Improvement of Visual Performance With Intravitreal Administration of 9-cis-Retinal in Rpe65-Mutant Dogs

Patricia M. Gearhart, DVM, PhD; Chris Gearhart, PhD; Debra A. Thompson, PhD; Simon M. Petersen-Jones, DVetMed, PhD

Objective: To determine the efficacy of intravitreal administration of 9-cis-retinal in restoring visual function in Rpe65-mutant dogs.

Methods: Intravitreal injection of 9-cis-retinal was administered in 1 eye of 7 Rpe65−/− dogs at a range of ages. Electroretinogram analysis and testing of visual performance was used to evaluate outcomes after a single injection and in 2 dogs after a second injection in the same eye.

Results: In 5 of 7 injected dogs, 9-cis-retinal injection resulted in increased rod electroretinogram responses and improved functional vision. Three injected dogs exhibited increased 33-Hz flicker amplitudes characteristic of cone-mediated responses. Electroretinogram improvement was no longer evident by week 10 postinjection in 1 dog monitored over time. A second injection of 9-cis-retinal was performed in the same eye of 2 of the 7 dogs and also resulted in rescue of visual function.

Conclusion: Our findings establish that 9-cis-retinoid therapy can restore visual function in a canine model of human disease resulting from RPE65 mutations.

Clinical Relevance: These positive proof-of-principle results provide support for the development of intravitreal devices for sustained delivery of 9-cis-retinal as a therapy for conditions resulting from failure of the visual cycle.


Leber congenital amaurosis is a genetically heterogeneous condition that is an important cause of severe visual disability in children and young adults.1 Mutations in RPE65 are responsible for approximately 6% of autosomal recessive Leber congenital amaurosis, often referred to as Leber congenital amaurosis type 2, with patients progressing to legal blindness in early adulthood.2

RPE65 is an essential component of the visual cycle where it acts as a retinoid isomerase to convert esters of vitamin A stored in the retinal pigment epithelium to 11-cis-retinol, which is then oxidized to 11-cis-retinal and transported to the retina where it combines with rhodopsin and cone opsins. Disruption of the Rpe65 gene in knockout mice results in a lack of 11-cis-retinal and electroretinogram (ERG) responses, downregulation of rhodopsin protein, and accumulation of retinyl esters in the retinal pigment epithelium.3

Groundbreaking research demonstrating the efficacy of gene therapy for inherited retinal degeneration centered around the Briard breed of dog that carries a naturally occurring 4-base pair deletion in Rpe65.4 Gene therapy was effective in rescuing the disease phenotype.5-8 These studies formed a critical foundation for work that has now progressed to phase 1/2 clinical trials of gene therapy in human patients with Leber congenital amaurosis, with the first reported outcomes showing promising results.9-11

As an alternative approach, studies with Rpe65 knockout mice have shown that retinoid supplementation strategies involving oral delivery of 9-cis-retinal, and intra-peritoneal injection of 11-cis-retinal, are effective in reconstituting active visual pigments and visual responses.12,13 Furthermore, a combination of retinoid and gene therapy was shown to be complementary and resulted in improved rescue compared with each treatment alone in mice.
with a failure of the visual cycle due to a lack of lecithin retinol acyltransferase.14 Oral retinoid therapy has entered phase 1b clinical trials, with early reports suggesting evidence of efficacy in patients with lecithin retinol acyltransferase mutations.15 Long-term administration of 9-cis-retinyl acetate in aging mice improved ERG responses and dark adaptation, leading to the suggestion that such therapies may protect against age-related retinal dysfunction.16 Despite its successes, gene therapy is unlikely to be the first-line treatment for all genetic defects affecting visual cycle function, especially those associated with disease that is less severe and of later onset (eg, mutations in RDH5 and RLBP1).17,18 In addition, adjunct therapies that augment other treatment paradigms are likely to be needed in specific circumstances, eg, in cases involving gene inactivation and where repeated injections of viral vectors might not be advisable. Because of these issues and other considerations relative to the development of gene therapy approaches,19 the purpose of our proof-of-principle study was to determine if retinoid therapy could rescue the Rpe65−/− dog phenotype. We now show that intravitreal injection of 9-cis-retinol can result in markedly improved ERG responses and visual performance in the Rpe65−/− mutant dog that can be reinitiated by repeated injection.

