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Circumferential flow of particles in the suprachoroidal space is impeded by the posterior ciliary arteries

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Abstract

Microneedle injection into the suprachoroidal space (SCS) enables targeted drug delivery for treatment of posterior segment diseases (e.g., posterior uveitis). This study sought to identify and characterize anatomical barriers to circumferential spread of particles in the SCS of rabbit and human cadaver eyes. These barriers could make targeting specific regions within the SCS challenging. A hollow microneedle (33-gauge, 750 μ m long) was used to inject fluorescent particles into albino New Zealand White rabbit eyes *ex vivo* at six different positions around the limbus and a limited number of conditions *in vivo*. SCS injections were also performed in human cadaver eyes 8 mm and 2 mm from the optic nerve (ON). Eyes were dissected and particle distribution was quantified. In rabbit eyes, injections made in the superior or inferior hemispheres (even when injected temporally immediately adjacent to the long posterior ciliary artery (LPCA)) did not significantly cross into the other hemisphere, apparently due to a barrier formed by the LPCA. The vortex veins had a minor effect on particle deposition, limited to only around the vortex vein root. In human eyes, the short posterior ciliary arteries (SPCAs) prevented circumferential spread towards the macula and ON. In conclusion, the rabbit LPCA and the human SPCA were anatomical barriers to particle spread within the SCS. Therefore, design of drug delivery protocols targeting the SCS need to account for barriers formed by anatomical structures in order for injected drug to reach target tissues.

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This potential conflict of interest has been disclosed and is overseen by Georgia Institute of Technology and Emory University.

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Keywords

Ocular drug delivery; Hollow microneedle; Suprachoroidal injection; Long posterior ciliary artery; New Zealand White rabbit; Short posterior ciliary artery

1 Introduction

Suprachoroidal delivery is being explored as a targeted ophthalmic drug delivery technique to treat posterior segment diseases that affect choroidal, retinal pigment epithelial, and/or retinal tissues (Chen et al., 2015; Einmahl et al., 2002; Gilger et al., 2013; Kadam et al., 2013; Olsen et al., 2006; Patel et al., 2012; Patel et al., 2011; Tyagi et al., 2012; Tzameret et al., 2014; Wang et al., 2012). The suprachoroidal space (SCS), which is a potential space between the sclera and choroid, is an attractive drug delivery site because it is directly adjacent to the diseased tissue in many posterior segment diseases, including age-related macular degeneration (AMD) and posterior uveitis (Ambati and Fowler, 2012; Gilger et al., 2013; Kim et al., 2014a). SCS delivery results in increased drug concentrations and bioavailability at the choroid while limiting drug concentrations elsewhere in the ocular globe compared with intravitreal injections (Kadam et al., 2013; Olsen et al., 2011; Patel et al., 2012; Patel et al., 2011; Wang et al., 2012).

The SCS can be accessed with a hollow microneedle that has a length similar to the thickness of sclera (Patel et al., 2012; Patel et al., 2011). The microneedle is designed to penetrate across the sclera and conjunctiva (and physically prevent penetration through the chorioretina) and inject material that can flow circumferentially within the SCS, thus bathing the choroid in drug. The safety and tolerability of microneedle injections into the SCS has been studied in an open-label Phase I/II clinical trial (NCT01789320) where triamcinolone acetate suspension was injected into the SCS to treat noninfectious posterior uveitis (Goldstein et al., 2015). In this clinical trial and supporting pre-clinical studies, the microneedle injection procedure was performed in a manner similar to an intravitreal injection, except it used a much shorter needle and therefore injected into the SCS rather than the vitreous; the procedure was well tolerated in animal and human subjects (Goldstein et al., 2015; Patel et al., 2012; Patel et al., 2011).

Since the chorioretina is typically not uniformly diseased (e.g., AMD affects the macula while sparing the peripheral retina), targeting drugs to specific regions of the chorioretina (i.e., to optimally treat the diseased region of the chorioretina) is of importance. Thus, understanding and controlling the movement of drugs within the SCS is needed to deliver drugs to the diseased tissue using the SCS as a conduit to get there. The purpose of this work is, therefore, to study the movement of particles injected into the SCS and, more specifically, identify barriers that impede circumferential flow in the SCS, which might influence the ability to target specific regions of the SCS.

