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The mechanical properties of *ex vivo* bovine and porcine crystalline lenses: age-related changes and location-dependent variations

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Abstract

The mechanical properties of *ex vivo* animal lenses from three groups – old bovine (25–30 months old, $n=4$), young bovine (6 months old, $n=4$), and young porcine (6 months old, $n=4$) eye globes – were evaluated. We measured the dynamics of laser-induced microbubbles created at different locations within the crystalline lenses. An impulsive acoustic radiation force was applied to the microbubble, and the microbubble displacements were measured using a custom-built high pulse repetition frequency ultrasound system. Based on the measured dynamics of the microbubbles, Young's moduli of bovine and porcine lens tissue in the vicinity of the microbubbles were reconstructed. Age-related changes and location-dependent variations in the Young's modulus of lenses were observed. Near the center, the old bovine lenses had a Young's modulus approximately 5 times higher than that of young bovine and porcine lenses. The gradient of Young's modulus with respect to radial distance was observed in the lenses from three groups.

Keywords

Crystalline lens; Young's modulus; Shear viscosity; Laser-induced microbubble; Acoustic radiation force; Ultrasound measurement; Presbyopia

Introduction

When a near object is in focus, the eye accommodates i.e. the crystalline lens becomes thicker and rounder in configuration for normal human eyes (Drexler et al., 1997; Strenk et al., 2004, 2005). With age, accommodation power degrades, resulting in presbyopia (Beers and Van der Heijde, 1994; Weale, 2000; Bailey et al., 2010). There is strong evidence that age-related changes in the mechanical properties of the lens play a major role in the development of presbyopia (Charman, 2008). To further evaluate the processes which result in accommodation changes and presbyopia development, a comprehensive understanding of the viscoelastic properties of the crystalline lens is required.

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To characterize the mechanical properties of the crystalline lens, different techniques have been developed (Kikkawa and Sato, 1963; Fisher, 1971, 1973; van Alphen and Graebel, 1991; Pau and Kranz, 1991; Heyworth et al., 1993; Assia et al., 1997; Soergel et al., 1999; Tabandeh et al., 2000; Heys et al., 2004; Weeber et al., 2005, 2007; Baradia et al., 2010; Sharma et al., 2011; Burd et al., 2011). Researchers reported that the stiffness of the lens as a whole object as well as variations of lens stiffness with respect to equatorial distance increase with age. Dynamic mechanical analysis has shown that in human lenses over the age of 50, stiffness of the nucleus is an order of magnitude greater than that of the cortex (Heys et al., 2004; Weeber et al., 2007). Furthermore, between ages of 14 and 78, an almost 1,000 times increase in stiffness of lens nucleus was reported (Heys et al., 2004). However, most of current techniques capable of measuring the lens elasticity are based on direct mechanical testing and usually can be used only *in vitro*.

To assess the mechanical properties of a crystalline lens *in vivo*, microbubble-based acoustic radiation force technique can be used (Milas et al., 2003a,b; Emelianov et al., 2004; Ilinskii et al., 2005; Erpelding et al., 2005, 2007a,b; Aglyamov et al., 2007; Hollman et al., 2007). In this technique, acoustic radiation force is applied to a microbubble created by laser-induced optical breakdown in the lens. The displacement of the microbubble is measured by ultrasound and used to evaluate lens elasticity. Compared to other methods, this non-invasive approach does not require contact with the crystalline lens, and therefore the intact lens can maintain its intrinsic mechanical properties during the measurements. In addition, remote assessments of the local viscoelastic properties of the crystalline lenses can also be achieved. The mechanical property changes in both porcine and human crystalline lenses with respect to age and locations in lenses were investigated using microbubble-based technique (Erpelding et al., 2007b; Hollman et al., 2007). In these studies, the local elastic properties were inversely proportional to the maximum displacement of the microbubble. However, the maximum displacement depends on the magnitude of acoustic radiation force. Therefore, to accurately measure the elastic properties of surrounding tissue, the magnitude of acoustic radiation force on the microbubble surface should be evaluated. However, estimation of the magnitude of acoustic radiation force is a difficult task given the unknown attenuation of sound in surrounding tissue. To overcome this limitation, we have previously proposed the use of the temporal characteristics of the dynamics of the microbubble (Ilinskii et al., 2005; Aglyamov et al., 2007; Karpouk et al., 2009; Yoon et al., 2011; Aglyamov et al., 2012). The temporal response of tissue interrogated by acoustic radiation force depends on the mechanical properties of tissue (Sarvazyan et al., 1998). We have demonstrated that the time of maximum displacement of the microbubble under impulsive acoustic radiation pressure is invariant even though the magnitude of acoustic radiation force changes. Therefore, the magnitude of acoustic radiation force, therefore, becomes just a scaling factor which determines the maximum displacement of a microbubble

