Short Communication

The relation between hydration and mechanical behavior of bovine cornea in tension

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ABSTRACT

The cornea is a transparent soft tissue covering the front of the eye. The biomechanical properties of the cornea have been commonly investigated by uniaxial tensile and inflation testing methods. The cornea like many other hydrated tissue swells when immersed in an ionic solution. Previous studies on hydrated tissues have shown that mechanical properties and hydration are closely related. The present study was designed to investigate the effects of thickness (hydration) variation due to swelling/dehydration on non-linear stress-strain response of the bovine cornea. Corneal strips were first air-dried and then soaked in a bathing solution until they reached an average thickness ranging from 0.3 mm to 1.1 mm. Based on their thickness, the samples were divided into different groups and uniaxial tests were performed to measure tensile properties. All experiments were done in mineral oil to prevent any hydration gain or loss during the tests. It was observed that swollen corneas had softer tensile properties in comparison with dehydrated ones. In particular, there was a significant difference between elastic tangent modulus of different groups (P<0.05). It was also shown that tensile behavior of bovine strips at any thickness within the range of 0.4–1.1 mm can be obtained from a single experiment conducted on samples with known thickness (hydration). The findings of the present study confirm that mechanical properties obtained from uniaxial tensile experiments are strongly dependent on thickness (water amount) of samples; therefore, careful attention must be taken in interpreting previous studies which did not fully control the thickness of specimens.

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1. Introduction

The cornea is a transparent tissue covering the front of the eye and is responsible for refracting about two thirds of incoming light. It also protects the eye against external forces such as those caused by eye rubbing. A detailed knowledge of corneal material parameters is essential for developing numerical models which could analyze/predict its mechanical response. From the posterior to anterior, the human cornea is composed of endothelium, Descemet’s membrane, stroma, Bowman’s layer, and epithelium. The stroma is the thickest layer and dominates the mechanical properties. In stroma, many flat sheets of regularly distributed collagen fibrils are embedded in a hydrated proteoglycan matrix. The

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collagen fibrils make up about 70% of cornea’s dry mass and are primarily arranged in a pseudo-regular lattice structure (Foster et al., 2005; Maurice, 1984). The second major component of the stroma is proteoglycans, which consist of a core protein and glycosaminoglycan (GAG) side chains. Corneal proteoglycans belong to the smallest small leucine-rich proteoglycan group whose core protein has a molecular mass of about 30–40 KD. Previous biochemical analysis studies have shown that chondroitin sulfate, dermatan sulfate, and keratan sulfate are prevalent glycosaminoglycans found in the cornea (Hassell and Birk, 2010; Tanihara et al., 2002). The glycosaminoglycans are linear carbohydrate polymers of repeating disaccharide units which become ionized under physiological conditions and carry fixed negative charges. The presence of these negatively charged ions induces strong electrostatic repulsion between the macromolecules and mighty swelling of the corneal stroma. The swelling properties of the cornea have tendency for the corneal stroma to swell when immersed in ionic solution. The swelling properties of the cornea can be strongly rely upon thickness and composition. Thus, it is expected that mechanical behavior of the cornea changes according to thickness (hydration) could explain the existing discrepancies in the mechanical properties of the cornea (Hassell and Birk, 2010; Tanihara et al., 2002). The presence of these negatively charged ions induces strong swelling tendency for the corneal stroma to swell when immersed in ionic solution. The swelling properties of the cornea can be strongly rely upon thickness and composition. Thus, it is expected that mechanical behavior of the cornea strongly rely upon thickness and composition. Thus, it is expected that mechanical behavior of the cornea would change according to thickness (hydration) could explain the existing discrepancies in the mechanical properties of the cornea (Hassell and Birk, 2010; Tanihara et al., 2002). The presence of these negatively charged ions induces strong swelling tendency for the corneal stroma to swell when immersed in ionic solution. The swelling properties of the cornea can be strongly rely upon thickness and composition. Thus, it is expected that mechanical behavior of the cornea would change according to thickness (hydration) could explain the existing discrepancies in the mechanical properties of the cornea.

