Human Transscleral Albumin Permeability and the Effect of Topographical Location and Donor Age

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PURPOSE. To quantify the permeability coefficient of albumin across human sclera and to assess topographical and age-related variation.

METHODS. Equatorial superotemporal scleral tissue from 15 donor eyes (mean age 60 years; range 39–84) was mounted in a modified Ussing chamber. Additional tissue was taken from the anterior and posterior superotemporal regions of six eyes, and equatorial superonasal, and inferotemporal regions of a further six eyes. Fluorescein isothiocyanate (FITC)-labeled, 0.412 mM, bovine albumin was placed in one hemichamber facing the internal scleral surface, and the rate of transscleral flux was determined over 24 hours, at 25°C, with a spectrophotometer.

RESULTS. Permeability coefficient for equatorial superotemporal scleral tissue at 25°C (±SD) was 0.85 ± 0.50 × 10−6 cm·s−1. The permeability coefficient adjusted for 37°C (±SD) was 1.43 ± 0.86 × 10−6 cm·s−1. The effect of donor age was assessed for the 15 equatorial superotemporal samples. Regression analysis showed a significant decline in scleral diffusion of albumin with increasing donor age (P = 0.0166). There was no significant difference in diffusion over the different topographical regions tested. The partition coefficient of permeability to albumin also showed a decline with increasing donor age (P = 0.001).

CONCLUSIONS. The permeability and partition coefficients of human sclera both significantly decline with increasing donor age. Permeability coefficient shows no significant variation over the different topographical regions tested. The decrease in albumin permeability with increasing donor age may have pharmacokinetic implications when considering transscleral diffusion of high-molecular-weight compounds. (Invest Ophthal mol Vis Sci. 2008;49:4041–4045) DOI:10.1167/iovs.07-1660

Transscleral protein movement is important not only for understanding normal and abnormal ocular physiology, but also for ocular pharmacotherapy, especially in the light of recent advances in intravitreous drug delivery. With the increased use of intravitreous injections of vascular endothelial growth factor inhibitors (anti-VEGF), comes the need for an increased knowledge of macromolecular protein flow characteristics across ocular structures.1–4 An improved understanding of ocular pharmacokinetics may enable drug modifications that enhance or prolong intravitreous drug activity, or allow extracocular administration of these novel agents. Animal and human studies in which the transscleral movement of several potential therapeutic agents are investigated depend on the flow characteristics of proteins moving into the eye, against the transscleral flow of water out of the eye.5–4 The movement of water out of the eye (scleral hydraulic conductivity) has been quantified in relation to age and topographic location.5 Understanding the effect these factors have on transscleral drug permeability is also important and may lead to targeting of certain topographic locations for drug delivery and appreciation of how drug effects vary with age. In this paper, we report the effect of age and topographical variation on the transscleral permeability of a key biological protein, albumin, in cadaveric human eyes. To our knowledge, this is the first report to quantify the human scleral permeability coefficient to albumin.

METHODS

Preparation of Tissue and Measurement of Transscleral Albumin Permeability

Fifteen human donor eyes (4 male and 11 female) were dissected to obtain full-thickness scleral specimens. Eyes were obtained from the U.K. Tissue Transplant Service (Bristol, UK), transported from the eye bank on ice, and stored at 4°C in the laboratory. The study was undertaken in accordance with the guidelines of the Declaration of Helsinki for research involving human tissue. The mean donor age was 60 years, with a range of 39 to 84 years. Those with known systemic conditions with ocular manifestations were excluded.

Sections of full-thickness sclera measuring approximately 8 by 9 mm were dissected from the equatorial superotemporal regions in all donor eyes. Areas under the rectus muscles and involving the vortex veins were avoided. Specimens with any suggestion of damage were excluded. An ophthalmic surgeon (OOA) carefully removed the episclera and choroid, with forceps, spring scissors, and a dissecting microscope. A 1-mm wide scleral section was removed from the edge of each specimen, with a razor blade. This section was used to determine scleral thickness, as described later.

The specimens were placed in a modified Ussing chamber6 with a 6-mm aperture. The screws that hold each half of the chamber together were tightened with a torque-range screwdriver (RS Components, Corby, UK), by applying the minimum force necessary to prevent leakage around the specimen (approximately 30 cN·m−1). The integrity of tissue clamped in this type of device has been demonstrated.5,7–9 Histology of clamped sclera has shown tissue compression to extend for a minimal distance into the central aperture, with the collagen fibrils remaining intact.5 Both hemichambers were filled with 750 µL phosphate-buffered saline (PBS; sodium chloride 137 mM, potassium chloride 2.7 mM, phosphate buffer 10 mM, pH 7.4 at 25°C; Sigma, Poole, UK), with penicillin 100,000 U·L−1, streptomycin 100 mg·L−1, and amphotericin B 250 µg·L−1 (Sigma-Aldrich, St. Louis, MO). In addition 0.412 mM fluorescein isothiocyanate (FITC) labeled bovine serum albumin (Sigma-Aldrich) was placed into the hemichamber facing the internal scleral surface. During both the setup and subsequent fluid removal stages, care was taken to keep the fluid volume in each hemichamber identical, to prevent the risk of harmful hydrostatic

