al., 1989; Mason and O'Leary, 1991; Pleshko et al., 1992). Depending on the measurement technique, tissue samples are sometimes embedded for example in paraffin or epoxy in order to enable sectioning. These sample preparation steps (fixation and embedding) are usually done, even though they may affect the protein structure (Pleshko et al., 1992; Yeni et al., 2006).

Small angle X-ray scattering (SAXS) studies have indicated that dehydration affects the collagen nanostructure, e.g., by decreasing the collagen d-spacing (Bigi et al., 1987; Wess and Orgel, 2000) and increasing the lateral packing of collagen (Fratzl et al., 1993). These changes were observed as a result of heating of air-dried rat tail tendons (Bigi et al., 1987), air-dried rat tail tendons (Wess and Orgel, 2000), or during vacuum drying of turkey leg tendon (Fratzl et al., 1993). Although these studies reveal substantial changes in collagen organization in response to dehydration, they do not report how e.g. overall collagen alignment of delamination are affected by dehydration. For comprehensive understanding of the effects of dehydration of collagen and the effects of fixation, especially in a scenario where samples are measured over a long period while they are drying in air without any external accelerator, extensive analysis of the structural changes is required.

In this study, we aimed to unravel the effect of 1) ethanol and formalin fixation and 2) 24-hour air-dehydration on tendon collagen alignment and organization on the nano-scale using scanning SAXS. Our hypothesis was that ethanol fixation alters collagen nanostructure while formalin fixation has only minor effects. Dehydration was hypothesized to cause shrinking and loss of alignment of collagen fibers. FTIR microspectroscopic imaging was utilized to evaluate

tendon molecular composition.

MATERIALS AND METHODS

Sample preparation

Achilles tendons from the left leg of female Sprague Dawley rats were carefully dissected (N = 12, 16 weeks old). The tendons were harvested together with the plantaris tendon and the gastrocnemius muscle, which was carefully scraped off before the tissue was frozen in NaCl. The tendons were randomly divided into 4 groups (N = 3 per group): 2 Untreated groups, Ethanol (ethanol fixation), and Formalin (formalin fixation). One untreated (Untreated dry) and both fixation groups (Ethanol and Formalin) were let dry in air during the measurement series, whereas the second group of untreated tendons (Untreated wet) was kept immersed in PBS and was considered as the control group. The ethanol and formalin samples were thawed 12 hours before treatment and subsequently immersed in 70% ethanol or formaldehyde for 48h. Untreated wet and dry samples were kept frozen and were thawed just prior to the SAXS measurements. All samples were kept immersed in the respective fluid until the measurements. All samples were placed in handmade Kapton pockets (Fig. 1A). The untreated wet samples were put in Kapton pockets that were sealed from all four sides of the pocket. The pockets for other samples were sealed on three sides leaving the top side open to allow evaporation. The animal study protocol was accepted by the local ethics authorities (Djurförsöksnämnden i Linköping, Dnr. 60-12).

