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Collagen Cross Linking and Fibril Alignment in Pericardium

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The influence of natural cross linking by glycosaminoglycan (GAG) on the structure of collagen in animal tissue is not well understood. Neither is the effect of synthetic cross linking on collagen structure well understood in glutaraldehyde treated collagenous tissue for medical implants and commercial leather. Bovine pericardium was treated with chondroitinase ABC to remove natural cross links or treated with glutaraldehyde to form synthetic cross links. The collagen fibril alignment was measured using synchrotron based small angle X-ray scattering (SAXS) and supported by atomic force microscopy (AFM) and histology. The alignment of the collagen fibrils is affected by the treatment. Untreated pericardium has an orientation index (OI) of 0.19 (0.06); the chondroitinase ABC treated material is similar with an OI of 0.21 (0.08); and the glutaraldehyde treated material is less aligned with an OI of 0.12 (0.05). This difference in alignment is also qualitatively observed in atomic force microscopy images. Crimp is not noticeably affected by treatment. It is proposed that glutaraldehyde cross linking functions to bind the collagen fibrils in a network of mixed orientation tending towards isotropic, whereas natural GAG cross links do not constrain the structure to quite such an extent.

1. Introduction

The Collagen I molecule is prevalent as the basis of many structural components in animals. It assembles with a complex hierarchical structure. This extracellular matrix forms resilient materials which are mechanically very tough[1]. This toughness is due in part to the highly fibrillar nature of collagen. Polypeptide molecules twist in left handed α-helical chains, and three of these in turn assemble with a right handed twist to form tropocollagens. Collagen fibrils are multiples of five tropocollagen strands thick and of extended length. The fibrils in turn may be assembled into larger fibres and a variety of structural motifs. There is great inherent strength and elasticity in each individual fibril. It is believed that the structure of materials composed of collagen I also require cross linking of the fibrils. This mechanically couples the fibrils restricting them from sliding past each other in order to achieve high strength[2].

In nature, these cross links between collagen fibrils are provided by proteoglycan bridges, predominantly decoran, forming shape modules[3, 4]. These proteoglycan bridges are elastic containing the glycosaminoglycan dermochondan sulfate[5, 6] . The way in which these connections might transmit force between fibrils to resist sliding forces has been modelled[7-11]. The energy absorbed by enthalpic transformations in the dermochondan can be significant[6, 12].

It has been found that the tensile elastic modulus of mouse tendon was reduced over much of the stress-strain curve when the natural glycosaminoglycan (GAG) content was lowered by the application of chondroitinase ABC while the ultimate tensile force and ultimate stress were relatively unchanged[13]. However, this is not universally agreed as other work has found no altered mechanical properties in tendon from the removal of GAGs[14, 15].

The GAG cross links associate with the collagen fibril at several different sites but is believed to always be associated with the Gly-Asp-Arg amino acid sequence[16].

Natural cross linking of collagen also increases with age due to glycation and has been shown to increase stiffness in connective tissues[17] and collagen gels[18] and increase brittleness in bones[19].

Methods of cross linking other than that found in nature can be used to modify the properties of collagen materials. Cross linking of bovine pericardium with glutaraldehyde either under strain or with no tension has been reported to result in a less extensible and stiffer material which is stronger than the untreated material[20, 21].

However, there is still much to learn about cross linking of collagen and the contribution these cross links make to the structure and mechanical properties of collagen tissues.

The arrangement of collagen fibrils, particularly the extent of alignment or anisotropy, is an important contributor to the strength of collagen materials. The structure-function relationship between collagen alignment and mechanical properties has been elucidated for a range of tissue types[22-26]. The orientation of collagen measured edge-on (alignment in-plane) has been shown in a range of mammal skins processed to leather to be correlated with strength[27, 28].

Small angle X-ray scattering (SAXS) is a powerful method for measuring the orientation of collagen fibrils in tissue[26, 29, 30]. Other methods may also be used such as small angle light scattering [31], confocal laser scattering [32], reflection anisotropy [33], and atomic force microscopy [34].