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pressure gradients developing across the sample. Evaporation was minimized by sealing the tops of the chambers with insulation tape. Each tape was pierced to prevent a pressure gradient developing across the sample. Small plastic-encased magnetic stirers were inserted into the bottom of each hemichamber. Experiments were conducted at 25°C, with the specimens protected from ambient illumination.

After a mean (±SD) of 11.11 ± 1.32 hours ($t_2$), 250 μL of fluid was simultaneously removed from both chambers. This process was repeated at a mean at 22.95 ± 1.66 hours ($t_2$). Any potential effect of leaching of glycosaminoglycans was minimized by limitation of the experimental duration to no more than 24 hours.10 Two samples were taken to eliminate any measurement error induced by a delay in the appearance of FITC-labeled bovine albumin in the second chamber, after commencing the experiment. Pilot studies, removing three samples over the same time period, had demonstrated the rate of increase in FITC-labeled albumin in the second chamber to be linear ($R^2 = 0.99$) and to occur after a significant time delay. The sample times were chosen as they occurred during the linear portion of diffusion, irrespective of the age of the donor. The maximum absorbance of FITC-albumin was at 490 nm and therefore this frequency was used for spectrophotometric quantification of each sample (UV-160; Shimadzu, Kyoto, Japan). The concentration of FITC-labeled bovine albumin was then calculated from predetermined standard curves.

**Topographical Variation in Permeability Coefficient to Albumin**

Experiments were repeated in 6 of the 15 eyes, using additional tissue taken from the anterior and posterior superotemporal regions. A further six eyes had additional tissue taken from the equatorial superonasal and equatorial inferotemporal regions.

**Measurement of Scleral Thickness**

Scleral thickness was measured in tissue removed from the main specimen (before mounting it in the Ussing chamber). The scleral specimen was fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer containing 10 g·L⁻¹ calcium chloride (final pH 7.4; Sigma-Aldrich). Samples were rinsed in sucrose, postfixed in 2% osmium tetroxide (Sigma-Aldrich) in 0.2 M sodium cacodylate buffer for at least 1 hour, dehydrated in a graded series of ethanol, and fixed in epoxy resin (Araldite CY212; Agar Scientific, Ltd., Cambridge, UK). The orientation of scleral specimens was preserved, and multiple semithin (1 μm) sections were cut, transferred to plain microscope slides containing a film of distilled water, and stained with toluidine blue. Scleral thickness was measured centrally and at either end of each section, with a light microscope fitted with an eye piece graticule (Leica Microsystems, Heerbrugg, Switzerland) and a calibration slide (Carl Zeiss, Oberkochen, Germany). An average of these readings was taken. Previous studies have shown little difference in scleral thickness, when measured by two different methods: fixed human scleral sections and ultrasonic measurement of unfixed tissue.11

**Permeability Coefficient**

The molar amount of FITC-albumin crossing the sclera between the two measured time points ($t_1$ and $t_2$) was determined. This amount was then used to calculate the flux of solute across the sclera. Flux is defined as the amount of solute crossing a plane of unit surface area, which is normal (perpendicular) to the direction of transport, per unit of time.12 The permeability coefficient, measured in centimeters per second, can then be determined based on the flux of the system divided by the concentration difference between the two chambers. The peak concentration of solute in the second chamber never achieved a level of more than 5% of the concentration in the first chamber over the measured time course of these experiments. Therefore, the concentration difference between the two chambers was taken to equal the concentration in the first chamber. This is in agreement with Olsen et al.13

The permeability coefficient (of FITC-albumin through human sclera) was calculated as follows:

$$\text{Permeability coefficient } = \frac{R_{\text{total}}(A \times t)}{[1/C]}. \quad (1)$$

where $R_{\text{total}}$ equals the total moles passing through the sclera during time $t$, $t$ (measured in seconds) is the period between the first and second sample (i.e., $t_2 - t_1$), $A$ is the area of the surface of the scleral sample (in square centimeters), and $C$ is the concentration of the original solute in the first chamber (moles per milliliter).14

**Partition Coefficient**

The partition coefficient was determined in six equatorial scleral samples. Scleral samples were equilibrated for 24 hours in 0.412 mM FITC labeled bovine serum albumin (Sigma-Aldrich) dissolved in PBS, containing penicillin 100,000 U·L⁻¹, streptomycin 100 mg·L⁻¹, and amphotericin B 250 μg·L⁻¹ (Sigma-Aldrich). The FITC-labeled albumin was then fully desorbed from the tissue by repeated washings with PBS.