Previous experiments show that fluid injected into SCS is limited by the scleral spur anteriorly and the optic nerve (ON) posteriorly (Kim et al., 2014b; Patel et al., 2012; Patel et al., 2011). Anatomically, the sclera and the choroid are tightly bound at these two sites, as they delineate the physical extent of the SCS. We hypothesize that there are additional

barriers that prevent circumferential flow in the SCS following microneedle injection. Other likely candidates include the posterior ciliary arteries and the vortex vein since the sclera and choroid are bound at these points (Einmahl et al., 2002). Although a previous study reported no anatomical barriers to spread of micro-bubbles within the SCS (Seiler et al., 2011), in that study injections were carried out at the superior (12 o'clock) position near the limbus, which may not optimally assess the existence of barriers throughout the eye, as shown in the present study.

2 Methods

2.1 Microneedle Injection and Tissue Processing

Enucleated albino New Zealand White rabbit eyes (Pel-Freeze, Rogers, AR) stored at -80°C were used for *ex vivo* rabbit experiments. Frozen eyes were used to facilitate the testing of many parameters, a subset of which were tested in an *in vivo* rabbit model. In all *ex vivo* eyes, no particles were detected in the vitreous, which suggests that the choroid had not degraded. When needed, frozen eyes were rapidly defrosted in a room-temperature water bath for ~30 min, and muscle, conjunctiva, and fascia were dissected from the ocular globe. The superior prime meridian of the eye (i.e., 12 o'clock) and the injection site were marked with a shallow cut in the adjacent cornea using a scalpel dipped in blue or red food coloring (McCormick, Hunt Valley, MD), respectively.

All injections were performed at room temperature ($\sim 23^{\circ}\text{C}$). SCS injections were accomplished using a 33-gauge hollow microneedle with a length of $\sim 750\text{ }\mu\text{m}$ (kindly provided by Clearside Biomedical, Alpharetta, GA) and a 250 μL glass chromatography syringe (National Scientific, Rockwood, TN). Injections were performed 3 mm posterior to the limbus, and occurred in 3 s. The fluid injected was 50 μL of 0.5% (w/v) 200 nm diameter red-fluorescent microspheres (580 nm excitation and 605 nm emission wavelength; FluoSpheres, Life Technologies, Carlsbad, CA) in Hank's Balanced Salt Solution (HBSS, Mediatech, Manassas, VA), unless otherwise specified. After waiting 1 min, the needle was withdrawn from sclera.

To arrest fluid flow in SCS, each eye was rapidly frozen 1 min after injection by submersion in 100% ethanol chilled over dry ice. Eight approximately equally-spaced, full-thickness cuts were made in the sclera from the posterior pole to limbus using a scalpel. This yielded eight scleral flaps (a.k.a., "petals") approximately equal in area. One cut was aligned with the injection site and one with the 12 o'clock position. The scleral flaps were splayed open to expose the frozen vitreous humor, lens and aqueous humor, which were removed with forceps and collected to verify no particles were in the vitreous. Three replicates were performed in each condition unless otherwise stated. The following experiments were performed.

2.1.1 Effect of injection site on spread in the SCS of rabbits—We performed injections at six positions around the limbus of *ex vivo* rabbit eyes (Figure 1A): at the superior (12 o'clock) and inferior (6 o'clock) prime meridians; infratemporal (7.5 o'clock) and supratemporal (10.5 o'clock) locations; and temporally directly superior and inferior the 90° meridian (9 o'clock). Injections were performed in the temporal hemisphere because, in

humans, the macula is temporal to the ON, which we knew limited flow from previous studies (Kim et al., 2014b; Patel et al., 2012; Patel et al., 2011).

2.1.2 Effect of IOP on spread in the SCS of rabbits—To change intraocular pressure (IOP), the eye was cannulated through the ON using a 25-gauge needle connected to a water column of predetermined height (Kim et al., 2014b). We tested three pressures: unpressurized, 10-12 mmHg (normotensive IOP in rabbits), and 22-25 mmHg (hypertensive IOP in rabbits). After confirming IOP using a rebound tonometer (iCare Tonovet, Helsinki, Finland), we performed an injection at either the temporal position (3 o'clock; directly above the temporal LPCA) or the superior position (12 o'clock) of that eye.

