Effects of Preservatives on Corneal Collagen Parameters Measured by Small Angle X-Ray Scattering Analysis

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Research note

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Abstract

Objective: Small angle X-ray scattering (SAXS) analysis is a sensitive method for determining the ultrastructure of collagen in various tissues. Little is known about how parameters measured by SAXS are affected by preservatives, commonly used to prevent autolysis and strengthen sample tissues. We determined the effects of formalin, glutaraldehyde, Triton X and saline on measurements of fibril diameter, fibril diameter distribution, and D-spacing of corneal collagen using SAXS analysis.

Results: Compared to control sheep and cats' corneas, frozen at -80 °C, those preserved in 5% glutaraldehyde and 10% formalin had significantly larger mean collagen fibril diameters, increased fibril diameter distribution and decreased D-spacing. Corneas preserved in Triton X had significantly increased collagen fibril diameters and decreased fibril diameter distribution. Corneas preserved in 0.9% saline had significantly increased mean collagen fibril diameters and decreased diameter distributions. Subjectively, the corneas preserved in 5% glutaraldehyde and 10% formalin maintained their transparency but those in Triton X and 0.9% saline became opaque. Subjective morphological assessment of transmission electron microscope images of corneas supported the SAXS data. Workers using SAXS analysis to characterize collagen should be alerted to changes that can be introduced by common preservatives in which their samples may have been stored.

Introduction

Collagen is a fibrous protein providing strength and structure to many tissues including the cornea. The basic unit of collagen is a repeating series of three amino acids which coil together in a triple helix, the basic collagen molecule. These molecules align in a staggered side-by-side fashion forming collagen fibrils with D-spaces representing areas of high and lower collagen molecule overlap. Collagen fibrils can be arranged in a mainly parallel fashion to give strength to tissue (tendon) or can be largely random forming a mesh-like structure providing flexibility and resistance to tear propagation (skin).(1) In the eye, collagen fibrils are responsible for maintaining the precise curvature of the cornea and have short-range ordering essential for corneal transparency.(2, 3)

Small angle X-ray scattering (SAXS) analysis is a sensitive method for the analysis of nanostructures. X-rays passing through a sample are diffracted by its components and the resulting scatter patterns provide information on their shape and size. Typically, SAXS analysis provides structural information on objects of 1-400 nm while being non-destructive and requiring minimal sample preparation.(4) Although SAXS has been used to analyse a wide range of biologicals, it has been particularly useful in studies of collagen in leather(5–8), tendon(9, 10) and the cornea(11–17), and in describing eye lesions.(18)

Although SAXS analysis can be done on unprocessed tissue, often samples for analysis are fixed in various preservatives to prevent autolysis and introduce rigidity necessary for tissue sectioning. Fixation brings about changes in collagen which have been described using high resolution transmission electron microscopy (TEM).(19) However, there is only limited information on the effects of fixation on collagen

parameters measured by SAXS analysis. Interfibrillar spacing in bovine corneas fixed in 2.5% glutaraldehyde in 0.9% saline was similar to that in fresh corneas (63.8 nm vs. 63.4 nm;$P > 0.2$) but D-spacing significantly decreased (65.0 nm vs. 64.5 nm;$P < 0.001$).(20) Subsequently, rat tendon stored in an unspecified formalin formulation for 48 hours had similar D-spacing to tendon stored in phosphate buffered saline.(21) Freezing of human corneas had no effect on X-ray scattering patterns.(15)

Knowing how fixation changes the ultrastructure is very important and the processing method needs to be chosen carefully to preserve features of interest in the studied tissue. To provide further information on the effects of commonly used preservatives on collagen parameters determined by SAXS analysis, treated and untreated sheep and cats' corneas were studied. TEM was performed to complement the SAXS data.

