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Effects of Storage Time on the Mechanical Properties of Rabbit Peripapillary Sclera After Enucleation

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ABSTRACT In the field of biomechanics, little research has been performed to evaluate the effect of storage time on the material properties of ocular tissues. Twenty-four rabbit eyes were divided into six groups with storage times from 3 to 72 hr. A tensile specimen was prepared from the inferior quadrant of each sclera and was subjected to a stress relaxation test. The data were analyzed using linear viscoelastic theory yielding four material parameters (E_0, instantaneous elastic modulus; E_∞, equilibrium elastic modulus; β, half-width of the Gaussian distribution; τ_m; mean relaxation time). No statistically significant differences were found in the material properties of each group, which suggests that sclera can be stored up to 3 days without risking mechanical deterioration.

KEYWORDS biomechanics; glaucoma; sclera

INTRODUCTION

Glaucomatous optic neuropathy is one of the three leading causes of blindness in the United States.[1] While intraocular pressure (IOP) plays a central role in the development of glaucomatous vision loss, other factors such as age, ischemia, and inflammation are likely important in the disease.[2]

In order to evaluate the effect of elevated IOP on the connective tissues of the posterior pole and optic nerve head, a computational biomechanical model of the posterior pole of the eye is currently under construction in our laboratory.[2] The ultimate goal is to fully characterize the biomechanical behavior of each tissue within and around the optic nerve head (ONH). To build these models, accurate values for material properties of the load-bearing tissues are necessary. Preliminary work has been done on the material properties of monkey and rabbit peripapillary sclera.[3,4]

The sclera is the outer shell and principal load-bearing tissue of the eye. Consisting primarily of collagen fibers[5] and essentially avascular, it provides constant protection for the adjacent intraocular tissues. Ninety percent of the sclera collagen fibers are collagen type I fibers, which provides the majority of the tissue's mechanical integrity and tensile strength.

The posterior sclera, just nasal to the fovea, contains the scleral canal, which is spanned by a fenestrated connective tissue structure known as the lamina cribrosa. The retinal ganglion cell axons that transmit visual signals from the

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retina to the brain pass through these fenestrations. The mechanical properties of the sclera immediately adjacent to this canal (the peripapillary sclera) likely influence the biomechanics of the optic nerve head and are therefore very important in engineering models used to determine to which extent the ONH will deform under changing IOP.

Most mechanical testing experiments on biologic soft tissues are conducted just after death to ensure the results reflect the specimen's original physiologic state as closely as possible. Human donor eyes are usually stored in phosphate-buffered saline (PBS) before they are released for research, however the effect of postenucleation storage time on the material properties of the load-bearing ocular tissues has not been rigorously characterized. The purpose of this study was to investigate the effects of postenucleation storage time on the mechanical properties of rabbit peripapillary sclera using linear-viscoelastic theory.

MATERIALS AND METHODS

Testing Protocol

Twelve mature New Zealand white rabbits were anesthetized with an intramuscular injection of ketamine/xylazine and sacrificed with an intravenous sodium pentobarbital injection. After enucleation, eyes were randomly divided into six groups (four samples per group) according to the postenucleation time (3, 8, 24, 36, 48, and 72 hr), cleaned from extraorbital tissues, and stored in a PBS solution at 4°C. Human donor eyes are generally available within 48 hr postmortem, therefore 72 hr was chosen as the maximum time limit. Other time points were added at 3, 8, 24, 36, and 48 hr to ensure that the time course of changes in the mechanical response of sclera, should they occur, would be detected. The sample size of four animals per group was identified in a power analysis as the minimum number of samples necessary to achieve statistical significance if changes in material properties were present.[3,4]

For each eye, the anterior chamber, vitreous, retina, and choroid were removed in order to isolate the sclera. Each scleral shell was then mounted on a fixed, 15-mm-diameter polyethylene sphere, and a custom-built cutting die (three mating stainless steel parts that compress two flexible razor blades into a precise dog-bone shape) was rotated over the surface of each scleral shell to generate a tensile test specimen (Fig. 1). A detailed