In our previous work, the mechanical properties of gelatin phantoms were estimated from the temporal characteristics of the motion of rounded objects within gelatin and found to be in good agreement with direct measurements (Ilinskii et al., 2005; Aglyamov et al., 2007; Karpouk et al., 2009; Yoon et al., 2011; Aglyamov et al., 2012). To increase both the sensitivity and the accuracy of the measurement by using impulsive acoustic radiation force, a high pulse repetition frequency (PRF) ultrasound system has been introduced (Yoon et al., 2012). We have validated this high PRF system successfully using *ex vivo* bovine crystalline lenses and compared the obtained data with results of independent mechanical tests (Yoon et al., 2012).

In this paper, we investigate age-related changes and location-dependent variations of the mechanical properties of bovine and porcine crystalline lenses. We generated the laser-induced microbubbles at various locations in the crystalline lenses by focusing a single

nanosecond laser pulse. Then, the dynamic behavior of the laser-induced microbubbles, displaced by an impulsive acoustic radiation force, was measured using the high PRF ultrasound system. We reconstructed the local Young's modulus of lens tissue by measuring the dynamics of the laser-induced microbubble and comparing it with the theoretically calculated values. We compared the viscoelastic properties of young and old bovine lenses to explore age-related changes. In addition, location-dependent variations of the mechanical properties were measured using bovine and young porcine lenses.

Materials and Methods

Lens preparation

All tissue samples were obtained from Sierra for Medical Science, Inc. (Whittier, CA). The eye globes were shipped overnight in a thermo-insulated box with ice packs. Three groups of tissue samples were used: old bovine (25-30 month old), young bovine (6 month old), and young porcine (6 month old) eye globes. In each group, four crystalline lenses ($n=4$, samples 1-4), excised from the eye globes, were used in the experiments. All the experiments were performed within 12 hours after the tissue samples arrived at our facility.

The lens was carefully extracted from an eye globe and the lens capsule was removed by making small tears at the lens equator. The lens was placed and secured in a lens holder filled with 5 ml of 6 wt % gelatin solution. The anterior of the lens was facing the bottom of the lens holder. During the experiments, the lens and the lens holder were kept in phosphate buffered saline (PBS, Sigma-Aldrich, Inc., St Louis, MO) to minimize changes in the mechanical properties of lens.

Figure 1a presents a coordinate system defined within the lens. Lenses from old bovine, young bovine, and young porcine eye globes had diameters (denoted by D in Fig. 1a) of 16 ± 0.5 mm, 12 ± 0.3 mm, and 10 ± 0.5 mm, respectively. Total thicknesses (denoted by h in Fig. 1a) from the anterior to the posterior part were 11 ± 0.6 mm, 9 ± 0.3 mm, and 7 ± 0.3 mm for old bovine, young bovine, and young porcine lenses, respectively.

Laser-induced microbubble generation

Laser-induced microbubbles with typical radii of $45\text{--}60\text{ }\mu\text{m}$ were generated along the S-axis (Figure 1a). No correlation was observed between the sizes of microbubbles and their locations within the lens. The typical life-time of the bubbles was between 40 minutes to 1 hour. Using the 3D translational stage attached to the lens holder with the crystalline lens inside, the distance d between the S-axis and anterior part (denoted by d in Fig. 1a) was set to 4 mm, 4 mm, and 3 mm in experiments with old bovine, young bovine, and young porcine lenses, respectively. The radial distances (denoted by S in Fig. 1a) were chosen as multiples of one fortieth of one inch ($S=\pm0.635\cdot k$ mm, $k=1,2,4,6,8$). Thus, laser-induced microbubbles were generated along the S-axis, d mm away from the anterior part of each crystalline lens.