Bovine pericardium is a suitable material to use as a model in investigating the effect of cross linking, both natural and synthetic, on mechanical properties. Bovine pericardium has an established use for heart valve leaflet replacement[35, 36]. The material requires high mechanical strength and a long performance life[37]. The orientation of collagen in pericardium heterograft materials for heart valve leaflets has been shown to affect the stiffness during flexing[38].

We investigate here the hypothesis that cross links, both natural (GAGs) and synthetic (glutaraldehyde), may constrain the alignment of the collagen fibrils to result in different extents of orientation in collagen tissues which in turn may partially explain the different physical properties of the materials.

2. Methods

2.1 Fresh Pericardium samples

Fresh bovine pericardium was obtained from John Shannon and from Southern Lights Biomaterials and stored in phosphate buffered saline (PBS) solution (Lorne Laboratories Ltd). The tissue was rinsed briefly in PBS solution. The tissue was then cut into rectangles of approximate dimensions 40-45 mm x 10 mm with the long axis taken from the long axis of the heart (as shown in Fig. 1). The method of decellularisation was based on Yang et al (2009)[39]. The pericardium was washed for 24 hr in a 1% octylphenol ethylene oxide condensate (Triton X-100, Sigma), 0.02% EDTA solution made up in PBS. This washing step was conducted under constant agitation at 4 °C. The samples were then rinsed in PBS buffer and stored in PBS. These are what we refer to as “native”. Subsequent processing of this material produced glutaraldehyde treated or chondroitinase ABC treated material. All samples were taken from one pericardium and randomly assigned to each treatment method.

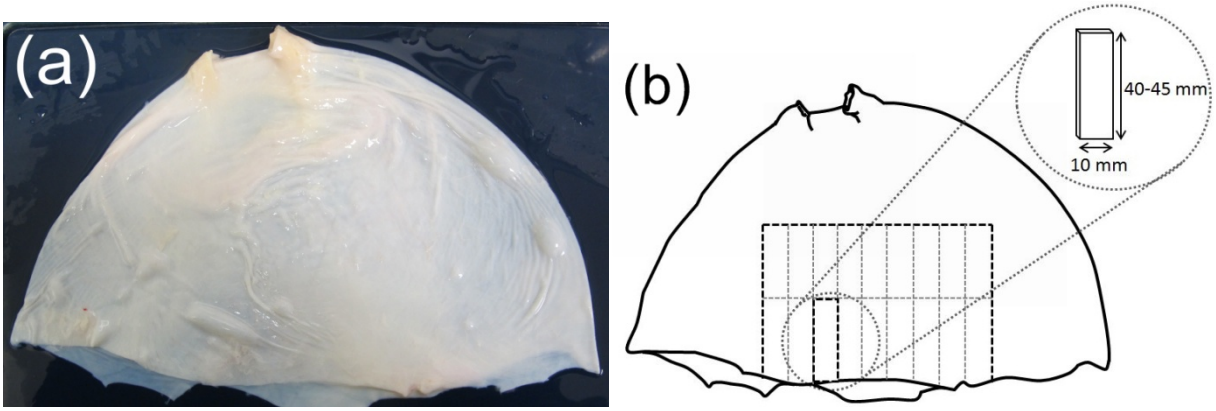

Fig. 1. Pericardium (a) ready to be cut for samples; (b) showing region used and sample size.

2.2 Glutaraldehyde treatment

The Triton treated pericardium was incubated with a 0.6% glutaraldehyde solution made up in PBS buffer at 4 °C for 24 h with constant agitation[40]. It was then stored in a sealed container in the solution of the same composition until SAXS measurements were performed. The total time in storage was 3-5 days.

2.3 Chondroitinase ABC treatment

Removal of GAG cross links was based on the method described by Schmidt et al. (1990)[41]. The Triton treated pericardium was incubated in 0.125 units of chondroitinase ABC per ml of buffer solution comprising of 0.05 M tris-HCl, 0.06 M sodium acetate and EDTA-free protease inhibitors (complete, EDTA-free protease inhibitor tablets, Roche Diagnostics, Mannheim, Germany) at approximately 27 °C for 24 h before rinsing and storing in 0.05 M tris-HCl and 0.06 M sodium acetate buffer in a sealed container at 4 °C.

