journal of the mechanical behavior of biomedical materials 133 (2022) 105300

Contents lists available at ScienceDirect

Journal of the Mechanical Behavior of Biomedical Materials

journal homepage: www.elsevier.com/locate/jmbbm

Effect of corneal collagen crosslinking on viscoelastic shear properties of the cornea

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ARTICLE INFO

Keywords:
Cornea
Shear properties
Collagen crosslinking treatment
Torsional shear experiments
Porcine eyes

ABSTRACT

The cornea is responsible for most of the refractive power in the eye and acts as a protective layer for internal contents of the eye. The cornea requires mechanical strength for maintaining its precise shape and for withstanding external and internal forces. Corneal collagen crosslinking (CXL) is a treatment option to improve corneal mechanical properties. The primary objective of this study was to characterize CXL effects on viscoelastic shear properties of the porcine cornea as a function of compressive strain. For this purpose, corneal buttons were prepared and divided into three groups: control group ($n = 5$), pseudo-crosslinked group ($n = 5$), and crosslinked group ($n = 5$). A rheometer was used to perform dynamics torsional shear experiments on corneal disks at different levels of compressive strain (0%–40%). Specifically, strain sweep experiments and frequency sweep tests were done in order to determine the range of linear viscoelasticity and frequency dependent shear properties, respectively. It was found that the shear properties of all samples were dependent on the shear strain magnitude, loading frequency, and compressive strain. With increasing the applied shear strain, all samples showed a nonlinear viscoelastic response. Furthermore, the shear modulus of samples increased with increasing the frequency of the applied shear strain and/or increasing the compressive strain. Finally, the CXL treatment significantly increased the shear storage and loss moduli when the compressive strain was varied from 0% to 30% ($p < 0.05$); larger shear moduli were observed at compressive 40% strain but the difference was not significant ($P = 0.12$).

1. Introduction

The cornea is the outer transparent part of the eye that bends incoming light and acts as a protective layer for the internal contents of the eyeball protecting them from intraocular injuries. The tissue is constantly subjected to intraocular pressure from the inside and external dynamic forces caused by eye rubbing. The corneal exact curvature, a requirement for proper light refraction, is a function of its mechanical properties. Thus, any changes in the structural integrity of the cornea caused by diseases or by trauma can have significant adverse effects on the quality of vision. From anterior to posterior, the cornea comprises of five layers, epithelium, Bowman's layer, stroma, Descemet's membrane, and endothelium. The stroma is the thickest layer and is primarily responsible for mechanical properties of the cornea. A proper understanding of the relation between corneal biomechanics and its shape is a great asset for guiding refractive surgeries and manipulating corneal topography.

Keratoconus, an eye disease associated with significant changes in corneal microstructure and biomechanics, is estimated to affect between 1 in 400 and 1 in 2000 individuals (Kennedy et al., 1986; Sharif et al., 2018). In keratoconus, the cornea loses its biomechanical integrity; thus, it thins and gradually becomes cone-like. Any change in the shape of the cornea interferes with its optical properties and causes the vision to become blurry and distorted. At initial stages of the disease, spectacles or contact lenses are used to obtain better vision. However, if keratoconus progresses such that excessive ectasia and thinning are observed, corneal transplantation is required to restore vision. Until recently, because of the absence of any treatment procedure and the unknown rate of progression of the disease, keratoconus patients could only improve their vision by alternating between the above options depending on the severity of their disease. However, corneal collagen crosslinking (CXL) is a newly FDA-approved treatment option to stop or slow down the progression of keratoconus.

CXL is a photochemical treatment therapy in which the cornea soaked in the photosensitizer riboflavin solution is subjected to ultraviolet-A (UVA) light (Spoerl et al., 1998). This procedure has been

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https://doi.org/10.1016/j.jmbbm.2022.105300
Received 15 February 2022; Received in revised form 8 April 2022; Accepted 27 May 2022
Available online 31 May 2022

shown to improve corneal structural properties and increase its resistance against collagenase digestion (Wollensak et al., 2003a). From the clinical perspective, stopping the progression of the disease means that individuals preserve their visual acuity and do not need corneal transplantation (Wollensak et al., 2003b). The strengthening effects of the CXL treatment have been commonly investigated by performing uniaxial tensile experiments (Wollensak et al., 2003a; Hatami-Marbini and Jayaram, 2018a; Hatami-Marbini, 2018; Wollensak and Iomdina, 2009; Lanchares et al., 2011; Chang et al., 2018). This is because the cornea has often been seen as an isotropic material in the literature. However, it is well known that the cornea has an inhomogeneous and anisotropic extracellular matrix composed of collagen fibrils that are uniformly organized in stacks of lamellae (Maurice, 1957; Meek, 2008; Hassell and Birk, 2010). This special lamellar microstructure is why in-plane and out-of-plane material properties of the cornea are significantly different from each other. Our previous studies have shown that the tensile modulus of the cornea is orders of magnitudes greater than its shear and compressive moduli (Hatami-Marbini, 2014; Hatami-Marbini and Etebu, 2013a; Hatami-Marbini and Rahimi, 2014).