To produce a microbubble inside the crystalline lens, we used a pulsed Nd:YAG laser (Polaris II, Fremont, CA) with 5 ns pulse duration, 532 nm wavelength, and 10 mJ energy. A custom-built objective lens with high numerical aperture ($NA=1.13$) and long working distance (8.0 mm) was designed to create spherical microbubbles located inside the outer cortex and the inner nucleus of the crystalline lenses (Aglyamov et al., 2008; Karpouk et al., 2008; Yoon et al., 2011, 2012). The size of the microbubble was monitored by an optical microscope (Dino-Lite AM411T, Wirtz, VA) operating at 230x magnification. The mean radius and standard deviation of each microbubble were estimated based on three measurements.

Experimental system and data processing

The present studies were performed using the experimental setup that was slightly modified from the one described in detail elsewhere (Yoon et al., 2012).

A crystalline lens was positioned in the lens holder. The holder was attached to 3D translational stage to control the locations of microbubbles generated by a focused laser beam (Fig. 1a). An ultrasound excitation transducer (3.7 MHz center frequency, f-number or focal ratio of 2 or f/2, 12.7 mm focal distance, 17% bandwidth, Valpey Fisher, Hopkinton, MA) was used to generate acoustic radiation pressure. Two ultrasound imaging transducers were used to transmit (T) pulses and to receive (R) echoes (25 MHz center frequency, f-number or focal ratio of 4 or f/4, 25.4 mm focal distance, about 50 % bandwidth, Olympus-NDT, Waltham, MA) and to measure bubble displacements. All transducers were positioned at the top of the cuvette and aligned to push and to track the microbubbles in a lens simultaneously. By separating ultrasound transducers T (transmit) and R (receive), a desired high pulse repetition frequency (PRF) set to 1 MHz was achieved (Yoon et al., 2011, 2012). The angle between T and R ultrasound transducers was 35° while the excitation transducer was in the middle. The foci of three transducers were aligned at the location of the microbubble.

As shown in Figure 1b, the main control unit was used to synchronize the sequence of pulse-echo pulses (ultrasound transducers T and R) and acoustic radiation pulse (ultrasound excitation transducer). The radiofrequency (RF) data storage unit, also controlled by the main control unit, was used to record transmitted pulses from transducer T and to capture the ultrasound echoes acquired by the ultrasound transducer R for off-line data processing. Therefore, RF raw data was comprised of a sequence of transmitted pulses and backscattered ultrasound echoes. The generation of the acoustic radiation pulse started with a delay of 60 μ s after the transmit/receive pulse sequence was launched. The duration of the acoustic radiation force pulse was 20 μ s and the voltage applied to the excitation transducer was 316 V.

Once the laser-induced microbubble was generated and the pulse sequence was initiated, arrival time of the first echo defined the initial location of the microbubble and the changes in the arrival times of the following echoes determined the microbubble displacement using a cross-correlation speckle tracking method (Lubinski et al., 1999). The kernel size and search window for cross-correlation tracking were 65 ns and 130 ns, respectively. RF raw data was filtered using a comb filter to remove or reduce the interference between the ultrasound transducer R and the ultrasound excitation transducer before the cross-correlation speckle tracking algorithm was applied. The displacement of a laser-induced microbubble under impulsive acoustic pulse was measured three times at each location within the lens, and averaged. For some points, displacements of microbubbles were not detectable likely due to misalignment between the bubble location and focus of the excitation transducer – those points were omitted from the analysis.

