

ARTICLE



Tolerability and tropism of recombinant adeno-associated virus vectors in the African green monkey (*Chlorocebus sabaeus*) anterior chamber

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While many studies have investigated the use of recombinant adeno-associated vectors (rAAV) in the posterior chamber for treatment of inherited retinal diseases, fewer studies have looked at rAAV's ability to transduce cells within the anterior chamber. This study focuses on evaluating the tropism and tolerability of three rAAV serotypes—rAAV2/6, rAAV2/9, and rAAV2/2[*MAX*]—expressing a green fluorescent protein (GFP) reporter following intracameral injection in the non-human primate (NHP) African green monkey (*Chlorocebus sabaeus*) model. Injection of high dose (1×10^{12} vg/eye) rAAV vector resulted in transient inflammation characterized by aqueous flare and cellular infiltrate that resolved without intervention in all serotypes. Post-mortem histology revealed widespread expression of GFP in cells of the trabecular meshwork and iris in high dose rAAV2/6, rAAV2/9, and particularly rAAV2/2[*MAX*] eyes, indicating that rAAV vectors of these serotypes have broad tropism for cells of the anterior chamber and may facilitate the treatment of blinding disorders, such as glaucoma.

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INTRODUCTION

The anterior chamber of the eye functions to focus light upon the retina to facilitate vision, consisting of the cornea, trabecular meshwork, iris, ciliary body, and lens. Anterior chamber dysfunctions, such as corneal opacities, endothelial dystrophies, and glaucoma together account for ~12% of global blindness and have limited treatment options [1]. While corneal dystrophies oftentimes have a defined genetic etiology, many diseases such as keratoconus, diabetic keratopathy, or glaucoma are complex multifactorial disorders characterized by abnormal alterations in downstream signaling pathways, such as changes in extracellular matrix depositions and the TGF β and Wnt signaling pathways [2–5]. Whether an anterior chamber disorder is caused by a single gene defect (e.g., Fuch's endothelial dystrophy) or has multiple genetic and environmental risk factors, diseases of the anterior chamber may be amenable to treatment through gene therapy approaches by gene augmentation or manipulation of pathway biology, respectively.

Traditionally, ocular gene therapy has primarily been focused on two major injection routes—intravitreal and subretinal—in order to target differing layers of the retina or retinal pigment epithelium (RPE) for the treatment of inherited retinal diseases, with relatively little research focused on the use of rAAV vector to transduce cells of the anterior chamber [6, 7]. While it is possible for rAAV injected intravitreally to transduce the anterior chamber owing to the posterior-to-anterior directional flow of the aqueous humor, intracameral injections offer a direct administration route to anterior chamber structures, such as the corneal endothelium,

iris, and trabecular drainage pathways without unwanted dilution of the vector in the vitreous [8]. While the majority of ophthalmic gene therapy studies in the past two decades have focused on targeting transgene delivery to cells of the retina for the treatment of neurodegenerative disease, there have been several attempts to define the tropism of rAAV for cells of the anterior chamber that together informed serotype selection for the present study [9, 10]. Specifically, rAAV serotypes rAAV2/2, 2/6 and 2/9 have been shown to transduce human trabecular meshwork cells in vitro with varying degrees of efficiency, and also to mediate efficient gene transfer to cells of the cornea in rabbits following epithelial debridement and topical application [11]. A small number of studies have also evaluated the tropism of self-complementary rAAV2/2 vectors in rat and non-human primate (e.g., *macaca fascicularis*) models, showing sustained transduction of corneal endothelium, iris, ciliary body, and trabecular meshwork [12, 13]. Lastly, efforts have also been made in mouse and rat models to improve the tropism of rAAV vectors delivered intracamerally through the use of capsid mutant serotypes, such as rAAV2/2(TrpYF), which contains three Y-F point mutations (Y444F, Y500F, Y730F) that decrease proteasomal degradation of the virion, leading to improved transduction of the corneal endothelium, chamber angle, and iris [14]. Here we aim to build upon these studies by directly comparing the tropism of multiple rationally selected rAAV serotypes in the NHP anterior chamber, including rAAV2/6, rAAV2/9 and rAAV2/2[*MAX*], a capsid mutant vector that contains several point mutations (Y272F, Y444F, T491V, Y500F, Y730F) and the 7m8 peptide insertion (N587-R588insLALGETTRPA)

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that further decrease proteasomal degradation and tissue penetrance.

The African green monkey (AGM), or Caribbean vervet, is an Old-World non-human primate species, a subpopulation of which was introduced to the West Indies in the 1600s, most notably St. Kitts, where a colony of AGMs reside at the St. Kitts Biomedical Research Foundation. Given their biologic isolation, St. Kitts AGMs are not carriers of pathogens common to continental NHPs such as simian immunodeficiency virus and Marburg virus, and as a species are not carriers of macacine herpesvirus I [15, 16]. Moreover, they exhibit a comparatively low prevalence of AAV exposure to multiple serotypes making them a favorable test system for AAV vector evaluation. In addition, having undergone a major population bottleneck during their introduction to the island, a substantial founder effect has occurred, leading to levels of homozygosity that enable genotype/phenotype correlations with respect to disease relevant genes, furthering their value for biomedical research. Herein, we investigate three rAAV serotypes injected intracamerally—rAAV2/2[*MAX*], rAAV2/6, and rAAV2/9—into the anterior chamber to determine both the safety and efficacy of transduction of the anterior chamber tissues in the AGM.