The torsional shear experiments have commonly been used to investigate the viscoelastic shear properties of soft tissues such as brain, skin, meniscus, and articular cartilage (Zhu et al., 1993; Hayes and Bodine, 1978; Bilston and Miller, 2011; Geerligs et al., 2011; Norberg et al., 2021). However, there are only a limited number of studies characterizing the shear properties of the cornea using this testing technique. Although, to the best of our knowledge, no previous studies determined corneal viscoelastic shear properties of collagen crosslinked corneas using torsional rheometry, Søndergaard et al. measured the corneal resistance to shear force after CXL therapy using a custom engineered biaxial biomechanical setup (Søndergaard et al., 2013). Despite valuable insight that this work provided, it was focused on static shear measurements; thus, it did not characterize viscoelastic shear properties of the cornea. In the torsional rheometry, the response of samples subjected to applied oscillatory angular deformation or torque is determined. Thus, the effects of CXL on viscoelastic shear properties, i.e. both viscous and elastic components, can be quantified.

The primary objective of the present work was to extend our previous study (Hatami-Marbini, 2014) and characterize the influence of the CXL procedure on viscoelastic shear properties of the cornea using oscillatory shear tests. For this purpose, we used the original CXL procedure, a.k.a. Dresden protocol, to crosslink corneal samples. We then performed oscillatory shear experiments to characterize the effects of the CXL therapy on corneal dynamic shear modulus. We performed both strain sweep and frequency sweep dynamic shear tests on the samples at various levels of axial compression. The stiffening effects of the CXL procedure on shear properties of the cornea were reported and discussed as a function of the applied frequency and shear strain at different levels of compressive strain.

2. Materials and methods

We brought porcine eye globes from a local abattoir to the laboratory by taking appropriate precautions. From the eye globes, corneal samples with approximately 2 mm scleral rims were prepared. The blunt edge of a scalpel was used to separate epithelial and endothelial layers. The samples were divided into three groups: control group (n = 5), pseudo-crosslinked group (n = 5), and crosslinked group (n = 5). The samples in the crosslinked group were crosslinked using the original CXL procedure. To this end, corneal scleral skirts were placed in 0.1% riboflavin and 20% dextran and were allowed to reach their equilibrium hydration/thickness state. The thickness variation during this period was measured by a pachymeter (DGH Technology, Inc., Pennsylvania) and the samples were removed from the solution when no significant thickness variation was observed between two consecutive thickness measurements. This initial soaking period was included in order to avoid significant thickness variation during instillation of hyperosmolar riboflavin solution drops. The samples were then placed on a hemispherical stage and irradiated with UVA light for 30 min with an intensity of 3 mW/cm^2 and a wavelength of 370 nm. During the UVA irradiation and in order to impregnate samples adequately with the photosensitize solution, extra drops of riboflavin dextran solution were instilled every 5 min. The samples in the pseudo-crosslinked group underwent the exact same procedure with the only difference that UVA light was turned off during the final 30-min-long step of the CXL protocol. All crosslinked and pseudo-crosslinked samples were washed in phosphate buffered saline (PBS) and a biopsy trephine was used to excise 8 mm disks from their center.

The oscillatory shear tests were performed using a DHR-2 rheometer (TA Instruments, Delaware) The oscillatory rheology is a commonly used technique for characterizing the viscous and elastic shear response of materials, Fig. 1. In this experimental method, the samples are subjected to oscillatory displacement and resulting forces are measured using a force transducer. The shear strain γ and shear stress τ can be represented as

$$\gamma = \gamma_0 \exp(i(\omega t - \delta)) \\ \tau = \tau_0 \exp(i\omega t) \quad (1)$$

where ω=2πf is the angular frequency, f is the ordinary frequency, δ is phase angle, t is time, τ_0 and γ_0 are shear stress and strain magnitude, respectively. The complex shear modulus G* relates the shear stress to shear strain. The storage modulus G′, and loss modulus G″ can be written as:

$$G' = |G^*|\cos\delta \\ G'' = |G^*|\sin\delta \quad (2)$$

where |G*| is the norm of complex shear modulus. The storage modulus represents the ability of materials to store energy elastically while the loss modulus denotes their ability to dissipate stress. The norm of complex modulus is a measure of materials' shear stiffness.