Reconstruction of the mechanical properties of bovine and porcine crystalline lenses using the microbubble approach was performed by finding the best fit between the experimental displacement profiles and the displacement profiles calculated by previously developed theoretical model (Ilinskii et al., 2005; Aglyamov et al., 2007; Karpouk et al., 2009; Yoon et al., 2011). In this approach, the time characteristics of the microbubble dynamics are used for elasticity and viscosity estimation. The time for a microbubble to reach its maximum displacement (t_{max}), measured in the experiment, was matched to t_{max} theoretically calculated by varying Young's modulus (E). The shear viscosity (η) was assessed by varying η to fit the decaying profiles of measured displacements using theoretical model. When the best fit has been reached, the values of both Young's modulus and shear viscosity

correspond to those of tissue. In addition, the magnitude of the acoustic radiation force (F_0) was chosen to match experimentally observed magnitude of the displacements. The detailed description of the Young's modulus and shear viscosity estimation procedure can be found elsewhere (Aglyamov et al., 2007, 2012; Karpouk et al., 2009; Yoon et al., 2011, 2012).

Results

Averaged ($n=3$) temporal displacement profiles of microbubbles in the cortex (thin line) and nucleus (thick line) of old bovine lenses are presented in Figure 2a. The radii of microbubbles in the cortex and nucleus of the lens were $47\pm 2\ \mu\text{m}$ and $54\pm 2\ \mu\text{m}$ (mean value and standard deviation), respectively and these measured values were used as input parameters in the theoretical model. Although we have previously developed an ultrasound technique to measure the size of laser-induced microbubbles (Aglyamov et al., 2008; Karpouk et al., 2008), in the current experiments the radii of the microbubbles were measured using an optical microscope. Theoretically calculated microbubble displacements are shown in Figure 2b. To match experimentally observed and theoretically calculated displacements, we chose the scaling factors of acoustic radiation forces (F_0) to be 500 mN for the microbubble in the cortex of the lens and 1000 mN for the bubble in the nucleus - the values of F_0 were not the same because the curvature of the lens surface and the distance between the lens surface and the microbubbles were different. Young's modulus and shear viscosity were determined by the best fit between measured and theoretically calculated displacements of the microbubbles. The reconstructed Young's modulus of the cortex was 2.9 kPa while the Young's modulus of the nucleus was 23.3 kPa. The shear viscosities in both cases were 1.3 Pa·s. Therefore, displacement profiles in Figure 2 demonstrate the differences of the behavior of microbubbles in crystalline lenses with different elasticities. As it has been observed in phantom experiments, maximum displacement (U_{max}) and time of maximum displacement (t_{max}) in stiff regions are smaller than those in soft regions (Yoon et al., 2011).

The location-dependent variations of Young's moduli in old bovine, young bovine, and young porcine lenses along the S-axis (Fig. 1a) are shown in Figures 3a-3c, respectively. The displacements of laser-induced microbubbles, shown in Figure 2a, correspond to the dotted and the solid arrows in Figure 3a. Dotted lines in Figures 3a-3c represent mean values of the Young's modulus at each point averaged over all samples. For the comparison of Young's modulus between bovine and porcine lenses and lenses from the same animals of different ages, dotted lines from Figures 3a-3c are plotted in Figure 3d. The measured values of Young's moduli for old and young bovine lenses over a $\pm 5.1\ \text{mm}$ distance ranged from 2.2 ± 0.1 to $25.9\pm 0.7\ \text{kPa}$ and from 1.6 ± 0.1 to $5.8\pm 0.2\ \text{kPa}$, respectively. The results show age-related increase of elastic modulus in bovine lenses. Near the $\pm 5.1\ \text{mm}$ area, Young's moduli of old and young bovine lenses are comparable; however, as one approaches the center, Young's modulus of old bovine lenses increases dramatically and the difference in Young's modulus between young and old lenses reaches about 5 times (Fig. 3d). For young porcine lenses, the Young's modulus over a $\pm 3.8\ \text{mm}$ distance varied from 1.2 ± 0.1 to $5.7\pm 0.2\ \text{kPa}$. Therefore, for both young and old bovine lenses and for young porcine lenses, the gradient of Young's modulus exists (Figs. 3a, 3b and 3c).