Methods

Samples

Clinically normal corneas were collected from two adult female sheep immediately after slaughter at an abattoir (NZ) and from an adult male and female cat necropsied at Massy University Veterinary School for reasons unrelated to the current study. The central areas of the corneas, having the most uniform collagen fibril arrangement(2, 22), were divided to provide duplicate samples for the following treatments. As freezing has no effect on X-ray scattering patterns(15), normal control samples consisted of two cat and two sheep samples immediately frozen in cling wrap at -80°C. Remaining samples were immersed in 2mL of: 5% glutaraldehyde (40mL(25% glutaraldehyde), 50mL(0.2M cacodylate buffer), 80mL(distilled water)), 10% formalin (100mL(37-40% formaldehyde), 900mL(distilled water), 4.0g(monosodium phosphate) and (6.5g)anhydrous disodium phosphate), Triton X (20mM(tris-aminomethane), 1mM(ethylenediaminetetraacetic acid), 1.25mL(10% Triton X) and 1.25mL(sodium deoxycholate)) or 0.9% saline (4.5g(sodium chloride) in 500mL(deionized water with heat sterilization)). After four days of preservation the samples were tested for transparency by subjectively observing a 4mm by 4mm cross (1-point black line) through the sample (see Supplementary Material) and analysed by SAXS (below). Immediately thereafter, samples were fixed in Karnovsy's fixative (2.0g(paraformaldehyde), 5.0mL(50% glutaraldehyde) and 20.0mL(0.2M cacodylate buffer); pH adjusted to 7.4 with 1M(sodium hydroxide)) for evaluation by TEM.

SAXS

At the SAXS/WAXS beamline of the Australian Synchrotron the samples were mounted flat-on to the X-ray beam (optical axis from anterior to posterior) and surface diffraction measurements performed using a 3x3 grid with 0.25mm spacing between points. A high-intensity undulator source from a cryo-cooled Si (111) double-crystal monochrometer was utilized with an energy resolution of 10^{-4}. Beam size was 250 x 80µm and total photon flux approximately $2x10^{12}$photons.s^{-1}. All diffraction patterns were calibrated with silver behenate and recorded at 12keV using a Pilatus 1M detector at 3337mm. Data was processed with

ScatterBrain software. D-spacing was calculated by comparing diffraction peak positions of the 5^{th} order peak with the calibrant to determine q-values, after background subtraction, using Gaussian approximations (Fig.1). Fibril diameters were determined over the full q-range ($0.01Å^{-1}$ - $0.1Å^{-1}$) (Fig.1) by applying the 'cylinder AR' model using "Irena", a macro developed for analysing particle size distributions in SAXS data(23) running in a data analysis tool (Igor Pro, Wavemetrics).The fibril diameter spread within a sample was determined from the scatter intensity patterns. The fibril diameter distribution was determined as the full width half maximum of the peak from the frequency vs fibril diameter histograms.

TEM

Corneas in Karnovsky's fixative were trimmed, post-fixed in osmium tetroxide (0.1M), dehydrated with ethanol washes, and embedded in epoxy resin (TAAB812, UK). Ultra-thin sections (70-90nm) were cut (LeicaEMUC7, DE), mounted on copper-grids, stained with uranyl acetate and lead citrate, and viewed in a CM10 TEM(Philips, NL) at 80kV.

Image Processing

A Graphical User Interface (GUI), written in C++, was used to measure the collagen fibrils in end-on TEM images. A pixel-to-nanometre scale factor was computed with the GUI and used to detect contours which, with Delaunay triangulation and Voronoi diagrams, enabled measurements of fibril diameters and distances to nearest neighbours.

Results

Transparency Test

The printed cross was clearly visible through the control corneas (see Supplementary Material) and those preserved in 5% glutaraldehyde and 10% formalin. It was less clearly visible through corneas preserved in 0.9% saline and not visible through corneas stored in Triton X.

SAXS

Scatter patterns and their associated intensity versus q-range plots are shown in Fig.1 for the sheep and cats' corneas treated with the various preservatives. The 5th order peak was used for measuring D-spacing and the full q-range ($0.01–0.1Å^{-1}$) for fibril diameter (Table.1). Relative to the controls, fibril diameters and distributions for both the sheep and cats' corneas preserved in 5% glutaraldehyde were significantly higher ($P<0.05$). However, they had significantly lower D-spacing than the controls ($P<0.05$). Similarly, corneas preserved in 10% formalin had fibril diameters and distributions significantly higher than the controls and D-spacing significantly lower than the controls ($P<0.05$). The fibril diameters for corneas preserved in Triton X were significantly greater ($P<0.05$) than controls and the largest recorded. The fibril diameter distributions, however, were significantly lower than for the controls. The D-spacing was increased in both the cats' and the sheep corneas but this was only significant in the latter ($P<0.05$).