2. Material and methods

A double blade device was used to excise 5 mm wide strips with 2–3 mm scleral tissue from nasal temporal direction of bovine eyes. All specimens were dissected from nasal temporal direction in order to avoid variation in measurements due to anisotropic material properties of bovine cornea (Boyce et al., 2007). The samples were tested using a custom-built micro tensile device composed of a linear stepper motor (Newmark system, Inc., CA), a submersible load cell (FUTEK, Inc., CA), and serrated grips, Fig. 1a. In order to investigate the effect of hydration, strips of seven different average thicknesses, i.e. 0.3, 0.35, 0.4, 0.5, 0.7, 0.9, and 1.1 mm, were tested. After dissection, the specimens were air-dried and were then immersed in Ophthalmic Balanced Salt Solution (OBSS) until the desired thickness of one of the groups was reached. The thickness was measured with a pachymeter (DGH Technology, Inc., PA) and the linear hydration–thickness relation, i.e. $H_w = 5.3t - 0.67$, was used to obtain the hydration at any thickness (Hedleys and Mishima, 1966). In this relation, $t$ is the thickness in mm and $H_w$ is hydration in mg water/mg dry tissue. In all hydration calculations, the thickness of the specimens at the beginning of the ramp loading was used. To confirm the accuracy of the above equation, the hydration of some of the samples was also obtained using the
The two-step preconditioning procedure including loading/unloading cycles and three successive relaxation tests dividing the recorded force by the initial cross-sectional area was taken as the initial length and the engineering strain was defined as the change in length divided by the initial length. Five samples in each group were tested using an engineering strain of 5% and a displacement rate of 2 mm/min. Furthermore, additional experiments were done on samples with average thickness of 0.9 mm to determine the effects of displacement rate, sample width, and corneal anisotropy. Finally, six corneal strips with average thickness of 0.9 mm were stretched up to failure in order to determine their behavior at high strain level. The stress was calculated from the familiar J-shaped strain–stress curves (Hoeltzel et al., 1992; Woo et al., 1972). In these expressions, ε is the engineering strain, σ is the stress, σ₀ is the initial tare stress and A, B, α, and β are fit parameters. The coefficients of determinations R² were calculated to determine the goodness of the fits. Furthermore, a P-value of 0.05 was selected as the significant level and one-way ANOVA was performed in order to determine the significant difference between mechanical properties of different groups. Data is reported as mean±standard deviation.

3. Results

The images from the camera confirmed that the thickness of samples that were soaked in oil (and not loaded) remained constant; minor transverse contraction was observed after stretching the specimens. Fig. 2 shows the stress–strain behavior of corneal samples with seven different thicknesses. The bovine strips with lower thickness showed a stiffer response. Both exponential and power-law function successfully curve-fitted the stress–strain curves of samples with thickness t>0.35 mm. The exponential fits are not shown but Table 1 gives the fitting parameters as well as the coefficients of determination. Fig. 3 depicts the influence of the loading rate, and sample dimensions, and corneal anisotropy on the measured properties. For this purpose, ten additional corneal strips with thickness of 0.9 mm were tested at displacement rates of 0.2 and 20 mm/min. A stiffer response was obtained with increasing the loading rate. Furthermore, five samples of width 3 mm and thickness 0.9 mm were tested; no significant difference was observed in the measured properties of 5 mm and 3 mm wide samples. Finally, the stress–strain response of five specimens from inferior superior (IS) direction with width 5 mm and average thickness 0.9 mm was measured. Consistent with previous observation (Boyce et al., 2007), the difference between the behavior of IS and NT specimens became significant (P<0.05) at strain larger than 3%. Fig. 4 plots the individual stress–strain curves of six corneal samples which have stretched up to failure. All tests resulted in the familiar J-shaped strain–stress curve, i.e. the strips showed a stiffer response with increasing the stretch.