Care was taken with all handling, cutting and treatment of the samples not to stretch the material as this might cause fibril alignment to change. The data presented here represents a duplication of this experiment with additional care taken in sample selection and handling in the second set of samples and SAXS analysis informed by the experience from the initial experiments.

2.4 GAG assay

An assay for sulfated GAGs was performed in triplicate for each of the sample treatments. GAGs were extracted with 1ml extraction reagent consisting of a 0.2 M sodium phosphate buffer at pH 6.4, containing 8 mg/ml sodium acetate, 4 mg/ml EDTA, 0.8 mg/ml cysteine HCl and 0.1 mg/ml papain enzyme (Carica papaya, Sigma, Biochemika, Enzyme no. 3.4.22.2). Each pericardium sample was

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incubated at 65 ºC for 26 hr. These samples were centrifuged and the supernatant containing the extracted GAGs collected. The concentration of GAGs in solution was determined with a Blyscan Sulfated Glyscosaminoglycan Assay kit (Bicolor, Carrickfergus, UK). GAGs were precipitated with 1 ml of dye reagent to 20 or 40 µl of supernatant diluted to 100 µl, mechanically inverted for 30 minutes, and then centrifuged. The unbound dye was drained off and 1 ml of Blyscan dissociation reagent was added to the precipitate, left to dissolve for approximately 10 minutes, and centrifuged. Absorbance was measured at a wavelength of 656 nm and compared with a standard curve.

2.5 SAXS analysis

In preparation for SAXS analysis, the pericardium was removed from the glutaraldehyde and tris-HCl, sodium acetate buffer solutions in which they had been stored. After soaking for at least 1 hr in buffered saline solution (Lorne Laboratories Ltd), pericardium strips were mounted and diffraction patterns recorded while the pericardium was wet. All diffraction patterns were recorded at room temperature.

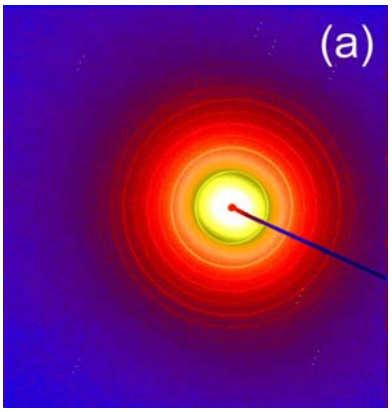

Fig. 2. Representative scattering pattern of pericardium

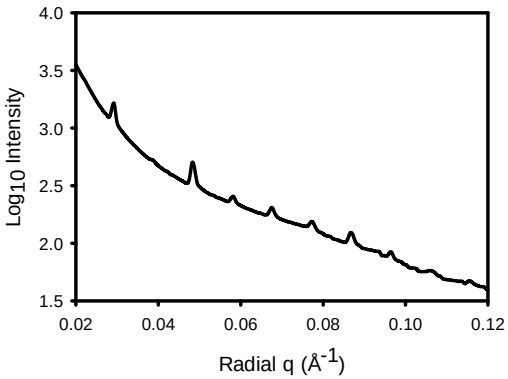

Fig. 3. Representative integrated scattering pattern of pericardium. The sharp peaks are due to diffraction for the D-spacing (at different orders).

Diffraction patterns were recorded on the Australian Synchrotron SAXS/WAXS beamline, utilizing a high-intensity undulator source. Energy resolution of 10^{-4} (e.g. 1 x 10^{-4} Å for 1 Å radiation) was obtained from a cryo-cooled Si(111) double-crystal monochromator and the beam size (FWHM focused at the sample) was 250 x 80 µm, with a total photon flux of about 2 x 10^{12} ph.s^{-1}. All diffraction patterns were recorded with an X-ray energy of 12 keV using a Pilatus 1M detector with an active area of 170 x 170 mm and a sample–to-detector distance of 3371 mm. Exposure time for diffraction patterns was 1 s and data processing was carried out using the software scatterBrainAnalysis V2.30[42].

The orientation index (OI) is used to give a measure of the spread of fibril orientation (an OI of 1 indicates the fibrils are parallel to each other; an OI of 0 indicates the fibrils are randomly oriented). OI is defined as (90° – OA)/90°, where OA is the minimum azimuthal angle range that contains 50% of the fibrils, based on the method of Sacks for light scattering[43] but converted to an index[27], using the

spread in azimuthal angle of one or more D-spacing diffraction peaks. The peak area is measured, above a fitted baseline, at each azimuthal angle.