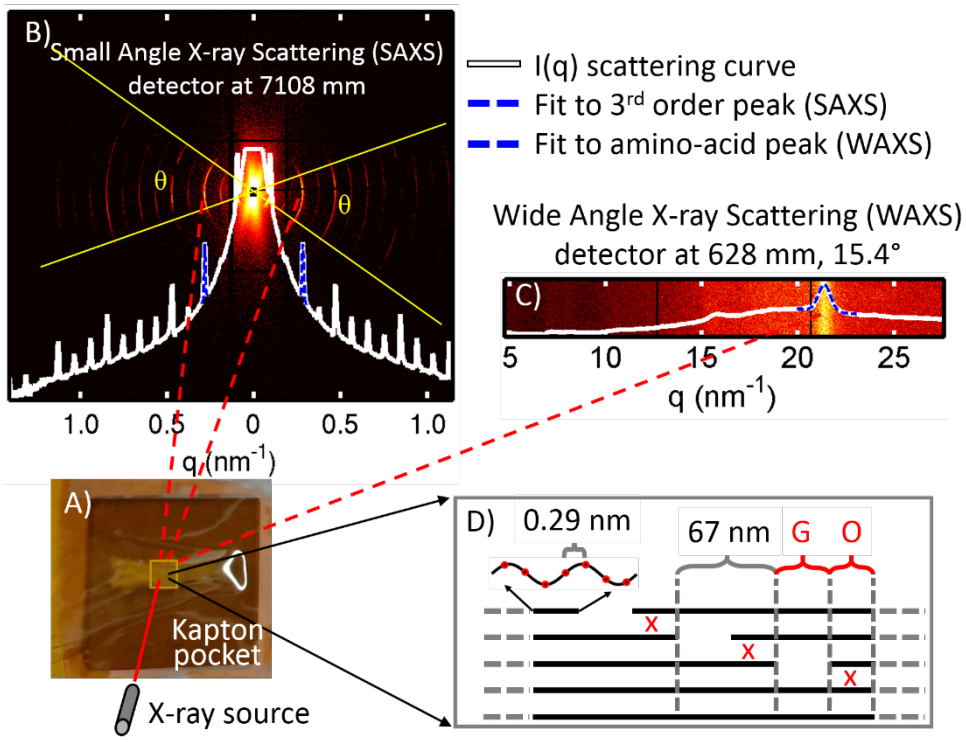

Figure 1. *Schematic illustration of the measurement setup. A) Tendons were placed into Kapton pockets. Ethanol and formalin fixed samples as well as untreated dry samples were placed without liquid and top of the pocket was not sealed (photo of tendon in Kapton pocket rotated 90 degrees counter-clockwise for presentation). The pockets with untreated wet samples were filled with PBS and sealed on all four sides. B) SAXS scattering was recorded with a Pilatus 2M detector. Gaussian curves were fit to the 3rd order collagen peaks for determination of d-spacing (peak location), delamination (peak FWHM), and alignment of the collagen fibers (peak intensity) for each measurement point. C) WAXS scattering was recorded with a Pilatus 300K detector. Intra amino-acid spacing of ~0.29 nm was determined from the peak. D) Schematic presentation of collagen nano-structure indicating the ~67 nm d-spacing periodicity, the gap (G) and overlap (O) regions, location of the collagen cross-links (X), and the ~0.29 nm intra amino-acid spacing.*

X-ray scattering

Identical regions of 1.26 x 1.26 mm^2 in the middle of each tendon were measured at time 0, 1,

2, 4, 8, 12, and 24 hours, at the cSAXS beamline at the Swiss Light Source (SLS), Paul Scherrer Institut (PSI), Villigen, Switzerland (Bunk et al., 2009). The 0 h measurement represents a fully hydrated state in all samples, since the samples were put to the Kapton pockets just prior the first measurement. A Pilatus 2M detector (Henrich et al., 2009) was used to record the 2D scattering patterns of the tendon using a raster-scan with 30 x 30 μm^2 spot size and scanning steps of 30 µm in a continuous line-scan mode (Fig 2B). Additionally, a background line-scan next to each sample on the respective Kapton pocket was recorded. The Kapton scattering in the analyzed q-region was negligible. The data were background corrected by subtracting the background scattering $I\_{BG}(q)$ from the tendon scattering $I(q)$. Exposure time of 50 ms and X-ray wavelength of 1.0 Å was used. The sample-detector distance was 7108 mm providing a q range of ~0.02-1.45 nm^{-1}. The beam center and the sample-detector distance were determined using a Silver-Behenate (AgBH) powder standard. WAXS scattering was recorded simultaneously with SAXS scattering using a Pilatus 300K detector. The sample-detector distance was 628 mm at 15.4 degree angle providing a q range of ~5-28 nm^{-1} (Fig. 1C).

Anisotropy of the collagen fiber orientation (θ, in degrees) was determined as the angular width of the collagen rings in the SAXS scattering patterns (Fig. 1B). The angle θ was determined for each measurement point as the full width of tenth maximum (FWTM) of the azimuthally integrated scattering using the intensity of the collagen rings. The $I(q)$ scattering curves, containing information about the collagen periodicity and orientation, were obtained by radially integrating the detector images over θ (Fig. 1B). Pixels blocked by the beam stop, dead or hot pixels and insensitive gaps between the detector modules were excluded from the analysis. The anisotropy and the azimuthal integration were determined on both meridional (axial) scattering of the beam stop.