4. Discussion

The primary purpose of the present study was to show the relation between corneal thickness (caused by hydration changes) and material properties in the uniaxial tensile testing method. To this end, bovine corneal samples of seven different thicknesses were tested and it was shown that swollen corneas had softer tensile properties in comparison with dehydrated specimens. The relation between the hydration and mechanical behavior of soft hydrated tissue has been previously studied (Hatami-Marbini and Etebu, 2013; Hjortdal and Jensen, 1995; Screen et al., 2006; Thornton et al., 2001); nevertheless, this is the first time, to the best of our knowledge, that the effects of corneal hydration in extensometry experiments have been investigated. This testing method has been widely used in the literature to determine the stress–strain behavior of the cornea; previous studies resulted in a wide range of variations for material parameters (Boschetti et al., 2012; Elsheikh and Anderson, 2005; Woo et al., 1972). The difference in reported material properties has often been discussed in terms of inherent differences in sample properties, experimental conditions, and testing protocols among others. The present study showed that lack of control on thickness (due to hydration/dehydration) of
The average thickness of bovine cornea has been estimated to be $0.78 \pm 0.06\text{mm}$ (Kim et al., 2004); thus, it is expected that the samples in these previous studies were swollen. Here, mineral oil was used to prevent swelling and keep the thickness constant. Although the mineral oil has been previously used as bathing fluid (Elsheikh and Anderson, 2005; Lari et al., 2012; Woo et al., 1972), we tested five additional corneal strips in OBSS in order to determine the possible effects of the bathing solution. The OBBS is an iso-osmotic solution commonly used in clinical applications to minimize swelling. A similar experimental protocol was used and the thickness of samples was obtained from the images of the side camera. The corneal samples had an average thickness of 1.03 mm at the beginning of the ramp loading. After completion of tensile experiments, the samples were allowed to relax in the solution for 10 min. The average thickness of the samples became 1.07 mm; this indicated that the swelling rate of the cornea in OBSS at this thickness was small. It is noted that Fig. 2 shows that experimental measurements in OBSS were between stress–strain curves of samples with thickness 0.9 and 1.1 mm. In Fig. 2, samples with different thicknesses had different cross-sectional areas. Therefore, considering the definition of stress, it is possible that variation in thickness of samples might have affected the interpretation of the results. In Fig. 5, we plotted the average force as a function of strain. It is seen that the measured force

<table>
<thead>
<tr>
<th>Thickness (mm)</th>
<th>Hydration (g water/g dry tissue)</th>
<th>$\alpha$ (MPa)</th>
<th>$\beta$</th>
<th>$R^2$ (%)</th>
<th>$A$ (KPa)</th>
<th>$B$</th>
<th>$R^2$ (%)</th>
<th>$\sigma_0$ (KPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.35</td>
<td>1.19</td>
<td>190.3 ± 14.5</td>
<td>1.60</td>
<td>0.04</td>
<td>99.36</td>
<td></td>
<td></td>
<td>437.6 ± 52.0</td>
</tr>
<tr>
<td>0.4</td>
<td>1.45</td>
<td>165.5 ± 27.4</td>
<td>1.64</td>
<td>0.06</td>
<td>99.73</td>
<td></td>
<td></td>
<td>342.2 ± 52.2</td>
</tr>
<tr>
<td>0.5</td>
<td>1.98</td>
<td>128.2 ± 20.7</td>
<td>1.66</td>
<td>0.06</td>
<td>99.55</td>
<td></td>
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<td>255.9 ± 27.8</td>
</tr>
<tr>
<td>0.7</td>
<td>3.04</td>
<td>70.0 ± 10.1</td>
<td>1.63</td>
<td>0.05</td>
<td>99.44</td>
<td></td>
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<td>156.6 ± 33.6</td>
</tr>
<tr>
<td>0.9</td>
<td>4.10</td>
<td>53.4 ± 8.8</td>
<td>1.69</td>
<td>0.07</td>
<td>99.62</td>
<td></td>
<td></td>
<td>93.1 ± 5.5</td>
</tr>
<tr>
<td>1.1</td>
<td>5.16</td>
<td>40.6 ± 3.2</td>
<td>1.69</td>
<td>0.06</td>
<td>99.32</td>
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<td>72.9 ± 14.7</td>
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<td>99.60 ± 20.0</td>
</tr>
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Fig. 3 – The effects of loading rate, $r$, and sample width, $w$, and corneal anisotropy on tensile stress–strain response of bovine corneal strips with average thickness 0.9 mm. The results of the previous study by Boyce et al., 2007 is also shown.