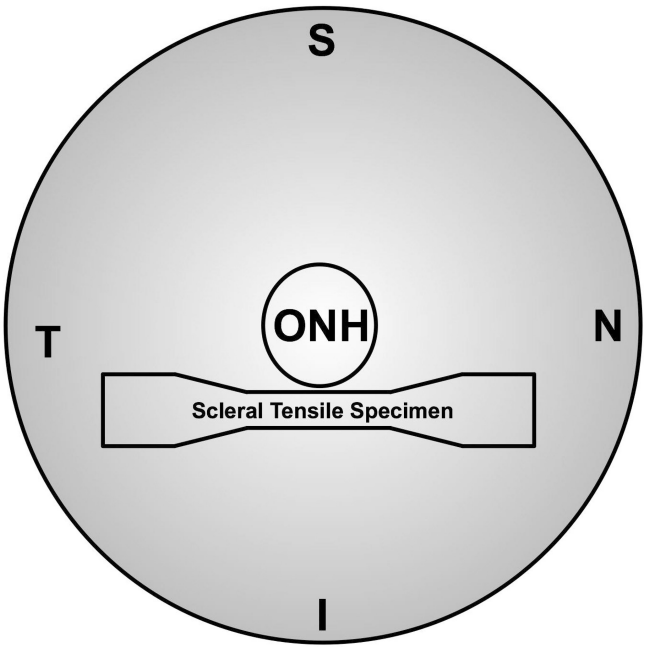

FIGURE 1 **Posterior view of the scleral shell of a left rabbit eye showing the position of the gauge length of a scleral tensile specimen harvested from the inferior quadrant.**

description of the tensile specimen preparation protocol has been reported previously.[3]

Each scleral specimen, generated with a gauge length of 8 mm, a gauge width of 3 mm, and an averaged thickness of 750 μm, was prepared from the inferior quadrant of the sclera with its center tangent to the optic nerve head (Fig. 1). The specimen was then clamped via custom-built soft tissue grips within an environment chamber maintained at 37°C and kept moist with an isotonic saline mist.[3] The sample was then slowly preloaded to 0.08 N using a material testing machine (MTS Renew 1122, Eden Prairie, MN, USA) equipped with a 2000 g load cell, then a double-armed, soft-contact extensometer (MTS Biomedical extensometer, model 632.32F, Eden Prairie, MN, USA) was applied to the central 5 mm of the specimen in order to measure the actual strain of the peripapillary sclera in the gauge section.

After extensometer placement, the specimen was subjected to a uniaxial preconditioning in tension, consisting of 10 cycles from 0 to a maximum of 1% strain at a rate of 1% per second. The specimen was allowed to recover for 360 s, and then a stress relaxation test was performed as follows. A 1%/s strain ramp to 1% strain was applied to the tissue and the stress relaxation behavior was recorded while displacement was held constant for 1000 s (Fig. 2). We based this strain range on our estimate that the posterior sclera shell experiences

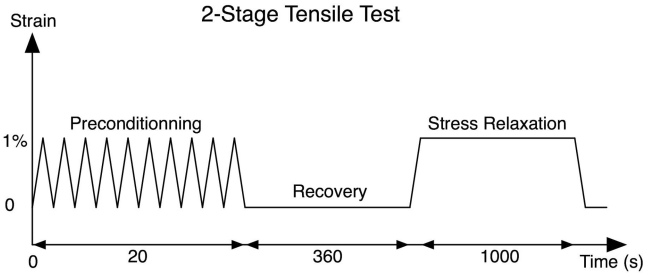

FIGURE 2 **Diagram showing the time course of the two-stage, uniaxial tensile testing protocol.**

a strain of the order 1% under normal physiologic conditions according to thin-walled, spherical pressure vessel theory.[6] Furthermore, our previous studies[3,4] have shown that a stress relaxation test is appropriate because the sclera's mechanical behavior under uniaxial tension can be accurately described with a time-dependent mathematical theory (i.e., linear-viscoelastic theory).

Mathematical Model

In order to quantify the relaxation phenomenon of the peripapillary sclera, we used linear-viscoelastic theory, which was initially described by Fung.[7] The model states that a viscoelastic material when subjected to a constant strain $\varepsilon_0$exhibits a stress relaxation response (Fig. 3) that can be expressed as

$$\sigma(\varepsilon_0, t) = G(t) \cdot \sigma^e(\varepsilon_0) \tag{1}$$

in which G is the reduced relaxation function, a normalized function of time, and σ^e is the elastic response function of the applied strain, ε_o. $\sigma^e(\varepsilon_0)$ can be described as the tensile stress instantaneously generated in the material when a step function of strain, ε_0,is applied to the specimen, so $\sigma^e(\varepsilon_0) = E_0\varepsilon_0$, with E_0 being the instantaneous elastic modulus of the material.

