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Microstructural Assessment of the Guinea Pig Sclera Using Quantitative Acoustic Microscopy

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Abstract—The objective of this initial study is to investigate acoustic microscopy as a tool to elucidate the relationship between microscopic structural properties of the sclera and its macroscopic mechanical properties in healthy and myopic conditions. Excised sclera from both eyes of one guinea pig were scanned with a custom-built scanning acoustic microscope (SAM). Prior to euthanasia and excision, the animal wore a plastic diffuser over the right eye for 7 days, resulting in -9.3 diopters of induced myopia. The left eye served as an untreated control. Upon excision, both eyes were flash-frozen, serially-sectioned at 12- μm intervals, and scanned using SAM. Maps of bulk modulus (K) and mass density (ρ) showed a distinctive layered structure in the sclera consisting of a thin outer layer with $K=2.47 \pm 0.09$ GPa and $\rho=1.06 \pm 0.02\text{g/cm}^3$ followed by three layers of successively decreasing K towards the vitreous body. In general, the myopic eye was less stiff at vertical distances between 1 and 4 mm from the optic nerve head. This study suggests that the guinea pig sclera has a layered structure with different values of K and ρ ; these differences may be related to the specific collagen-network structure of each layer.

Keywords—Scanning acoustic microscopy; myopia; guinea pig; sclera; layers

I. INTRODUCTION

The guinea pig is an accepted animal model of myopia and an emerging model of high myopia with similarities to human myopia [1, 2]. Globally, myopia affects up to 2.5 billion persons and this prevalence is rising and considered epidemic in some populations [3]. Although minimal levels of myopia are considered a minor inconvenience, high (pathologic) myopia can lead to irreversible vision loss. High myopia is variously defined as more than -5.0 or -6.0 diopters (D) of near-sightedness and is associated with sight-threatening pathology in 70% of patients. The excessive eye elongation underlying myopia likely occurs in the sclera, a collagen-rich tissue providing form and stability to the mammalian eye. Studies showed that the sclera is a viscoelastic structure and is

more flexible and less load-bearing in myopes than in normal cases [4, 5]. In mammalian models, scleral thinning and tissue loss occur during development of myopia; increased collagen degradation and decreased scleral collagen synthesis are evidenced by reduced scleral dry weight and reduced glycosaminoglycan content [6, 7]. Decreased collagen-fiber diameter and collagen crosslinking [8] occur in mammalian models as well as in high-myopia patients [8].

In this preliminary study, scanning acoustic microscopy (SAM) was investigated as a tool to elucidate the relationship between microscopic structural properties of the sclera and its macroscopic mechanical properties in a form-deprivation (FD) guinea pig model of high myopia. SAM is currently the only method that can provide estimates of the mechanical properties at very fine resolutions and possibly over the entire eye. Other methods that have been utilized for evaluating the elastic properties of *ex vivo* ocular tissues, including strip extensimetry, thermal shrinkage, gel-electrophoresis, differential scanning calorimetry, inflation testing, and atomic force microscopy (AFM) all have technical limitations. Strip-extensimetry and inflation testing, for example, are limited to assess the stiffness on a macroscopic scale only and preclude measurements of the finer structures. To date, the link between macroscopic and microscopic mechanical and structural properties of the sclera remains unclear. Such information potentially could lead to novel targeted diagnostic or treatment approaches.

SAM has become a reliable technology for assessing the acoustical and mechanical properties of soft tissue utilizing frequencies between 100 MHz and 1.5 GHz. Various soft tissues in diseased and normal states have been investigated [9–12]. However, few studies have reported SAM measurements of ocular tissues. In a study by Marmor et al., the retinas of two human eyes were imaged at a 1-GHz center frequency with approximately 1- μm spot size [13]. High contrast was found among the acoustic properties of different retinal structures. Beshtawi et al. [12] used a 761-MHz SAM-device to compare

speed-of-sound at the micrometer-scale in crosslinked corneas with controls and found good agreement between histology and speed-of-sound in the treated cases. The current study uniquely applied SAM measurements to assess the properties of scleral tissues extracted from myopic guinea pig eyes.