The partition coefficient ($K$) was determined as follows:

$$K = \frac{C_{A_{\text{in tissue}}}/C_{A_{\text{in equilibrating solution}}}}{C_{A_{\text{in tissue}}}/C_{A_{\text{in equilibrating solution}}}}. \quad (2)$$

where $C_{A_{\text{in tissue}}}$ is the concentration of FITC-labeled albumin in the tissue obtained from the wash fluid, and $C_{A_{\text{in equilibrating solution}}}$ is the concentration in the equilibrating solution (0.412 mM). Partition coefficients were measured on paired samples at both 25°C and 37°C. To minimize the effect of potential leaching of glycosaminoglycans, we limited the experimental duration of equilibration to no more than 24 hours.10,15 FITC-labeled albumin was shown to be fully equilibrated by this point.

The measurement of the partition coefficient assumes homogeneity in a tissue, which is actually heterogeneous.16 As with many experiments in biological tissues, this problem can never be fully resolved. However, it remains reasonable to attempt to measure the partition coefficient by using techniques previously reported in the literature.10,15

**Adjustment for Temperature**

Experiments were performed at 25°C. The permeability coefficient was adjusted to provide a value at 37°C. This was performed as follows.

The relationship between diffusion coefficient and permeability coefficient was determined as follows:

$$D = P/K. \quad (3)$$

where $D$ is diffusion coefficient (in square centimeters per second), $P$ is the permeability coefficient (in centimeters per second), $K$ is the partition coefficient (in units) and $l$ is the thickness of the sclera across which flux is measured (in centimeters).12,15 Einstein’s relationship5,17 can thus be adapted to the following:

$$P_{37} = P_{25}(K_{37}/K_{25})(T_{25}/T_{37})(\eta_{25}/\eta_{37}). \quad (4)$$

where $P$ is the permeability (in square centimeters per second), $K$ is the partition coefficient, $T$ is the absolute temperature (in Kelvins), and $\eta$ is the viscosity of water at 25°C (0.950 mPa·s) and 37°C (0.680 mPa·s). Therefore:

$$P_{37} = 1.453 \times P_{25}. \quad (5)$$

**Statistical Tests**

The paired, two-tailed t-test was used to compare means. The coefficient of determination $R^2$ and analysis of variance were used for linear...
Regression. Kolmogorov-Smirnov assumptions tests were used to confirm Gaussian distribution. $P < 0.05$ was considered significant.

**RESULTS**

**Transscleral Permeability Coefficient and Age**

The permeability coefficient of albumin, using equatorial superotemporal scleral tissue at 25°C (±SD) was $0.85 ± 0.50 \times 10^{-6}$ cm $\cdot$ s$^{-1}$. The permeability coefficient for equatorial superotemporal scleral tissue adjusted for 37°C (±SD) was $1.21 ± 0.70 \times 10^{-6}$ cm $\cdot$ s$^{-1}$. The effect of donor age was assessed for the 15 equatorial superotemporal samples (Fig. 1). Regression analysis showed a significant decline in scleral permeability coefficient to albumin with increasing donor age (linear $R^2 = 0.37; P = 0.016$).

**Transscleral Permeability Coefficient and Scleral Thickness**

The relationship between scleral permeability and scleral thickness was assessed for the 15 superotemporal equatorial scleral specimens. There was no statistically significant relationship between albumin diffusion and scleral thickness (linear $R^2 = 0.0030; P = 0.87$). There was no statistically significant relationship between age and scleral thickness (linear $R^2 = 0.0025; P = 0.88$).

**Transscleral Permeability Coefficient and Topographic Location**

There was no significant difference in permeability coefficient over the different topographic regions tested (Fig. 2).

The topographic variation in scleral thickness was assessed for the 12 eyes used for topographic studies. There was no statistically significant variation in mean scleral thickness when comparing the equatorial superotemporal region with the anterior and posterior superotemporal regions ($P = 0.17$ and 0.25, respectively). The mean scleral thickness of the anterior and posterior superotemporal regions was significantly different ($P = 0.0012$). When comparing the superotemporal equatorial region with the superonasal and inferotemporal equatorial regions, no significant difference was found ($P = 0.86$ and 0.067, respectively). The mean scleral thickness of the superonasal and inferotemporal equatorial regions was significantly different ($P = 0.031$; Table 1).