A Gaussian curve was fitted to the 3rd order collagen peak (Fig. 1B) in order to determine collagen periodicity (d-spacing, Fig. 1D), alignment of the collagen fibers (peak intensity), and

delamination of the collagen fibers (peak full width at half maximum, FWHM) (Yang et al., 2015). Gaussian curve was fitted also to the 2nd order collagen peak for the determination of the overlap/d-spacing ratio. The variation in the ratio is caused by sliding of laterally and axially adjacent molecules. The overlap/d-spacing ratio can be determined from the relative integrated intensities of two successive SAXS collagen peaks (Bianchi et al., 2016; Bigi et al., 1987; Fessel et al., 2014; Sasaki and Odajima, 1996) using

$$\frac{I_{n+1}}{I_n} = \left(\frac{n}{n+1}\right)^2 \left[\frac{\sin\left((n+1)\pi\frac{O}{D}\right)}{\sin\left(n\pi\frac{O}{D}\right)}\right]^2$$

where I_i is the integrated intensity of the i^{th} SAXS collagen peak, n is the order of the peak, O is the overlap length, and D is the d-spacing. From the radially integrated WAXS $I(q)$ scattering curves, the intra amino-acid spacing of ~0.29 nm was determined from the location of the fitted Gaussian curve to the measured peak (Fig. 1D).

In order to evaluate the effect of drying, the measured regions at each time-point were spatially matched and downscaled by a factor of 6 resulting in 49 data-points per sample per time-point. Each parameter at each data-point was then normalized to the matching 0 h data-point. Subsequently, data-points were pooled for each group (147 data-points per group). From the normalized pooled data-points, scatter-plots with mean ± standard deviation (SD) regions were generated (Fig. 2). All analysis were done with in-house written scripts in Matlab (R2014a, The MathWorks, Inc., MA).

FTIR

After SAXS measurements, all samples were embedded in paraffin following standardized protocol for histological analysis. Thin sections of the mid-tendon were cut and the paraffin was removed for the FTIR measurements (D7 beamline at MAX-IV, Lund, Sweden). From each

tendon section, a region of 0.3 x 0.3 mm^2 with 64 x 64 measurement points was chosen using a light microscope and a spatial map of that region was measured. From the IR spectra, collagen content (amide I peak area, 1725-1585 cm^{-1}) and collagen maturity (1660/1690 cm^{-1}, mature/immature crosslink ratio) were determined (Boskey and Pleshko Camacho, 2007; Paschalis et al., 2001). Some of the tendons could not be sectioned because of their fragility. Thus, unfortunately no FTIR data is available for any ethanol treated samples.

Statistical analyses

Univariate general linear model was used to test differences between SAXS and WAXS parameters of different groups at 0 h and 24 h, by using each parameter (one at a time) as the dependent variable and samples (3 per group) and groups as fixed factors. Same model was used to test differences between parameters within each group at different time points by using each parameter (one at a time) as the dependent variable and samples (3 per group) and time-points as fixed factors. All statistical analyses on SAXS and WAXS data were done using the downscaled data-points, 49 points per sample per time-point. Statistical model residuals were normally distributed. For FTIR analyses, Mann-Whitney U-test was used to test for differences in averaged collagen content and mature/immature cross-link ratio between the groups. For all analyses, $p \leq 0.05$ was considered statistically significant. All statistical analyses were done using IBM SPSS Statistics (v21, IBM corp., Armonk, NY).

RESULTS

Effects of ethanol and formalin fixations with untreated (0 hours)

Immediately after removal of the samples from the immersions, the d-spacing and peak intensity were slightly higher ($p < 0.001$ for all) in the ethanol samples compared to formalin or untreated samples (Fig. 2A,B). The peak FWHM was similar in all samples (Fig. 2C). The

overlap/d-spacing ratio was lower ($p < 0.001$ for all) in ethanol and formalin samples compared to untreated samples (Fig. 2D). Amino acid spacing was lowest in ethanol samples and highest in formalin samples (Fig. 2E) with all differences being significant ($p < 0.001$). There were no differences between untreated wet and untreated dry samples in the measured parameters immediately after removal of the samples from the immersions, except peak intensity was slightly higher in untreated wet compared to untreated dry samples ($p < 0.001$).