### METHODS

#### ANIMALS

Seven Rpe65−/− dogs were used in this study (Table 1). All procedures were approved by the Institutional Animal Care and Use Committee. Prior to 9-cis-retinol injections, baseline vision testing of each eye and bilateral ERG recordings were made. Following 9-cis-retinol injections, dogs were kept in the dark with dim red light used during cleaning, care, and maintenance. In 2 dogs (dogs 5 and 6), a second injection of 9-cis-retinol was made into the same eye. Ophthalmoscopically normal Rpe65+/+ crossbred dogs of similar ages were used as controls for normal ERG responses.

#### ANESTHESIA

Dogs were anesthetized by premedication with acepromazine maleate (0.1-0.3 mg/kg) intramuscularly, induction with thiopental sodium (6-12 mg/kg intravenously), and maintenance with isoflurane (1% to 2% delivered in oxygen via an endotracheal tube).

#### 9-cis-RETINAL PREPARATION AND INJECTION

9-cis-Retinal (RS754, Lot 026K1125; Sigma Chemical, St Louis, Missouri) was resuspended in ethanol at 344mM and purity was evaluated by normal-phase high-performance liquid chromatography. The concentration was calculated from the 373-nm absorbance maximum and the extinction coefficient 36 068M−1 cm−1 (in ethanol).20 For injections, 4 µL of 9-cis-retinol stock solution (391 µg) was mixed with lactated Ringer solution to make 100 µL. Four-microliter ethanol in 100-µL lactated Ringer solution was used as a control vehicle injection. All retinoid manipulation was performed under dim red light.

#### 9-cis-RETINAL INJECTION

**Standard Intravitreal Injection**

In the early part of the study (dogs 1-4), 9-cis-retinol was delivered by a conventional intravitreal injection. With the dog under general anesthesia and in lateral recumbency, the ocu-

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### Table 1. Details of the Dogs Used in the Study and ERG Outcomes Using a Measure of Rod and Cone Function

<table>
<thead>
<tr>
<th>Dog No./ Sex/Age at Injection, wk</th>
<th>Eye</th>
<th>Treatment</th>
<th>Vision Testing Technique</th>
<th>Pretreatment Dark-Adapted b-Wave Amplitude, µV at 0 log cdS/m²</th>
<th>1 wk Postinjection Amplitude, µV at 0 log cdS/m²</th>
<th>Pretreatment 33-Hz Flicker Amplitude, µV</th>
<th>1 wk Posttreatment 33-Hz Flicker Amplitude, µV</th>
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<tr>
<td>1/M/28</td>
<td>OD</td>
<td>Intravitreal 9-cis-retinal</td>
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<tr>
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**Second Injection**

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<tr>
<th>Dog No./ Sex/Age at Injection, wk</th>
<th>Eye</th>
<th>Treatment</th>
<th>Vision Testing Technique</th>
<th>Pretreatment Dark-Adapted b-Wave Amplitude, µV at 0 log cdS/m²</th>
<th>1 wk Postinjection Amplitude, µV at 0 log cdS/m²</th>
<th>Pretreatment 33-Hz Flicker Amplitude, µV</th>
<th>1 wk Posttreatment 33-Hz Flicker Amplitude, µV</th>
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</tbody>
</table>

Normal control dogs (n=9), mean (SD) | 46 (18) | 21 (9) |

**Abbreviations:** ERG, electroretinogram; NT, not tested.

*See text for details.*

*Shows the ERG results from 18 Rpe65+/+ eyes of dogs of similar breeding.*
lar surface was prepared for injection using dilute (1 in 50) povidone-iodine solution. The dorsolateral bulbar conjunctiva was grasped with forceps and the globe was rotated ventrally. A 27-gauge needle on the Hamilton syringe containing the material for injection was inserted approximately 7 mm posterior to the sclera, angled posteriorly so as to miss the lens, and the material was injected.