Shear viscosity was also evaluated with the developed microbubble approach. Mean and standard deviations of shear viscosity are shown for each lens sample (Fig. 4a) and all lens samples (Fig 4b). Generally, old bovine lenses had the highest shear viscosity ($1.32\pm 0.12\ \text{Pa}\cdot\text{s}$) and young porcine lenses had the lowest values ($0.87\pm 0.08\ \text{Pa}\cdot\text{s}$). Young bovine lenses had medium shear viscosity values ($1.0\pm 0.12\ \text{Pa}\cdot\text{s}$). Shear viscosity of lenses in three groups are statistically significantly different as determined by the student's t-test results after comparing p-values of three combinations: old and young bovines, young porcine and

bovine, and old bovine and young porcine ($p < 0.01$ for all cases, Fig. 4b). Therefore, it appears that microbubble-based approach can differentiate shear viscosity values of lenses from different groups.

Discussion

The laser-induced microbubble-based approach has several advantages when measuring the viscoelastic properties of the crystalline lenses. First, this approach can assess the local mechanical properties by measuring the viscoelastic properties of a small volume around the bubble within the crystalline lenses. Secondly, microbubbles can be created at any location within the crystalline lens thus allowing investigation of the spatial variations of the mechanical properties within a lens. Finally, the proposed method potentially allows measuring the viscoelastic properties of a crystalline lens without dissecting it and, therefore, without disturbing its structure and natural environment. As was shown in our previous work, the generation of a single laser-induced microbubble in the crystalline lens did not produce significant changes in the elasticity of the surrounding tissue (Yoon et al., 2012).

The lens capsule was removed because phosphate buffered saline (PBS, Sigma-Aldrich, Inc., St. Louis, MO) permeated the lens capsule. It was difficult to produce a common focal point for optical and ultrasound parts when permeated PBS occupied the space between the lens capsule and the lens substance. It was possible that removal of the capsule affected postmortem changes in the mechanical properties of the crystalline lens. However, these changes are minimal according to the results of our previous work (Yoon et al., 2012).

We observed location-dependent variations of Young's modulus within all three groups of old and young bovine lenses, and young porcine lenses. The Young's modulus of old bovine lenses continuously increases approximately 10 fold from their outer cortex to their nucleus. The young bovine and the young porcine lenses do not have dramatic spatial changes in elasticity compared to old bovine lenses; however, they also demonstrate spatial variation of Young's modulus, which is in agreement with the results reported by other groups (Erpelding et al., 2007b; Reilly and Ravi, 2009). Qualitative measurements of viscoelastic properties of young (6 months old) and old (2–5 years old) porcine lenses were performed (Erpelding et al., 2007b). Even though no absolute values of Young's modulus were provided, the tendency of the changes of mechanical properties of porcine lenses was found. The central part of the lens was stiffer than the cortex and the stiffness of young porcine lenses was less than that of old porcine lenses. Quantitative measurements of Young's modulus of young (6 months old) porcine lenses showed a similar tendency with the highest Young's modulus at the center and the lowest values at the periphery (Reilly and Ravi, 2009). In human studies, it was reported that age and location dependences of the mechanical properties of lenses were more drastic than those in animal crystalline lenses (Heys et al., 2004; Weeber et al., 2007). Therefore, the results from animal studies cannot be directly translated to humans. However, the results reported here show that the proposed microbubble-based approach can be directly used to measure the mechanical properties of the human crystalline lenses, which may provide the fundamental information to develop the model of accommodation and the treatment plans for presbyopia.

The preliminary results of shear viscosity measurements of lenses from three groups (young bovine, old bovine, and young porcine lenses) show that the microbubble-based approach can be used to identify the shear viscosity of a crystalline lens. The microbubble-based approach can differentiate shear viscosity in different groups (Fig. 4b). Shear viscosity, as measured with the microbubble-based approach, changes from species to species significantly but does not vary much within the lens or between species of the same age.

Future studies will be performed to verify the precision and the accuracy of the microbubble approach as a technique for shear viscosity assessment.