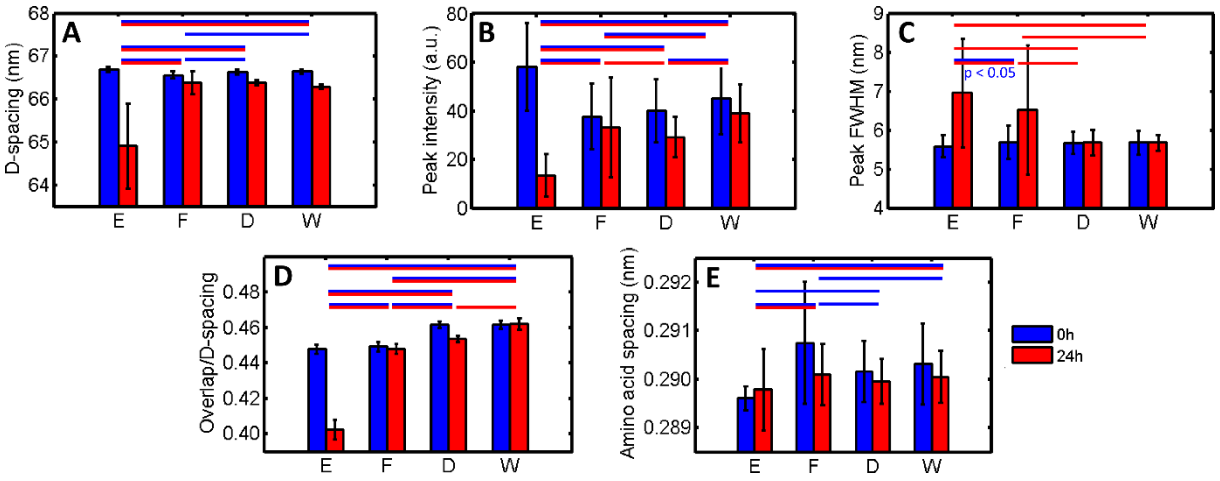

Figure 2. *A) D-spacing, B) 3rd order collagen peak intensity, C) 3rd order collagen peak FWHM, D) overlap/D-spacing ratio, and E) amino acid spacing at 0 h (blue) and 24 h (red) time-points in ethanol (E), formalin (F), untreated dry (D), and untreated wet (W) samples. Statistically significant differences between groups (p < 0.001) at 0 h are indicated with blue line and at 24 h with red line. For example in A), the blue line spanning from E to D indicates a statistically significant differences at 0 h between ethanol and untreated dry samples. The blue lines spanning from E to F and F to D indicate statistically significant differences at 0 h between ethanol and formalin, and formalin and untreated dry samples, respectively.*

Effect of dehydration within each group

During 24 hours of air-dehydration, the most dramatic changes were observed in the ethanol samples showing 2.7 % and 76.9 % decrease in d-spacing and peak intensity, respectively. The decreases in all other samples were less than 10 % of the decrease measured in the ethanol

samples (Fig. 3A,B). However, the decrease in the d-spacing was significant in all samples ($p < 0.001$) and the decrease in peak intensity was significant in all ($p < 0.001$) except formalin samples. FWHM increased significantly ($p < 0.001$) in ethanol (24.9%) and formalin samples (13.9 %) but no changes were found in the untreated samples (Fig. 3C). The overlap/d-spacing ratio decreased dramatically in ethanol samples ($p < 0.001$, 10.1%). However, a limited but still significant decrease ($p < 0.001$, 0.02 %) was also observed in untreated dry samples (Fig. 3D). No significant change in overlap/d-spacing ratio was found in formalin or untreated wet samples. A slight increase in amino acid spacing was observed in ethanol samples, whereas it decreased in other samples (Fig. 3E). All changes were significant ($p < 0.001$), although the absolute change was small (< 0.1 %). Typical SAXS patterns and the respective $I(q)$ curves for all sample groups at 0 and 24 hours are presented in Figure 4.

