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Susan E. Pearce-Kelling

Tomas S. Aleman
University of Pennsylvania, aleman@mail.med.upenn.edu

Amanda Nickle
University of Pennsylvania

Alan M. Laties
University of Pennsylvania, laties@mail.med.upenn.edu

Gustavo D. Aguirre
University of Pennsylvania, gda@vet.upenn.edu

See next page for additional authors

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Abstract

Purpose: D-*cis*-diltiazem, a calcium channel blocker, has been reported to enhance photoreceptor survival in the *rd* mouse, a model of retinitis pigmentosa (RP) resulting from mutation of the *PDE6B* gene. We tested the hypothesis that diltiazem treatment would similarly rescue the canine *rcd1* model of RP, which is also caused by a null mutation in the *PDE6B* gene.

Methods: D-*cis*-diltiazem was delivered orally twice daily to *rcd1* affected dogs beginning at 4 weeks of age; untreated age-matched *rcd1* dogs served as controls. At 14 weeks, electroretinograms (ERG) were performed on all animals; 14 dogs were euthanized at this age, and 2 dogs at 25 weeks of age. Eyes were enucleated, fixed, and processed for routine histological examination.

Results: No significant differences were found in ERG or histopathologic parameters between diltiazem-treated and untreated *rcd1* dogs. Neither *rcd1* group showed a rod b-wave; ERGs evoked by single white flashes (dark- or light-adapted) and flicker were also identical between groups. Similarly, treated and untreated animals did not differ in the degree of preservation of the photoreceptor layer, confirmed in cell counts within the outer nuclear layer.

Conclusions: Treatment of *rcd1* affected dogs with D-*cis*-diltiazem did not modify the photoreceptor disease when results were assessed using either ERG or histopathologic criteria. The positive photoreceptor-rescue effect of calcium channel blockers reported in the *rd* mouse was thus not generalizable to another species with retinal degeneration due to mutation in the *PDE6B* gene. Caution needs to be exerted in extrapolation to the comparable human forms of RP.

Disciplines
Comparative and Laboratory Animal Medicine | Eye Diseases | Medicine and Health Sciences | Ophthalmology | Veterinary Medicine

Author(s)
Susan E. Pearce-Kelling, Tomas S. Aleman, Amanda Nickle, Alan M. Laties, Gustavo D. Aguirre, Samuel G. Jacobson, and Gregory M. Acland

Calcium channel blocker D-*cis*-diltiazem does not slow retinal degeneration in the *PDE6B* mutant *rcd1* canine model of retinitis pigmentosa

Susan E. Pearce-Kelling,[1] Tomas S. Aleman,[2] Amanda Nickle,[3] Alan M. Laties,[2] Gustavo D. Aguirre,[1] Samuel G. Jacobson,[2] Gregory M. Acland[1]

[1]*Baker Institute, College of Veterinary Medicine, Cornell University, Ithaca, NY;* [2]*Department of Ophthalmology, University of Pennsylvania, Philadelphia, PA;* [3]*Retinal Disease Studies Facility, University of Pennsylvania, Kennett Square, PA*

Purpose: D-*cis*-diltiazem, a calcium channel blocker, has been reported to enhance photoreceptor survival in the *rd* mouse, a model of retinitis pigmentosa (RP) resulting from mutation of the *PDE6B* gene. We tested the hypothesis that diltiazem treatment would similarly rescue the canine *rcd1* model of RP, which is also caused by a null mutation in the *PDE6B* gene.