The function$G$, which satisfies$G(0) = 1$, can be expressed as

$$G(t) = \frac{1 + c\int_{-\infty}^{+\infty} \Phi(\log\tau)\exp(-\frac{t}{\tau})d\log\tau}{1 + c\int_{-\infty}^{+\infty} \Phi(\log\tau)d\log\tau} \tag{2}$$

in which c is a relaxation ratio determined by $c = G(0)/G(\infty) - 1$, τ is the relaxation time, and Φ is the log distribution function of the relaxation time,[8] which satisfies

$$\int_{-\infty}^{+\infty} \Phi(\log\tau)d\log\tau = 1 \tag{3}$$

We chose a log-normal distribution function for $\Phi(\log\ \tau)$ (Gaussian distribution)[9] which can be expressed as

$$\Phi(\log\tau) = \frac{1}{\beta\sqrt{\pi}}\exp\left(-\frac{(\log\tau - \log\tau_m)^2}{\beta^2}\right) \tag{4}$$

where β is the half-width of the Gaussian distribution, and τ_m is the mean relaxation time constant (i.e., the most probable value of τ).

As suggested by Wagner,[10] the variable $z = \log(\tau/\tau_m)$ can be introduced in Eq. (4) and satisfies $dz = d\log\tau$.

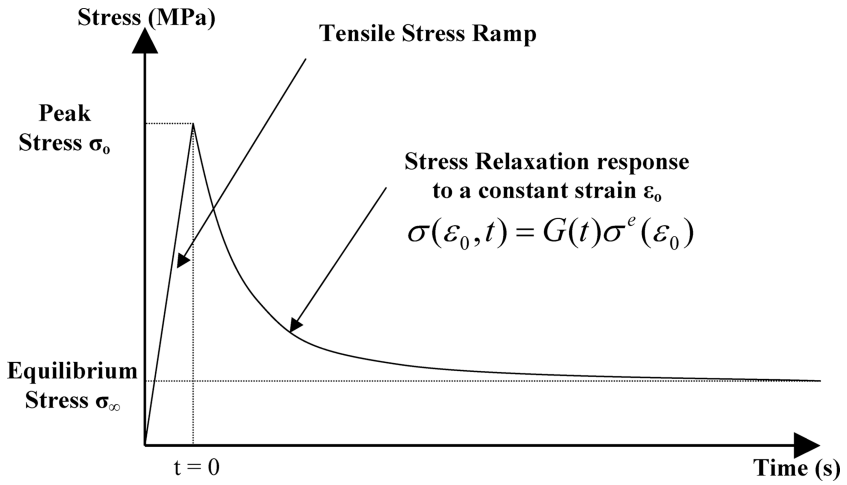

FIGURE 3 **A typical stress relaxation response of soft tissue. In this study, the specimen was stretched at a strain rate of 1%/s to the peak strain of 1%. At time $t = 0$, the displacement was fixed (no further stretching of the specimen) and the stress monitored until the specimen reached stress equilibrium (1000 s).**

TABLE 1 **Material properties of rabbit peripapillary sclera listed by postmortem group (mean value ± standard deviation)**

Postmortem storage time (hr)	E_o (MPa)	E_∞ (MPa)	β	τ_m (s)
3	28.73 ± 7.12	3.30 ± 0.43	1.47 ± 0.21	14.50 ± 4.00
8	24.35 ± 7.52	3.77 ± 1.09	1.64 ± 0.29	20.81 ± 10.88
24	30.33 ± 6.91	3.17 ± 1.29	1.38 ± 0.15	11.99 ± 5.22
36	25.73 ± 3.88	3.00 ± 0.89	1.55 ± 0.15	10.27 ± 3.36
48	22.96 ± 7.59	2.65 ± 1.84	1.54 ± 0.06	18.76 ± 14.57
72	29.32 ± 3.76	3.20 ± 0.51	1.27 ± 0.14	16.29 ± 9.41

After simplification, the stress relaxation response can be written as

$$\sigma(t) = \frac{\sigma_0}{1+c}\left(1 + \frac{c}{\beta\sqrt{\pi}}\int_{-\infty}^{+\infty} \exp\left(-\frac{z^2}{\beta^2} - \frac{t}{\tau_m 10^z}\right) dz\right) \tag{5}$$

where σ_0 is defined as the peak stress (Fig. 3) and is directly obtained from the experimental data.