2.1.3 Effect of injection volume on spread in the SCS of rabbits—To determine if injection volume would change the flow patterns following a microneedle injection, injections of 50, 100, and 200 μ L were performed. Before each experiment, the eye was pressurized to 10-12 mmHg. Injections were performed either at the superior position (12 o'clock) or the temporal position (9 o'clock) directly superior to the LPCA.

2.1.4 Effect of particle size on spread in the SCS of rabbits—To study the effect of particle size on spread, we injected one of three mixtures: 0.5% (w/v) 200 nm red fluorescent particles, 0.5% (w/v) 20 nm red fluorescent particles, or 0.025% sulforhodamine B dye (559 Da; 565 nm excitation and 585 nm emission wavelength). The excitation/emission spectra of these three mixtures were comparable. The eyes were pressurized to 10-12 mmHg. Fifty microliters of one of the three formulations were injected at the superior position (12 o'clock) or the temporal position (9 o'clock), directly superior to the LPCA.

2.1.5 Effect of bevel orientation on spread in the SCS of rabbits—To study the effect of bevel orientation on spread, injections were performed at the superior position (12 o'clock) with the bevel either directed temporally or nasally. Before each experiment, the eye was pressurized to 10-12 mmHg.

2.2 *In vivo* injection into the SCS of rabbits

All *in vivo* experiments were carried out in albino New Zealand White rabbits (Charles River, Wilmington, MA) and were approved by the Georgia Institute of Technology Institutional Animal Care and Use Committee. Practices complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Both eyes were used in this study, since the growing body of evidence on SCS injections (pre-clinical animal and human clinical data) indicates that SCS injections were well-tolerated and there have been no reports on vision loss associated with SCS injection.

Animals (N=3 per group) were anesthetized with a subcutaneous injection of ketamine and xylazine. A 50 μ L injection of 200 nm fluorescent particles in HBSS was made either in the supratemporal position (10.5 o'clock) or in the temporal position (9 o'clock), directly superior to the LPCA. A superior injection was infeasible as that would have required puncturing through the superior rectus muscle. In some cases, green fluorescent fundus images of the eye after suprachoroidal injection were obtained with a RetCam II with fluorescein angiography attachment (Clarity Medical Systems, Pleasanton, CA). In this case,

a 50 μ L injection of 200 nm green fluorescent particles was made in the supranasal quadrant. Animals were euthanized with an injection of pentobarbital through the ear vein after two days. The eyes were enucleated and processed as described above.

2.3 Dissection of ocular globe of rabbits and humans

To better visualize attachments between choroid and sclera, we dissected *ex vivo* rabbit eyes and human cadaveric posterior poles (Georgia Eye Bank, Atlanta, GA) under a stereoscope (Olympus SZX16, Tokyo, Japan) with a camera (Olympus DP71). The human donors had no history of ocular disease.

2.3.1 Effect of injection site on spread in the SCS of cadaver eyes—Based on findings from the dissections, we hypothesized that the short posterior ciliary arteries (SPCA) would impede particle spread. We tested this hypothesis by injecting particles within and without the SPCA ring. Although this was a different injection site than those used with previous injections (in this and previous studies) and also not clinically relevant, injections made at these positions would adequately test the barrier function of the SPCAs.

Human cadaveric ocular globes (Georgia Eye Bank, Atlanta, GA) with no history of posterior segment disease were obtained 1-3 days after death. We performed injections at two positions: (a) 2 mm from the ON and (b) 8 mm from the ON ($N = 4$ per group), which were on either side of the SPCAs (Hayreh, 2004). To process the eye, the cornea was removed and the eyes were cut, starting anteriorly and leaving the ON and surrounding tissue untouched. Since the eyes were pigmented, the retina and choroid were removed prior to imaging. Other procedures are described above.

2.4 Image acquisition and analysis, and statistics

We took two photographs of each mounted eye using a Canon 60d dSLR digital camera (Canon, Melville, NY). Camera settings were held constant at an aperture of 1/2.8 and shutter speed of 1/15 s. All images were captured in the same windowless room with the same light sources. The first image was taken with room light and no filter; the second image was taken with green light (Bluewind Multicolor RGB, HitLights, Baton Rouge, LA) and an optical bandpass filter (610 ± 10 nm; Edmunds Optics, Barrington, NJ) mounted on the camera. This lighting and filter were chosen to match the fluorescence properties of the fluorescent particles used.