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Finding treatments that would slow progression of the relentless loss of vision in human retinitis pigmentosa (RP) is a major goal of research into these genetically heterogeneous inherited retinal degenerations. The ideal therapy would be simply and safely administered and would be immediately available for use by patients. Hope was raised recently that one such pharmaceutical treatment for RP may be forthcoming with the report that D-*cis*-diltiazem, an available oral calcium channel blocker used for human cardiac disease, may have efficacy to rescue photoreceptors in the *rd* (retinal degeneration) mouse model of human RP [1]. The pathophysiology of retinal degeneration in the *rd* mouse has been explored over decades, and the molecular basis of the disease is now known to be a mutation in the *PDE6B* gene [2-6]. An advantage of discovering measurable benefit in this murine model of RP is that testing can be promptly advanced to a large animal model, the *rcd1* (rod-cone dysplasia 1) dog, also known to have severe retinal degeneration caused by a similar deficiency in cyclic guanosine monophosphate (cGMP)-phosphodiesterase (PDE) activity resulting from an autosomal recessive *PDE6B* mutation [7-10]. Further, if therapeutic effect

is mutant gene-specific, there are RP patients known to share the same molecular causation [6,11-13].

Prompted by the report of neuroprotection with D-*cis*-diltiazem in the *rd* mouse [1], we tested the hypothesis that rescue may be similarly detectable in the *rcd1* dog. Histopathologic and electroretinographic criteria were used to evaluate *rcd1* dogs fed the human cardiac medication (Cardizem™) during a relatively early period of the retinal disease in this species.

METHODS

Animals: All experimental procedures were conducted in accordance with institutional guidelines and those of the Institute for Laboratory Animal Research (Guideline for the Care and Use of Laboratory Animals) and the US Public Health Service (Public Health Service Policy on Humane Care and Use of Laboratory Animals). Seventeen *rcd1*-affected dogs were used for this study. The animals were the product of 4 different litters born within one month of each other, representing affected x affected and affected x carrier matings. Disease genotype of the latter group was established by molecular diagnostic testing for the mutation in *PDE6B* causing *rcd1* [14]. Schedules for initiation of dosing, electroretinography, enucleation, and termination of the study were adjusted so that animals of comparable ages were studied. The dogs were

Correspondence to: Samuel G. Jacobson, M.D., Ph.D., Scheie Eye Institute, Department of Ophthalmology, University of Pennsylvania, 51 North 39th Street, Philadelphia, PA 19104; Phone: (215) 662-9981; FAX: (215) 662-9388; email: jacobsos@mail.med.upenn.edu

maintained in indoor runs with controlled cyclic illumination. Following weaning from the mother, the dogs were fed *ad libitum* with commercial dog food diets (Purina Puppy Chow, Ralston Purina, St. Louis, MO; Hi Tor beef, Triumph Pet Industries, Warwick, NY).

Treatment Regimen: Beginning at 4 weeks of age, 10 *rcd1*-affected dogs had oral administration of D-*cis*-diltiazem (Cardizem™) tablets or sustained release capsules at a dose of 40 mg/kg/day (tablets = divided doses 2 times/day for 5 days; sustained release capsules = one time/day weekends). Seven age-matched *rcd1*-affected dogs served as untreated controls. At this age, the retinal photoreceptors show only the earliest stages of visual cell degeneration [8,15]. The daily dose and frequency of administration were approximately the same (40 mg/kg) as those used in the *rd* mouse (21 and 54 mg/kg) study [1]. This dose is more than 6.6-10 fold higher than the dose currently used in the clinical management of dogs with cardiac disease (R. Gleed, Cornell University, personal communication, October 1999), and just below the 50 mg/kg single daily dose that produces severe toxicity in dogs when administered daily for 30 days (Hoechst Marion Roussel, personal communication, October 1999). Prior to the initiation of dosing, the dogs were weighed and amount of drug to be administered determined. This amount was adjusted on a weekly basis based on the animals' weight. Treatment was continued until 14 weeks of age (n=8) and 25 weeks of age (n=2).