A genetic optimization algorithm[11] was employed to curve-fit the stress calculation of Eq. (5) with the experimental data, thus leading to unique estimates of the three model parameters: β,τ_m and c.

From the estimated model parameters, two elastic moduli, the instantaneous elastic modulus E_0 and the equilibrium elastic modulus E_∞, were determined as follows

$$E_0 = \frac{\sigma_0}{\varepsilon_0} \tag{6}$$

$$E_\infty = \frac{E_0}{1+c} \tag{7}$$

Statistical Analysis

An analysis of variance (ANOVA) was employed to compare the four material parameters (β, τ_m, E_0, andE_∞) among the six groups ($n = 4$ each for postmortem times of 3, 8, 24, 36, 48, and 72 hr) and to determine the effects of postenucleation storage time on the mechanical properties of rabbit peripapillary sclera.

RESULTS

Mean stress relaxation curves were plotted for each group (Fig. 4) ($n = 4$ samples per group). Mean value ± standard deviation of each of the four material parameters (E_0, instantaneous elastic modulus; E_∞, equilibrium elastic modulus; β, half-width of the Gaussian distribution; τ_m, mean relaxation time) are shown in Table 1. By ANOVA, no statistically significant difference was detected in the material properties of

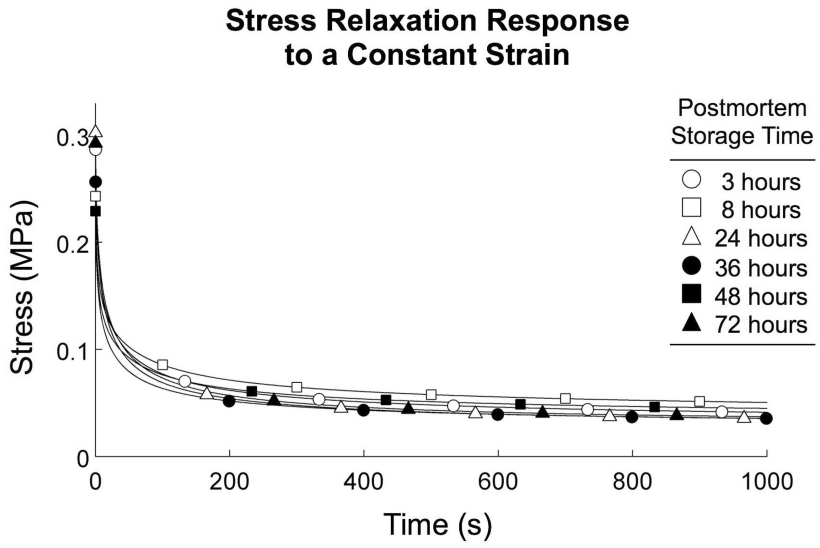

FIGURE 4 **Average stress relaxation responses of rabbit peripapillary sclera (inferior quadrant only) for postmortem storage times of 3, 8, 24, 36, 48, and 72 hr.**

the peripapillary sclera between the groups ($p > 0.05$ for each of the four parameters).

DISCUSSION

In this report, we quantified the viscoelastic behavior of rabbit peripapillary sclera by using four viscoelastic parameters. Eyes were divided into six groups according to the time separating the animal sacrifice from the beginning of the test (postmortem time of 3, 8, 24, 36, 48, and 72 hr). No significant differences in the viscoelastic properties of rabbit peripapillary sclera from each group were detected, which indicates that rabbit sclera can be stored in PBS at 4°C for up to 72 hr without causing any detectible change in its mechanical properties.

