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Collagen cross-linking treatment effects on corneal dynamic biomechanical properties

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ABSTRACT

Cornea is a soft tissue with the principal function of transmitting and refracting light rays. The objective of the current study was to characterize possible effects of the riboflavin/UVA collagen cross-linking on corneal dynamic properties. The original corneal cross-linking protocol was used to induce cross-links in the anterior portion of the bovine cornea. A DMA machine was used to conduct mechanical tensile experiments at different levels of tensile strains. The samples were divided into a control group ($n = 5$) and a treated group ($n = 5$). All specimens were first stretched to a strain of 5% and allowed to relax for twenty minutes. After completion of the stress-relaxation experiment, a frequency sweep test with oscillations ranging from 0.01 to 10 Hz was performed. The same procedure was repeated to obtain the stress-relaxation and dynamic properties at 10% strain. It was observed that the collagen cross-linking therapy significantly increased the immediate and equilibrium tensile behavior of the bovine cornea ($P < 0.05$). Furthermore, for all samples in control and treated groups and throughout the whole range of frequencies, a significantly larger tensile storage modulus was measured at an axial strain of 10% compared to what was obtained at a tensile strain of 5%. Finally, it was noted that although this treatment procedure resulted in a significant increase in the storage and loss modulus at any axial strain and frequency ($P < 0.05$), it significantly reduced the ratio of the dissipated and stored energy during a single cycle of deformation. Therefore, it was concluded that while the riboflavin/UVA collagen cross-linking increased significantly corneal stiffness, it decreased significantly its damping capability and deformability. This reduced damping ability might adversely interfere with corneal mechanical performance.

The cornea transmits and reflects the light while protecting the inner contents of the eye. Corneal collagen cross-linking therapy has recently been introduced as a potential treatment option for halting the progression of keratoconus (Caporossi et al., 2010; Mazzotta et al., 2007; Wollensak, 2006). In this procedure, the strength and stiffness are increased by using the photosensitizer riboflavin solution and ultraviolet (UVA) to induce cross-links in the anterior portion of the corneal stroma (Søndergaard et al., 2013; Spoerl et al., 1998; Wollensak and Iomdina, 2009; Wollensak et al., 2003b). Till to date, the influence of this therapeutic intervention on corneal hydrodynamic behavior, curvature, collagen fibril diameter, keratocytes, enzymatic digestion, and endothelial cells has been studied (Bottos et al., 2008; Bottós et al., 2010; Coskunseven et al., 2009; Hayes et al., 2013; Spoerl et al., 2007, 2004; Wollensak et al., 2007, 2003a, 2004a, 2004b). Since the primary objective of this treatment option is to biomechanically strengthen the cornea, its effectiveness is directly related to the amount of improvement in biomechanical properties. The mechanical tests such as the strip extensometry have been commonly used to assess the stiffening effect of the riboflavin/UVA treatment on static (equilibrium) mechanical properties. Nevertheless, from the mechanics point of view, the cornea is a rate-dependent viscoelastic material and there is little known about the possible effects of collagen cross-linking on dynamic properties. In fact, the cornea may be subjected to vibrations of order 0.1–100 Hz in *in vivo* conditions (Kaplan and Bettelheim, 1972). In addition to vibrations coming from the external objects (such as those caused in a car accident or by an explosion), sudden variations in the intraocular pressure, and rapid contraction and relaxation of muscle fibers could apply dynamic loadings. Therefore, it is important to characterize the influence of riboflavin/UVA collagen cross-linking on dynamic and viscoelastic response of the cornea. The dynamic mechanical analysis (DMA) is a powerful experimental technique

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for investigating viscoelastic properties of materials. In this testing method, the viscoelastic properties are investigated by applying a cyclic strain (stress) and measuring the resulting stress (strain). This testing method has been widely used to characterize material properties of different tissues such as ligament, brain tissue, blood vessel, lens, and tendon (Fallenstein et al., 1969; Patel et al., 1973; Schechtman and Bader, 1994; Tanaka et al., 2007; Weeber et al., 2005); nevertheless, fewer studies used this technique to investigate the dynamic properties of eye components (Bettelheim and Wang, 1976; Kaplan and Bettelheim, 1972; Weeber et al., 2005). For example, a recent study used the DMA to examine regional variations of dynamic mechanical properties of the sclera (Palko et al., 2011). Furthermore, dynamic compression and shear experiments were used to show that vitreous and lens have a relatively high viscosity and could therefore dissipate a large portion of the external energy (Bettelheim and Wang, 1976; Weeber et al., 2005). The tensile and shear dynamic properties of the cornea and its relation with temperature and hydration have also been studied (Hatami-Marbini, 2014b; Kaplan and Bettelheim, 1972).