After the images were acquired, the red channel of the fluorescent images was thresholded to capture the coverage of the fluorescent particles (ImageJ, National Institutes of Health, Bethesda, MD). The measurement tool in ImageJ was then used to find, for each petal: (a) the area, (b) the average intensity and (c) standard deviation, and (d) the percentage of the area of each petal that was above the threshold. Alternatively, we used the measurement tool in ImageJ to find the percentage of thresholded area that was superior and inferior to the LPCAs.

Further analysis was performed using Matlab (Mathworks, Natick, MA) and Prism (Graphpad, La Jolla, CA). Values are presented as the mean $\pm$ standard error of the mean

(SEM), unless otherwise specified. Unpaired Student's t-test and two-way ANOVA analysis were performed to determine statistical significance ($\alpha = 0.05$).

3 Results

3.1 Effect of injection site on spread in SCS of rabbits

After injecting particles at different positions around the limbus, we identified the LPCA as a barrier to circumferential spread of particles in the SCS of rabbit eyes *ex vivo* (Figure 1). Injections at the superior (Figure 1B), supratemporal (Figure 1C), infratemporal (Figure 1F), and inferior (Figure 1G) positions resulted in similar particle distributions, where the spatial distributions of particles were roughly normal (i.e., not skewed) (see Figure 1, right column for quantification of particle per petal). Furthermore, the spatial distribution of particles was centered near the injection site. Vortex veins, typically located in the supratemporal and infratemporal quadrants, did not produce a noticeable effect on circumferential particle spread when visualized via flat mounts.

In contrast, injections at the temporal (9 o'clock) position (Figures 1D and 1E) had markedly different patterns of spread compared with the others ($p < 0.0001$, 2-way ANOVA). In particular, >95% of particles injected on one side of the 90° meridian did not cross to the other side (i.e., injection superior to the LPCA resulted in few particles in the inferior hemisphere, and *vice versa*). This resulted in a skewed distribution with the tail directed away from the 90° meridian (Figure 1D and 1E, right column), which indicates an anatomical barrier that impedes flow near the 90° meridian.

We reanalyzed the eyes presented in Figure 1 to further examine barrier function at the site of LPCA. We calculated percentage of area with fluorescent signal exceeding background threshold levels above and below LPCA for each injection site (Figure 2) and found they were significantly different ($p = 0.0009$, 2-way ANOVA), despite their close proximity. For each of the three injection sites above LPCA (i.e., Figure 2B, C and D), 96±3% (mean±SD) of thresholded area was in the superior hemisphere of the eye. Likewise, in each of the three injections below LPCA (i.e., Figure 2E, F and G), 98±3% (mean±SD) of thresholded area was found in the inferior hemisphere. From these data, we conclude that barriers at the site of LPCA impeded circumferential flow into the opposite hemisphere.

3.2 Effect of IOP, injection volume, and particle size on spread in SCS

The effects of IOP, injection volume and particle size on spread in the SCS were evaluated. We found no statistical difference in particle spread at the three different IOPs tested (unpressurized, normotensive IOP, and hypertensive IOP) (Figure 3A, $p=0.85$, 2-way ANOVA).

Varying injection volume did not result in a statistically significant difference in particle spread (Figure 3B, $p = 0.59$, 2-way ANOVA). It is important to note that the eye was able to accommodate the entire 200 µL volume when injected at the 12 o'clock position but not when the injection occurred temporally above the LPCA (i.e., there was leakage, supporting the hypothesis that the LPCA formed a barrier to flow). Furthermore, particles injected at 12 o'clock had entirely covered the superior hemisphere and could be found partially in the

inferior hemisphere. On the other hand, the 200 μ L injection above the LPCA had a smaller coverage area compared to the injection at 12 o'clock. The presence of LPCA might have decreased the expandability of SCS enough so that 200 μ L could not be injected temporally.