**Partition Coefficient and Age**

The partition coefficient of albumin, using equatorial scleral tissue at 25°C (±SD) was $0.62 ± 0.17$. Regression analysis showed a significant decline in the scleral partition coefficient with increasing donor age (linear $R^2 = 0.98; P = 0.001$).

**Table 1. Mean Scleral Thickness for Each Topographical Location**

<table>
<thead>
<tr>
<th>Topographic Location</th>
<th>Mean Scleral Thickness (mm ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superotemporal anterior (n = 6)</td>
<td>$0.57 ± 0.23^*$</td>
</tr>
<tr>
<td>Superotemporal equatorial (n = 6)</td>
<td>$0.81 ± 0.28$</td>
</tr>
<tr>
<td>Superotemporal posterior (n = 6)</td>
<td>$1.05 ± 0.29^*$</td>
</tr>
<tr>
<td>Equatorial superonasal (n = 6)</td>
<td>$0.62 ± 0.086^†$</td>
</tr>
<tr>
<td>Equatorial superotemporal (n = 6)</td>
<td>$0.63 ± 0.11$</td>
</tr>
<tr>
<td>Equatorial inferotemporal (n = 6)</td>
<td>$0.76 ± 0.087^‡$</td>
</tr>
</tbody>
</table>

* Statistically significant difference between superotemporal anterior and posterior regions.
† Statistically significant difference between equatorial superonasal and inferotemporal regions.
was no significant difference between partition coefficients measured at 25°C and 37°C (0.62 ± 0.14; $P = 0.96$).

**DISCUSSION**

The purpose of this study was to determine whether age and topographic location had any influence on human trans-scleral permeability coefficient to albumin. The mean permeability coefficient (±SD) for equatorial superotemporal scleral tissue at 25°C was 0.85 ± 0.50 × 10⁻⁶ cm·s⁻¹. This value declined significantly with increasing age and was independent of both scleral thickness and topographic location. The mean partition coefficient for albumin at 25°C was determined to be 0.62 ± 0.17 and showed a significant decline with increasing age.

The adjusted scleral permeability coefficient of albumin at 37°C (1.21 × 10⁻⁶ cm·s⁻¹) was of a magnitude similar to that recorded in previous reports concerning rabbit sclera and albumin (5.49 × 10⁻⁶ cm·s⁻¹). To our knowledge, no other reports have quantified either albumin permeability coefficient or partition coefficient in human sclera. The use of Einstein’s relationship is consistent with previous reports of its use in scleral and neural tissue. The application of a homogeneous phase equation to heterogenous tissue such as sclera has limitations. As a result its use has been strictly limited, to allow comparison with other relevant research, and does not influence the main conclusions of the study.

The question as to whether scleral permeability changes with age has been the subject of conflicting reports. Early reports described no appreciable decline in scleral permeability with age. Olsen et al. used ¹⁴C-inulin (molecular mass, 5.25 kDa) and found no difference, but this molecule is appreciably smaller than albumin. In contrast, Boubriak et al. determined that human scleral hydration and partition coefficient decreased with increasing age. They therefore postulated that transscleral diffusion would decrease with increasing age, although they did not directly measure scleral diffusion over a wide enough age range to link the two directly. Our results support their work by presenting a direct and significant association between decreasing scleral permeability and increasing age.

As human sclera ages, various changes occur. These include increased collagen cross-linking and glycation, leading to reduced scleral compliance and hydration. Although ocular rigidity increases with age, scleral thickness has not been shown to vary significantly. The reduction in interfibrillary distance may have more influence on larger molecules such as albumin, as opposed to smaller molecules such as inulin, accounting for the discrepancy between our results and those of Olsen et al. A similar preferential effect on larger molecules has been demonstrated with regard to partition coefficient. Boubriak et al. postulated that this effect was due to a reduction in interfibrillary space available for molecular storage with smaller molecules still being able to access the tissue more effectively.

We found no significant difference between albumin diffusion over various topographic locations, despite the variation in scleral thickness over the various locations. Scleral thickness has been shown to vary from the front to the back of the eye, with the posterior sclera being almost twice as thick as the anterior sclera. Posterior sclera contains a looser weave of collagen fibrils, greater hydration, and a higher diffusion coefficient per unit thickness. These factors explain our finding no significant variation in scleral permeability in relation to topographic location or scleral thickness.

**CONCLUSIONS**

We present the first quantitative assessment of human scleral albumin permeability coefficient. The results showed that albumin permeability decreases with increasing age. The effect of age on scleral permeability is relevant when considering transscleral administration of existing and potential therapeutic compounds. The lack of topographic variation suggests that drug delivery to the anterior scleral surface may be as effective as the more complex procedures that deposit depot injections in a more posterior location.

**References**