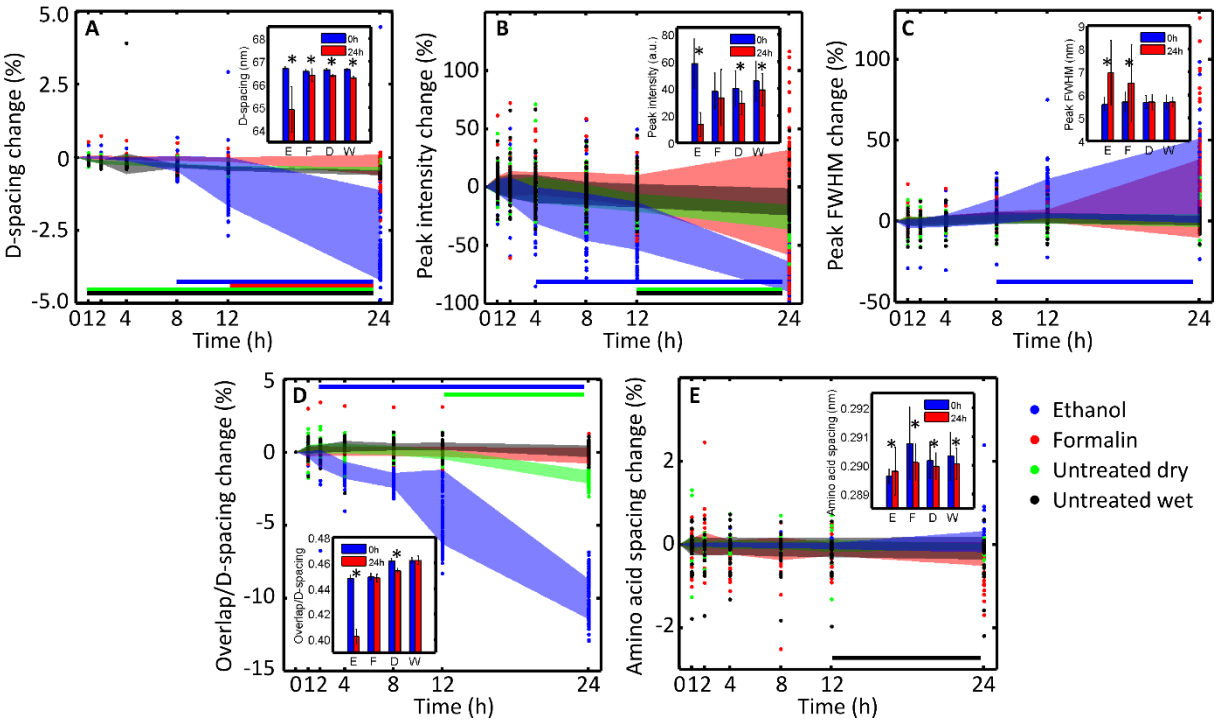

Figure 3. *Relative change of A) d-spacing, B) peak intensity, C) peak FWHM, D) overlap/d-spacing, and E) amino acid spacing at each downscaled measurement point. Value at each point is normalized to the value of the respective measurement point at 0 h time. Colored areas represent the mean ± SD for each sample group as a function of time. Significant changes (* p*

< 0.001) between 0 h and 24 h in parameter values are indicated in subplots within each plot A-E). Moveover, time-points with significant difference (p < 0.001) in comparison to 0 h time-point are indicated with horizontal lines with colors respective to the group colors. For example in A), blue line spanning from 8 h to 24 h indicates statistically significant changes in D-spacing in ethanol samples from 8 hours to 24 hours when compared to 0 h time-point. The green line spanning from 1 h to 24 h indicates statistically significant changes in D-spacing in untreated dry samples from 1 hour to 24 hours when compared to 0 h time-point. If a colored line is not present, the statistically significant change is found only at 24 h time-points (compared to 0 h time-point, if indicated in the subplot).