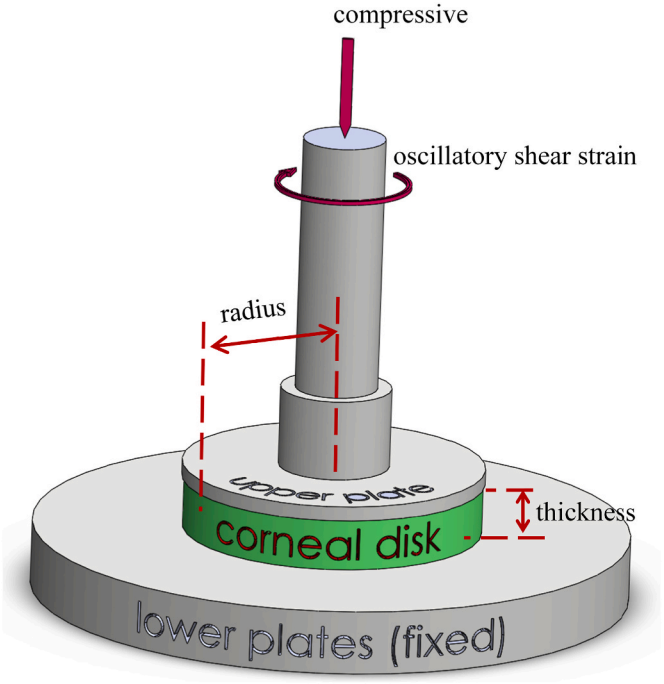

Fig. 1. *A* schematic plot of the dynamic shear experimental setup. The corneal disks of 8 mm diameter were placed between the lower and upper plates of a rheometer in order to measure their dynamic shear properties at different levels of compressive strain. The strain sweep (with amplitude of 0.01% to 10%) and frequency sweep (with frequencies of 0.01 Hz to 2 Hz) torsional shear experiments were performed.

The dynamic torsional shear experiments were done using the protocol that we used in our previous work (Hatami-Marbini, 2014). Briefly, all corneal disks were placed in PBS solution until their thickness reached about 1000 μm. Then, they were mounted in the rheometer's submersion chamber filled with PBS solution. In order to prevent any slippage, sandpaper was used at upper and lower loading plates of the rheometer. The physiological swelling pressure of the porcine cornea is about 52 mm-Hg (Hatami-Marbini et al., 2013). The constant tare stress of 3.5 KPa was used in order to determine shear properties at relevant levels of physiological swelling pressure. After applying the tare load, the samples were subjected to strain sweep experiments with shear strains of 0.01%–10% and constant frequency of 1 Hz followed by frequency sweep experiments at shear strain magnitude of 0.2% and frequencies of 0.01–2 Hz. The engineering strain ε was defined as $\Delta t/t_0$ where t_0 is the thickness of samples at tare stress and Δt is the change in their thickness. The rheometer head was brought down with a displacement rate of 1 μm/s to compress corneal disks to 10% strain. After allowing the samples to relax for 30 min at this compressive strain, they were subjected to another set of strain and frequency sweep experiments. The above procedure was repeated until shear properties at ε = 0%, 10%, 20%, 30%, and 40% were characterized. From the experimental measurements, shear storage and loss moduli were calculated at each compressive strain. Furthermore, the engineering compressive stress was calculated from dividing the axial force F at the end of the 30 min relaxation period of each ramp compressive strain by the initial cross-sectional area A_0 of the samples. The one-way ANOVA tests were done to compare the experimental data obtained for different groups. The p-value of 0.05 was used to find any statistically significant variation. Furthermore, the Shapiro-Wilk test was used to confirm that experimental measurements were normally distributed.

3. Results

The thickness of the samples at the beginning of experiments, i.e., after applying the tare load, was 930 ± 50 μm, which agrees with the estimated thickness obtained from the equilibrium compressive stress versus thickness relation (Hatami-Marbini et al., 2013). The storage and loss moduli of samples in the control and crosslinked groups as a function of magnitude of the applied shear strain are shown in Fig. 2. The measured shear modulus was a function of applied axial compressive strain and increased with increasing compressive strain for all samples. At all levels of compressive strain and for both crosslinked and untreated samples, the variation of loss and storage moduli was almost constant (the range of linear viscoelasticity) when the applied shear strain was small ($\gamma_0 < 1\%$). Fig. 3 shows the variation of shear modulus as a function of frequency at different compressive strain levels. The storage modulus increased with increasing frequency and/or increasing compressive strain. The results for samples in the pseudo-crosslinked group are not shown for brevity but their behavior was similar to the response of untreated samples.

4. Discussion

The present study was done to characterize the effects of CXL treatment on corneal dynamic shear properties. The strengthening effects of this treatment procedure have been commonly investigated by performing uniaxial tensile experiments (Hatami-Marbini and Jayaram, 2018a; Hatami-Marbini, 2018; Wollensak and Iomdina, 2009; Lanchares et al., 2011; Chang et al., 2018). This testing technique examines the mechanical response of isotropic materials and has also been used to characterize the nonlinear viscoelastic response of the cornea (Boyce et al., 2007; Elsheikh and Anderson, 2005; Hoeltzel et al., 1992; Boschetti et al., 2012; Hatami-Marbini and Rahimi, 2015a). However, the cornea is a transversely isotropic material with significantly different in-plane and out-of-plane material properties (Hatami-Marbini and Maulik, 2016; Pitre et al., 2020; Bryant and McDonnell, 1996). The shear stiffness of the cornea has been previously studied and it was found that it is significantly lower than its tensile stiffness