Four samples were prepared of native material, three with treatment by chondroitinase ABC for 24 h and three with treatment by glutaraldehyde. For each sample one diffraction pattern was recorded at each of nine positions.

From each pattern (an example is shown in Fig. 2) the OI was calculated from the azimuthal spread of the 5th collagen diffraction peak (as seen in Fig. 3 at around 0.05 $Å^{-1}$).

2.5 Atomic Force Microscopy

Small square sections were cut from the native, chondroitinase ABC and glutaraldehyde treated pericardium samples and mounted onto 12 mm diameter magnetic metal discs with double sided tape. The samples were left to air dry for a few h before being imaged. A Nanoscope E (Veeco) atomic force microscope with a JV scanner was used with x-y calibration to ±3% completed just prior to imaging. CSG01 cantilevers (NT-MDT, Russia) with a force constant of about 0.05 N/m were used for contact mode imaging.

2.6 Histology

Samples of pericardium were cut and frozen flat in a Leica CM1850 UV cryogenic microtome at -30 ºC before being mounted on microtome disks using embedding medium for frozen tissue specimens. 10 µm thick cross-sections were cut and transferred to glass microscope slides. The mounted sections were stained as per the protocols of the Picrosirius Red Stain Kit (Polysciences, Inc.) before being placed in 70% ethanol for 45 s and left to air dry for several h. Optical images were recorded on a Nikon Eclipse TE2000-U microscope fitted with a Nikon Digital Sight DS-Fi2 camera and cross-polarising filters.

2.7 Tensile Properties

Three rectangular sections of pericardium with the long axis of the sections equivalent to the long axis of the heart were cut from each of three pericardium sacs and treated with glutaraldehyde or chondroitinase ABC or left as native tissue. From these, samples were cut using a press knife and stress-strain curves were measured by uniaxial strain using an Instron 4467 with the sample mounted vertically at a rate of 100 mm/min according to standard ISO 3376:2011. Thickness was measured using method BS EN ISO 2589:2002 but with reduced pressure. Elastic modulus was determined for the linear region of the stress-strain curve.

2.8 Statistical analysis

Statistically significant differences between treatment mean OI values, GAG content and tensile properties were tested for using One Way ANOVA implemented in SigmaPlot 12.0 with a significance level, alpha, of 0.05. If statistical differences were found (P = <0.001), pairwise multiple comparisons were performed using the Holm-Sidak method in SigmaPlot 12.0 where the overall significance level used was 0.05. Pairwise comparisons with P-values less than 0.05 were considered to be significantly different.

3. Results

3.1 Chondroitinase ABC GAG removal

The GAG assay found that approximately 81% of GAGs were removed with chondroitinase ABC treatment (Fig. 4) which can be considered a success. As expected, glutaraldehyde treatment did not remove the GAGs, showing similar GAG content to the native material. Therefore the chondroitinase treated samples do represent pericardium with most of the GAGs removed.

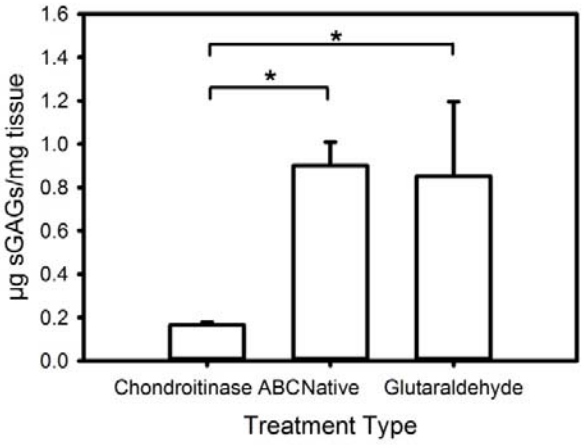

Fig.4. GAG assay for pericardium for triplicate samples (error bars for 95% confidence intervals). Pairs that are significantly different (P<0.001 for α = 0.05) are shown by a *.