Preretinal 9-cis-Retinal Injection

For the latter part of the study, we developed and used an injection technique to deposit the 9-cis-retinal on the surface of the retina. Under general anesthesia, the dog was positioned in dorsal recumbency with the neck flexed to achieve a horizontal cornea when the eye was in a primary gaze position. The eye was prepared with dilute (1 in 50) povidone-iodine solution, an eyelid speculum was fitted, and the globe was positioned using conjunctival stay sutures of 4-0 silk. A subretinal injector (Retinaject; SurModics Inc, Irvine, California) was used for the preretinal injections. The injector was inserted through the pars plana region and advanced toward the retina under direct visualization through an operating microscope (Zeiss OpM1 Operating Microscope; Carl Zeiss, Inc, Thornwood, New York) using a Machemer Magnifying Vitrectomy contact lens (Ocular Instruments Inc, Bellevue, Washington). The 39-gauge extendable cannula of the Retinaject was advanced until it was at the retinal surface. At that point, the room lights and the microscope light were turned off and the injection was made.

ERG RECORDINGS

Electroretinograms were performed in dog 1 at 1 hour after injection and then every week for 4 weeks. In dog 2, ERGs were performed at 1 hour, then every week for 8 weeks, and then every 2 weeks until 18 weeks. In all other dogs, ERGs were performed 1 week after injection. Dark-adapted intensity series; 5-Hz rod flicker, light-adapted intensity series; and 33-Hz cone flicker ERGs were recorded as previously described except that ERG-Jet lens electrodes (The Electrode Store, Enumclaw, Washington) were used.

ERG DATA ANALYSIS

The a- and b-wave amplitudes were measured as previously described. Electroretinogram amplitudes were plotted as a function of light stimulus. For the flicker responses, amplitude (trough to peak) and implicit times (flash onset to peak amplitude) were measured.

VISION TESTING

This was performed 1 week after treatment. For dogs 1 through 4, a subjective assessment of vision was performed by observing treated animals moving through an obstacle course of traffic cones with either the treated or untreated eye patched. The dog was filmed using a Sony TRV85 video camera with Nightshot (Sony, San Diego, California). Rpe65−/− dogs maintain some vision in bright lighting conditions for the first few weeks of life. We therefore assessed vision at a lighting level at which all untreated Rpe65−/− dogs were unable to successfully negotiate the obstacle course. The video recording of the vision testing sessions was assessed by 2 independent observers unaware of the treatment status of the dog.

For dogs 5 and 6, objective vision testing was performed according to a method that we have previously described. Briefly, this uses a testing device consisting of a chamber with 4 exit tunnels. One random tunnel was open for each run of the test. The first choice of exit tunnel and the time taken to exit were recorded. Performance was analyzed under 3 lighting intensities (35-45 [room light], 1-1.5, and 0.02-0.04 cd/m2). Each eye was tested separately by masking each eye in turn.

STATISTICAL ANALYSIS

Paired-sample t tests were used to test for differences in ERG amplitudes between 9-cis-retinal–treated eyes and vehicle-treated eyes before and after injection of the vehicle. For vision testing outcomes, mean time to exit, and mean number of correct exits, paired-sample t tests were again used to test for differences between 9-cis-retinal–treated eyes and vehicle-treated eyes. Paired-sample t tests were chosen instead of more complex tests because of the relatively low sample size of this study. Data were considered significant at P < .05.