Of all the preservatives, samples in 0.9% saline had values closest to those of controls with no significant differences between the D-spacing of the sheep and cats' corneas. Compared to controls, however, fibril diameters in both species were significantly higher ($P<0.05$) and diameter distributions significantly lower.

TEM

It should be noted that the steps used in processing the samples for TEM affect the morphology of collagen(21) and it was not therefore possible to perform quantitative comparisons of results obtained by SAXS analysis and TEM. However, subjective morphological assessment of the TEM images strongly supported the SAXS data showing, for example, that fibril diameters in the 5% glutaraldehyde, 10% formalin samples were significantly smaller than the saline and control samples while the Triton X samples were significantly larger than the saline and control corneas (Fig.2). Additionally, visual inspection of Fig.2 suggests significant variation in the interfibrillar spacing/distance, number of fibrils in a given area, and the amount of interfibrillar matrix between the Triton X and saline samples with the controls, and, to a lesser degree, the samples preserved in 10% formalin and 5% glutaraldehyde. As with the SAXS analyses, fibril diameters were significantly larger in the Triton X samples and significantly smaller in the saline samples compared to the control samples (Fig.2). Epithelial and endothelial cells, when visible in the 10% formalin and 5% glutaraldehyde preserved corneas, had normal morphology. No cells were seen in sections showing the anterior and posterior areas of corneas preserved in Triton X and saline.

TEM Image Processing

A pattern of fibril diameter distribution like that in the SAXS study was noted (Table.1). The diameters of the fibrils in both the sheep and cats' normal controls were significantly smaller than those in the samples preserved in 5% glutaraldehyde, 10% formalin, Triton X and saline.

Discussion

While sophisticated modern techniques enable detailed analyses of the nanostructure of biological materials, the processing required before analysis often leads to significant changes in the shape and size of different tissue components. Although there is reasonable data on the changes brought about by processing for TEM(19, 20, 24, 25), there is only fragmented data on the effects of tissue processing on SAXS analysis. Our study has expanded this data and shows commonly used preservatives introduce significant changes in collagen parameters that can be measured with SAXS. Formalin and glutaraldehyde significantly decreased D-spacing and increased collagen fibril diameters. Both formalin and glutaraldehyde are relatively small molecules, which can readily penetrate collagen, forming cross linkages that bind the collagen molecules together and decrease the D-spacing. Before such cross links can form, however, it has been suggested that the hypotonic fixative solution moves into the fibrils and causes them to swell.(20) The swollen fibrils are expected to be larger, leading to increased fibril diameter.

This is consistent with our findings of increased fibril diameters in samples stored in formalin and glutaraldehyde.

With TEM, the fibrils in 5% glutaraldehyde and 10% formalin samples appeared to have relatively uniform diameters with short-range order interfibrillar spacing; shown to be essential for optical transparency(2, 3) which was noted in these samples in the transparency testing. Further, epithelial and endothelial cells in corneas preserved in 5% glutaraldehyde and 10% formalin appeared normal, another requirement for corneal transparency. The presence of specialized water-soluble structural proteins (crystalline proteins) and high levels of enzymes such as aldehyde-dehydrogenase and transketolase in the cytoplasm of the epithelial cells(26) results in refractive indices of the cytoplasm and cell organelles within a range that does not produce scattering of light.

Triton X is a non-ionic detergent used to produce implantable acellular matrix scaffolds from heart valves(27), tendons(28), and ligaments.(29) In removing proteoglycans and the intercellular matrix between collagen fibrils, the Triton X likely facilitated the entry of its 0.9% saline diluent into fibrils causing them to swell, increasing the fibril diameter as seen in SAXS analyses of the samples. In TEM sections, the fibrils also appeared larger with considerable variation in interfibrillar spacing, very irregular packing and poor short-range order, all consistent with the lack of transparency noted in the transparency testing.

Storage in 0.9% saline only resulted in a significant increase in collagen fibril diameter and fibril diameter distribution. This most likely was because the cornea is normally maintained in a slightly dehydrated state by endothelial cells on its inner surface.(30, 31) 0.9% saline is relatively hypotonic to the cornea and, with the loss of endothelial and epithelial cells we noted, water would have moved into the corneal samples increasing the hydration status of the fibrils, causing them to swell and have larger fibril diameters and distributions as we found in our SAXS analysis and TEM.(9, 32) The resultant mild corneal odema would have interfered with the optimal regular spacing and size of fibrils required for normal transparency(33, 34) and explains the loss of clarity of these corneas in the transparency test.