Electroretinography: Full field electroretinograms (ERG) were recorded on all 17 *rcd1*-affected dogs at about 14 weeks of age. For 10 of the dogs, this recording was after 10 weeks of treatment with D-*cis*-diltiazem and these results were compared with ERGs from the group of untreated dogs (n=7). For the treatment group, no medication was given after the last dose on the day before ERG testing to permit time (>20 h) for drug clearance, which is reported to be about 2.5 h [16]. The ERGs were performed using a computer-based system (EPIC-XL, LKC Technologies, Inc., Gaithersburg, MD) and bipolar contact lens electrodes (Hansen Ophthalmics, Iowa City, IA). Animals were anesthetized with an intramuscular injection of acepromazine (0.08 mg/kg) followed by intravenous doses of ketamine (15 mg/kg). Pupils were dilated with cyclopentolate (1%) and phenylephrine (2.5%). In fully dark-adapted (overnight) dogs, a dim blue flash (W47A, -1.9 log scot-cd.s.m^{-2}) was used to elicit a rod ERG b-wave. To elicit a maximal mixed rod and cone response, single white flashes of light (0.8 log scot-cd.s.m^{-2}) were used. Following light adaptation, cone ERGs were recorded on a rod desensitizing white background (1.5 log cd.m^{-2} with 1 Hz; 0.6 log cd.m^{-2} background with 29 Hz); 5-20 responses were averaged for light-adapted recordings. Waveforms were measured conventionally [17]. Treated and untreated groups were compared using amplitude criteria with unpaired two-tailed t-tests. Results are presented as mean values±standard deviation (SD).

Histopathology: At 14 weeks of age (treated=8; untreated=6), and 25 weeks (treated=2), dogs were euthanized with a barbiturate overdose and their eyes enucleated and processed for microscopic examination. Both eyes from the dogs at the 14 week time point and one eye each from the dogs at the 25 week time point were fixed in Bouin's solution, embedded in paraffin, 5 μm sections were cut from the central (pupil/optic nerve) block and stained with hematoxylin and eosin. The fellow eyes from the two dogs at the 25 week time period were fixed in formaldehyde/glutaraldehyde and post fixed in osmium tetroxide. After embedding in Epon, 1 μm sections were cut from the central superior and inferior meridians, and stained with azure II, methylene blue. Fixation and sectioning methods are published [18].

Photoreceptor rescue was assessed by evaluating the structural integrity of the visual cell layer by an examiner masked as to the treatment status of the dogs. For quantitative evaluation of rescue, nuclei in the outer nuclear layer (ONL) were counted in three regions from both the superior and inferior quadrants (area 1 was central, area 2 was equatorial, and area 3 was peripheral). These areas are located within 2000 μm from the optic nerve head, midway between the optic nerve head and the ora serrata, and within 2000 μm from the ora serrata, respectively [19]. At three locations within each section, the number of rows of photoreceptor nuclei was counted [15] and the average of these values used in the analyses. Differences between treated and untreated groups both by retinal area, and for all areas, were analyzed using the unpaired two-tailed t-test. Results are presented as mean values±SD.
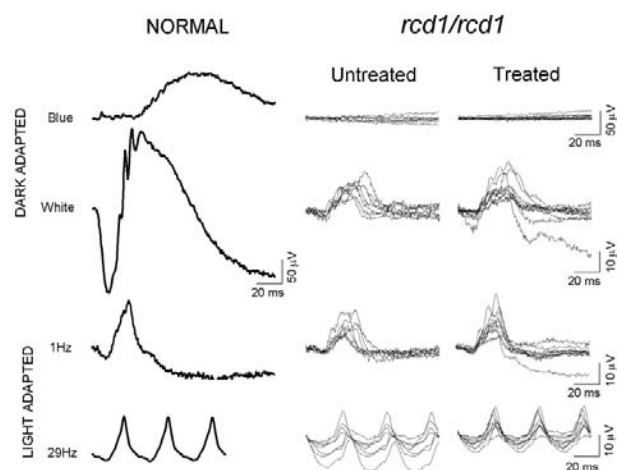

Figure 1. Rod, mixed cone-rod, and cone electroretinograms. Rod, mixed cone-rod, and cone electroretinograms in a representative normal dog and the untreated and D-*cis*-diltiazem-treated *rcd1*-affected dogs in this study. All ERG responses of *rcd1* dogs are shown by overlaying the waveforms from each group. Stimulus onset is at trace onset. Calibrations for amplitude and timing are below and to the right of the responses.