Effects of ethanol and formalin fixations with untreated (24 hours)

After 24 hours of air-dehydration the d-spacing and peak intensity were significantly lower ($p < 0.001$ for all) in ethanol samples than in formalin or untreated samples (Fig. 2A,B). No statistical differences were found between other groups in d-spacing (Fig. 2A). Peak intensity was highest in untreated wet samples and lowest in untreated dry samples (Fig. 2B). All differences between each group were significant ($p < 0.001$). Peak FWHM was highest in ethanol samples but was elevated also in formalin samples (Fig. 2C). Both were significantly higher ($p < 0.001$) compared to untreated samples. No differences between in peak FWHM in untreated dry and untreated wet samples were observed. After 24 hours of dehydration the overlap/d-spacing ratio was lowest in ethanol samples and highest in untreated wet samples (Fig. 2D). All differences between each groups were significant ($p < 0.001$). The amino acid spacing was significantly lower ($p < 0.001$) in ethanol samples compared to formalin and untreated wet samples (Fig. 2E). There were no significant differences in amino acid spacing between other groups after 24 hours of dehydration.

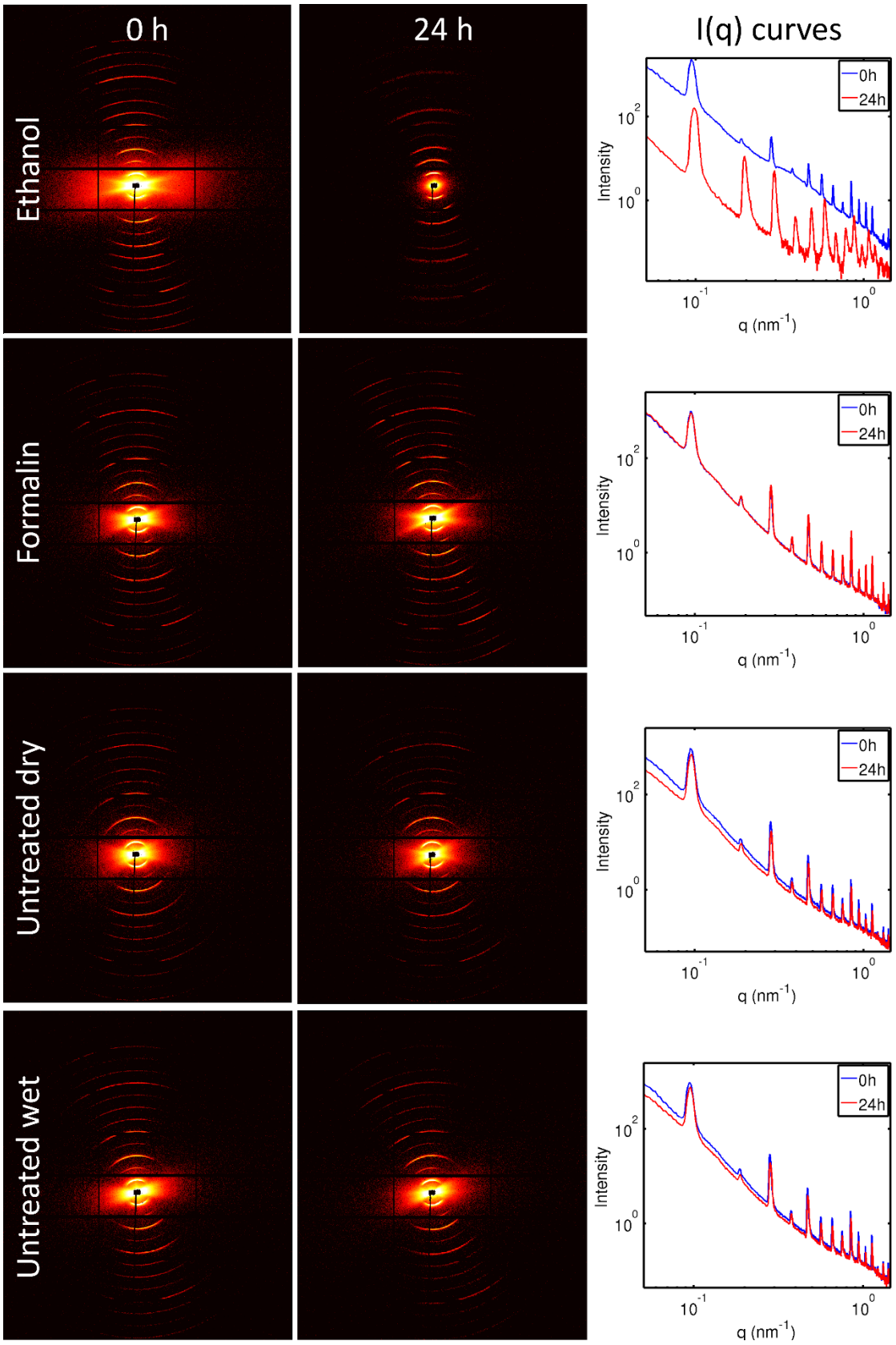

Figure 4. *Typical SAXS scattering patterns and respective I(q) curves of all sample groups at 0 h and 24 h time-points.*

Compositional analysis

FTIR analysis confirmed there were no differences in collagen content or collagen maturity (mature/immature collagen cross-link ratio) between any groups (Table 1). However, due to embedding and cutting issues, no data was available for ethanol treated samples.

Table 1. Collagen content (amide I peak area) and collagen maturity (1660/1690 cm^{-1} mature/immature collagen cross-link ratio) in different groups shown as mean ± standard deviation. No data was available for ethanol samples. No significant differences were found between the groups.

	Ethanol	Formalin	Untreated dry	Untreated wet
Collagen content	no data	39.7 ± 5.1	43.0 ± 16.2	42.8 ± 2.0
Collagen maturity	no data	1.98 ±0.05	1.97 ± 0.12	1.99 ± 0.06

DISCUSSION

In this study, the effect of ethanol and formalin fixation, and 24 hours of air-dehydration on the organization of collagen in rat Achilles tendon was investigated. Generally, ethanol fixation resulted in dramatic changes in collagen organization when compared to untreated samples, especially after dehydration. Further, it was found that formalin fixation preserves the collagen organization but that there were clear differences in comparison to the untreated/unfixated samples especially after 24 h of dehydration. Untreated samples that were left to air-dry for 24 hours showed some moderate changes in collagen