No differences in particle spread were detected when particle size was varied (Figure 3C, $p=0.28$, 2-way ANOVA) with >90% of delivered compounds retained in the superior hemisphere. We conclude that particle size did not significantly affect particle spread circumferentially past LPCA. Moreover, since very little sulforhodamine B was found in the inferior hemisphere, LPCA appears to physically prevent fluid flow into the opposite hemisphere and did not just sieve particles. Indeed Gu et al. noted that saline injected into the suprachoroidal space of guinea pigs did not cross the "horizontal midline" and was not found in the inferior hemisphere (Gu et al., 2015).

3.3 Effect of bevel orientation on spread in SCS

When the bevel was directed either temporally or nasally during injection, particle spread was indistinguishable (data not shown). This result was expected, since the needle length, including bevel, is designed to penetrate only conjunctiva and sclera.

3.4 In vivo injection into SCS

Guided by findings in the *ex vivo* eye, we performed injections in the rabbit eye *in vivo* at the supratemporal position or temporal position above LPCA, and found no statistical difference between these injection sites ($p = 0.71$, 2-way ANOVA). The supratemporal position was chosen instead of the superior position (as in the *ex vivo* eyes) because a superior injection would require puncturing the superior rectus muscle. In eyes injected supratemporally, $87 \pm 13\%$ (mean $\pm$ SD) of thresholded area was above LPCA and in eyes injected temporally, $92 \pm 12\%$ (mean $\pm$ SD) of thresholded area was above LPCA (Figure 4A-F). As indicated by the large error bar, one of the eyes injected temporally had particles present in the inferior hemisphere (Figure 4F).

We next visualized the rabbit fundus (Figure 4G) and imaged spread of green-fluorescent particles (Figure 4H) after supranasal injection in the rabbit eye *in vivo* using an ophthalmic digital imaging system. Since these rabbits were albino, the choroid was readily visible, with the inner retinal vessels shadowing the choroid. The LPCA could be seen (Figure 4G, blue arrowheads) after penetrating through sclera anteriorly (rightmost arrowhead) and as it traversed deep to sclera, sending small branches through sclera. When visualized with fluorescein angiography, the injected green fluorescent particles were clearly seen in SCS. Fluorescence was shadowed by the inner retinal and choroidal vessels, confirming particles' localization in SCS between choroid and sclera. The microneedle injection (Figure 4H, white arrowhead) occurred in the supranasal quadrant and spread until flow was impeded by LPCA and its branches (blue arrowheads) anteriorly, as indicated by bright fluorescence superior to LPCA and dimmer fluorescence elsewhere. When we imaged the superior region of the eye, the area immediately adjacent (within 0.5 mm) to the vortex vein was devoid of fluorescent particles (Figure 4I). We hypothesize that vessels feeding into the vortex vein were sufficiently dense to prevent particle deposition or, alternatively, particles deposited

were efficiently cleared. Thus, the vortex veins had a minor effect on particle deposition that was visible on fundus exam but not flat mounts.

3.5 Dissections of *ex vivo* rabbit eyes and human eyes

Previous studies have reported that branches of the LPCA pass through sclera via multiple tightly packed perforations (Okubo et al., 1990; Rucker et al., 1956). To confirm this anatomy in this study, we dissected *ex vivo* rabbit eyes to better visualize the path of LPCA as it penetrated through sclera. During dissection, choroid easily separated off sclera for most of the globe, consistent with a potential space between these tissues, but was strongly attached to sclera where LPCA penetrated through sclera near and anterior to the equator (Figure 5). The vortex vein did not tightly adhere sclera and choroid together, possibly due to tissue processing. We hypothesize that multiple sites of adhesion associated with penetration of branches of LPCA across SCS in the rabbit eye are responsible for LPCA's barrier to circumferential flow.

The literature indicates that in human eyes, the LPCA anatomy is different from rabbit eyes (Rucker et al., 1956). Dissection in human cadaver eyes demonstrated that the LPCA penetrated through sclera more posteriorly (relative to the equator) than in the rabbit eye, and then travelled within SCS for at least 5 mm before anastomosing with the circulus arteriosus (Figure 6A). Notably, the two LPCAs penetrated into SCS at just one location and did not have multiple branches penetrating into SCS, as seen in the rabbit eye. It is possible that the presence of a section of LPCA running within SCS may be able to facilitate, rather than impede, distention of SCS during microneedle injection in humans, especially anterior to the equator.

