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The Impact of Intraocular Pressure on Elastic Wave Velocity Estimates in the Crystalline Lens

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Abstract

Intraocular pressure (IOP) is believed to influence the mechanical properties of ocular tissues including cornea and sclera. The elastic properties of the crystalline lens have been mainly investigated with regard to presbyopia, the age-related loss of accommodation power of the eye. However, the relationship between the elastic properties of the lens and IOP remains to be established. The objective of this study is to measure the elastic wave velocity, which represents the mechanical properties of tissue, in the crystalline lens ex vivo in response to changes in IOP. The elastic wave velocities in the cornea and lens from seven enucleated bovine globe samples were estimated using ultrasound shear wave elasticity imaging. To generate and then image the elastic wave propagation, an ultrasound imaging system was used to transmit a 600 µs pushing pulse at 4.5 MHz center frequency and to acquire ultrasound tracking frames at 6 kHz frame rate. The pushing beams were separately applied to the cornea and lens. IOP in the eyeballs was varied from 5 to 50 mmHg. The results indicate that while the elastic wave velocity in the cornea increased from 0.96 ± 0.30 m/s to 6.27 ± 0.75 m/s as IOP was elevated from 5 to 50 mmHg, there were insignificant changes in the elastic wave velocity in the crystalline lens with the minimum and the maximum speeds of 1.44 ± 0.27 m/s and 2.03 ± 0.46 m/s, respectively. This study shows that ultrasound shear wave elasticity imaging can be used to assess the biomechanical properties of the crystalline lens noninvasively. Also, it was observed that the dependency of the crystalline lens stiffness on the IOP was significantly lower in comparison with that of cornea.

Keywords

elastography; crystalline lens; acoustic radiation force; elastic wave; intraocular pressure
I. Introduction

The biomechanical properties of ocular tissues are known to play a significant role in many dysfunctional conditions, such as glaucoma, keratoconus, and presbyopia. Glaucoma is a disease that damages the optic nerve, which can lead to blindness. Previous studies have shown that the stiffness of sclera surrounding the optic nerve head is related to glaucoma by significantly influencing the neural tissue (Burgoyne et al., 2005; Langman et al., 2005; Pavlatos et al., 2016; Sigal et al., 2005). Keratoconus is a visual distortion created by the change in corneal rigidity, causing the cornea to bulge from its normal shape (Zhang et al., 2015; Ruberti et al., 2011). Lastly, presbyopia is the loss of the ability of the eye to change focus onto near objects; most of the previous studies investigated age-related stiffening of the crystalline lens (Charman, 2008; Weale, 2000; Glasser et al., 2001; Heys et al., 2004; Pau and Kranz, 1991; Wu et al., 2015; Yoon et al., 2013; Glasser, 2008). Yet, the exact relationship between the mechanical properties and the impaired functionalities of the eye remains to be established.

Currently, there are no methods to restore the true dynamic accommodative function of the eye. Medical procedures to restore a normal near visual function will significantly improve the quality of life for millions of individuals worldwide who are presbyopic. Most of the investigational approaches for restoration of accommodation are based on the theory that the presbyopia is caused mainly by the progressive hardening of the lens (Glasser, 2008). Therefore, one of the critical barriers to the development and improvement of these procedures is that there is currently no technique to assess elastic properties of the crystalline lens in vivo and in situ.

The complex anatomy of the eye makes it challenging to access the mechanical characteristics of the crystalline lens. In our previous work, we measured elasticity distribution in animal lenses extracted from the eyeballs using bubbles generated within lens (Aglyamov et al., 2007; Yoon et al., 2012, 2013). Also, we showed that the biomechanical properties of the crystalline lens can be assessed in situ using a combination of the acoustic radiation force and optical coherence tomography (OCT) imaging (Wu et al., 2015). In order to measure the elastic property of the soft tissue noninvasively, there have been efforts to use ultrasound shear wave elasticity imaging (Sarvazyan et al., 1998; Bercoff et al., 2004; Chen et al., 2004; Nightingale et al., 2003). Ultrasonic shear wave elasticity imaging, which generates the shear wave using the acoustic radiation force and measures the speed of wave propagation, has been used to measure the mechanical properties of various soft tissues including breast, liver, and muscles (Tanter et al., 2008; Chen et al., 2013; Eby et al., 2013). In a homogeneous and unlimited medium, the relationship between elastic shear modulus (µ) and shear wave speed (c) is \( \mu = \rho c^2 \); where \( \rho \) is the density of the medium (typically density of soft tissue is assumed to be 1,000 kg/m\(^3\)) (Landau and Lifshits, 1959; Sarvazyan et al., 1998). Thus, for stiffer tissues the shear wave speed is higher. Recent studies demonstrated that ultrasound shear wave elasticity imaging can also noninvasively assess the mechanical properties from the layered structure of the eye while providing quantitative elasticity maps of cornea (Nguyen et al., 2014; Shih et al., 2013; Tanter et al., 2009; Nguyen et al., 2011; Touboul et al., 2014). Given that cornea is thin and surrounded by liquids, the elastic waves propagating in the cornea are Lamb waves, which are elastic waves guided by layers.