Limitations

Our SAXS analysis of scattering of the X-ray beam as it passed through the anterior to posterior (optical axis) of the cornea gave us an average picture of the collagen layers across the cornea. Recent studies have shown the collagen layers in the cornea are not uniform, instead there are variations in collagen fibril size and direction that occur at various depths in the cornea.(35) Access to facilities enabling the study of micro-focus X-ray data on cross sections of the cornea would have provided us with more precise data on collagen changes at various depths in the cornea, rather than an overall average.

List Of Abbreviations

SAXS, small angle X-ray scattering

TEM, transmission electron microscopy

GUI, graphical user interface

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

SK designed the experiment, collected and analysed the small angle x-ray scattering data, and was a major contributor in writing the manuscript. LP collected and analysed the transmission electron microscopy images. JS analysed the transmission electron microscopy measurements and contributed in writing the manuscript. FN developed the image analysis tool and analysed images. HW assisted with small angle x-ray scattering data collection and analysis. PK was a major contributor in designing the experiment and writing the manuscript. All authors read and approved the final manuscript.

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Table 1

Due to technical limitations, table 1 is only available as a download in the supplemental files section.

Figures

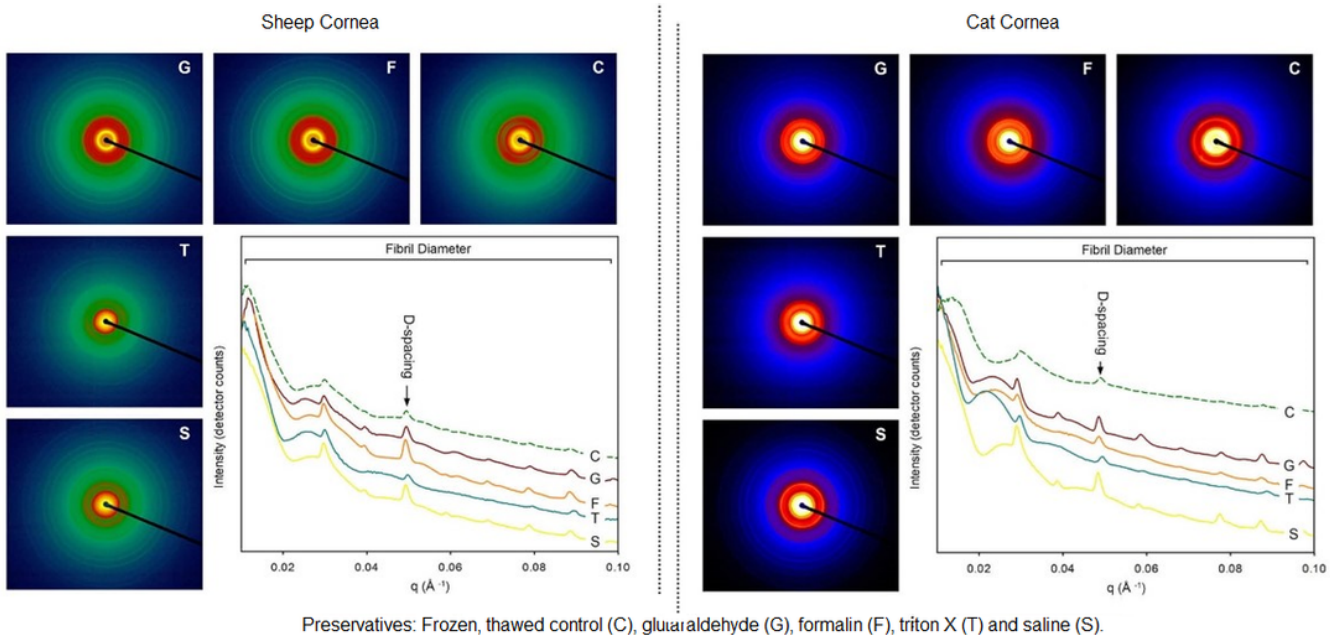

Figure 1

Photo images of 2D small angle X-ray scattering patterns produced by frozen and thawed control corneas and the preserved corneas. The graph shows the intensity profiles over the measured q-range for all the samples. Arrow indicates peak (0.045–0.055 Å-1) used to determine D-spacing and the full q-range (0.01–0.1 Å-1) for the fibril diameter.