However, we found that the choroid was tightly attached to the sclera in a region surrounding the ON and roughly corresponding to sites of the SPCA penetration into the eye. The six-to-twelve SPCAs found in the human eyes formed an ellipsoid around the ON with a major radius of ~5 mm and minor radius of ~1 mm (Figure 6B), which is consistent with previous findings (Hayreh, 2004). Since retina had peeled off choroid, we were unable to locate the macula in relation to SPCA. However, it is likely that the macula was within the ellipsoid demarcated by SPCAs (Mok and Lee, 2002). This suggests that in human eyes, flow within SCS may also be impeded, but not anteriorly by LPCAs, as seen in the rabbit eye, but more posteriorly by SPCAs in the human eye. This might affect the ability of an injection in the SCS to target the macula due to a possible barrier formed by the SPCAs.

3.6 Microneedle injection into human cadaver eyes

To test the hypothesis that the SPCAs impede flow in the human eye, we performed injections inside the SPCA ring (2 mm from the ON) and outside the SPCA ring (8 mm from the ON) on human cadaver eyes. The SPCAs could be found between the chosen injection sites at ~3 mm from the ON. Although these injection site were not clinically relevant, they allowed us to assess the barrier function of the SPCAs; we reasoned that if the SPCAs formed a barrier when an injection was made immediately adjacent to the vessels, then an injection made near the limbus (i.e., >20 mm from the ON) would experience similarly impeded flow, provided particles could flow that far.

The particle distributions of these conditions were statistically different ($p = 0.0035$, 2-way ANOVA). When injection occurred within the SPCA ring, $66 \pm 8\%$ (mean $\pm$ SD) of particles were within 5 mm of the ON. When injection occurred outside the SPCA ring, $18 \pm 7\%$ (mean $\pm$ SD) of particles were found within 5 mm of the ON. These data indicated that the SPCAs provided a barrier to circumferential spread towards the ON and macula when an injection occurred more anteriorly than the SPCAs.

4 Discussion

The SCS is an attractive site of drug delivery targeted to the chorioretina (Chen et al., 2015; Einmahl et al., 2002; Gilger et al., 2013; Kadam et al., 2013; Olsen et al., 2006; Patel et al., 2012; Patel et al., 2011; Tyagi et al., 2012; Tzameret et al., 2014; Wang et al., 2012).

However, targeting within the SCS is important as well, since sites of disease are often localized to specific regions of the chorioretina, such as localization of AMD to the macula and localization of tumors at their sites of growth. This study addressed the possible existence of barriers to flow in the SCS due to its anatomy, more specifically due to sites where the posterior ciliary arteries, but not the vortex vein, cross the SCS and thereby limit its distention. This study showed that sites where the LPCA crosses the SCS in the rabbit eye and where the SCPA crosses the SCS in the human eye were associated with limited flow of particles in the SCS. The vortex veins had a minor effect on particle deposition, limited only to near the vortex vein root. In the rabbit eye, the LPCA barrier limited flow of particles between the upper and lower hemispheres of the eye, which was similar to distribution of saline seen with guinea pigs (Gu et al., 2015). In the human eye, the SPCA barrier limited flow of particles posteriorly to the macula and ON.

We believe that previous studies have not reported this barrier because they were not designed to look for it and, in some cases, used animal models with different anatomy (Patel et al., 2012; Seiler et al., 2011). One studies mentions injected fluid not crossing the midline but does not offer an explanation as to why this is (Gu et al., 2015). Other studies have shown that formulations designed to keep SCS open after injection allow particles to redistribute within SCS and cover both hemispheres of the eye (Kim et al., 2015), although re-analysis of particle spread indicates lower particle concentration overlaying LPCAs compared with elsewhere (data not shown).

Vortex veins, another likely candidate, did not have a large effect on particle distribution. Any effect by the vortex vein on particles spread was not apparent when viewed in flat mount, but was visible when imaged fundoscopically *in vivo*. We noted that few particles were deposited within 0.5 mm of the vortex vein upon fundusoscopic exam. It is important to note that each vortex vein penetrates through sclera as a single large vessel, which does not form a large region of physical attachment between sclera and choroid (Bill, 1962). We posit that morphological differences between how branches of LPCAs penetrate sclera at multiple locations, whereas each vortex vein penetrates at just a single vessel, govern the ability of vessels to block circumferential flow.