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Therefore, studies have shown that the relationship between shear modulus and velocity is more complex than the simple relationship ($\mu = \rho c^2$) when applied to evaluate elasticity in the corneal tissue (Han et al., 2017; Han et al., 2015; Tanter et al., 2009).

Numerous studies revealed that the internal pressure and the mechanical properties of tissues can significantly influence each other. Most of biological tissues demonstrate highly nonlinear stress–strain behavior. Elastic nonlinearity results to stiffening of the tissue under internal pressure. For example, escalation of internal pressure of bladder in combination with tissue stiffening can result in bladder dysfunction (Nenadic et al., 2013), and hepatic venous pressure can predict clinical hepatic diseases such as cirrhosis (i.e., stiffening of the liver) (Rotemberg et al., 2012). Studies also investigated the effect of intraocular pressure (IOP) on the mechanical properties of the eye and demonstrated that corneal and sclera stiffness grew with increasing IOP (Litwiller et al., 2010; Li et al., 2016; Pavlatos et al., 2016; Han et al., 2017; Touboul et al., 2014). However, to the extent of our knowledge, there was no investigation of the effect of IOP on the mechanical properties of the crystalline lens.

Given that the location of the crystalline lens is in the anterior segment of the eye, non-invasive measurements of lens elasticity with regard to IOP changes will promote comprehensive understandings of the mechanism of presbyopia, as well as help in developing new approaches for treatments. In this paper, we assess the mechanical properties of the crystalline lens using ultrasound shear wave elasticity imaging and we believe it is the first time the speed of elastic waves in the crystalline lens ex vivo in response to IOP changes is measured. It should be noted that the elastic waves propagating in the crystalline lens are Rayleigh waves (i.e., surface waves) and, therefore, the elastic waves in the cornea and the lens are different types of elastic waves due to boundary conditions (Aglyamov et al., 2015; Nenadic et al., 2011). Therefore, in this paper, the waves generated in both cornea and lens (Lamb and Rayleigh waves in cornea and lens, respectively) are referred to as elastic waves and their speeds of elastic waves are compared as functions of IOP.

II. Materials and Methods

Seven enucleated eye globes (Sierra for Medical Science, Inc., Whittier, CA, USA) of 25–30 months old bovines were used. As shown in Fig. 1, each eyeball with the cornea facing upright was placed in a tank filled with saline solution. In order to vary and measure the IOP, the eyeballs were cannulated with two needles inserted into the anterior chamber. One needle was connected to a saline-filled syringe via tubing to increase the pressure in the eyeball. The second needle was connected to a pressure transmitter (Keller AG, WinterThur, Switzerland) via tubing to measure the IOP. Thus, IOP was increased by infusion of saline from the syringe, and was monitored by the pressure transmitter in real-time.