II. MATERIAL AND METHODS

A. Animal and sample preparation

In this preliminary study one neonatal guinea pig had a translucent diffuser in front of the right eye for 8 days (from 4-13 days of age), to induce form deprivation myopia [14]. After 8 days, refractive error was measured in dilated eyes (1% cyclopentolate) and eye length measured using high-frequency ultrasound in anaesthetized animals (isoflurane 1.5% in oxygen). In this preliminary study, sections from the sclera of the FD myopic (*M*) and untreated contralateral (*C*) eyes of this animal were analyzed. The difference between *M* and *C* was -9.3 D on the day of euthanasia. Specifically, at 13 days of age, the diffuser was removed and the animal was sacrificed and the eyes were enucleated and flash-frozen in liquid nitrogen and stored at -80°C until processed. Thawed eyes were oriented and embedded in medium (Tissue-Tek optimum cutting temperature, O.C.T.). A cryomicrotome (Leica Biosystems, Buffalo Grove, IL, USA) was used to take 12- μ m thick vertical sections (superior/inferior) of the FD-treated and contralateral control eye. Adjacent, 6- μ m sections were taken and used for H&E staining and histological analysis (Fig. 1a).

B. SAM Measurements

The slides were scanned according to the same protocol and SAM system as described previously [11, 15]. Briefly, the sectioned slides were mounted face down on a three-axis, high-precision, scanning stage (Newport, UTM100CC1HL) and 2D raster-scanned in 2- μ m increments. A 300-MHz monocycle pulser (GEOZONDAS, GZ1120ME-03) together with an F-1.16, 250-MHz transducer (Fraunhofer, Ingbert, Germany) with a 160-MHz bandwidth and a 450- μ m radius of curvature was used to generate ultrasonic signals. The transducer's lateral beam width and resolution was 7 μ m. A 12-bit HD oscilloscope was used to digitize the amplified (MITEQ, Hauppauge, NY, USA) signals at 2.5 GHz (Teledyne Lecroy, Chestnut Ridge, NY, USA). Deionized and degassed water was used as the coupling agent between the sample and the transducer. The temperature was measured pre- and post-scan using thermocouples. Custom LabVIEW software was used to control the system.

C. Data Processing

To generate maps of acoustic properties a custom, frequency-domain and model-based approach was employed. The approach has been detailed in our previous publication [15] and is based on the study by Hozumi et al. [9]. Briefly, the reflected signals (*s*) comprising reflections from the sample surface (*s₁*) and sample-glass-plate-interface (*s₂*) were modeled and fitted by a summation of two reference signals with different phase, frequency-dependent attenuation, and amplitude. Reference signals (*s₀*) were obtained from measurements at locations devoid of tissue sample (i.e., glass-

reflection only). By mapping *s* into the frequency domain using a fast-Fourier-transform algorithm (FFT) and by normalizing with the Fourier transform of *s₀* ($FFT(s_0) = FFT(s_1 + s_2) / FFT(s_0)$) it can be shown that sample thickness, speed of sound (*c*, in m/s), attenuation (α , dB/MHz/cm) and acoustic impedance (*Z*, in MRayl), can be directly calculated [9]. Bulk modulus (*K* in Ga) and mass density (ρ , in g/cm³) were calculated using $\rho = Z/c$ and $K = c \cdot Z$, respectively [16]. Acoustic parameter maps were smoothed using a 2D median filter.

Amplitude images (i.e. the maximum of the Hilbert transformed signals) were individually thresholded to exclude the glass plate reference from the analysis. Next, the sclera and optic nerve were outlined manually to create a mask excluding irrelevant tissue sections and preparation artifacts.