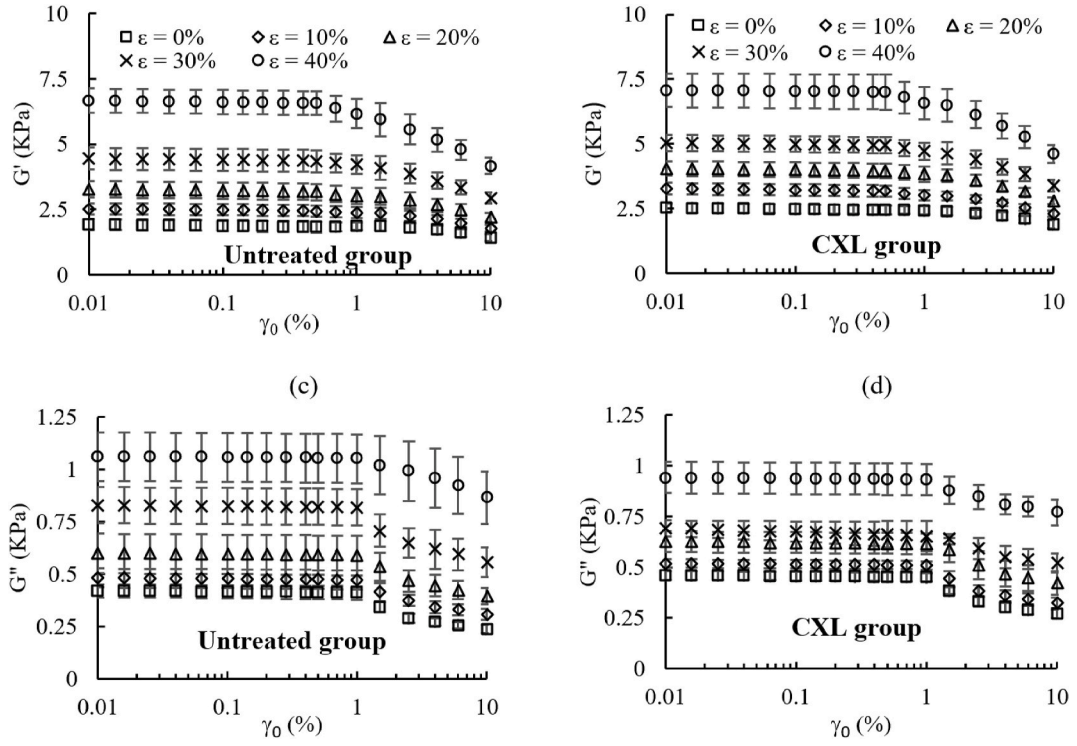

Fig. 2. The effect of CXL treatment on the variation of storage shear modulus G' (a and b) and loss shear modulus G" (c and d) as a function of amplitude of the applied shear strain γ_0. The dependence of storage and loss shear moduli on the applied compressive strain ε is also shown. The strain sweep experiments were done at a frequency of 1 Hz. The symbols denote the average of experimental measurements and error bars indicate one standard deviation.