To generate acoustic radiation force and measure the elastic wave propagation in tissue, an ultrasound imaging system (V1, Verasonics Inc., Kirkland, WA, USA) equipped with an L7-4 linear array transducer (128 elements) was employed. The ultrasound transducer was positioned at the top of the eyeball such that both the cornea and the lens can be visualized as shown in Fig. 1. A pushing pulse with 600 μsec pulse duration and 4.3 MHz center
frequency was used to remotely disturb the cornea and the crystalline lens. The pushing pulses for the cornea and the lens were focused separately at the cornea and the lens. Figure 2 presents a B-mode image showing the anatomy of the bovine eyeball. To acquire a high quality B-mode image, 100 multi-angle ultrasound plane waves at 5.6 MHz center frequency were transmitted swiping the $-16^\circ$ ~ $16^\circ$ sector. Both the anterior and posterior sides of the cornea and the lens are clearly visible in the image. In order to generate acoustic radiation force in the cornea, a pushing beam was focused on the corneal stroma. A pushing beam for the lens was focused on the anterior surface of the lens through the pupil where the iris was open. Both the corneal stroma and the pupil are clearly identified in the B-mode image in Fig. 2. After the pushing pulse, ultrafast ultrasound images were acquired from three plane waves (angles at $-4^\circ$, $0^\circ$, and $4^\circ$, and center frequency at 5.6 MHz) sequentially transmitted at 6 kHz frame rate and coherently compounded (Montaldo et al., 2009). In order to observe the propagation of the elastic wave without on-target interrogation due to a pushing beam, we applied a pushing pulse on the left side of our region-of-interest (ROI, dashed lined box in Fig. 2) and the tracking pulses on right side of the pushing pulse. Propagation of the elastic waves was measured in nasal-to-temporal direction for both the cornea and lens. While the IOP in the eye was increased from 5 to 50 mmHg in 5 mmHg intervals, ultrasound IQ data were collected from the successive pushing and tracking events. For each IOP, three measurements were performed separately for the cornea and crystalline lens. The average value and standard deviation of the thicknesses estimated from ultrasound images of seven bovine eyeball samples were 10.91 ± 0.37 mm and 1.36 ± 0.18 mm for the lens and cornea, respectively.

To estimate the velocity of elastic waves, axial displacements were first estimated using a two-dimensional autocorrelation approach (Loupas et al., 1995) with kernel size measuring two and a half ultrasound wavelengths. Then the displacement estimates were averaged from three neighboring frames in time. Propagation of the elastic wave was visualized by superimposing the axial displacements on the B-mode image. In order to measure the shear wave velocities, the lateral time-to-peak method (Palmeri et al., 2008; McLaughlin and Renzi, 2006a, b) was employed to calculate the group velocity of the wave by tracking the times needed for the wave to reach the peak axial displacements at laterally-offset positions separated by one ultrasound wavelength (i.e., 0.275 mm). Linear regression was then performed on a slope of the time-to-peak displacement versus the lateral position, and a 95% confidence level was used to exclude any outliers. The velocities were calculated over the regions of the cornea and the lens where the waves were propagating to the right side of the shear wave excitations. For each sample and at each level of IOP, three estimates were obtained and the mean and standard deviation of the elastic wave velocities were calculated.

### III. Results

Elastic waves propagating through the cornea and the crystalline lens in the eye globe under 5 mmHg IOP are shown in Fig. 3 and Fig. 4, respectively. The acoustic radiation pushing beam was applied on the cornea (Fig. 3) and at the upper surfaces of the lens (Fig. 4) and propagation of elastic waves was monitored. B-mode images (Figs. 3(a) and 4(a)) present ROI measuring 10.5 mm by 10.5 mm in axial and lateral directions. The pushing force was applied to the left side of the cornea (Fig. 3(a)) or the lens (Fig. 4(a)), and the axial
displacements associated with an elastic wave propagating to the right from the push position are displayed in Figs. 3(b)–(d) and Figs. 4(b)–(d) for cornea and lens, respectively. The spatial distributions of the axial displacements superimposed on the B-mode image are displayed for three different times after the pushing pulse was applied (0.50 msec, 1.17 msec, and 1.84 msec in Figs. 3(b) and 4(b), 3(c) and 4(c), and 3(d) and 4(d), respectively). Here, the time when the first tracking pulse started immediately after the pushing pulse corresponds to 0. After cornea was interrogated, it was clearly seen that the peak of displacements was sequentially moving from left to right of the cornea as shown in Figs. 3(b)–(d). Although not as noticeable as that through the cornea, the wave propagating through the lens was also observed after the lens was pushed as shown in Figs. 4(b)–(d). In Fig. 5, axial displacements as a function of time are plotted for the cornea (Fig. 5(a)) and for the lens (Fig. 5(b)) at 2.5 mm, 3.3 mm, and 4.1 mm lateral positions. Given a lapse between pushing and tracking, the displacements at 0 ms were not zero. From these sequences, we could calculate the speed of the wave using the time-to-peak method. The estimated speeds from this sample in Fig. 4 were approximately 1.23 m/s and 1.45 m/s for the cornea and the lens, respectively. Thus, the lens has slightly higher elastic wave velocity than the cornea but these velocities are comparable. In addition, due to the attenuation of elastic waves, wave-induced displacements diminished as the wave propagates in the lateral direction away from the origin of the wave.