organization, which were less pronounced in untreated samples that were kept hydrated. Differences between untreated dry tendons and untreated wet tendons were evident especially after 12 h of air-dehydration. When the untreated samples were kept hydrated, the overall collagen nanostructure was preserved better and showed less variation than the ethanol or formalin fixed samples as well as untreated samples that were air-dried. Although the untreated wet samples were kept hydrated throughout the

experiment, similar changes in collagen nanostructure are evident as in the other untreated samples that were let air-dried. This could be due to possible minor degradation of the tissue.

The dehydration driven shrinkage of tendons is anisotropic, mainly in the direction of the collagen fibers. This can be seen in the decrease in collagen alignment (peak intensity) in all samples whereas the delamination (peak FWHM) in untreated samples was not altered during 24 hours. In ethanol and formalin samples the delamination increased during 24 hours suggesting a more isotropic shrinking. However, the reasons behind drying of ethanol and formalin fixed samples are different and are discussed below.

Ethanol has been shown to change the secondary structure of collagen (N L Pleshko et al., 1992a). The recorded SAXS patterns suggest changes also in collagen organization and alignment. Immediately after removing the samples from ethanol (0 h), the results indicate a greater alignment of collagen fibers compared to other samples. However, during 24 hours of dehydration the results show dramatic shrinking (decreased d-spacing), reduced alignment (decreased peak intensity), delamination of the collagen fibers (increased peak FWHM), and decreased overlap/d-spacing ratio. These changes, driven by tissue dehydration, are pronounced especially in the ethanol fixed samples, but are evident also in other samples. Amino acid spacing was lowest in ethanol samples and was the only group where it increased during the dehydration, while it decreased in other samples. The changes are most likely pronounced in ethanol treated samples compared to untreated samples because ethanol replaces the collagen bound water and evaporates at a faster rate than water. Overall, in fully hydrated state (0 h) ethanol samples exhibited lowest amino acid spacing, largest d-spacing and lowest overlap/d-spacing ratio. Thus, it seems that in fully hydrated samples the shorter amino acid spacing is coupled with longer d-spacing. However, during dehydration this relation seems to vanish, which is probably due to variation in evaporation of water bound at different sites of collagen (Bigi et al., 1987). The effects of dehydration on collagen is complex since it is affected by a