The primary objective of the present study was to perform the dynamic analysis and investigate how riboflavin/UVA collagen cross-linking procedure affects the viscoelastic biomechanical properties of the bovine cornea. For this propose, five bovine samples were used for the control group and five for the collagen cross-linked group. The samples were prepared by first using a scissor to dissect corneal scleral rings from the intact eyeglobes obtained from a local slaughterhouse. After removing the corneal epithelium, the original collagen cross-linking procedure was used to induce cross-links (Wollensak et al., 2003b). Briefly, two UV LEDs with 365 nm wavelength (Roithner lasertechnik, Vienna, Austria) were used with a resistor in series; the resistor was chosen in order to have an intensity of 3 mW/cm^2 in the 2 cm distance. The desired intensity of 3 mW/cm^2 at a distance of 2 cm was calibrated with a UVA meter (Solartech, Michigan, USA). The treatment duration was 30 min and drops of riboflavin solution (0.1% riboflavin and 20% dextran T500) were applied every 5 min on the anterior surface of specimens. During the treatment period, the samples were placed on a spherical stand in order to mimic their *in vivo* curvature, Fig. 1. The initial hydration of samples is expected to affect the strengthening effect of the riboflavin/UVA corneal cross-linking. Therefore, prior to the collagen cross-linking treatment, samples were immersed in the 0.1% riboflavin and 20% dextran T-500 solution and their thickness was measured regularly with a pachymeter (DGH Pachette 3, DGH Technology, Pennsylvania, USA) until an equilibrium swelling state was reached. This equilibrium state was taken to be established when the difference in thickness for two subsequent measurements was less than 10%. A custom-built two bladed device was then used to prepare corneal strips (width of about 4–5 mm) from the nasal-temporal direction. Previous studies have shown that hydration significantly affects the mechanical properties of the cornea (Hatami-Marbini and Etebu, 2013b; Hatami-Marbini and Rahimi, 2014b). Therefore, prior to mechanical testings, the specimens were air-dried or swelled in OBSS (ALCON, Texas, USA) to reach to an average thickness of 900 μm. This is done in order to ensure that all experiments were done on samples with *almost* similar hydration. An RSA-G2 DMA machine (TA Instruments, Delaware, USA) was used to conduct the dynamic testings. Sandpapers were glued to the grips of the device to prevent possible slippage and the submersion chamber was filled with the solution. The distance between the grips was approximately 5–7 mm in order to be able to test the cross-linked portion of the treated samples. Prior to the experiments, all samples were subjected to a preconditioning procedure consisting of five loading/unloading cycles and three stress relaxation steps (Hatami-Marbini and Rahimi, 2014a, 2014b). This preconditioning step was included in order to have samples with a similar stress history and remove possible contribution of the load-history from the measured properties. Before performing the experiments, the strips were allowed to recover for five minutes. Fig. 1a shows a schematic plot of the experimental procedure. Following the preconditioning and recovery period, 100 mN tare force was applied to remove any slack and determine the initial length. The samples were then stretched to a strain of 5% and 10% with a displacement rate of 5 mm/min. At each level of strain, the strips were allowed to relax for 1200 s before preforming the frequency sweep experiments with oscillation frequency ranging from 0.01 to 10 Hz and a strain amplitude of 0.1%. In the dynamic mechanical analysis (DMA), the viscoelastic properties are found by applying a cyclic strain $\varepsilon = \varepsilon_0 \sin(2\pi ft)$ and measuring the resulting stress. Within the range of linear viscoelasticity, the stress can be written as $\sigma = \sigma_0 \sin(2\pi ft + \delta)$ where σ_0 is the stress amplitude, f is the frequency of oscillation, t is time, and δ is the phase lag between stress and strain. For a purely elastic solid and viscous fluid, the phase lag will be 0° and 90°, respectively. For a viscoelastic material, the complex tensile modulus $E^* = \sigma/\varepsilon$ can be written as $E^* = E' + iE''$, where the tensile storage modulus E' and tensile loss modulus E'' measure the stored and dissipated portions of the energy, respectively. One way ANOVA test was used to assess the possible significant difference between measured properties; p-values less than 0.05 were considered to be significant.

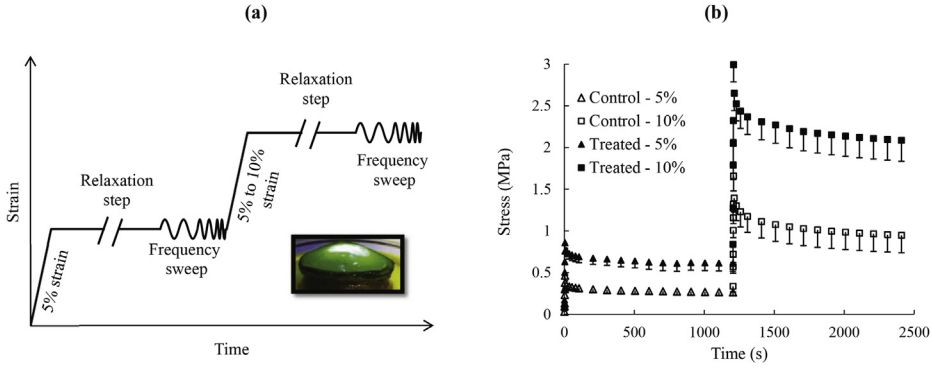

Fig. 1. (a) A schematic plot of the experimental procedure. After applying the preconditioning and the 5 min recovery period, the samples were subjected to a 5% strain with a displacement rate of 5 mm/min and were allowed to relax for 20 min. Frequency sweep experiments with a strain amplitude of 0.1% and frequencies ranging from 0.01 Hz to 10 Hz were then conducted. A similar procedure was repeated to obtain the dynamic properties at 10% strain. The inset shows a typical cross-linked corneal scleral ring. (b) The stress-relaxation behavior of the normal (control) and riboflavin/UVA collagen cross-linked (treated) groups. The symbols denote the average and the bars represent one standard deviation.