Figure 5 shows the shear wave propagation through the cornea and the lens of the bovine eyeball where cornea (Fig. 6) and lens (Fig. 7) were measured at 30 mmHg IOP. After the pushing pulse was applied on the cornea (Figs. 6(b)–(d)), the displacement peak travels quickly and almost reached the end of the cornea in 1.84 msec. However, the displacement in the lens moved slower than in the cornea as shown in Figs. 7(b)–(d). The axial displacement profiles at different times after the pushing pulse was applied in the eye under 30 mmHg IOP are plotted in Fig. 8 for lens (Fig. 8(a)) and for cornea (Fig. 8(b)) at 5.2 mm, 5.7 mm, and 6.3 mm in lateral direction. Because the propagation of the wave in the cornea was faster at 30 mmHg than at 5 mmHg, lateral positions in farther distances (5.2, 5.7 and 6.3 mm) than those for 5 mmHg (2.5, 3.3, 4.1 mm) are displayed. The estimated elastic wave velocities were approximately 4.78 m/s and 1.83 m/s for the cornea and the lens, respectively. Compared to the measurements at 5 mmHg, it is clear that the shear wave velocity in cornea greatly increases (approximately 4 times) while the velocity in lens does not. Figure 9 shows the time-to-peaks as a function of lateral offset positions using the displacements of the cornea and the lens for an eye under 30 mmHg IOP. The linear regression analysis showed that the slope for the lens (0.76 s/m) is higher than that for the cornea (0.20 s/m), where the wave velocity is the inverse of the slope. For this eye globe, the confidence levels of the regression were 97.6% and 97.0% for the cornea and the lens, respectively.

The dependences of the speed of elastic wave on IOP ranging from 5 mmHg to 50 mmHg are summarized for the cornea and the lens in Fig. 10. For a single eyeball, the mean and standard deviation of wave velocities from three measurements per IOP are shown in Fig. 10(a). The mean and standard deviation of elastic wave speeds averaged over seven eyeball samples are presented in Fig. 10(b). While wave velocities increased from 0.96 ± 0.30 m/s to 6.27 ± 0.75 m/s in the cornea as IOP increased from 5 to 50 mmHg, no significant changes
in wave velocities (minimum 1.44 ± 0.27 m/s at 5 mmHg and maximum 2.03 ± 0.46 m/s at 20 mmHg) were observed in the crystalline lens. Thus, the dependency of the speed of elastic waves on the IOP is much less for the lens than for the cornea.

IV. Discussion

This study shows that the influence of IOP on lens stiffness can be noninvasively assessed by the ultrasound shear wave imaging. The velocity started from a slightly higher value in the lens than in the cornea at 5 mmHg, but there was no significant change in the lens with increasing IOP up to 50 mmHg (Fig. 8). A positive correlation was also noted between the frequency of the displacement profile in the cornea and IOP increment (Fig. 5(a) and Fig. 8(a)), while it was steady in the lens (Fig. 5(b) and Fig. 8(b)). Given that a higher frequency response of the displacement profile indicates tissue stiffening (Deffieux et al., 2009; Litwiller et al., 2010; Muller et al., 2009; Tanter et al., 2009), this result demonstrates that the stiffness of the lens is less dependent on the IOP than the stiffness of the cornea. One possible explanation for this result is that the lens deformation during our experiments was significantly lower than deformation of the cornea. Therefore, nonlinear elastic properties of the lens did not play a significant role in the process of the elastic wave propagation, in contrast to those of the cornea. This is in agreement with Rotemberg et al. (2012), in which the effect of hepatic pressurization on liver shear wave speed was investigated in constrained and unconstrained conditions.