number of factors. This has been discussed elsewhere (Wess and Orgel, 2000).

Formalin fixation has been shown to have only minor effects on the secondary structure of collagen (Bot et al., 1989; Mason and O'Leary, 1991; N L Pleshko et al., 1992a), and based on our data when comparing to the untreated samples, also collagen nanostructure is preserved. However, during 24 hours of dehydration, although the average values are similar to the untreated samples, the spatial variation of alignment and delamination of the collagen fibers increase substantially. These increases in variation are most likely caused by increased internal strains as a result of drying (Bertinetti et al., 2015; Masic et al., 2015). Since formalin fixes the tissue strongly by creating new cross-links which stabilize the tissue (Kiernan, 2000; Puchtler and Meloan, 1985), the drying could possibly results in internal strains inside the tissue which can be observed as increased variation in collagen fiber alignment and delamination. The increase in spatial variation is also somewhat seen in the d-spacing, but not in amino acid spacing, or overlap/d-spacing ratio.

The effect of dehydration on untreated samples were surprisingly small. After 24 hours of air-drying, the collagen fibers were less aligned (lower peak intensity) in the untreated dry tendons compared to the wet tendons. Moreover, the overlap/d-spacing ratio decreased in untreated dry tendons, whereas it stayed constant in the untreated wet samples. In both groups, the d-spacing decreased slightly. In the Kapton pockets the samples were in contact with the Kapton film on both side, meaning that the samples were not fully in air, which could have slowed down the dehydration process. Thus, the alterations in collagen nanostructure in untreated dry tendons may be interpreted to represent the initial stage of dehydration. In untreated dry samples the decrease in the overlap/d-spacing ratio is subtle but distinguishable from untreated wet and formalin samples where the decrease in d-spacing is evenly distributed to the overlap and gap regions. In ethanol samples the decrease in the overlap/d-spacing ratio, as well as in the d-spacing, is striking. The d-spacing and the overlap/d-spacing ratio are

decreasing in ethanol and untreated dry samples, suggesting that shrinkage of the tissue is mostly dominated by the decrease of the overlap region rather than gap region.

One limitation in this study is the low number of samples, three per group. However, this is partly compensated with the fact that the samples were homogeneous and from each sample, 1762 different point measurements were taken. By spatially downscaling the measured maps (to minimize the error caused by misalignment of the measurement areas between time-points), time-dependent changes could be evaluated spatially in each time point. Secondly, based on the results complete dehydration of the samples was not achieved. Even though the Kapton pockets were left open on one side, it is possible that the pockets were still tight enough to maintain some humidity in the sample environment and prevent complete dehydration. Unfortunately, water content of the samples could not be determined during or directly after the measurements at the synchrotron beamline. A longer dehydration time would potentially yield more information about the collagen organizational changes due to only drying. However, a longer time could have led to natural degradation of the non-fixed samples, and the interpretations become less relevant for other preparation protocols for collagen based tissues.

In conclusion, tissue fixation needs to be chosen carefully and with the research question in mind in order to preserve the features of interest in the tissue. Ethanol fixation is not recommended when studying collagen nanostructure and alignment, while formalin fixation may be applied, as long as the measurements are done shortly after taking the sample from the immersion. Untreated samples exhibit smallest changes in the nanostructure if the sample is kept hydrated or the measurements are done shortly after removal from the immersion. This study also demonstrates that collagen nanostructure of untreated tendons in PBS immersion can be studied for over at least 24 hours without severe degradation.

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DISCLOSURES

The authors have no conflicts of interest.

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