Results from our *ex vivo* studies agreed with *in vivo* rabbit studies, suggesting that particle distribution was insensitive to live processes, such as blood flow, for the injection

formulation tested (i.e., particles suspended in HBSS). Previous studies using particles suspended in HBSS have indicated that particle spread occurs at the time of injection, after which the particles do not move (Kim et al., 2014b, c; Kim et al., 2015; Patel et al., 2012; Patel et al., 2011).

Dissection of the choroid from the sclera demonstrated that the choroid is strongly attached to the sclera where multiple branches of the LPCA penetrate through sclera to anastomose with the choroid. These branches of the LPCA may act like staples to secure sclera and choroid together, thus physically impeding fluid flow. We hypothesize that it is possible to bypass this barrier by (i) flowing anteriorly or posteriorly around attachment sites, (ii) distending the choroid between the vessels, or (iii) tearing the small vessels. Further studies are needed to test this hypothesis.

Human eye dissection was consistent with prior studies (Hayreh, 2004; Krohn and Bertelsen, 1997; Okubo et al., 1990), which indicated that the LPCA pierces the sclera more posteriorly and does not tightly adhere the sclera to the choroid, as compared to rabbit eyes. In contrast, SPCAs do tightly adhere choroid to sclera in a ring around the ON. This ring likely encompasses the macula, which may make SCS delivery to the macula and ON more difficult in humans. Consistent with these findings, Olsen et al. found that there were more and “stronger connections” between sclera and choroid in the macular region of porcine eyes (Olsen et al., 2006), which is where the SPCAs penetrate through the sclera (Ninomiya and Inomata, 2006).

Limitations of this study include the use of *ex vivo* rabbit eyes, inability to determine kinetics of particle movement, lack of blood flow in *ex vivo* experiments, and inability to study temporal effects on LPCA's barrier function. Furthermore, the conclusion of anatomy affecting particle spread (expected to be driven by convective flow) cannot be applied to distribution of small molecules (expected to be driven by both convection and diffusion). These barriers may not affect drugs diffusing within the SCS since they are on different length scales. Factors, such as physiochemical properties and clearance, are more likely to affect drug distribution (Kadam et al., 2013; Olsen et al., 2011). More comprehensive testing of anatomical barriers present in the SCS of human eyes is warranted.

In conclusion, we found that circumferential fluid flow in the SCS was impeded by the LPCA in rabbit eyes *ex vivo* and *in vivo*. It appeared that multiple tightly clustered vessels perforating through the sclera (such as those seen with branches of the LPCA in New Zealand White rabbits) result in strong attachment between sclera and choroid, and that these strong attachments form a physical barrier to circumferential flow. On the other hand, singular large vessels (e.g., vortex veins) had a minor effect on impeding circumferential particle spread. Human cadaver eyes exhibited a similar pattern of tightly clustered vascular perforations at the SPCAs near the ON, which impeded particle spread near the ON. This may make specifically targeting the SCS overlaying the macula and ON more difficult. Development of drug delivery and other applications requiring fluid flow in the SCS should take these anatomical barriers into account and either accept limitations they may impose or develop strategies to overcome or circumvent them.

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- Microneedles enable targeted drug delivery to specific regions within the suprachoroidal space.
- Anatomical barriers can impede spread of particles within the suprachoroidal space.
- Circumferential spread of particles after suprachoroidal injection is blocked at the midline by long posterior ciliary arteries in rabbits.
- Circumferential spread of particles after suprachoroidal injection is blocked near the optic nerve and macula by short posterior ciliary arteries in humans.
- Vortex veins do not appear to block circumferential spread of particles.

Figure 1.