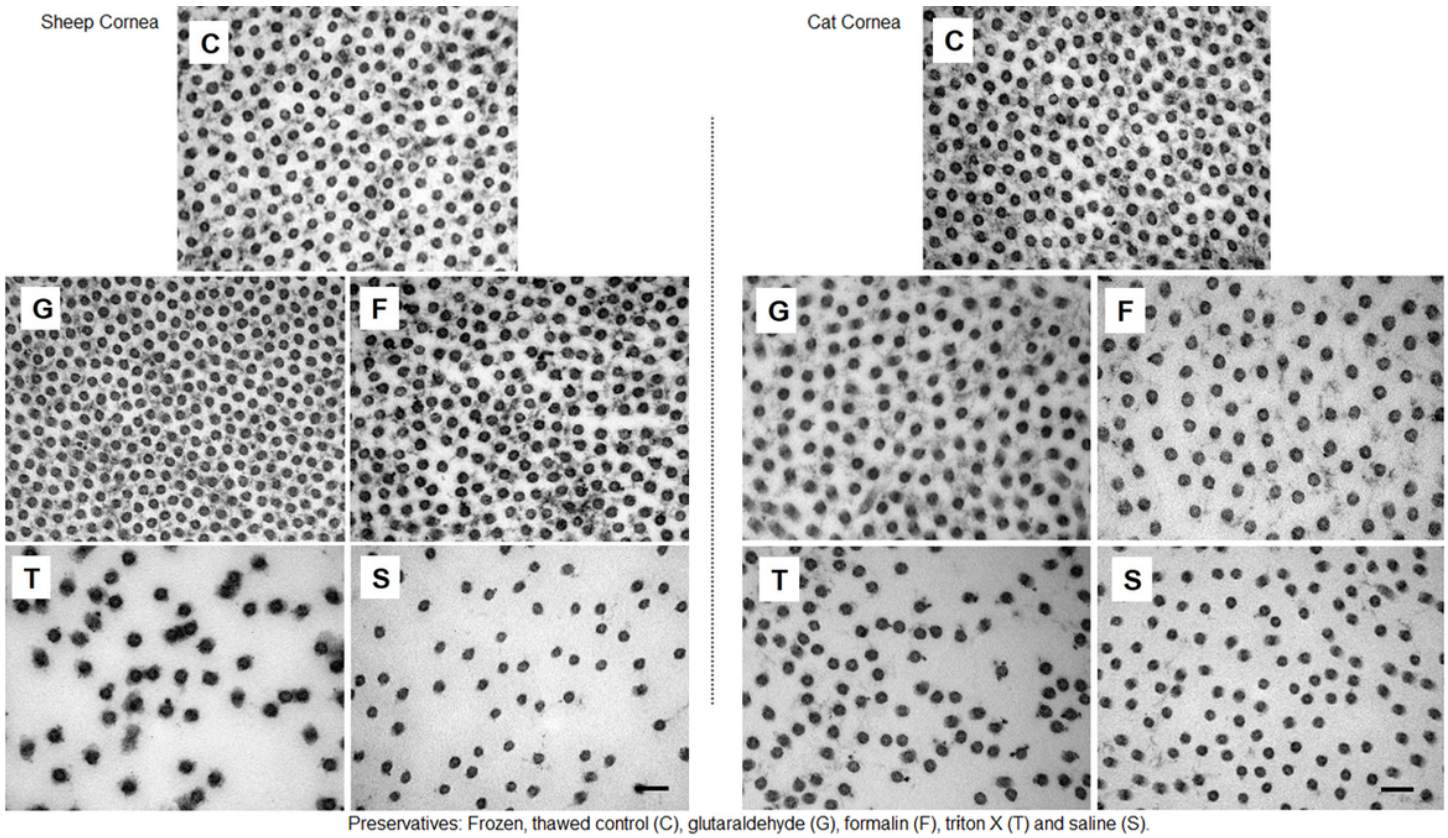

Figure 2

Transmission electron micrographs depicting collagen fibril cross-sections in the stroma of sheep (left) and cat (right) corneas freeze/thawed or preserved for 5 days followed by fixation in Karnovsky's fixative and processing for TEM.

Supplementary Files

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- Table1.png
- SupplementaryMaterial.docx