RESULTS

The medication was well tolerated in all but two of the treated dogs. One dog developed drug-associated toxicity 12 days after the start of dosing. This was characterized by depressed activity including inability to stand and walk, hypoten-

sion, and labored breathing. These clinical signs lasted 5 days. Diltiazem treatment was discontinued when the adverse effects became apparent with no medication administered between days 12 through 21 of the study. Once treatment was resumed, there was no recurrence of the drug-associated reactions. A second dog showed similar but milder adverse effects beginning on drug day 24, and lasting 2 days. Treatment was withheld for 1 day, and the effects disappeared and did not recur after treatment was resumed.

Electroretinograms (ERGs) from a representative normal dog and the two groups of *rcd1*-affected dogs at 14 weeks of age are illustrated (Figure 1). The dim blue flash elicits a rod b-wave in the normal of about 100 µV [20-23], but there was no detectable rod b-wave to this stimulus in either the diltiazem treated or untreated *rcd1*-affected dogs. The mixed rod and cone ERG in response to the bright white flash in the normal dog has an a-wave of 150 µV and a b-wave of 250 µV. Affected animals in both groups have very reduced but measurable b-waves. Cone ERG b-waves in response to the 1 Hz stimulus in the normal dog were about 30 µV while affected animals had reduced amplitudes. Unlike the normal animal, dark- and light-adapted waveforms are very similar in affected animals suggesting a cone origin of the dark-adapted ERGs to the maximal stimulus. Responses to 30 Hz flicker were also reduced in amplitude compared to the normal. These findings confirm the presence of rod and cone functional abnormalities in the disease [24]. Inspection of the waveforms showed no obvious amplitude or timing differences between diltiazem-treated and untreated *rcd1*-affected dogs.

Histologic examination of the retinas showed no apparent differences in the structural preservation of the photoreceptor layer between treated and untreated *rcd1*-affected dogs (Figure 2). Normal control dogs had a well developed photo-

receptor layer with elongated inner and outer segments, and an ONL approximately 10 nuclei in thickness. In contrast, the *rcd1*-affected dogs, whether diltiazem treated or not, showed the same degree of photoreceptor and ONL loss at 14 weeks of age. In both groups, the outer segment layer was fragmented and, for the most part, lost, and the inner segments were diminutive. The remaining photoreceptors were mainly cones, and these appeared as broadened and club-shaped, possibly due to the disappearance from the photoreceptor mosaic of the adjacent rods which provided lateral support. In the 25 week old treated animals, there was further reduction in ONL thickness. Variation in outer segment length occurred between sections and retinal regions (at 14 and 25 weeks), but a qualitative survey showed no consistent differences between treated and untreated retinas at the ages examined.

ERG and histopathological data from all 14 week old *rcd1*-affected dogs with and without diltiazem treatment are summarized in Figure 3. For the three measurable ERG responses (Figure 3A), the group treated with diltiazem (n=10) did not differ statistically from the group that was untreated (n=7). Dark-adapted ERG b-wave amplitudes in response to the maximal white stimulus in untreated (14±4 µV) and treated (15±8 µV) animals were not significantly different (p=0.68). Light-adapted 1 Hz ERG amplitudes in the untreated (14±4 µV) and treated (14±7 µV) groups were not different; 29 Hz amplitudes of the untreated (11±4 µV) and treated (11±5 µV) groups
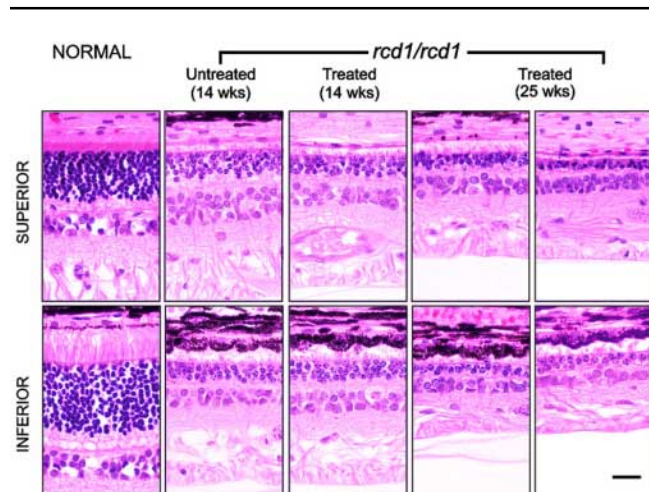