METHODS

Vector production

Vector production was carried out following a previously described protocol [17]. Briefly, rAAV vectors were manufactured in adherent HEK293T (ATCC no. CRL-11268; Manassass, VA, USA) cells seeded in hyperflasks (Corning, Corning, NY) using a triple plasmid transfection approach, wherein each cell receives: (1) a plasmid containing inverted terminal repeats flanking a self-complementary green fluorescent protein (GFP) transgene cassette, (2) a helper plasmid coding for adenovirus helper genes necessary for cis-packaging, and (3) a Rep/Cap plasmid expressing viral replicatory and capsid genes necessary for packaging of each vector serotype, namely rAAV2/2[*MAX*], rAAV2/6, or rAAV2/9. Cell lines used were not tested for mycoplasma contamination. Plasmids were transfected using polyethylenimine (PEI, Polysciences, #N23966-100, PA, USA) in a 1:1:1 equimolar ratio based on plasmid size to a total of 500 µg DNA per hyperflask (Corning, Corning, NY). Following a 72 h incubation to allow for viral production, cells were recovered from each flask by manual displacement and concentrated via centrifugation (370,000 × g, 1.5 h). rAAV virions were recovered from the resulting HEK293T cell pellets using repetitive (4×) freeze-thaw lysis before being purified using an iodixanol density gradient separation, as described previously [17, 18]. The purified vector was then concentrated through buffer exchange as described previously and titered via a PicoGreen assay (Thermo Fisher Scientific, Waltham, MA) [17, 19]. Following purification and titration, all vector preparations were screened to confirm the absence (<5EU/ml) of endotoxin (Endosafe Nexgen-PTS, Charles River, Wilmington, MA) and then assigned an anonymous identifier (e.g., 'A', 'B' or 'C') so as to mask the injecting surgeon and in vivo investigators with respect to vector serotype and dose ('low' or 'high'). As such no randomization was necessary during ocular exams.

Animals and anesthesia

12 adult African green monkeys (*Chlorocebus sabaeus*) exhibiting normal health were screened by slit lamp exams and tonometry to identify animals with normal ocular anatomy and IOPs greater than 18 mmHg. Exams were performed under sedation with ketamine (8 mg/kg) and xylazine (1.6 mg/kg) and sensitivity to anesthesia associated IOP fluctuations was assessed by measuring IOP 5 and 15 min after administration and any animals exhibiting greater than a 4 mmHg decrease over this time period were excluded. Neutralizing antibody (NAb) screening was additionally conducted to select animals with an absence of AAV2, AAV6 and AAV9 NABs at a serum dilution of 1:20 by luciferase inhibition assay. Animals with an absence of NABs were chosen to complete the study (*N*=6, 5 male and 1 female (B353)). All experiments adhere to the Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmic and vision research.

Intracameral injection

Under anesthesia, 0.5% proparacaine hydrochloride was applied topically followed by lid speculum placement and aseptic preparation of the eye

with 5% Betadine solution rinsed with a sterile saline. Each eye was injected with 100 µL of vector suspended in HBSS + 0.014% Tween-20 using a 31-gauge 5/16" needle placed into a 0.3 mL syringe. The needle was inserted temporally 2 mm anterior to the limbus in the coronal plane and when removed, a topical triple antibiotic (neomycin, polymyxin, bacitracin) ophthalmic ointment was applied.

Tonometry

Tonometry was completed at baseline, and days 0 (dosing), 3, 7, 14, 21, 28, 25, and 42 (Table 3) with three measurements at each time point obtained at a consistent circadian interval (between 8 and 10 am). IOP measurements were recorded within 5 min of sedation with the animal placed in the supine position using a TonoVet tonometer (Icare, Oy, Finland) applying the dog calibration setting.

Slit lamp biomicroscopy

Slit lamp exams were completed at baseline, and days 0, 3, 7, 14, 21, 28, 35, and 42 post-injections. The non-human primate ophthalmic exam scoring system [20] was applied to quantitatively define conjunctival congestion, swelling, and discharge, pannus, corneal clouding, inflammatory keratic precipitates, aqueous cell, hypopyon, fibrin strands, aqueous flare, iris hyperemia, iris exfoliation, iris synechia, anterior and posterior lens deposits and opacity, vitreous cell and haze, retinal vasculitis, and papillitis. Each of these parameters were scored according to incident severity with scores summed to derive a total clinical score (Supplemental Table 1).

Pachymetry

An Accutome AccuPach 5 ultrasound pachymeter (Keeler, Malvern, PA) was used to measure corneal thickness at baseline, days 7, 21, and 42 post-injections, with three measurements of corneal thickness obtained in succession to derive an average measure per eye per time point.

Optical coherence tomography (OCT)

OCT was completed at baseline and days 7, 21, and 42 using a Heidelberg Spectralis OCT Plus (Heidelberg, Germany) with eye tracking and HEYEX image capture and analysis software. The macula, peri-macula, temporal arcades, and optic nerve were imaged by obtaining a scan encompassing the central posterior pole at a dense scan interval and retinal thickness and volume derived from the HEYEX software, following automated segmentation.

General ophthalmic imaging

Color fundus imaging and anterior segment photography was performed with a Topcon TRC-50EX (Topcon, Livermore, CA) retinal camera equipped with Canon 6D digital imaging hardware and New Vision Fundus Image Analysis software. Color fundus photos were captured with a shutter speed of 1/25 s, ISO of 400, and flash of 18.