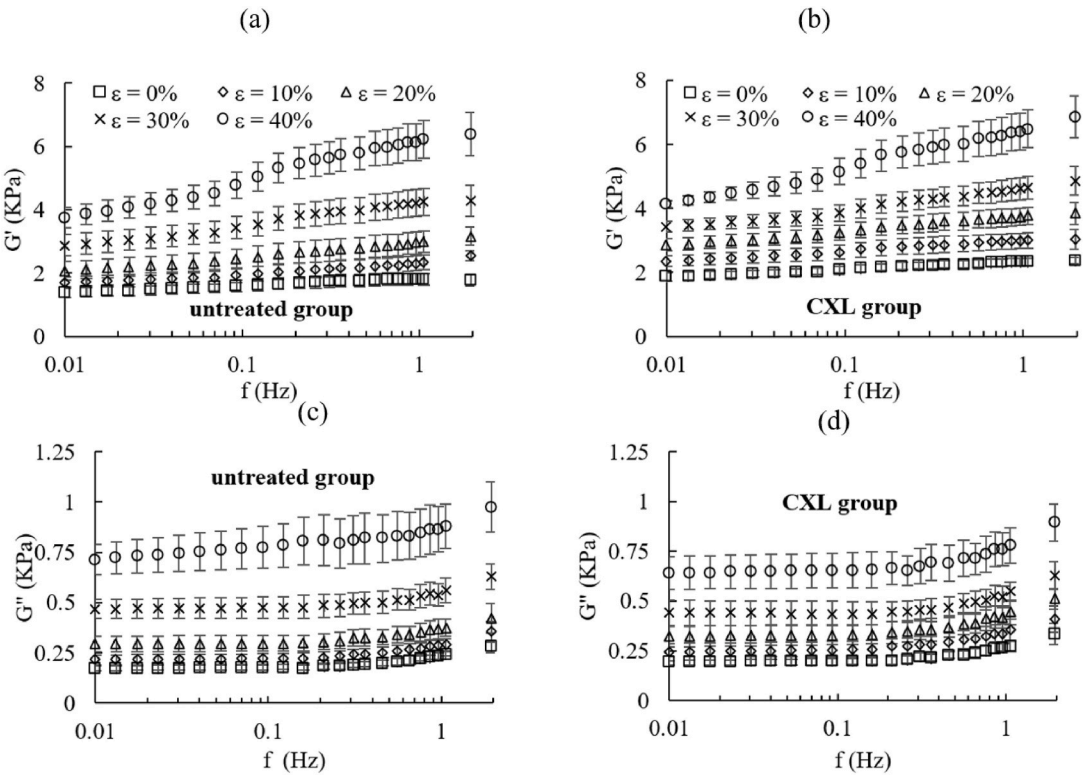

Fig. 3. The effect of CXL treatment on the variation of storage shear modulus G' (a and b) and loss shear modulus G" (c and d) as a function of frequency. The dependence of storage and loss shear moduli on the applied compressive strain ε is also shown. The frequency sweep experiments were done within the range of linear viscoelastic at shear strain magnitude of $\gamma_0 = 0.2\%$. The symbols denote the average of experimental measurements and error bars indicate one standard deviation.

(Hatami-Marbini, 2014; Petsche et al., 2012; Sloan et al., 2014). However, viscoelastic shear properties of collagen crosslinked corneas have not been characterized. The shear stiffness of the cornea is a measure of its resistance against shear deformation that, for example, is caused by eye rubbing (McMonnies, 2009; Liu and Lin, 2009). Experimental measurements of corneal shear properties and possible stiffening effects of CXL are very limited in the literature (Søndergaard et al., 2013). A better understanding of the effects of the CXL therapy on corneal shear properties is necessary and it could be helpful for recent efforts in characterizing the mechanical response of cornea using optical coherence elastography (OCE) (Ramier et al., 2020; Lan et al., 2021). OCE uses a mechanical loading apparatus to produce elastic shear waves, whose velocity is proportional to shear stiffness, and an optical coherence tomography imaging system for detecting the tissue response.

The strain sweep experiment results, Fig. 2, showed that the cornea has a linear viscoelastic shear behavior if the magnitude of the applied shear strain is less than critical shear strain γ_{cr}. Within the range of linear viscoelasticity, the measured shear response is independent of shear strain amplitude. We estimated the critical shear strain as the shear strain for which the average shear modulus decreased by 5% of its initial value at $\gamma = 0.01\%$. Fig. 2 shows a qualitatively similar variation of the shear modulus with shear strain at all levels of applied compressive strain. We plotted the normalized storage shear modulus as a function of the shear strain in Fig. 4. A characteristic curve, independent of the compressive strain and CXL treatment, was found. The exact value of the critical shear strain γ_{cr} slightly depends on the applied compressive strain and whether the samples were crosslinked or not. However, a linear viscoelastic response was observed for all samples when $\gamma < 1\%$, which is in agreement with previous studies on cornea, articular cartilage, and brain tissue (Hatami-Marbini, 2014; Zhu et al., 1993; Bilston and Miller, 2011). Fig. 4 also shows that when the applied shear strain becomes larger than the critical shear strain, the complex shear moduli

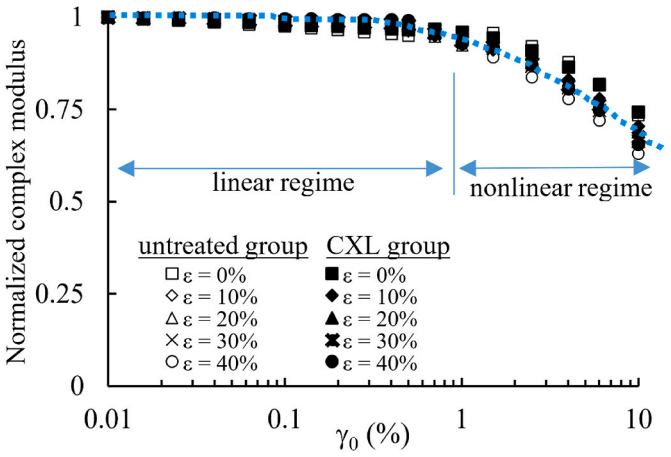

Fig. 4. The normalized complex modulus of crosslinked and untreated samples as a function of the shear strain magnitude in strain sweep experiments. The experiments were done using compressive strain from 0% to 40% and at a frequency of 1 Hz. A characteristic curve, shown by the dashed line, independent of the compressive strain and whether the samples were crosslinked, is observed. The filled and unfilled symbols show the measurements obtained for the corneal disks in crosslinked and untreated groups, respectively. The range of linear viscoelasticity is also shown.

decrease nonlinearly. The corneal tensile modulus, as measured by uniaxial strip testing and inflation experiments, increases nonlinearly with increasing the applied strain (Elsheikh and Anderson, 2005; Boschetti et al., 2012). This nonlinear increase is because of nonlinear mechanical response of collagen fibrils and their rearrangement in the loading direction. However, collagen fibrils play a lesser role in defining

corneal shear properties and the interfibrillar proteoglycan matrix and their interaction with collagen fibrils are primarily responsible for the corneal shear response. With increasing the shear strain magnitude, the matrix domain gets damaged resulting in a lower shear modulus, i.e. shear moduli decrease nonlinearly with increasing shear strain. It is noted that the same response was observed in both crosslinked and control samples.