Although we separated the experiments for the cornea and the lens, it was observed that the pushing beam affected both the cornea and lens. As the cornea was pushed, there was a wave generated on the lens (Figs. 3 and 6) and vice versa (Figs. 4 and 7). Because the lens and the cornea are close to each other and coupled by the aqueous humor in between, the generation of unwanted elastic waves was unavoidable. Thus, to estimate the speed, the secondary wave generated outside of the pushing position was excluded. The pushing pulse generated elastic waves propagating to both right and left sides of the pushing position. For visualization purpose, we overlaid the displacements on the B-mode image where we chose the displacements on the right side of the pushing position in the image.

In the previous investigations, lens stiffness was mostly involved as an age-related connection with presbyopia (Glasser et al., 2001; Heys et al., 2004; Wu et al., 2015; Pau and Kranz, 1991; Loupas et al., 1995; Aglyamov et al., 2015). Yoon et al. (2013) measure the Young’s modulus an shear viscosity from extracted bovine and porcine lenses in mature (25–30 months old) and young (6 months old) ages using laser induced microbubbles. Wu et al. (2015) investigated the young (2–3 months old) and the mature (over 6 months old) rabbit eyes to estimate the Young’s modulus an the shear viscosity using optical coherence elastography. These studies concluded that lens stiffness is highly correlated with age. In our study, we chose only one age group (25–30 months old) to focus on IOP dependence instead of the age dependence. Thus in order to investigate IOP influence on lens stiffness with regard to presbyopia, we will perform experiments for various age groups in the future studies.
Several studies had reported the relationship between the IOP and the elastic wave speed of the cornea. Using ultrasonic shear elastography, Tanter et al. (2009) showed that elastic wave speed in the porcine cornea ranged from 4 to 6 m/s, and Nguyen et al. (2014) acquired higher group shear velocity at 20 mmHg (shear speed ~ 7 m/s) than that at 10 mmHg (shear speed ~ 2.5 m/s) in the porcine cornea. Also, Litwiller et al. (2010) used magnetic resonance elastography to measure the shear wave speed in bovine corneas with varying IOPs from 0.85 mmHg (shear speed: 1.43 m/s) to 9.05 mmHg (shear speed: 2.29 m/s). Li et al. (2016) calculated the group velocity in the porcine cornea approximately 1.5 m/s, 2.5 m/s, 4.0 m/s and 5.0 m/s at IOPs of 15 mmHg, 20 mmHg, 25 mmHg, and 30 mmHg, respectively. Touboul et al. (2014) assessed corneal stiffening with supersonic shear wave imaging technology for the rabbit model of corneal collagen cross-linking (CXL) in IOP range of 15–50 mmHg, and demonstrated increment in the Young modulus values from 34 to 95 kPa, and from 36 to 133 kPa for untreated and CXL-treated corneas, respectively. In our recent study, the mechanical properties of the untreated and CXL-treated porcine corneas were quantified at various IOPs based on optical coherence elastography (OCE) measurements and the modified Rayleigh-Lamb equation. We have demonstrated that the increases in Young’s modulus were approximately from 52 to 157 kPa for untreated cornea, and from 87 to 281 kPa for CXL-treated cornea, as IOP was elevated from 15 to 30 mmHg (Han et al., 2017). These previous studies demonstrated that the velocities in the cornea quickly rise with increasing IOP, a finding further supported by our investigation (Fig. 10). Their results also align with our images showing the propagation of the axial displacement at different times where the peak of displacements in the cornea moves faster at 30 mmHg (Figs. 6 (b)–(d)) than at 5 mmHg (Figs. 3 (b)–(d)). Therefore, it clearly demonstrates that the cornea becomes stiffer with higher IOP. In addition, we can confirm that the velocity estimates in our experiments are in a similar order of magnitude as those of the previous studies.

While IOP can dramatically affect the elastic properties of the cornea and sclera, results of our study imply that we can expect much smaller effect of IOP when measuring the elasticity of the crystalline lens in vivo. Such in vivo studies will require high frequency ultrasound imaging or optical methods (e.g., OCT), which can provide significant advantages in terms of spatial resolution for clinical ophthalmic applications. Thus, in our future studies we are planning further investigation of the impact of IOP on the elasticity measurements in the crystalline lens using high frequency ultrasound imaging and OCE.