Euthanasia and tissue collection

Animals were euthanized with ketamine/xylazine followed by sodium pentobarbital delivered intravenously (100 mg/kg), after which eyes were enucleated and a suture placed in the limbal conjunctiva at the 12:00 o'clock position to maintain orientation during histology. Globes were then intravitreally injected with 500 µL of 4% paraformaldehyde (PFA) to facilitate reinflation, and the globe submerged in 4% PFA. After 6 h, eyes were transferred to PBS with 0.05% sodium azide and sent to Medical College of Wisconsin for tissue processing.

Tissue processing

Eyes were quartered and cryoprotected in 30% sucrose overnight. Tissue was then submerged in optimal cutting temperature media (Sakura, Torrance, CA) and frozen on dry ice before being sectioned on a Leica CM1860 cryostat (Leica, Buffalo Grove, IL) in 12 µm sections onto Fisherbrand Superfrost Plus slides and left to dry overnight. Sections were subsequently stored at −20 °C until use, at which point they were thawed for 2 h at room temperature, and the optimal cutting temperature media washed off using PBS. Sections were then incubated 1:200 with anti-GFP antibody (Millipore, MAB2580, St. Louis, MO) and counterstained with Hoechst 33342 for nuclei visualization. Sections were imaged with a Nikon Eclipse 80i confocal microscope (Nikon, Tokyo, Japan) at 20×, and processed as maximum intensity projections and merged in FIJI.

Table 1. Serum testing of AGMs for rAAV neutralizing antibodies.

AAV6 Nab					Assigned	AAV9 Nab					Assigned	AAV2 Nab					Assigned
Serum Dilutions					Group	Serum Dilutions					Group	Serum Dilutions					Group
ID	1:5	1:20	1:80		ID	1:5	1:20	1:80		ID	1:5	1:20	1:80				
B342	-	-	-	2/6	B342	-	-	-	x	B342	+	-	-	x			
B343	-	-	-	x	B343	-	-	-	x	B343	+	+	-	x			
B344	-	-	-	x	B344	-	-	-	x	B344	+	+	-	x			
B346	-	-	-	x	B346	-	-	-	x	B346	+	+	-	x			
B347	-	-	-	2/6	B347	-	-	-	x	B347	+	-	-	x			
B350	-	-	-	x	B350	+	-	-	x	B350	-	-	-	MAX			
B353	-	-	-	x	B353	-	-	-	2/9	B353	+	-	-	x			
B354	-	-	-	x	B354	-	-	-	2/9	B354	+	-	-	x			
B361	-	-	-	x	B361	-	-	-	x	B361	+	-	-	MAX			
B366	-	-	-	x	B366	-	-	-	x	B366	+	+	-	x			
B367	-	-	-	x	B367	-	-	-	x	B367	+	+	-	x			
B368	-	-	-	x	B368	-	-	-	x	B368	+	-	-	x			

12 AGMs were tested for pre-existing neutralizing antibodies specific to the capsids of rAAVs 6, 9, and 2. Two AGMs per serotype (indicated in bold) were assigned to treatment and study enrollment, noted in blue.

RESULTS

Intracameral administration of rAAV causes transient inflammation that resolves without pharmacological intervention in naïve AGMs

To limit the potential for an adaptive immune response following rAAV vector injection, sera from twelve NHP subjects were screened prior to enrollment to confirm the absence of NABs raised against wild-type AAV2, AAV6, or AAV9 infections (Table 1). Six subjects (B342, B347, B353, B354, B350 and B361) with no detected NABs for respective serotypes at serum dilutions of 1:20 were subsequently selected and assigned to receive intracameral injections of either buffer (control, $N = 1$ eye) or rAAV2/2[**MAX**], rAAV2/6 or rAAV2/9 at either a low (1×10^{11} vg/eye) or high (1×10^{12} vg/eye) dose ($N = 2$ eyes per group; see Table 2). Slit lamp examinations were completed on all subjects at baseline (one day prior to injection), immediately following injection (day 0) and at day 3, 7, 14, 21, 28, 35, and 42 post-injection (Table 3); images were graded based on the applied non-human primate ophthalmic exam scoring system [20] by observers masked to treatment. Despite the seronegative status of the subjects, aqueous cell scores increased in all rAAV treated eyes regardless of dose or serotype immediately following injection (day 0) and was maintained until day 3, indicative of an acute innate immune response to the vector capsid (Fig. 1B–D). Importantly, this inflammation resolved by day 7 in all animals without pharmacological intervention. Between days 14 and 28 in high dose rAAV treated eyes, a secondary, potentially adaptive phase of inflammation was observed, but this also resolved without pharmacological intervention by the end of the study at day 42 (Fig. 1B–D). Total clinical scores, including aqueous cell and various metrics of conjunctival, corneal, iris, lens, and posterior chamber health (Fig. 1F–H) remained generally low (total clinical score < 10) in all treated eyes, with the exception of B342 OD, which received an injection of high dose rAAV2/6 (Fig. 1F). This eye exhibited a total clinical score of 15 at day 3 due to cell and fibrin strand presence, keratic precipitates, as well as iris hyperemia and anterior lens deposits, which resolved progressively between days 5 and 42 (Fig. 1F). Eyes treated with rAAV2/9 (animals B353 and B354) exhibited total clinical score fluctuations between 0 and 7 with varying levels of cell and fibrin strand presence in addition to iris hyperemia and anterior lens deposits (Fig. 1G). rAAV2/2[**MAX**] treated eyes presented clinical scores of ≤ 7 wherein most activity was seen at day 0 immediately following vector administration. These findings mostly resolved by day 14, however, a secondary

Table 2. AGM treatment assignments.