SCS injections at different injection sites, shown in the right eyes *ex vivo*. [A] Representative images showing spread of fluorescent particles in eyes at the tested injection sites. Eyes are all positioned in the same anatomical orientation – superior is on top and temporal is right. Injections were performed at the superior (12 o'clock, b); supratemporal (10.5 o'clock, b); temporal, directly superior (d) and inferior (e) to the long posterior ciliary artery (9 o'clock); infratemporal (7.5 o'clock, f); and inferior (6 o'clock, g) positions. [B-G] Quantification of the average concentration of particles in each scleral flap for the tested injection sites. Distribution of particles is shown after 50 μ l of HBSS containing 200-nm particles was injected into SCS at different sites (white arrow). Sup. = superior (12 o'clock); Temp. = temporal (9 o'clock); Inf. = inferior (6 o'clock); Nas. = nasal (3 o'clock). Since the eye is spherical, leftmost and rightmost bars are adjacent.

Figure 2. Percentage of particles above versus below LPCA. Data from images like those in Figure 1 were re-analyzed to find the percentage of SCS area with fluorescent signal above threshold, indicating presence of injected particles. Injections were carried out in right eyes *ex vivo* using 50 μ l of HBSS containing 200-nm particles. Data show mean $\pm$ SEM from n=3 replicates.

Figure 3.

Effect of IOP (A), injection volume (B), and particle size (C) on LPCA barrier function. The fraction of eyes in which particles were detected inferior to LPCA is shown above each tested condition. Data show mean±SEM from n=3-5 replicates. (A) Injections were carried out in left eyes *ex vivo* using 50 μl of HBSS containing 200-nm particles. The percentage of SCS area with fluorescent signal above threshold is shown in unpressurized eyes, eyes at physiological IOP (10 - 12 mmHg), and eyes with hypertensive IOP (22 – 25 mmHg). (B) Injections were carried out in right eyes *ex vivo* using 50, 100 or 200 μl of HBSS containing 200-nm particles. Pound sign (#) indicates that significant leakage out the injection site occurred. (C) Injections were carried out in right eyes *ex vivo* using 50 μl of HBSS containing 200 nm fluorescent particles, 20 nm fluorescent particles, or a small molecule fluorescent dye (559 Da ≈ 1 nm).

Figure 4.

Distribution of particles in SCS after injection into rabbit eyes *in vivo*. (A-F) The path of LPCA (dotted line) and injection site (white arrow) are shown, based on examination of tissue using brightfield optics (not shown). Supratemporal (A, B, C) and temporal, above the LPCA, (D, E, F) injections are shown. One eye (F) had some particles present in the inferior hemisphere presumably by flowing anteriorly bypassing the LPCA. Temporal injections were carried out in left (D, E) and right (F) eyes *in vivo* using 50 μ l of HBSS containing 200-nm particles. RetCam image after supranasal injection illuminated with white light (G) and fluorescein angiography attachment (H and I). Particles were not found near the root of the vortex vein (I). White arrow = injection site; ON = optic nerve; VS = visual streak; blue arrowheads = LPCA with its branches.

Figure 5.

Image of rabbit sclera showing LPCA perforations through sclera. The temporal LPCA is a branch of the ophthalmic artery that penetrates through sclera near the equator to supply choroid, ciliary body, and iris (Gray, 1918). In the rabbit, the LPCA branches before penetrating through sclera; these LPCA branches appeared to strongly adhere choroid to sclera during dissection. Dashed lines indicate the path of LPCA. Arrows indicate sites where LPCA branches perforated sclera. The representative image was taken with a brightfield stereoscope of an *ex vivo* rabbit eye after dissection. No SCS injection was performed.

Figure 6.

Images of the posterior pole of a representative human cadaver eye showing LPCA and SPCAs. (A) Choroid was lifted off sclera and folded over to show the LPCA, which penetrated more posteriorly than in the rabbit and traveled within SCS for at least 5 mm before anastomosing with the ciliary body. (B) Retina/choroid was removed to show SPCAs, which are vessels originating from the ophthalmic artery that penetrate sclera to form a ring around the optic nerve and macula (Gray, 1918). Images were taken with a brightfield stereoscope of a representative human cadaver eye after dissection. No SCS injection was performed.

Figure 7. Images of human cadaver eye after microneedle injection (A) 2 mm from the optic nerve (ON), and (B) 8 mm from the ON. Solid gray circle denotes location of ON, dashed line denotes location of SPCAs and white arrow denotes injection location. (C) Quantification of images following injection within the SPCA ring and without the SPCA ring. ** indicates $p < 0.005$.