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3.2 Histology

The picrosirius red stained sections of each of the treated samples show a similar level of crimp in each sample type (Fig. 6). Crimp is the wavy structure of collagen fibrils which is typically seen in tendon and pericardium (with a period of 25-45 μm in pericaridum[23]) but not as prominently in skin. The chondroitinase ABC treated sample and native sample are the most similar. The glutaraldehyde treated pericardium has the appearance of a more open structure (which may be because it did not microtome as well) and it has some variation in colour. While picrosirius red is intended as a specific stain for collagen with Type 1 collagen showing as red, other factors can affect birefringence and the resulting colour under cross-polarised filters, such as fibril thickness and the availability of free basic amino acid binding sites. The sulfonic acid groups of the picrosirius red dye molecule bind to the free amino acid residues on collagen, as do the aldehyde groups of glutaraldehyde; therefore binding of glutaraldehyde to these sites will inhibit dye binding and may result in decreased birefringence. The presence of colours other than red in the glutaraldehyde treated samples does not therefore indicate other types of collagen present, but rather, modification to the type I collagen[44-46].

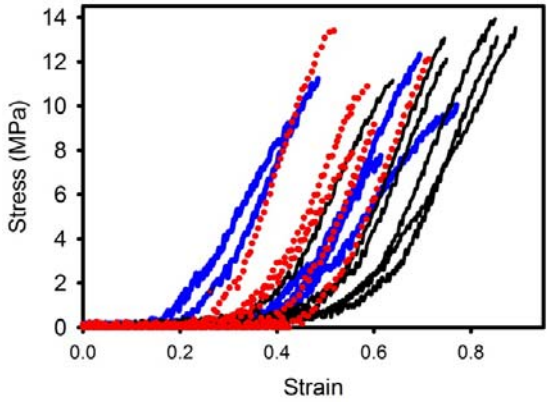

Fig. 5. Stress strain curves for native pericardium (blue thick lines); chondroitinase ABC treated pericardium (red dotted lines); glutaraldehyde treated pericardium (black thin lines).

3.2 Tensile properties

The tensile properties of the pericardia samples had a high variability (Fig. 5, Table 1). There is a foot region of variable length followed by an approximately linear region until the material reached its ultimate tensile stress and broke (the failure region is not shown). The chondroitinase treatment perhaps increases the elastic modulus, in agreement with other studies[17], however with the small sample size this difference cannot be considered statistically significant ($P = 0.043$, $t = -1.9$, for $\alpha = 0.05$). The stress at failure may be higher for glutaraldehyde, also in keeping with other studies[20, 21], but this also cannot be considered statistically significant ($P = 0.012$, $t = -3.1$, for $\alpha = 0.05$).The only statistically significant difference between the mechanical properties of the treatment types is the strain at failure, which is higher for the glutaraldehyde treated material ($P = 0.026$ $t = -2.6$ for $\alpha = 0.05$).

Table 1. Tensile properties of pericardium (with 95% confidence intervals)

Sample	Elastic Modulus in linear region (MPa)	Stress at Failure (MPa)	Strain at Failure (%)
Native	40 ± 12	10.2 ± 2.2	60 ± 17
Chondroitinase ABC	52 ± 13	10.8 ± 2.7	60 ± 9
Glutaraldehyde	50 ± 6	12.8 ± 1.1	79 ± 10

3.3 SAXS

The pericardium gives good scattering patterns with clearly defined diffraction rings due to the D-spacing periodicity (Fig. 2). The integrated intensity plots show well defined peaks corresponding to the collagen D-period (Fig. 3). The odd numbered peaks have a much higher intensity than the even numbered peaks, a characteristic attributed to a fully hydrated sample[47]. This provides some reassurance that the samples are maintained in the hydrated state during collection of the diffraction patterns, as intended.