For each scan the optic nerve head (ONH), superior (*S*), and inferior (*I*) areas were specified and separated into three equidistant and equally-sized ROIs. These 1 mm-long ROIs (i.e. R1 to R3) had average starting distances of 1, 2 and 3 mm from the ONH horizontal midline (Fig. 1c). R3 of all scans exhibited a distinct layered structure and was, therefore further divided into four sub ROIs specifying layer 1 to 4 (i.e., L1 to L4) from posterior to anterior. By comparing parameter maps for *c*, *K*, and ρ , each layer was manually selected. All data processing was performed using MATLAB programming language (The MathWorks Inc., Natick, MA, USA). Averages for each ROI were calculated and used to generate means and standard deviations for each ROI and layer. Statistical results were considered significant for p-values less than 0.05.

Fig. 1. H&E histology (a) and amplitude (b) image of the superior section of the sclera from the treated eye (Sc) with marked ROI 1 to 3 (c). (a) and (b) correspond to the black rectangular region in (c). Anterior (ANT), posterior (POS)

III. RESULTS

Average acoustical and material properties of eyes (i.e. *C*, *M*), regions (i.e. *S*, *I*) and ROIs are summarized in Table I. Fig. 2 and Fig. 3 show maps of bulk modulus and mass density of the scanned sections.

TABLE I. AVERAGE MATERIAL PROPERTIES (MEAN $\pm$ STANDARD DEVIATION)

R	L	Co	K (GPa)	ρ (g/cm ³)	c (m/s)	Z (MRayl)	α (dB/MHz/cm)
1	I	C	2.63 $\pm$ 0.07	1.03 $\pm$ 0.03	1596 $\pm$ 31	1.64 $\pm$ 0.03	11.1 $\pm$ 0.8
1	I	M	2.67 $\pm$ 0.05	0.99 $\pm$ 0.01	1642 $\pm$ 22	1.62 $\pm$ 0.01	9.3 $\pm$ 0.7
1	S	C	2.47 $\pm$ 0.06	1.01 $\pm$ 0.02	1560 $\pm$ 35	1.58 $\pm$ 0.01	12.1 $\pm$ 2.2
1	S	M	2.60 $\pm$ 0.02	0.98 $\pm$ 0.01	1627 $\pm$ 10	1.60 $\pm$ 0.01	9.3 $\pm$ 0.5
2	I	C	2.70 $\pm$ 0.04	0.98 $\pm$ 0.01	1659 $\pm$ 18	1.62 $\pm$ 0.01	9.7 $\pm$ 0.7
2	I	M	2.67 $\pm$ 0.03	0.97 $\pm$ 0.01	1655 $\pm$ 12	1.61 $\pm$ 0.01	8.7 $\pm$ 0.8
2	S	C	2.66 $\pm$ 0.06	0.98 $\pm$ 0.01	1648 $\pm$ 16	1.61 $\pm$ 0.02	10.7 $\pm$ 1.4
2	S	M	2.60 $\pm$ 0.05	1.01 $\pm$ 0.01	1602 $\pm$ 16	1.62 $\pm$ 0.01	9.7 $\pm$ 0.7
3	I	C	2.74 $\pm$ 0.03	0.97 $\pm$ 0.01	1679 $\pm$ 11	1.63 $\pm$ 0.03	8.4 $\pm$ 1.3
3	I	M	2.63 $\pm$ 0.07	0.98 $\pm$ 0.01	1636 $\pm$ 20	1.61 $\pm$ 0.02	7.9 $\pm$ 2.0
3	S	C	2.69 $\pm$ 0.07	1.00 $\pm$ 0.01	1643 $\pm$ 23	1.63 $\pm$ 0.01	9.2 $\pm$ 0.3
3	S	M	2.57 $\pm$ 0.02	1.03 $\pm$ 0.01	1577 $\pm$ 6	1.63 $\pm$ 0.01	7.9 $\pm$ 1.1

Abbreviations are ROIS (R) with (R1 to R3), location (L) Inferior (I), Superior (S), Condition (Co) Myopic (M) and control (C)

N-Way ANOVA revealed that the superior sclera is 0.08 GPa less stiff when compared to inferior. In the control eye the furthest ROIs (R2 and R3) are stiffer compared to ROI closer to the ONH (R1). In general no significant difference was found between the two eyes (i.e. C, M). However, if only the furthest ROI (R3) is considered then the myopic eye was less stiff by 0.2 GPa and 0.07 GPa in the S-region and I-region, respectively.