Fig. 4 – Stress strain behavior of six bovine corneal strips with average thickness 0.9 mm up to failure. The average failure strain and stress were about at 31% and 6.9 MPa, respectively.

Fig. 5 – The average measured force of bovine corneal samples with different thicknesses. It is seen that the force and thickness of specimens were closely related.

specimens has significantly contributed to this existing variation. For instance, Boyce et al. and Hoeltzel et al. used bovine samples with thickness of about 1.15–1.30 mm and 1.53 mm, respectively (Boyce et al., 2007; Hoeltzel et al., 1992).
increased with decreasing thickness, for instance, the average force at 5% strain increased from 1.54 N to 3.21 N when thickness changed from 1.1 mm to 0.3 mm. This observation could be explained in terms of biphasic and composite nature of the cornea. In uniaxial experiments conducted on single phase materials, the measured force due to a constant strain is directly proportional to the cross-sectional area of the specimens, i.e. the force increases with increasing the cross-sectional area. From the mechanics viewpoint, the cornea is considered as a composite domain composed of a collagenous phase (with relatively high stiffness) and a proteoglycan-based matrix phase (with fairly low stiffness). The ratio of the weight of water to the tissue dry weight gives the corneal hydration $H_w$. Due to the one-to-one hydration–thickness relation, any increase in thickness is associated with an increase in the volume of water inside the tissue (Hatami-Marbini et al., 2013; Hedbys and Mishima, 1966). Previous X-ray scattering experiments have shown that with increasing the hydration (and when $H_w \leq 1$ mg water/mg dry tissue), the diameter of collagen fibrils remains almost constant and the excess water increases the distance between the collagen fibrils (Fratzl and Daxer, 1993). Therefore, the general rule of mixture theory can be used to conclude that increasing the thickness (hydration) should cause a reduction in the overall stiffness of the corneal samples; which is in agreement with the experimental observations of this study. It needs to be mentioned that although the above discussion provides a general explanation for the relation between hydration and tensile properties, future studies are required to elucidate the underlying molecular-level mechanisms (Cheng et al., 2013; Hatami-Marbini and Pinsky, 2009).

The mechanical behavior of the cornea is rate-dependent (Elsheikh et al., 2011). In Fig. 3, we investigated the effect of loading rate by testing corneal samples with thickness of 0.9 mm, which is close to the physiological value. It was observed that with increasing the loading rate, the tensile stiffness was increased. This observation is in agreement with previous studies (Boyce et al., 2007; Elsheikh et al., 2011). The effect of the width of samples is also shown in this figure. Although the tensile stress of 3 mm specimens at $\varepsilon > 3\%$ was slightly higher than that of the 5 mm wide samples, their difference was not significant. This higher stress might be probably because of anisotropic properties of bovine cornea, i.e. strips from inferior–superior direction are stiffer than those from the nasal–temporal direction, Fig. 3 (Boyce et al., 2007). Fig. 3 shows that the results of the present study were in good agreement with those from Boyce et al. (2007). In addition to variations in the results because of the intrinsic difference in specimens, it should be noted that experimental protocols were not exactly the same, i.e. Boyce et al. (2007) conducted stress-controlled tensile tests while those in the current study were displacement-controlled. The large strain behavior of corneal specimens and their typical failure response is plotted in Fig. 4. The overall behavior of the bovine cornea at large strain was similar to that of the porcine cornea (Boschetti et al., 2012). Nevertheless, the failure strain was...
31%±2% and failure stress was 6.9 MPa±1.0 MPa, which are higher than what previously reported for the porcine cornea. The cornea like many other biomaterials exhibited close to a J-shaped stress–strain curve. In other words, a low stress is initially required for achieving large deformation. In the present study, this phase was not as significant as what has been reported for other soft tissues, e.g., skin; this is possibly because of the initial preconditioning procedure. As the stress is increased, the stress–strain first becomes non-linear and then almost linear. These phases of deformation might have occurred because of straightening out and reorienting collagen fibrils (Veronda and Westmann, 1970).