In Fig. 5, we plotted the average measured shear storage modulus obtained from strain sweep experiments within the range of $0 < \gamma < 1\%$ and compared shear properties of samples in different groups. The shear modulus increased significantly in all samples with increasing axial compression ($P < 0.05$). No significant difference was found between the shear modulus of corneal buttons in pseudo-crosslinked and untreated groups; however, crosslinked samples had significantly larger shear modulus than untreated samples when the compressive strain was lower than 30%. Although at 40% compression, the shear modulus of crosslinked samples was larger than that of untreated samples, the difference was insignificant ($P = 0.12$). The possible reason for this insignificant difference will be discussed later.

The corneal extracellular matrix is composed of collagenous lamellae that are stacked on top of each other. The collagen fibrils in adjacent lamellae run in different orientations but lie primarily parallel to corneal surface, especially in the posterior and middle regions of the stroma. The individual lamellae consist of collagen fibrils of almost uniform diameter that are embedded in a hydrated matrix composed of proteoglycans. Because of its unique architecture, the corneal stroma behaves as a transversely isotropic material, i.e. its in-plane tensile and out-of-plane compressive properties are (orders of magnitudes) different (Hatami-Marbini, 2014; Hatami-Marbini and Etebu, 2013a). The proteoglycans consist of negatively charged glycosaminoglycans, which attract water molecules inside corneal extracellular matrix and fill the gap between collagen fibrils. The hydrated proteoglycan matrix and its interaction with collagen fibrils provide the cornea with shear strength. The

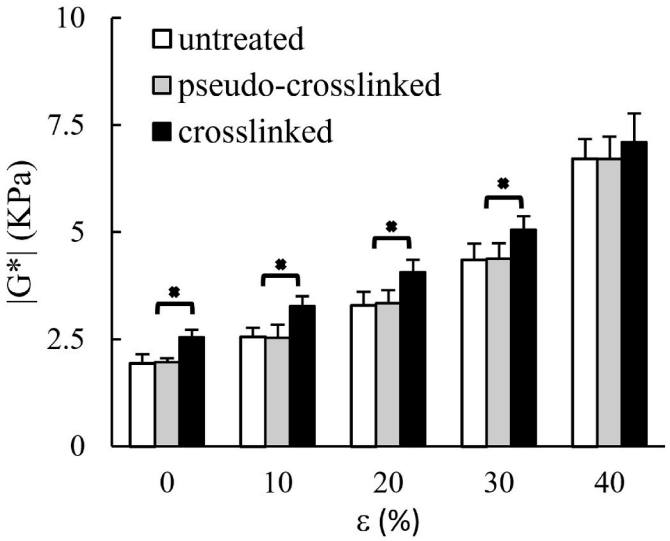

Fig. 5. The effect of corneal collagen crosslinking on the complex shear modulus $|G^*|$, averaged over the range of linear viscoelasticity, at different levels of compressive strain ε. The strain sweep experiments were done at frequency 1 Hz. No significant difference was observed between the shear response of pseudo-crosslinked and untreated samples; however, the crosslinking treatment significantly increased the magnitude of complex shear modulus when the compressive strain was less than 40% (no significant difference was observed at $\varepsilon = 40\%$). The asterisk indicates significant differences between the two groups ($p<0.05$). The complex shear modulus of all samples increased significantly with increasing the compressive strain (no asterisk was shown for these groups so that the plot does not get crowded). The error bars indicate one standard deviation.

three-dimensional electron microscopic reconstructions of the cornea have shown that the proteoglycan network binds and connects adjacent collagen fibrils (Lewis et al., 2010). In particular, proteoglycans are attached to collagen fibrils via their core proteins while their glycosaminoglycan side chains form interfibrillar duplexes acting as tiny ropes tying collagen fibrils together (Scott and Bosworth, 1990), Fig. 6. With increasing the compressive strain, short-range interactions between proteoglycans, glycosaminoglycan side chains, and collagen fibrils become more pronounced resulting in large shear resistance to the applied deformation. We found that the complex shear modulus of untreated samples varies between 2 KPa and 7 KPa, which agrees with previous studies (Hatami-Marbini, 2014). It is noted that the shear modulus reported by Søndergaard et al. for untreated porcine samples was less than 1 KPa, which we believe is because they used a significantly different testing technique, i.e. a custom engineered biaxial biomechanical setup (Søndergaard et al., 2013). They also reported shear modulus of about 2 KPa for human cornea, which is significantly lower than what was found previously using the torsional shear rheology for human donor cornea (Petsche et al., 2012).