Animal ID	Treatment	Eye	Dose (vg/eye)	Volume
B347	rAAV2/6	OD	Uninjected Control	100 μ L
		OS	1×10^{11}	
B342	rAAV2/6	OD	1×10^{12}	
		OS	1×10^{11}	
B353	rAAV2/9	OD	1×10^{12}	
		OS	1×10^{11}	
B354	rAAV2/9	OD	1×10^{12}	
		OS	1×10^{11}	
B361	rAAV2/2[MAX]	OD	1×10^{12}	
		OS	1×10^{11}	
B350	rAAV2/2[MAX]	OD	1×10^{12}	
		OS	1×10^{11}	

Animals underwent 100 μ L intracameral injections with one of three rAAV serotypes: rAAV2/6, rAAV2/9, or rAAV2/2[**MAX**]. Each right eye (OD) was injected with a high dose of vector, while left eyes (OS) were injected with a low dose of vector. One eye, B347 OD, was left as an uninjected control eye.

phase was also found at day 28 in B361 OD, which was treated with high dose rAAV2/2[**MAX**] and was found to have increased scores, reflecting presence of keratic precipitates and iris hyperemia (Fig. 1H). In depth findings, indicating individual values for each parameter investigated, can be found in Supplemental Table 1.

Intracameral administration of rAAV temporarily alters intraocular pressure

To assess whether intracameral administration of rAAV2/2[**MAX**], rAAV2/6, or rAAV2/9 in the NHP anterior chamber affected intraocular pressure, rebound tonometry was completed on all subjects at baseline (one day prior to injection), immediately following injection (day 0), and at 3, 7, 14, 21, 28, 35, and 42 days post-injection (Table 3). Intraocular pressure readings were found to be steady in all baseline and injected eyes across the time course and exhibited only minor fluctuations that remained within the normal IOP range (12–22 mmHG) of the NHP (Fig. 2A–D)

Table 3. Timeline of ocular assessments.

Evaluation	Timeline								
	Baseline	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
Vector Administration	-	X	-	-	-	-	-	-	-
Tonometry	X	X	X	X	X	X	X	X	X
Pachymetry	X	-	-	X	-	X	-	-	X
Slit Lamp	X	X	X	X	X	X	X	X	X
Retinal Photos	X	-	X	-	X	-	-	-	X
Optical Coherence Tomography	X	-	-	X	-	X	-	-	X

All AGMs underwent baseline tonometry, pachymetry, slit lamp biomicroscopy, fundus photography, and optical coherence tomography. Various assessments were completed at differing timepoints listed throughout the study. Baseline imaging assessments were performed ~7 weeks prior to dosing. This study concluded at day 42, wherein all assessments were repeated.

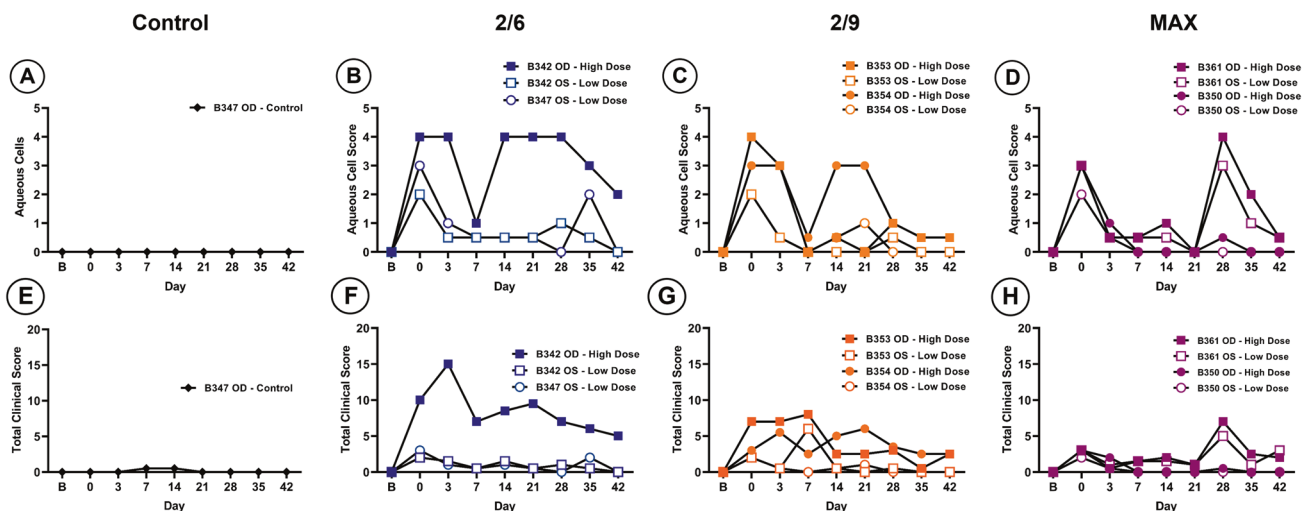


Fig. 1 Slit lamp biomicroscopy of the anterior chamber for indications of inflammation. All eyes except for the uninjected control (A) exhibited a transient phase of inflammation immediately after vector administration (B–D). In most animals this phase resolved by day 7. One high dose treated eye in each treated group (B342, B354, and B361) exhibited a second phase of inflammation that occurred at days 14 and 28, possibly coinciding with gene expression. In rAAV2/2[*MAX*] treated eyes, both high and low dose treated eyes in subject B361 showed a second phase of inflammation (D). Total clinical scores derived from multiple parameters showed increased incidence of clinical findings high dose treated eyes (F–H), but was only minimally evident in the control eye (E). Most instances of inflammation resolved by day 42 without intervention.