RESULTS

ERG RESPONSES OF TREATED RPE65-MUTANT DOGS

In 5 of the 7 Rpe65−/− dogs, injection of 9-cis-retinal resulted in restoration of dark-adapted ERG responses of normal shape and with a reduced (improved) response threshold (Figure 1). In the 2 dogs that had an ERG recorded at 1 hour after 9-cis-retinal injection, only 1 showed an ERG improvement (data not shown) at that point. However, both dogs showed an ERG improvement at 1 week after treatment (this was the next point at which an ERG was recorded). The mean dark-adapted intensity response curve of the 5 dogs with improved ERG tracings (Figure 2) shows a lowering of threshold for a-wave of about 1.5 log units and for b-wave of about 2.5 log units and a significant increase in amplitude compared with the vehicle-injected eye. Although significantly improved, ERG amplitudes remained significantly decreased compared with the normal control dogs (Figure 2).

Of the 4 dogs injected by a standard intravitreal injection (dogs 1-4), 2 (dogs 1 and 2) showed an ERG rescue and 2 (dogs 3 and 4) showed no rescue. We considered variability of positioning of the 9-cis-retinal injection within the vitreous as a possible cause for these differing results. To eliminate this possibility, we developed a technique to place the injected material at the retinal surface. Dogs 5, 6, and 7, and the second injections in dogs 5 and 6, were performed using this technique and all showed ERG rescue at 1 week after injection.

Dogs 1 and 2 had weekly ERGs performed for the first 4 weeks. There was a progressive decline in ERG rescue (Figure 1 shows the comparison between rescue at 1 and 4 weeks posttreatment in dog 1). Dog 2 was followed up for a longer period by ERG, and the 9-cis-retinal– and vehicle-injected eyes had comparable ERG responses by 10 weeks following treatment (data not shown).

Rod responses were evaluated using the dark-adapted b-wave amplitude at 0 log cd/deg2, a flash intensity at which untreated Rpe65−/− canine eyes had no response, or at most a very small response (<3 µV). The
amplitudes at this flash intensity pretreatment and post-treatment are shown in Table 1.

To evaluate cone responses, the amplitudes of 33-Hz flicker responses were analyzed. By this measure, cone ERG rescue was not as dramatic as that of rod-mediated responses (Table 1). Three of the 7 dogs had an improvement in 33-Hz flicker amplitudes above the levels seen in all Rpe65−/− dogs preinjection and in all eyes following vehicle injection.

SECOND ADMINISTRATION OF 9-cis-RETINAL

Dogs 5 and 6 received a second administration of 9-cis-retinal in the same eye. There was an improvement in ERG responses at 1 week following treatment (Figure 3 and Table 1). The dark-adapted ERG after the second treatment had a slightly higher response threshold compared with that after the first injection. However, there was no significant difference in the a-wave amplitudes between the 2 treatments. The b-wave amplitudes following the second treatment were significantly lower at 2 of the dimmer flash intensities (−2.0 and −0.8 log cd/s/m²; P = .04 and .04, respectively) compared with the first. There was no significant difference between treatments in b-wave amplitudes at other flash intensities (P values ranged from .05 to .47). The treated eyes had significantly larger a- and b-wave amplitudes at all flash intensities compared with the vehicle-injected eyes (P values ranged from .049 to .001).

VISION ASSESSMENT
IN TREATED Rpe65−/− DOGS

Independent observers blinded to the treatment status of the dogs found that both dogs 1 and 2 negotiated the obstacle course in dim lighting conditions with fewer collisions with obstacles and more rapidly when the 9-cis-retinal–injected eye was uncovered compared with when the vehicle-injected eye was uncovered (video, http://www.archophthalmol.com). There was no difference in visual performance detected between 9-cis-retinal–treated and vehicle-treated eyes of dogs 3 and 4. These results correlated with the ERG findings. To provide data for statistical analysis of vision testing, we developed an objective vision test as described earlier.22 For dogs tested with this technique (Table 2), at the 2 lower–light intensity ranges (1-1.5 cd/m² and 0.02-0.04 cd/m²), there was a statistically significant improvement in both mean selection of the correct exit tunnel (P = .003 and P = .03, respectively) and the mean time to exit the device (P = .008 and P = .02, respectively) with the treated eye uncovered compared with the vehicle-treated eye uncovered. In normal room lighting (35-45 cd/m²), there was no significant difference in mean correct choice and mean time to exit (P = .19 and P = .15).