There exists a one-to-one relation between average thickness and hydration of the bovine cornea, i.e. \( H_w = 5.3 t - 0.67 \) (Hedbys and Mishima, 1966). From this relation, the average hydration of different groups was 0.92, 1.19, 1.45, 1.98, 3.04, 4.10, and 5.16 mg water/mg dry tissue at thicknesses of 0.3, 0.35, 0.4, 0.5, 0.7, 0.9, and 1.1 mm, respectively. For samples in groups with thicknesses 0.3, 0.35, and 0.4 mm, the hydration was also calculated from the definition of hydration, i.e. the ratio of water weight divided by the dry tissue weight; this resulted in average hydration of 0.99, 1.14, and 1.38 mg water/mg dry tissue, respectively. It is seen that the Hedbys and Mishima’s hydration–thickness relation gives a very good estimate for the average hydration of the samples. Using the stress–strain curves shown in Fig. 2, we calculated the tangent modulus as a function of strain. Fig. 6a shows that overall there was a significant difference between tangent modulus of different groups (\( P < 0.05 \)). It is noted that a similar hydration-dependent behavior has been observed in tendons; i.e. the stiffness of tendon was reduced with increasing hydration (Screen et al., 2006). The extracellular matrix of the cornea like many other soft tissues is primarily composed of collagen fibrils and proteoglycans. In particular, regularly distributed collagen fibrils are surrounded by a proteoglycan matrix. The presence of proteoglycan creates a strong tendency for the tissue to swell when immersed in a water-based solution. As schematically shown in Fig. 6b, with increasing thickness (caused by hydration changes), the bonds between collagen fibrils and proteoglycan side chains possibly break and a softer mechanical response is obtained (Cheng et al., 2013; Hatami-Marbini and Pinsky, 2009; Scott, 1991). Similarly, with increasing the thickness, the total fixed charge density (and subsequently the osmotic pressure) decreases. Therefore, it can be proposed that hydration-dependent properties of the cornea are due to the molecular-level interactions between collagen fibrils and proteoglycans.

The experimental measurements were curve-fitted with an exponential \( \sigma = A(e^{0.01} - 1) + \sigma_0 \) and a power-law \( \sigma = w \theta + \sigma_0 \) function. Both of these expressions were able to represent the nonlinear stress–strain response with \( R^2 \) greater than 99%. The thickness (hydration) had a significant effect (\( P < 0.05 \)) on material constants \( A \) and \( \alpha \) of the exponential and power-law functions, respectively. Nevertheless, parameters \( B \) and \( \beta \), which represent the nonlinearity of the stress–strain curves, were almost independent of thickness. Based on this observation, we plotted the normalized stress–strain behavior of different categories in Fig. 7. For this purpose, we used the stress at 5% strain, \( \sigma_{5\%} \), to normalize the stress values.