[21, 22]. A minor increase in IOP was observed immediately following vector administration on day 0–3 in all treated animals, as expected following the introduction of 100 μ l buffer (HBSS + 0.014% Tween) containing either a low (1×10^{11} vg/eye) or high (1×10^{12} vg/eye) dose of rAAV vector into the anterior chamber. Importantly, this increase returned to baseline by day 3 and no relationship was observed between subsequent IOP fluctuations and vector dose or serotype (Fig. 2A–D).

Intracameral rAAV administration does not impact retinal thickness or fundus appearance

Optical coherence tomography was carried out throughout the study (Table 3) to assess the effects of intracameral rAAV injection on retinal morphology. Retinal thicknesses in the macula and surrounding retina were observed to be maintained throughout the course of this study, with only minor percent changes in thickness (Fig. 3B–H). Importantly, there was no observable trend in retinal thickness measurements at any sector as either a function of vector dose or serotype compared to the uninjected control eye (B347 OD), indicating that intracameral injection of the evaluated doses of rAAV does not alter central retinal thickness. Similarly, fundus photography of the posterior pole including the macula and optic disk revealed no evidence of pathologies (e.g., detachments, disc pallor, pigmentary alterations) when comparing

baseline (pre-injection) and day 42 images in any groups, regardless of treatment (control vs. rAAV), dose (low vs. high) or serotype (rAAV2/2[*MAX*], rAAV2/6 or rAAV2/9) (Fig. 4A–N).

Intracameral rAAV administration does not consistently alter corneal thickness

Pachymetry was completed at various time points (Table 3) to determine if intracameral administration of rAAV affects corneal thickness in the NHP (Fig. 5). Only one animal (B347 OS) that received a low dose of rAAV2/6 showed a decrease in thickness (28%) which occurred between day 21 and day 42 (Fig. 5B). rAAV2/9 treated eyes (B353 OD & OS) showed an 18 and 19% increase, respectively, in corneal thickness at day 7 that resolved by day 42 without intervention (Fig. 5C). *MAX* treated eyes exhibited consistent corneal thickness throughout all timepoints. (Fig. 5D).

rAAV transgene expression persists within the iridocorneal angle and iris after 42 days

42 days post-injection all subjects were euthanized, and the globes recovered for histological examination in order to determine the cellular tropism of each rAAV serotype. Each of the high dose serotypes injected demonstrated GFP expression evident in the cells surrounding the iridocorneal angle and iris (Fig. 6E–H). High dose injected *MAX* AGMs exhibited the highest

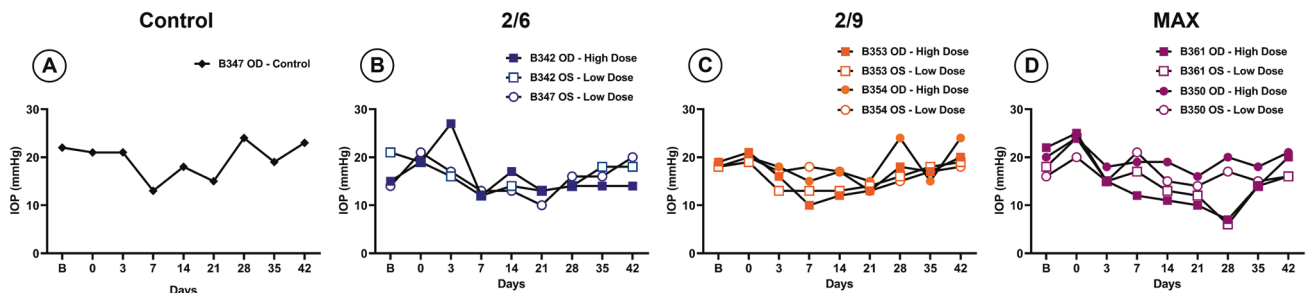


Fig. 2 Intraocular pressures as measured by rebound tonometry. A transient increase in IOP was seen immediately following vector injection at day 0 (B–D). This observation was not evident in the uninjected control eye (A). One rAAV2/2[*MAX*] treated animal (B361) exhibited a decrease in IOP at day 28 that resolved by day 35 (D).