Figure 2. Normal and affected retinal histology. Retinal histology in a representative normal dog (age 21 weeks) and *rcd1*-affected dogs: one untreated animal (age 14 weeks) and two D-*cis*-diltiazem-treated animals (ages 14 and 25 weeks). Upper and lower panels represent superior and inferior regions of the retina (area 2), respectively. Calibration bar at lower right, 20 µm.
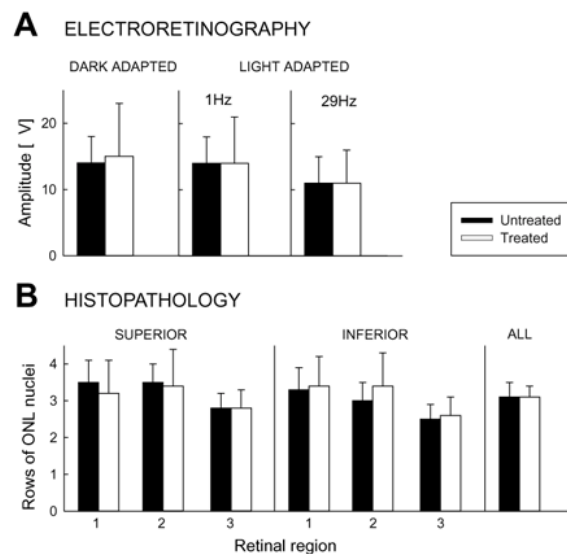

Figure 3. ERG and retinal morphometric comparisons of treated and untreated dogs. ERG and morphometric comparisons of D-*cis*-diltiazem-treated (white bars) and untreated (black bars) *rcd1*-affected dogs (14 weeks of age). **A**: Histograms of average ERG amplitudes for the three measurable responses. **B**: Histograms of average number of rows of photoreceptor nuclei remaining in *rcd1* dogs with or without D-*cis*-diltiazem treatment. Central (area 1), equatorial (area 2, as shown in Figure 2), and peripheral (area 3) regions were analyzed in both superior and inferior retina. Summary histograms of all data are at right. Error bars represent SD.

were also not significantly different (p=0.9).

Morphometry (Figure 3B) was consistent with ERG. There was no statistically significant difference in rows of ONL nuclei between treated (n=10) and untreated (n=6) 14 week old *rcd1*-affected dogs in any of the six retinal areas or in the combined totals of all retinal areas (Figure 3B). In no single area did the mean number of ONL nuclei in treated and untreated groups differ by more than 0.4 rows; in all six areas examined, there was no statistical difference between the groups (p=0.3 in all cases). When the retinal areas were combined and comparison made by groups, ONL nuclei in the treated (mean=3.1, SD=0.4 rows) and untreated (3.1±0.3 rows) groups also did not differ (p=0.9). In the two treated dogs examined at 25 weeks of age, the retinal disease had progressed still further, and the ONL was reduced to 1.5 and 1.6 rows of nuclei respectively. Untreated *rcd1*-affected animals at age 21 weeks (n=4) showed 2.0 rows of nuclei (SD=0.3); and at 27 weeks (n=1) there were 1.9 rows of nuclei. This degree of severity of the retinal degeneration in untreated animals was expected from the natural history of the disease previously described [8,15,25].