The exact mechanisms of CXL at the molecular level, i.e. the nature of crosslinks and their location in corneal extracellular matrix, have not yet been fully understood. The corneal collagen crosslinking involves mainly carbonyl-based crosslinks that mainly occur within collagen molecules themselves and between collagen and proteoglycan core proteins (McCall et al., 2010; Zhang et al., 2011). The in vitro swelling studies on crosslinked corneas suggested that crosslinking occurs within and between molecules on the surface of collagen fibrils, and within proteoglycans surrounding them (the interfibrillar space) (Hayes et al., 2013). The CXL significantly increases the tensile tangent modulus by about 80–100% (Wollensak et al., 2003a; Hatami-Marbini and Rahimi, 2015a). Here, we observed that the CXL treatment increased the storage shear modulus by 32%, 29%, 24%, 14%, and 7% at compressive strain $\varepsilon = 0$, 10%, 20%, 30%, and 40%, respectively. The previous studies from our groups and others suggest that corneal shear stiffness is primarily due to the proteoglycan-rich matrix and its interaction with collagen fibrils (Hatami-Marbini, 2014; Hatami-Marbini and Etebu, 2013a; Ramier et al., 2020). Thus, the CXL therapy improved the mechanical properties of interfibrillar and interlamellar matrix, Fig. 6. This hypothesis agrees with a recent study that reported CXL caused a significant decrease in solute diffusion in porcine cornea (Hepfer et al., 2021). The permeability of the cornea is proportional to the porosity of its ultrastructure, i.e. it has an inverse relation with corneal mechanical properties. As the collagen and proteoglycans matrix become further packed with increasing compression, the corneal permeability decreases and its structural integrity increases (Hatami-Marbini and Etebu, 2013b).

The effect of CXL procedure on frequency dependent shear properties of corneal samples is shown in Fig. 3. The frequency sweep experiments were done within the range of linear viscoelasticity. With increasing the frequency of the applied shear strain, the storage shear modulus increased, Fig. 7. Furthermore, the storage shear modulus increased significantly at any constant frequency with increasing the compressive strain. Finally, the CXL therapy significantly increased the frequency dependent shear properties of porcine corneal samples, Fig. 7. Under physiological conditions, the cornea is constantly subjected to dynamic loadings because of eye movement and intraocular pressure fluctuations. The cornea in above instances undergoes dynamic loadings with a wide range of frequencies. Increasing the frequency from 0.1 Hz to 2 Hz increased the average shear storage modulus at $\varepsilon = 0\%$ from 1.4 KPa to 1.8 KPa for untreated corneal samples and from 1.8 KPa to 2.4 KPa for the crosslinked group. No significant difference between complex modulus of untreated and pseudo-crosslinked samples was found ($P > 0.05$). The shear storage modulus quantifies the overall stiffness of a material. Thus, the CXL therapy increased the shear stiffness of the cornea and the amount of energy that it could store elastically in a cycle of deformation. The loss modulus is a measure of damping or energy

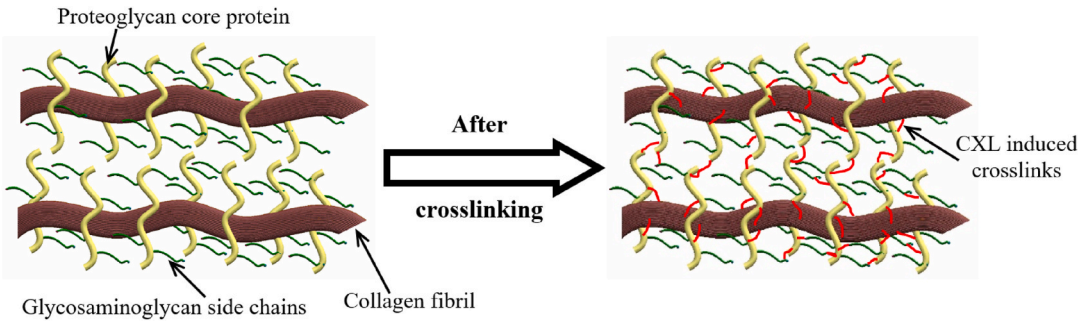

Fig. 6. A schematic showing that the CXL treatment creates additional crosslinks in corneal extracellular matrix increasing its structural integrity. The exact location of these crosslinks is not known but they possibly form between proteoglycans core proteins and collagen molecules, within collagen molecules, between glycosaminoglycan side chains and core proteins of proteoglycans, and between the proteoglycan core proteins (Hatami-Marbini and Etebu, 2013b).