One of the possible explanations for this result is that sclera is predominately avascular, has a low cell density, and consists mainly of collagen fibers (75% to 80% of its dry weight[5]). Because of the preponderance of extracellular matrix (ECM), the necrosis of scleral cells (mainly fibroblasts) is unlikely to affect the mechanical properties of the tissue. While this is likely true for the sclera, prolonged storage time might have an impact on highly perfused tissues with a high cell density, such as retina, choroid, nerve fiber layer, blood vessels, and musculature. In such tissues, the anchoring junctions mechanically joining these cells could deteriorate rapidly due to postmortem cell necrosis, which could disturb the integrity of the tissue and result in a change of mechanical properties.[11]

It has been shown that sclera from humans and rabbits have similar organic composition and similar proteoglycan content and collagen organization.[13] As a result, mechanical testing of ocular load-bearing tissues from human donor eyes that have been collected and stored in PBS for up to 72 hr should yield reliable material property data. There have also been some studies that relied on histomorphometric measures of IOP-induced tissue deformation in human donor eyes that were fixed at pressure via cannulation.[14] Our results suggest that as long as the fixation was performed within 72 hr postmortem, the results from these studies should provide insight into the true deformation of the load-bearing tissues.

Downs[3] reported both rabbit peripapillary sclera instantaneous and equilibrium elastic moduli to be 11.6 MPa and 0.79 MPa in average, respectively. These values are about three times lower than the values obtained in this study and these differences likely arise from the improvement of both experimental protocol and computational fitting of the material parameters that have been used in this study. First, the tensile tests were performed on a much more accurate test frame fitted with a high-sensitivity, low-noise load cell, which yielded improved stress relaxation curves with a significantly higher signal-to-noise ratio. Second, an improved relaxation spectrum (Eq. 4) was employed to estimate the viscoelastic stress relaxation, which produced a stress relaxation curve that more closely matched the experimental data, resulting in better estimates of material property parameters.

Several limitations warrant further discussion. First, the initial strain ramp phase (1 s) used in the stress relaxation experiment was excluded in the model simulation. As in similar experiments of this kind, stress relaxation can take place during the initial strain ramp phase, which likely results in an error in the instantaneous elastic modulus reported in Table 1. We investigated the effects of including the initial ramp phase in the model and found that the computed peak stress (at $t = 0$ in Fig. 3) showed an error as large as 25% when compared with the experimental peak stress value (data not shown). We believe that ignoring the tensile ramp phase in the computational fitting of parameters provides the best estimate for the material parameters.

Second, the scleral thickness was assumed to be uniform at 750 μm for all specimens.[3] The potential variation in the scleral thickness in each sample could introduce errors in the stress calculation, which would likely affect the reported values of the elastic moduli, E_0 and E_∞. To minimize the effects of varying scleral thickness on our results, all scleral samples were harvested from the inferior quadrant of the posterior pole, and were randomized to treatment. Previous histomorphometric characterizations of scleral thickness have indicated that the sclera is composed of two principle layers: the dense lamellar load-bearing sclera, and the loose, disorganized episclera.[15,16] Only the dense scleral tissue should be attributed thickness for the purpose of material property determination through mechanical testing. Unfortunately, these layers are inseparable via dissection in fresh sclera, and it remains unclear how one might measure the thickness of the load-bearing layer alone in fresh tissues.

Third, the sclera has complex anisotropic characteristics produced from the multidirectional arrangement of

collagen fibers embedded in the ground substance.[17,18] It has been shown that the collagen and elastin fibers of the sclera are predominately oriented circumferentially to the ONH in the immediate peripapillary region. [19] As our specimens were harvested from the immediate peripapillary region (Fig. 1), it is likely that the load-bearing fibers in the gauge section of our tested samples were predominately oriented along the axial direction of the tensile test in the study. Therefore, the data presented here likely represent the mechanical properties of the sclera in the direction of the collagen fibers. A multiaxial mechanical testing protocol is necessary to describe the three-dimensional behavior of sclera with a multidirectional arrangement of collagen fibers, and this approach is in development in our laboratory.

Finally, the rabbit eyes in this study were enucleated, placed in PBS, and stored at 4°C within 2 hr after death. However, in the case of human cadaver eyes, it is most common for 6 to 48 hr to pass between the time of death and the time of enucleation. Our study was not designed to test the effect of time to placement in PBS, during which the tissue may undergo undetermined degradation.

Biomechanical testing and modeling of the human sclera will contribute to our understanding of glaucomatous optic nerve head damage, myopia, and a host of other ocular disorders. The results from this study suggest that for human cadaver eyes obtained soon after death, scleral viscoelastic material properties should be stable for postmortem storage times (in PBS) of up to 72 hr.

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