Values of mass density were found to be lower by 0.02 g/cm³ in the I-region when compared to S-region. N-Way ANOVA revealed significant variations of mass density with respect to the ROIs. The myopic eye had significantly increased values of ρ in the superior section of R2 and R3 but lower values in R1 for both I and S-region. The furthest ROIs measured away from the optic nerve (R2 and R3) showed a distinctive layered structure in terms of K and ρ (Fig. 2 and Fig. 3). L1 showed the highest mass density (1.06 ± 0.02 g/cm³) but lowest bulk modulus (2.47 ± 0.09 GPa). Fig. 4 shows boxplots summarizing the results from ANOVA analysis.

Across R2 and R3, L2 and L3 were not significantly different in mass density with an average ρ of 0.97 ± 0.04 g/cm³. The highest values for K were found in L2 and L3 with $K_{L3}=2.94 \pm 0.2$ GPa and $K_{L2}=2.81 \pm 0.2$ GPa. The layered structure was not visible in the H&E stained images (Fig. 1). When the layers were compared between myopic and control eye, only L2 was found to be significantly less stiff by 0.2 GPa in the myopic eye. The mass density was significantly higher in

the superior sections of the myopic eye in layer 2 3 and 4 by an average of 0.038 g/cm³.

Fig. 2. Bulk modulus of myopic (M) and control (C) eye, at superior (S) and inferior region (I) with zoomed in areas and one example showing the segmented layered structure.

Fig. 3. Mass density of myopic (M) and control (C) eye, at superior (S) and inferior region (I) with zoomed in areas and one example showing the segmented layered structure.

IV. DISCUSSION

This study shows a layered structure in the guinea pig sclera with differing properties in ρ and K, which may correspond to the structure of the collagen network. These layers are typically not seen in H&E-stained, light-microscopy images, but may be associated with differences in collagen diameter and orientation in the inner and outer sclera. Other studies using electron microscopy have separated the vertebral sclera into three inner layers of the stroma plus an outer thin but dense layer referred to as epi sclera [17], which is in accordance with our findings. Curtin et al. found that the outer

and middle layers have similar distributions of fibril diameters (161 and 148 nm, respectively), while the innermost layer has a much smaller average diameter of 96 nm [18]. An average smaller fibril diameter likely leads to a weaker and less stiff sclera tissue. This is supported by our findings showing higher stiffness values in L2 and L3 and significantly lower values in L1 (Fig. 4a). In general it was reported that smaller fiber diameters and collagen crosslinking are associated with myopic conditions [4, 5]. A smaller fiber diameter might lead to a less stiff tissue at the microscopic level leading to a weaker and less load bearing sclera in myopic conditions as found in other studies. In fact our results suggest a less stiff but denser sclera in regions further from the ONH. The larger densities might be explained by an increased cross linking. We hypothesize that smaller fiber diameters are associated with less stiff sclera leading to a weaker tissue in myopia conditions that cannot be solely explained by scleral wall thinning. Interestingly our study found the largest difference of K between myopic and control eye in L2 emphasizing an important role of sclera micro structure under myopic conditions.

Fig. 4. Box plots showing variation of bulk modulus (K) and mass density of the four layers found in the sclera

Currently we are investigating a much larger set of data including both horizontal and vertical scans from 20 neonatal guinea pigs with different levels of myopia which will allow conclusions to be made in relation to possible regional differences induced by myopia. Further studies could consider other modalities which enable visualization of the nanostructure of the sclera, such as electron microscopy, in combination with SAM at higher frequencies (≥ 500 MHz). This will provide spatial resolutions exceeding $4 \mu\text{m}$, and allow investigation of the relation between microstructural organization of the collagen fibers and the mechanical outcome at the microscopic level.

In general, fine-resolution biomechanical properties of soft tissues provided by SAM may be invaluable for investigating ocular tissues in diseases, such as high myopia, where tissue elasticity and mechanical properties are believed to be an important factor.

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