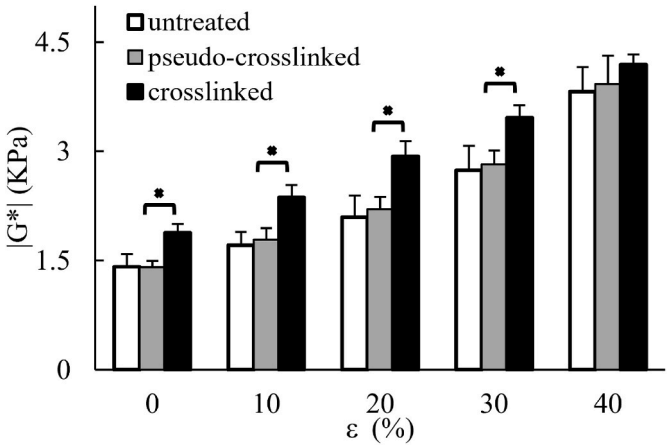

Fig. 7. The effect of corneal collagen crosslinking on the complex shear modulus, obtained from torsional shear tests done at shear strain 0.2% and at frequency 0.01 Hz, as a function of the applied compressive strain. No significant difference was observed between the shear response of pseudo-crosslinked and untreated samples; however, the crosslinking treatment significantly increased the magnitude of complex shear modulus when the compressive strain was less than 40% (no significant difference was observed at $\varepsilon = 40\%$). The asterisk indicates significant differences between the two groups ($p<0.05$). The complex shear modulus of all samples increased significantly with increasing the compressive strain (no asterisk was shown for these groups so that the plot does not get crowded). The error bars indicate one standard deviation.

dissipation capability of materials. The collagen crosslinking treatment decreased the damping ability of the cornea. The reduced damping means that dynamic processes will be continued over longer times, i.e. less energy dissipation and more oscillation. The significant reduction of damping ability of cornea because of the CXL procedure has been previously reported using dynamic uniaxial testing technique (Hatami-Marbini and Rahimi, 2015b). The present study confirmed that the CXL treatment has a similar effect on shear properties of the cornea. Our find, which agrees with a previous study that used wave based OCE to show a loss of tissue viscosity after crosslinking (Han et al., 2017).

There are a number of applications in which the range of corneal deformation is large. For example, Ocular Response Analyzer, an air puff tonometer, measures the inward and outward motion of corneal apex to quantify its hysteresis. The present work did not fully characterize the nonlinear viscoelastic shear properties of the cornea when the tissue is subjected to large deformation.. Furthermore, we did not consider the effect of age or breed of the animals. The samples may not have been from different animals, i.e., a pair might have come from the same animal; we did not consider this possibility in the statistical analysis. Another drawback could be the use of porcine corneas instead of human corneas (Zeng et al., 2001; Hatami-Marbini and Jayaram, 2018b). The microstructure of samples may have been damaged during the dissection procedure and during torsional shear experiments. In particular, possible adverse effects of sandpaper may have prevented us to have an accurate estimate of shear properties at large compressive strain levels. This could be why we did not see a significant CXL effect at 40% compressive strain. Although we believe that the measured loss modulus is primarily related to energy dissipation in the material, other dissipative phenomena, such as damage mechanisms and the friction between the sandpaper and samples, may have played a role. This means that reported moduli from this study may include the effects of these other dissipative phenomena. The increase in corneal shear stiffness as a function of the ultraviolet intensity, riboflavin concentration distribution, and treatment time can be obtained using theoretical models of corneal crosslinking procedure in future studies (Schumacher et al., 2012). Furthermore, the present work reported shear properties using an in vitro experimental technique and did not characterize in vivo properties. We expect corneal shear properties to be a function of intraocular pressure (IOP) since we found a significant correlation between shear moduli and compressive strain. Future studies are needed to better understand the relation between IOP and mechanical properties. The Although Shapiro-Wilk test showed that the data were normally distributed, the low number of corneal samples should be taken into account when considering mechanical measurements of the present work. Finally, previous studies have clearly shown that mechanical properties of anterior and posterior parts of the cornea are significantly different (Hatami-Marbini and Jayaram, 2018a; Hatami-Marbini, 2018; Petsche et al., 2012). Not considering inhomogeneous properties of the cornea may be seen as a other limitation of the current work.

In summary, the present study characterized the effects of the CXL therapy on viscoelastic properties of the cornea as a function of compressive strain by performing dynamics torsional shear experiments. In addition to the compressive strain, the shear properties were dependent on the shear strain magnitude and loading frequency. The corneal samples showed linear viscoelastic shear behavior when the applied shear strain was less than 1%. Within the range of linear viscoelasticity, the measured shear response was independent of shear strain amplitude. The complex shear modulus decreased nonlinearly when the applied shear strain increased from 1% to 10%. Furthermore, the complex shear modulus increased nonlinearly with increasing the loading frequency and compressive strain. The present work showed that althgouh the CXL treatment significantly increased the storage shear modulus of corneal samples, it caused significant reduction of their damping ability. The stiffening effect of CXL treatment on shear storage modulus was less than its influence on tensile properties and was inversely proportional to the applied compressive strain. The above observations for the effects of the CXL therapy on corneal viscoelastic properties were explained in terms of changes in corneal microstructure caused by this treatment procedure. The findings of the present work on corneal viscoelastic shear

properties can be used for both analyzing and developing better numerical models for experimental measurements that are obtained using in vivo techniques such as Ocular Response Analyzer and optical coherence elastography.

CRediT authorship contribution statement

Hamed Hatami-Marbini: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Md Esharuzzaman Emu:** Writing – review & editing, Writing – original draft, Validation, Software, Methodology, Investigation, Formal analysis, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

This work has been supported in part by grants from the National Science Foundation (1635290) and the National Institutes of Health (R21 EY030264).

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