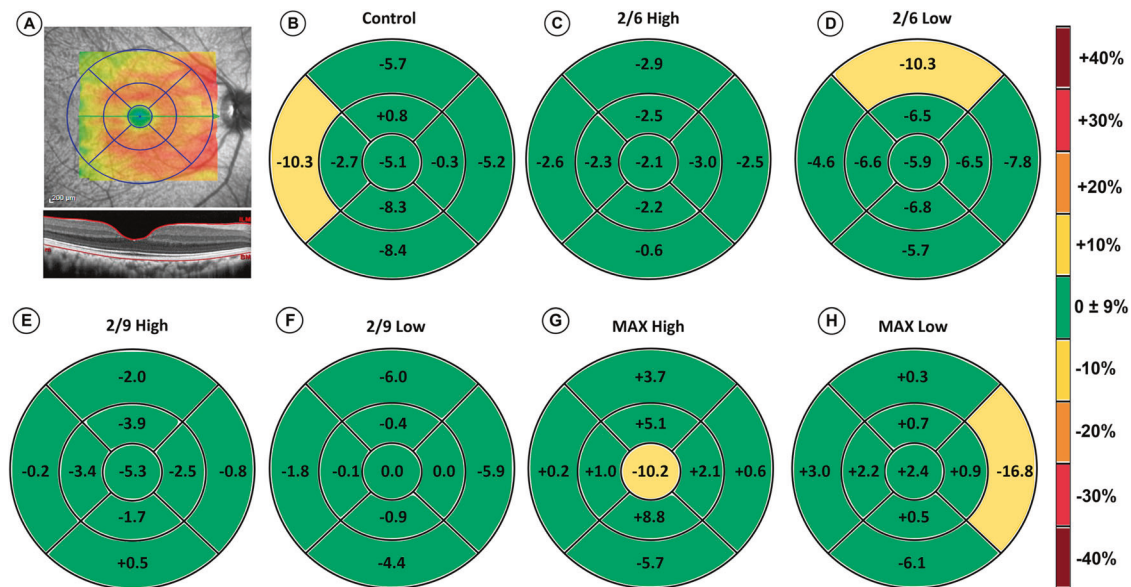


Fig. 3 Average percent change in retinal thickness from baseline to day 42 for each treatment group. A representative cSLO and OCT scan indicates the examined areas (A). Retinal thicknesses stayed largely consistent throughout the study with most deviations staying within 9% of baseline measurements (C, E, F). Exceptions to this trend are seen in the control eye (B), rAAV2/6 low treated group (D), and both rAAV2/2[*MAX*] treated groups (G, H).

number of transduced cells within the angle (Fig. 6H). While no transduction was evident within the cornea or retina (Fig. S2B–D and N–P) of high dose treated AGMs, transduction was evident in the iris (Fig. S2F–H), ciliary body and ciliary muscles of the high dose treated animals (Fig. S2J–L). No evidence of GFP expression was found in any low dose treated iridocorneal angles for any serotype (Fig. S1D–F). In addition, no GFP expression was evident in the cornea, ciliary body, or retina of low dose treated AGMs (Fig. S3B–D, J–L, and N–P respectively). Trace amounts of GFP expression was evident within the iris of low dose treated groups (Fig. S3F–H).

DISCUSSION

Numerous congenital and acquired diseases involve dysfunction or death of anterior chamber cells, including various endothelial dystrophies (e.g., Fuch's) and open-angle glaucoma, one of the leading causes of blindness worldwide. As such there is a need to develop methodologies to target gene delivery to cells of the anterior chamber in order to facilitate long-term treatment of chronic diseases; unfortunately, despite extensive work in recent decades focusing on improving subretinal and intravitreal delivery of genetic material using a variety of viral vectors (e.g., rAAV, lentivirus and adenovirus) and non-viral vectors (e.g., naked DNA or nanoparticles), limited work has focused on exploring gene

delivery in the anterior chamber. Herein, we evaluated three rAAV serotypes following intracameral injection in a clinically relevant large animal model, the AGM, focusing firstly on evaluation of both anterior and posterior chamber health using comprehensive ocular examinations consisting of retinal imaging, slit lamp biomicroscopy, pachymetry, tonometry, and secondarily on cellular transduction using post-mortem histology. Over a period of 42 days post-dosing, no major complications were observed following intracameral rAAV administration, with all noted events (e.g., transient IOP increase, aqueous cell, etc.) resolving naturally without pharmacological or surgical intervention. Expression of the GFP reporter transgene was observed in all high dose rAAV injected eyes, predominantly in cells of the trabecular meshwork and iris, indicating that rAAV vectors may be promising tools for the treatment of open-angle glaucoma and other anterior segment pathologies.

Slit lamp biomicroscopy defined a self-limited inflammatory response immediately after vector administration that was more evident in the high dose treated eyes across all serotype groups, as revealed by aqueous cell scoring and total clinical scores. It is possible that the immune system responded to vector injection itself, as similar immediate trends of inflammation have been seen previously in studies where rAAV has been intravitreally injected in humans and other species, including NHPs, dogs, sheep, and rodents, despite being sero-negative [23, 24]. Alternatively, it is

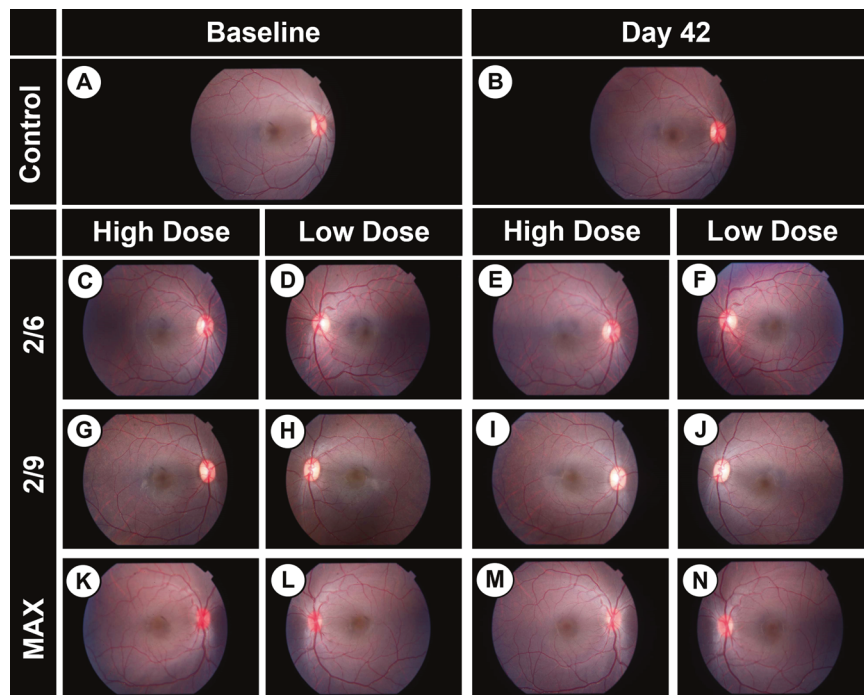


Fig. 4 Representative fundus photography at baseline and day 42. No changes were observed in any eyes between baseline and day 42 in either the control (A, B), high dose treated eyes (C, E, G, I, K, M), or low dose treated eyes (D, F, H, J, L, N).