COMMENT

Our findings establish that intravitreal administration of 9-cis-retinal is effective in restoring useful visual function in Rpe65−/− deficient dogs. Using optimized injection protocols, we consistently obtained improved responses that were attributable to rod photoreceptor function. These results provide strong support for the potential of retinoid delivery as a possible treatment for RPE65 loss of function.

Previous studies of Rpe65−/− mice established the effectiveness of systemic administration (both oral and intravenous) of 9-cis-retinal for restoring ERG activity13,23 and repeated oral administration was also effective.24 Intraperitoneal injection of 11-cis-retinal was also shown to improve the ERG function of Rpe65−/− mice25 and when administered to young animals, corrected the subcellular mislocalization of cone opsin resulting from chromophore loss and associated with cone cell death.26 However, the doses of retinoid required for rescue were extremely high, leading to concerns over potential toxic
effects and motivating us to develop an effective approach for limited and local delivery. The dose used in the current study (about 400 µg of 9-cis-retinal per eye) translates to about 20 µg/kg of body weight, which is considerably lower than the dose used for systemic administration in mice, which was between 250 and 2500 µg per mouse, which is in the region of 1.6 to 16 /H11003 /H11004 µg/kg of body weight.13,23

Electroretinogram analysis of Rpe65-mutant dogs treated with 9-cis-retinal showed increased dark-adapted ERG amplitudes in 5 of 7 treated dogs and cone flicker responses in 3 of 7 treated dogs. Rod responses were also compared at a flash intensity (0 log cdS/m²) that is less than the response threshold for most untreated Rpe65-mutant dogs (although in a few dogs a b-wave of <5 µV could be recorded). The lack of ERG at this intensity is similar to that reported by others.3 Vision testing correlated with the recovery of dark-adapted ERG responses. Cone function was assessed by analyzing 33-Hz flicker amplitudes rather than the single-flash light-adapted responses. The flicker responses were selected because the dramatically reduced rod sensitivity in RPE65-deficient animals could mean that normal rod-suppressing background illumination might not completely eliminate responses from untreated rods. Responses from these rods might contaminate the light-adapted single-flash responses. Convincing improvements in 33-Hz flicker responses in 3 of the 7 treated dogs provide strong evidence of cone rescue. Our results suggest that a fraction of the cone cell population

Figure 2. Dark-adapted mean a- and b-wave electroretinogram intensity response plots (log/log scale) for normal dogs of similar breeding (n=9), the treated dogs with rescue (n=5), and the vehicle-injected eyes of the same dogs (n=5). Error bars are ± 1 SD. The mean a-wave (A) and mean b-wave (B) amplitudes for the treated eyes are significantly greater than those of the vehicle-injected eyes at each flash intensity (P<.05).

Figure 3. Result of the second 9-cis-retinal injection in the same eye for dogs 5 and 6. The mean (SD) a-wave (A) and b-wave (B) dark-adapted intensity response amplitudes are shown. In each graph, the solid line is the mean result from the first 9-cis-retinal injection, the long dashed line is the mean result from the second 9-cis-retinal injection, and the short dashed lines are the mean results from the vehicle-injected eyes. The second injection was performed 29 weeks after the first for dog 5 and 20 weeks later for dog 6. Electroretinograms before the second injection showed that response had returned to baseline following initial rescue by the first injection.
remains responsive to intravitreal retinoid in Rpe65−/−
dogs up to at least 82 weeks of age (dog 6, second injection).
This observation is noteworthy in the context of previous studies showing that cone cell death begins in
Rpe65−/− mice at a few weeks of age.30,31 Early cone cell
death is also evident by in vivo imaging of patients with
RPE65-null mutations, including young children32,34; however,
a subpopulation of cone cells appears to retain normal
structure and function for longer.