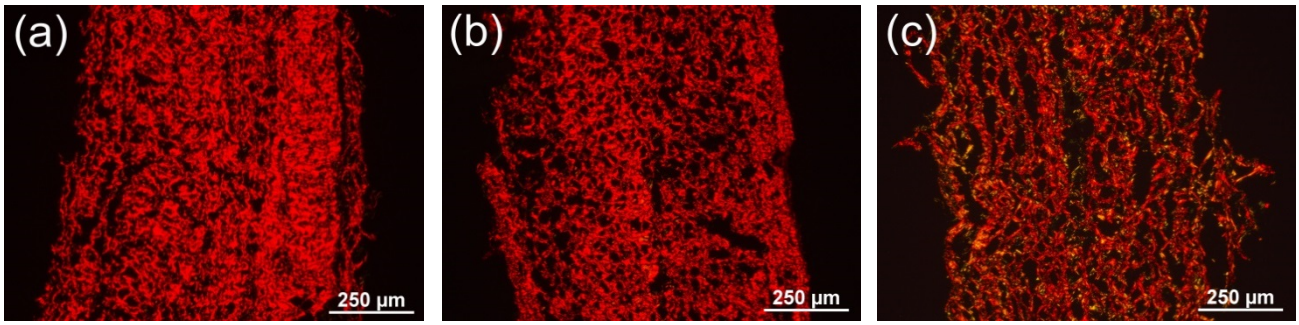

Fig. 6. Picrosirius stained sections of pericardium treated with a) chondroitinase ABC; b) native; c) glutaraldehyde

3.4 OI

The distribution of orientation of the fibrils can be seen with a plot of the intensity (we use the peak area) of any of the collagen diffraction peaks (Fig. 7). A narrow peak in this plot is indicative of more highly aligned collagen fibrils, as seen for the native and chondroitinase treated tissue, whereas broader peaks such as that for glutaraldehyde indicate a more isotropic arrangement. This can be quantified as an orientation index, OI. We calculate first an orientation angle (OA) which is defined as the minimum angle which contains 50% of the fibrils[48]. From this the OI is calculated as (90° – OA)/90°.

The OI calculated for the three treatments provide different average OI values (Table 2, Fig. 8). There is a statistically significant difference in the OI between the glutaraldehyde treated material and the other two materials but the difference in the OI between the native and chondroitinase treated pericardium does not pass the significance test. Previously we have compared chondroitinase ABC treatment for 48 h and 24 h with diffraction patterns recorded and analysed, however the OI obtained from the 48 h treated samples was not significantly different from that obtained after 24 h, probably indicating that most of the GAGs were removed already by 24 h of treatment (not shown here).

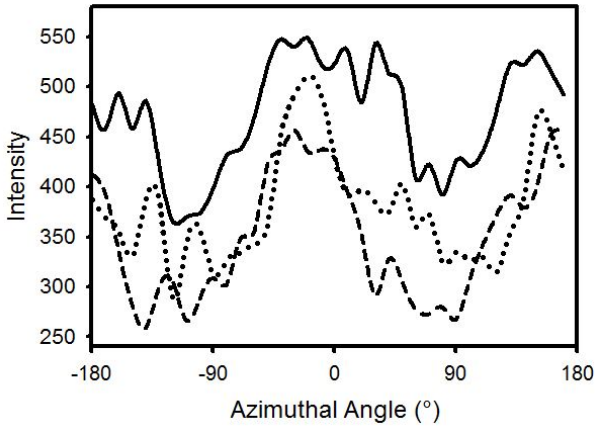

Fig. 7. Representative azimuthal intensity variation plots of the fifth collagen D-period diffraction peak for pericardium. The width of the central peak represents the spread in fibril orientation. Solid line, glutaraldehyde; dotted line, native; dashed line, chondroitinase ABC.

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Table 2. Orientation Index obtained for pericardium samples.

Sample	Number of diffraction peaks analysed (N)	Mean OI	95% confidence interval
Chondroitinase ABC	27	0.208	0.032
Native	36	0.192	0.021
Glutaraldehyde	27	0.117	0.021

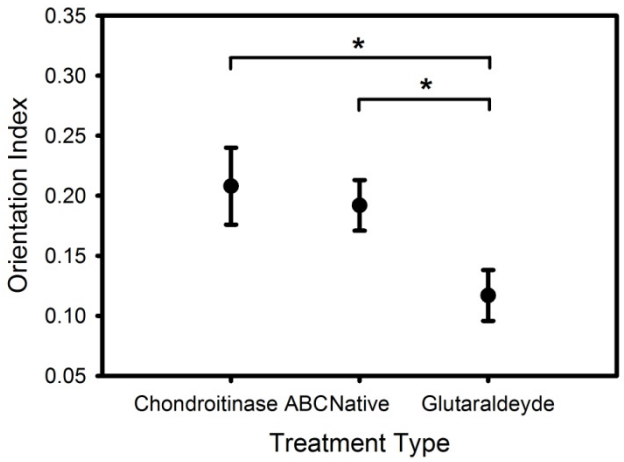

Fig. 8. Orientation index for each of the three levels of cross linking (error bars for 95% confidence intervals). Pairs that are significantly different ($P<0.001$ for $\alpha = 0.05$) are shown by a *.