Interestingly, the data for samples with \( t \geq 0.4 \) mm collapsed into a master curve given by \( \sigma_m = \epsilon_\beta \). In this expression, \( \sigma_m = (\sigma - \sigma_0)/(\sigma_{\%} - \sigma_0) \) and \( \epsilon_\beta = \epsilon / 0.05 \) denote the normalized stress and strain, respectively. The existence of this master curve implies that the thickness- (hydration-) dependent stress–strain behavior of bovine strips at any thickness within 0.4–1.1 mm may be represented by a single experiment at a given thickness. In other words, once the parameter \( \beta \) is determined by curve-fitting the stress–strain curve of corneal strips with a known thickness (and even in a swollen state), the above equation can be used to obtain the tensile properties of corneal strips at any desired thickness. This is an important observation as it enables comparing the results of different experimental studies on samples with different thicknesses (hydration). Nevertheless, it should be noted that the present study only confirmed the existence of this master curve for the selected experimental configuration; its existence in other experimental set-ups remains to be investigated in future studies. In this plot, we also showed the results of tests that were done on corneal specimens with average thicknesses of 0.3 and 0.35 mm (hydration of about 0.99 and 1.14 mg water/mg dry tissue). The stress–strain curve for these specimens was different than those obtained for other groups. Specifically, it was observed that the stress–strain relation for samples with thickness 0.3 mm was almost linear. As discussed earlier, previous studies have shown that below the critical hydration of about 1 mg water weight/mg dry weight, the collagen fibrils start to lose water and become dehydrated (Fratzl and Daxer, 1993). This drying of the collagen fibrils is associated with a structural transformation in corneal microstructure which might be responsible for the significant change that was observed in the tensile behavior, Figs. 2 and 7. It is noted that the exponential and power-law relations that were used to represent the experimental measurements are phenomenological expressions. The cornea is a nonlinear viscoelastic and inhomogeneous material and future research is required to develop a multi-scale multiphasic constitutive model for its mechanical behavior (Hatami-Marbini, 2013).
A limitation of the present study is that samples in each group do not necessarily have similar hydration. In other words, it is possible to have intrinsically thin or thick samples. Nevertheless, the "average" corneal thickness is fairly constant in different animals. For example, the average thickness of bovine cornea was estimated at 0.78 mm with a standard deviation of 0.06 mm (Kim et al., 2004). Therefore, the chance of having a dramatically thinner/thicker sample is not very high. Furthermore, previous swelling studies as well as ours have shown that "on average" the hydration of samples increases with thickness (Hatami-Marbini et al., 2013; Hedbys and Mishima, 1966). Because of the almost 0.2 mm difference in thickness of samples in various groups, it can be assumed that the difference in properties of these groups was mainly due to the amount of water. The uniaxial tensile tests on corneal strips are not able to represent in-vivo mechanical properties of the cornea as a whole. In uniaxial tension tests, the specimens do not necessarily have their natural pre-stressed conditions, damages to the internal microstructure during excision could produce errors, and air-drying the specimens might have modified the natural microstructure of the samples. Furthermore, the in-vivo thickness of tested samples was not known in the present study; therefore, its findings (like many other similar studies) do not necessarily represent the mechanical properties of the original tissue. Moreover, although every effort was made to fully align the strips with the axis of tension and prevent slippage at the grips, the experimental measurements might have still been affected by these factors. Despite its limitations, the uniaxial testing method is well-suited for comparative studies and could be used to investigate the influence of different parameters (e.g., age, breed, and type of samples) on corneal mechanics. For instance, Elsheikh and coworkers used the strip extensometry tests to investigate effects of the loading rate and anatomical orientation on corneal biomechanical properties (Elsheikh and Alhasso, 2009; Elsheikh et al., 2011). They performed tensile experiments on corneal strips dissected from vertical, horizontal, and diagonal directions and demonstrated that the corneal response is nonlinear and anisotropic. Similarly this testing technique has been used to estimate the effects of collagen cross-linking on mechanical properties of the cornea (Wollensak et al., 2003). Despite the wide application of this experimental technique in corneal field, the present study showed that the hydration of samples possibly affected the results of most of previous experimental characterization studies which used uniaxial tests.

In summary, the present research work was a step forward in clarifying the important roles of hydration (thickness) changes due to swelling/de-swelling on tensile properties of the cornea. It was shown that swollen corneal samples had softer tensile properties in comparison with dehydrated ones. Therefore, careful attention must be taken in interpreting experimental studies that do not fully control the thickness. In spite of this important conclusion, this study did not investigate the viscoelastic inhomogeneous properties of the cornea and did not present a microstructure-based model for the experimental observations. Future studies are undergoing in our laboratory to extend the findings of this work and investigate the non-linear inhomogeneous poro-viscoelastic constitutive properties of the cornea.

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REFERENCES