The visual testing results correlated closely with the
ERG findings. We have previously reported that in bright
room-lighting conditions young Rpe65-deficient dogs have
reasonable vision, allowing them to exit from our vision
testing device22 and to negotiate an obstacle course (data
not shown). In fact, the condition in the Swedish Briard
was first described as a congenital stationary night blindness.
35 We have shown that as the room lighting is lowered
from normal levels they start to have problems seeing
and at lower lighting levels are unable to see the exit
from the vision testing device.22 Following successful treatment (as assessed by ERG outcome), the visual performance was significantly improved at lower lighting conditions (Table 2 and video).

The failure of intravitreal injection to provide evidence of rescue (by ERG or vision testing) in 2 dogs may reflect the initial injection technique used, in which there was potential for considerable variation of site of 9-cis-retinal deposition. This could vary from close to the retinal surface to midvitreous. 9-cis-Retinal is very hydrophobic, and therefore, there is probably limited diffusion from the injection site. We theorized that variability in the site of deposition of 9-cis-retinal could have accounted for the variability in whether rescue was achieved. Development of a technique to accurately place the injected material at the retinal surface resulted in ERG-detectable rescue in all eyes injected in this fashion (1 eye injected once and 2 eyes injected twice in dogs 5-7). Additional experiments to look at diffusion of the retinoid within the vitreous body would be required to test our theory for the reason for failure of treatment in dogs 3 and 4. Clearly, the method of injection, injection site, dose given, and diffusion characteristics of the retinoid have the potential to significantly impact outcomes and will form the basis for future refinements of the approach.

The improvements in ERG function obtained following a single injection of 9-cis-retinal were lost by 10 weeks following injection indicating that repeated administra-
tion or development of a sustained delivery device will be required. Importantly, we found that repeated 9-cis-retinal injection into a previously treated eye resulted in rescue as assessed by ERG and vision testing. The dark-adapted ERG a- and b-wave intensity response curves were similar between the first and second treatments, but there did appear to be slight differences at lower flash intensities with an increase in response threshold and a significant reduction in b-wave amplitude at 2 of the low-intensity flashes. The cone ERG results were different between the injections, with dog 5 having a lower cone ERG recordable at the second injection compared with the first, whereas dog 6 had no detectable cone ERG improvement at the first injection but a good improvement after the second injection. It is possible that the proximity of the injection to the regions of highest cone density (area centralis and visual streak36) coupled with limited retinoid diffusion may have accounted for some of this apparent disparity in cone rescue. This warrants further study.

The RPE65-deficient phenotype is characterized by a progressive loss of cone photoreceptors, which could contribute to the difference in cone responses between the 2 points. There appear to be species differences in the rate of cone degeneration, with mice losing cones rapidly in early life30 whereas humans have an early cone loss but have a population of cones that are maintained for decades.32,33 Cone survival in Rpe65-deficient dogs has not been studied in detail, but ultrastructural studies in younger dogs showed that cone outer segments were better preserved than rod outer segments,35 and in a single older (7-year-old) dog that was examined, cones were preserved in the central retina and had intact outer segments.38 In view of the apparently slow cone degeneration in the dog model, it seems unlikely that cone degeneration made a large contribution to the reduced cone ERG rescue seen after the second injection in dog 6.

The approach reported herein is the first, to our knowledge, to show the efficacy of intravitreal administration of 9-cis-retinal in an RPE65-deficient animal model and describes a technique for accurate injection of the retinoid at the retinal surface. Our long-term goal is the development of sustained-release delivery of retinoids as an alternative treatment for patients with failure of the visual cycle.

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### Table 2. Vision Testing Results

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<th>Light Intensity, cd/m²</th>
<th>Frequency of Correct Tunnel Choice (7 runs)</th>
<th>Time to Exit Device, s</th>
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<td>Vehicle Injected</td>
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<tr>
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*Significant at P < .05.