Precise *in vivo* measurements of the mechanical properties of the crystalline lens would critically help to develop the mechanical model of accommodation and clinical treatments of presbyopia (Glasser, 2008). Laser-induced incisions, created by femtosecond laser (femtosecond lentotomy), can make the hardened crystalline lens softer thus allowing to focus a near object clearly (Schumacher et al., 2008; Ripken et al., 2008; Lubatschowski et al., 2010). Potentially, the microbubble-based approach could be combined with femtosecond lentotomy to measure the mechanical properties of the crystalline lens *in vivo* for guidance and better outcome of the surgery.

Conclusions

The laser-induced microbubble approach enables remote and accurate measurements of the mechanical properties of *ex vivo* bovine and porcine crystalline lenses. Age-related changes in Young's modulus were observed in bovine crystalline lenses. The Young's modulus in the central region of old bovine lenses is much higher than that of young bovine and porcine lenses. Generally, the absolute values of Young's modulus of old bovine lenses are larger than those from young bovine lenses. Location dependence of Young's modulus with the central regions being stiffer compared to the peripheral regions was found in all groups: young and old bovine lenses and young porcine lenses.

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Figure 1.

(a) Coordinate system defined within the lens. The top and bottom diagrams show a sagittal section and an equatorial section of the lens, respectively. Laser-induced microbubbles were

created along the S-axis and the center location (solid dot) corresponds to $S=0$. (b) A schematic view of the experimental setup. A crystalline lens was positioned in the lens holder with anterior surface facing down, and a microbubble was produced by a laser beam focused inside a lens. An excitation transducer (3.7 MHz) was used to produce an acoustic radiation force pulse and initiate microbubble displacement (dash-dotted line and arrow). The motion of a microbubble was tracked by two 25 MHz ultrasound imaging transducers (T and R) - the separation of the transmit (T) and receive (R) transducers allowed for high pulse repetition frequency (up to 1 MHz). The train of pulses generated by ultrasound transducer T and echoes reflected from the surface of a microbubble and traveling to ultrasound transducer R are shown as dashed lines and arrows. The size of the laser-induced microbubble was monitored by an optical microscope.

Figure 2.

(a) Averaged ($n=3$) displacements of microbubbles created in the cortex (thin line) and nucleus (thick line) of old bovine lenses. The displacements of microbubbles were induced by a 20 μs acoustic pulse applied at time $t = 60 \mu\text{s}$ time point. (b) Theoretically calculated displacements of the microbubbles in the medium with Young's modulus (E) of 2.9 kPa (cortex, thin line) and 23.2 kPa (nucleus, thick line). The time of maximum displacement of microbubble is indicated by t_{max} . The dotted vertical lines indicate the start and end of the acoustic radiation force pulse. The radii of the microbubbles in the cortex and nucleus of the lenses were $47 \pm 2 \mu\text{m}$ and $54 \pm 2 \mu\text{m}$, respectively. In theoretical calculations, a shear viscosity of 1.3 Pa·s was used for both bubbles. The scaling factors resulting in 500 mN (cortex) and 1000 mN (nucleus) acoustic radiation forces (F_0) were used in the theoretical

calculations to achieve the best fit between measured displacement and displacement calculated using the theoretical model.

Figure 3.

Young's modulus measurements in (a) old bovine (25–30 months old), (b) young bovine (6 months old), and (c) young porcine (6 months old) lenses. Radial distance is measured along the S-axis (see Figure 1a), and 0 mm radial distance corresponds to the center of the lens. Four samples were used for each group. Dotted lines in (a), (b), and (c) are mean values averaged over all samples. (d) Dependence of Young's modulus on radial distance. Measurement points in (d) are reproduced from plots (a), (b), and (c). Examples of detailed displacement profiles of the laser-induced microbubbles in old bovine lenses (dotted and solid arrows in (a)) are shown in Figure 2a. Three measurements were performed at each point, the error bars represent plus/minus one standard deviation.

Figure 4.

(a) Shear viscosity measurements in old and young bovine and young porcine lenses. The horizontal axis represents the sample number, and the vertical axis is shear viscosity. (b) Averaged shear viscosity values of all samples from each group. Error bars represent plus/minus one standard deviation. The differences of shear viscosity between the groups are statistically significant ($p < 0.01$) as determined by student's t-test.