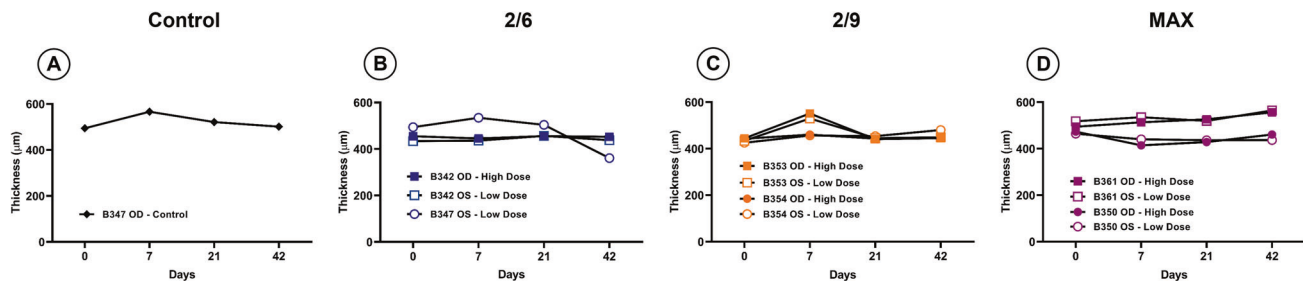


Fig. 5 Average corneal thicknesses as determined by pachymetry. Corneal thickness remained fairly constant with minor fluctuations throughout the study. One low dose treated rAAV2/6 eye (B347 OS) (B) showed the largest decrease in thickness at day 42. An increase in thickness was noted at day 7 in both control and rAAV2/9 treated eyes in subject B353 (A, C). All other measurements remained similar to baseline (A–D).

possible that even the low endotoxin levels present within our vector preparations may be sufficient to elicit an immediate inflammatory response, despite detected levels being below thresholds considered acceptable for human use (< 5 EU/mL). Specifically, previous studies have shown a similar degree and time course of inflammation in AGMs, with resolution within 18 days, when purified endotoxin was administered intravitreally, suspended in a vehicle (PBS), at a level of 0.08 EU/eye [20]. Indeed, this level of endotoxin is similar to that contained within each of our vector preparations used herein (0.0723–0.0851 EU/eye).

While the injection of foreign material—rAAV capsids containing extremely low-levels of endotoxin—into the anterior chamber likely explains the acute inflammation observed, many of the NHPs in this study exhibited a secondary phase of inflammation occurring between days 14 and 21. The likeliest explanation of this secondary inflammatory peak is the triggering of an innate immune response to expression of the fluorescent reporter transgene itself, where GFP has been shown to be immunogenic [25]. The timing of the secondary inflammatory peak fits well with our understanding of expression kinetics from a self-complementary vector genome, as employed in this study, where

it is expected that transgene expression occurs at a faster rate than when a single-stranded expression cassette is used [26]. This observation would also be in line with other previous studies, which have shown that when compared to an injection of empty vector capsid, injection of rAAV vectors expressing a transgene triggers a larger immune response. Another contributing factor to the observed second peak of immune response may be our choice of fluorescent reporter transgene, where GFP is known to be at least mildly toxic in the central nervous system and rAAV-derived GFP expression (e.g., after intrathecal delivery) has previously been shown to cause a progressive inflammatory response in NHPs due to sustained expression of non-self protein [27, 28]. These findings have been corroborated in several additional studies, where T-cell mediated inflammation following GFP expression in the eyes or liver of dogs and NHP models has been reported, indicating that we may potentially have observed a lesser secondary immune peak if we had either selected a less toxic reporter gene or used a humanized version of GFP to reduce recognition of non-self [29–31]. Importantly; however, despite one or two phases of inflammation being observed in several injected eyes, total clinical scores returned to baseline by study conclusion at day 42 without

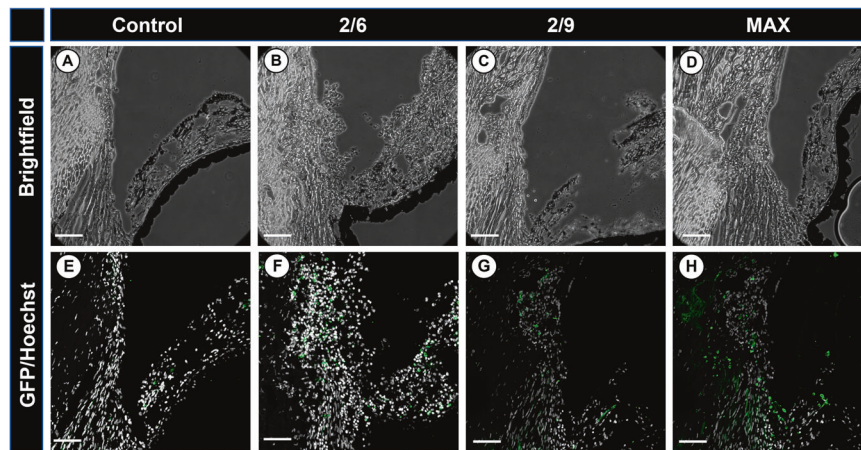


Fig. 6 Representative brightfield and confocal microscopy of the control and high dose treated eyes. Transduction of the iris and iridocorneal angle was observed in rAAV2/6 (**B, F**) and rAAV2/9 (**C, G**) treated eyes, with most prominent transduction observed in rAAV2/2[**MAX**] treated eyes (**D, H**). A small amount of non-specific staining is seen in the control eye (**A, E**). Scale bar = 100 μ m.

pharmacological intervention (e.g., topical/systemic antibiotics or immune suppressants) indicating that intracameral injection of rAAV is well tolerated in NHPs, at least in AAV naïve subjects.