DISCUSSION

Photoreceptor rescue by D-*cis*-diltiazem in the naturally-occurring *PDE6B* murine model of RP was the first successful use of a pharmaceutical to alter the abnormal cellular mechanisms resulting from a mutant gene causing retinal degeneration [1]. For this reason, it represented a landmark observation in the history of investigation of the cGMP cascade, its perturbation in disease, and strategies to modify the pathophysiology. The deficiency of c-GMP PDE in photoreceptors of the *rd* mouse resulting from a null mutation in the *PDE6B* gene leads to a cascade of events culminating in rod and then cone cell death [26]. The suspected mechanism of diltiazem's neuroprotective effect of photoreceptors involved a blockade of L-type voltage-gated calcium channels, theoretically preventing activation of apoptosis by high levels of calcium influx [1,27]. The present study began with the simple goal of confirming and extending the important initial observation in *rd* mice by applying the pharmaceutical treatment to a naturally-occurring canine disease with similar molecular causation. Because there are patients that share this molecular cause with the *rd* and *rcd1* animal models, proof that a calcium channel blocker rescued photoreceptors in both species could lend support to consideration of a clinical trial in this form of RP. It would seem prudent now, considering our negative result in the *rcd1* model, to perform further animal experiments, rather than accelerate forward to human intervention.

On the other hand, different results in different species must be viewed for the complexity that they represent. In the two sets of experiments in the *PDE6B* mutant animals, there are not only species differences but also model differences and variations in the exact conduct of the individual experiments, all of which could complicate comparison of final results. For example, D-*cis*-diltiazem provided measurable protection in later stages of the murine disease model [1]. It may

be that this treatment can retard degeneration at ages greater than 25 weeks in the canine model. However, the absence of photoreceptor rescue in the *rcd1* dog in contrast to the *rd* mouse is not likely to be due to differences in either the initiation of dosing, or alternatively, differences in bioavailability of drug to the target tissue. The 4 week age chosen for the beginning of treatment in the dog is comparable to postnatal day 9 in the mouse. Both species display arrested retinal photoreceptor differentiation and the very early stages of rod cell death are apparent [6,15,20,28]. Likewise, the dosing used appears comparable in terms of actual daily drug administration. Evidence that we are close to the maximal tolerable dose is based not only on the information made available by the drug manufacturer (Hoechst Marion Roussel, personal communication, October 1999), but also on the fact that two dogs showed drug-associated toxicity, which disappeared with temporary cessation of therapy, but was definitely severe. Time was allowed for clearance of the drug from the circulation [16] before ERGs were performed, but whether there are more prolonged effects that could complicate interpretation of ERG results needs to be determined [1].

Whether providing confirmation or contradiction, studies like the present one represent an early attempt to follow a stepwise, conservative, and logical path en route to clinical trials of human retinal degeneration. Decades of basic and clinical scientific work in the field of retinal degenerations now permit comparing response to intervention in small animals with that in large animals having a similar molecular cause of disease before embarking on human trials. In this regard, we are not restricted to small and large animal models of human autosomal recessive RP due to *PDE6B* mutation. There are genetically-engineered murine and naturally-occurring avian forms of early-onset retinal degeneration from abnormal retinal guanylate cyclases [29,30], genetically-engineered murine and naturally-occurring canine models of autosomal recessive early-onset retinal degeneration from mutations in *RPE65* [31-33], and certain genetically-engineered mice, rats, and swine with rhodopsin gene mutations modeling human dominant RP [34-36]. Promising results in murine and canine models of the *RP3* form of X-linked RP may also become the pre-clinical path for human trials of this severe form of retinal degeneration [37,38].

The current study represents to our knowledge the fourth report of use of calcium channel blockers to intervene in a hereditary retinal degeneration. The *Drosophila* retinal degeneration B (rdgB) mutant was reported to show successful rescue [39], while a line of P23H rhodopsin mutant rats were recently found to show no neuroprotective effect when the exact same protocol used in the *rd* mouse was studied in this animal model of RP [27]. In conclusion, our results should signal the beginning of greater depth of investigation of the effects of calcium channel blockers and related drugs in both the canine and murine *PDE6B* mutant models. The hope that an available oral medicine could slow the visual loss of this specific molecularly-defined subset of RP patients is too attractive to abandon without further study.

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