Although the speeds of the elastic waves in the cornea and the lens are compared in this study, it is noted that Lamb wave velocity for thin structures is lower than surface wave velocity (Nenadic et al., 2011; Nguyen et al., 2011). Therefore, direct comparison of the speeds in the cornea and lens in terms of elastic modulus is hardly possible. To reconstruct the elastic properties based on the results of the shear wave elasticity imaging, appropriate mechanical models are required. There were studies that estimated the Young’s modulus in consideration of the complex structure of the eye using shear spectroscopy with the Lamb wave theory (Han et al., 2015; Tanter et al., 2009; Han et al., 2017), using finite element model derived from an anatomical image of the eye (Litwiller et al., 2010), and using the laser induced microbubbles generated at different locations of the crystalline lens (Yoon et al., 2013). As a future study, we will investigate a mechanical model to characterize the elastic modulus based on the anatomical properties of the eye.
For the ex vivo experiment, we used bovine eyes that generally have a large size and a large pupil opening to the crystalline lens. This choice allowed us to use relatively low frequency ultrasound to measure elastic wave propagation in the lens. For the bovine eyes, the thickness of the lens was significantly larger than the thickness of the cornea and wavelength. Therefore, we have weakly dispersive Rayleigh wave propagating on the lens surface. However, human lenses are smaller than bovine ones and the geometric factors could significantly influence to the wave propagation by introducing additional wave dispersion in human lenses. Besides, literatures (Heys et al., 2004; Weeber et al., 2005) reported that the mechanical properties of human lenses were more dependent on age and anatomical location compared to those of animal studies. While our results acquired from bovine eyes may not be conclusive for the human case, they clearly demonstrate that the IOP dependence of the lens in comparison to the cornea can be obtained using ultrasonic shear wave imaging. This information can provide better understanding of the lens and contribute to new treatment approaches of dysfunctional lenses.

V. Conclusion

Shear wave elasticity imaging was demonstrated as a promising tool for noninvasive assessment of the biomechanical properties of the lens. In contrast with cornea, the increase in IOP does not result in significant changes in the elastic wave velocity of the crystalline lens.

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References


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Fig. 1.
Experimental setup.
Fig. 2.
B-mode image of a bovine eyeball. Area indicated by a dashed rectangle shows region of interest.
Fig. 3.
Propagation of elastic waves through the cornea of a bovine eyeball at 5mmHg IOP. (a) the acoustic radiation pushing pulse was applied to cornea as depicted in a B-mode image. Axial displacements overlaid on the B-mode image (b) 0.50 msec, (c) 1.17 msec, and (d) 1.84 msec after the pushing pulse.
Fig. 4.
Propagation of elastic waves through the lens of a bovine eyeball at 5mmHg IOP. (a) the acoustic radiation pushing pulse was applied to crystalline lens as shown in an ultrasound B-mode image. Axial displacements overlaid on the B-mode image (b) 0.50 msec, (c) 1.17 msec, and (d) 1.84 msec after the pushing pulse.
Fig. 5.
Profiles of axial displacements at 5 mmHg IOP in (a) cornea and (b) lens measured 2.5 mm, 3.3 mm, and 4.1 mm away from the location of the pushing pulse.
Fig. 6.
Propagation of elastic waves through the cornea of a bovine eyeball at 30 mmHg IOP. (a) the acoustic radiation pushing pulse was applied to cornea as depicted in a B-mode image. Axial displacements overlaid on the B-mode image (b) 0.50 msec, (c) 1.17 msec, and (d) 1.84 msec after the pushing pulse.
Fig. 7.
Propagation of elastic waves through the lens of a bovine eyeball at 30 mmHg IOP. (a) the acoustic radiation pushing pulse was applied to crystalline lens as shown in an ultrasound B-mode image. Axial displacements overlaid on the B-mode image (b) 0.50 msec, (c) 1.17 msec, and (d) 1.84 msec after the pushing pulse.
Fig. 8.
Profiles of axial displacements at 30 mmHg IOP in (a) cornea and (b) lens measured 5.2 mm, 5.7 mm, and 6.3 mm away from the location of the pushing pulse.
Fig. 9.
Lateral time-to-peaks and the lines fitted by regression as a function of lateral position using displacement data from cornea and lens at 30 mmHg IOP. The elastic wave velocity is the inverse of the slope from the regression. R is the regression value.
Fig. 10.
Comparison between elastic wave velocities in cornea and lens with an incremental pressure (a) measurements obtained from a single bovine eyeball sample, and (b) measurements obtained from seven bovine eyeball samples.