One of the primary considerations we investigated was whether intracameral administration of vector would cause any changes in intraocular pressure, as this may limit future downstream applications of intracameral rAAV delivery in certain indications, such as development of gene therapy for open-angle glaucoma, where IOP is already elevated. IOP increased in all animals immediately following injections; however, this observation was expected owing to the acute increase in aqueous volume following injection of 100 μ L fluid into the anterior chamber. While efforts were made to control for circadian and diurnal fluctuations in IOP by recording measurements at the same time of day, IOP fluctuations were observed throughout the course of the study; however, these measurements remained within the normal range of 12–22 mmHg observed in the AGM and other NHP species and are likely to represent normal pressure variations [20]. We observed two deviations outside of the normative range during the study—B361 OS (low dose rAAV2/2[**MAX**] reached an IOP of 6–7 at day 28 and B342 OD (high dose rAAV2/6) that exhibited an IOP of 27 at day 3. These incidents corresponded to peaks of inflammation, as evidenced by high aqueous cell and total clinical scores—indicating that the large IOP fluctuations observed were due to inflammatory responses, which can lead to either decreased or increased pressure, reflecting decreased aqueous production and outflow obstruction, respectively [32–34].

As the vector in this study was injected intracamerally, we also completed pachymetry to determine if any changes in corneal thickness occurred. Previous studies indicated that the average normal corneal thickness for NHPs such as the rhesus macaque is ~456 μ m for males and 449 μ m for females [35]. However, AGMs have been shown to have corneal thicknesses between 406–535 μ m in males, and 393–574 μ m in females [16]. Corneal thicknesses remained consistent with baseline findings, with most measurements being within the acceptable range for AGMs. Two exceptions to normal corneal thickness were found in B347 OS (low dose rAAV2/6) wherein thickness decreased by 28% between days 21 and 42, and so falling out of normal range for male AGMs, and possibly reflecting technical variability.

While we were primarily focused on metrics of anterior chamber health, it is also important to investigate the health of the posterior chamber. Retinal thicknesses were generally stable throughout the study in all animals when comparing baseline measurements to day 42 measurements, and critically, measurements of foveal thickness ranged from 239 to 278 μ m, which align

with the previous reported normal range in the AGM of $274 \pm 8 \mu$ m [22] by histological measures and in the cynomolgus monkey by OCT of $244 \pm 21 \mu$ m and $286.27 \pm 53 \mu$ m [36, 37]. Of note, most animals maintained changes in retinal thickness that were similar to that of the control eye, and color fundus photography revealed no differences between baseline and day 42, with an absence of observed adverse retinal findings.

To determine tropism of each serotype in the AGM anterior chamber, histology of the anterior chamber was completed to assess distribution of transgene expression. GFP expression was evident within the angle, iris, and ciliary body of the high dose treated eyes with each serotype, with rAAV2/2[**MAX**] treated eyes showed the highest intensity of staining within the angle and iris compared to eyes administered rAAV2/6 or rAAV2/9. Interestingly, this transduction pattern is similar to that reported previously in brown Norway rats, wherein transgene expressed was primarily restricted to cells of the iris and iridocorneal angle [38]. By contrast, in all low dose treated eyes no GFP expression was evident within the angle, with only sparse transduction evident within the iris. One possible explanation for the absence of GFP expression is that the low dose viral vector load falls below a threshold (or multiplicity of infection) necessary to efficiently transduce cells within the anterior chamber iridocorneal angle. Another possibility is that while the inflammatory response was overall less severe in low dose treated eyes, T-cell mediated cytotoxicity may still possibly result in a decrease in the levels of GFP below the threshold for visible detection, leading to a situation where cells may have been successfully transduced, but are not obviously fluorescent [29–31]. While it is not possible to distinguish in the present study between these options, the possible role of inflammation in diminishing transgene expression highlights the importance of considering prior immune exposure to rAAV when selecting prospective subjects, where it has been shown that ~72%, 46%, and 70% of individuals tested marked seropositive for antibodies for AAV2, 6, and 9 respectively [39].

In conclusion, treatment of NAb negative African green monkeys with intracameral injection of rAAV2/6, rAAV2/9, and rAAV2/2[**MAX**] packaging a self-complementary GFP transgene resulted in minimal adverse events, such as inflammation, that resolved within 6 weeks of dosing. Posterior and anterior chamber health was found to remain generally unaffected with minimal changes in retinal and corneal thicknesses observed. Future research investigating transduction with therapeutic transgenes will determine if transduction of the iridocorneal angle is sufficient to achieve a therapeutic effect.

DATA AVAILABILITY

The data necessary to evaluate the conclusions within this paper are present in the paper and/or supplementary materials. Any other requests may be communicated to the corresponding author.

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AUTHOR CONTRIBUTIONS

Conceptualization: DML, MEO, MSL, KJC. Investigation: KJC, KZI, ZDG, MEO. Writing (Original Draft): KJC, DML, MEO. Writing (Revision and Editing): KJC, DML, MSL, MEO, KZI, ZDG.

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICAL APPROVAL

All animal experiments were approved by the Medical College of Wisconsin's Institutional Animal Care and Use Committee and adhere to the Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmic and vision research.

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