3.5 Atomic Force Microscopy

Atomic force microscopy provided clear images of collagen fibrils on the fibrous (outer) surface of the pericardium (Figure 9). AFM provides small area images of a diverse surface so that unbiased selection of images can be difficult. We have selected one image of each material that is generally representative of that sample. The glutaraldehyde treated sample clearly had more of a collagen fibril network with fibrils not so often seen in parallel. In contrast the native material and the pericardium treated with chondroitinase ABC contained many aligned collagen fibrils.

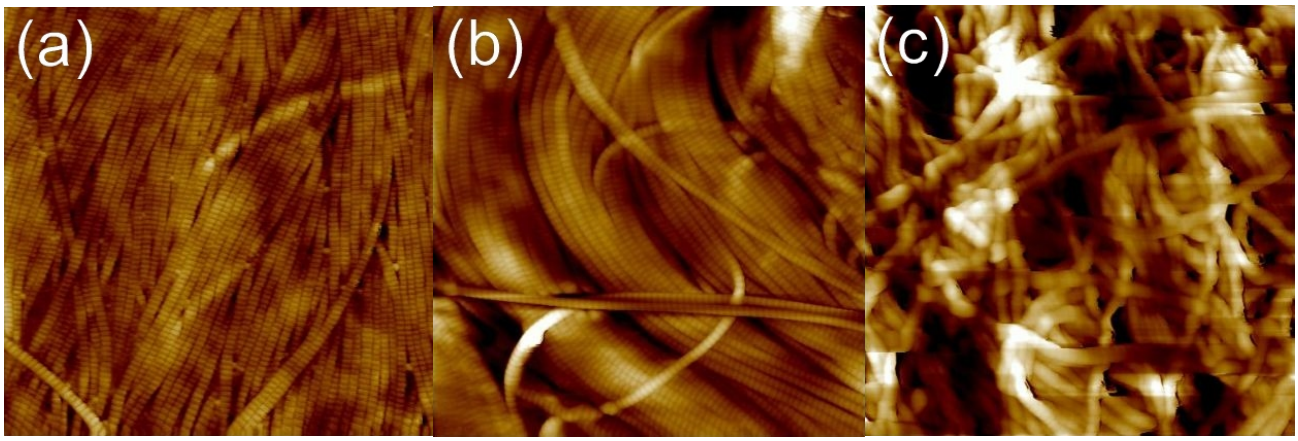

Fig. 9. Atomic force microscopy height images for (a) native bovine pericardium (b) chondroitinase ABC treated pericardium (c) glutaraldehyde treated pericardium. Images are 5 µm square.

4. Discussion

We have found an effect on collagen fibril alignment with cross linking.

Native tissue containing GAG cross links has a moderate degree of fibril alignment. When these cross links are removed by treatment with the enzyme chondroitinase ABC the alignment of the fibrils does not show a significant change. When cross links are added, in the form of glutaraldehyde, the alignment of the fibrils decreases, becoming more isotropic with a network like structure forming. These changes do not appear to be associated with a change in crimp. Glutaraldehyde cross links therefore appear to have a direct effect on the arrangement of the collagen fibrils whereas native GAG cross links do not have a statistically significant effect on alignment for tissue that is not under any mechanical load.

Glutaraldehyde has long been used as a cross linking agent for collagen, reacting primarily with ε-amino groups of lysine and hydroxylysine located on the outer surface of the triple helix region. Such links have been reported to occur both intramolecularly and intermolecularly depending on the treatment conditions and may involve some polymerisation of the glutaraldehyde to link greater distances[49-51]. Here we have shown that this network structure means not just a cross linked network of collagen but that the collagen fibrils also rearrange into a less aligned, more isotropic network structure under the action of glutaraldehyde cross linking without the application of external force. This chemically induced restructuring results in a decrease in the OI.

We have not specifically investigated the heterogeneity with depth, however the treatment time was ample to enable glutaraldehyde to penetrate the tissue fully[50]. In other work on glutaraldehyde treatment of pericardium, the variation of OI with depth through the glutaraldehyde treated pericardium tissue has been investigated and the OI did not vary greatly throughout the thickness, although a comparison was not been made with untreated pericardium[52].

In contrast to glutaraldehyde cross links, proteoglycan (containing GAG) cross links are reported to occur solely on the outer surface of collagen fibrils, forming both axially and orthogonally with the majority located orthogonally between adjacent fibrils by the interaction of GAG side chains localised on the surface of collagen fibrils in mature tissues[53, 54]. More specifically, it is believed proteoglycan cross links are associated with the gap region of the collagen D-spacing, binding to a single tropocollagen molecule[53, 54]. We propose that these GAG bridges do not constrain the fibrils in a somewhat unaligned network structure in a higher energy state; these links appear only to form between adjacent fibrils at specific locations. Removal of these links therefore does not result in relaxation of some kind and fibrils do not spontaneously realign into a lower energy state and adopt some sort of preferred alignment. However, we suggest that the removal of the GAG links by chondroitinase ABC may give the potential for fibrils in the treated pericardium to become more easily aligned under tension.

This understanding of structural changes with treatment also has consequences for the preparation of materials for medical applications such as the treatment of bovine pericardium for heart valve repair, or ovine forestomach extracellular matrix material for surgical scaffolds[55]. The modifications imposed on the native tissue due to the processing of the material, sometimes including glutaraldehyde cross linking, may be better understood in terms of the structural changes that lead to altered physical properties. A careful balance of cross linking is then is required to achieve the properties required for in-service applications.

Conclusions

We have found that the extent and nature of cross linking present in pericardium has an impact on the collagen fibril orientation. When additional cross links with glutaraldehyde are added the fibrils form more of a network structure. We suggest that formation of cross links via glutaraldehyde addition progressively constrains the fibrils into a random network. The relationship between cross linking and fibril alignment provides a perspective on the importance of cross links in determining the structure of tissues. This could have relevance both in the preparation of new biomaterials and in the understanding and treatment of ageing and disorders in human tissues.

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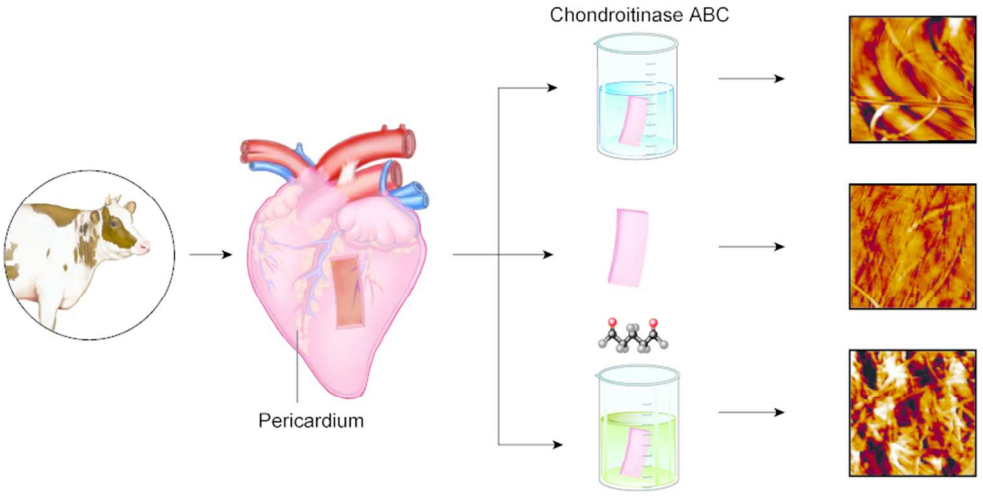

Cross linking was found to be a factor in collagen fibril alignment in pericardium tissue
80x40mm (300 x 300 DPI)