

Published in final edited form as:

Curr Gene Ther. 2013 August ; 13(4): 275–281.

Identification of a Therapeutic Dose of Continuously Delivered Erythropoietin in the Eye Using An Inducible Promoter System

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Abstract

Erythropoietin (EPO) can protect the retina from acute damage, but long-term systemic treatment induces polycythemia. Intraocular gene delivery of EPO is not protective despite producing high levels of EPO likely due to its bell-shaped dose curve. The goal of this study was to identify a therapeutic dose of continuously produced EPO in the eye. We packaged a mutated form of EPO (EPOR76E) that has equivalent neuroprotective activity as wild-type EPO and attenuated erythropoietic activity into a recombinant adeno-associated viral vector under the control of the tetracycline inducible promoter. This vector was injected into the subretinal space of homozygous postnatal 5-7 day *retinal degeneration slow* mice, that express the tetracycline transactivators from a retinal pigment epithelium specific promoter. At weaning, mice received a single intraperitoneal injection of doxycycline and were then maintained on water with or without doxycycline until postnatal day 60. Intraocular EPO levels and outer nuclear layer thickness were quantified and correlated. Control eyes contained 6.1 ± 0.1 (SEM) mU/ml EPO. The eyes of mice that received an intraperitoneal injection of doxycycline contained 11.8 ± 2.0 (SEM) mU/ml EPO-R76E. Treatment with doxycycline water induced production of 35.9 ± 2.4 (SEM) mU/ml EPO-R76E in the eye. The outer nuclear layer was approximately 8 μ m thicker in eyes of mice that received doxycycline water as compared to the control groups. Our data indicates that drug delivery systems should be optimized to deliver at least 36mU/ml EPO into the eye since this dose was effective for the treatment of a progressive retinal degeneration.

Keywords

Dose; erythropoietin; gene therapy; inducible promoter; intraocular; recombinant adeno-associated virus; retinal degeneration

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CONFLICT OF INTEREST

Tonia S. Rex has a patent application filed for the use of EPO-R76E as a neuroprotective agent. No commercialization has occurred.

INTRODUCTION

Significant progress has been made recently in treating certain forms of inherited retinal degenerations with gene specific approaches [1-3]. However, over 200 genes and loci are known to cause retinal degenerative diseases [4], and the most common causes of blindness in the U.S., age-related macular degeneration and glaucoma, are complex, multifactorial diseases. Due to this complexity, the use of a neuroprotective agent to block cell death and preserve retinal cell function regardless of the genetic cause is appealing.

We, and others, have shown that erythropoietin (EPO) is neuroprotective to photoreceptors and retinal ganglion cells [5-13]. Treatment is effective regardless if EPO is delivered systemically or intraocularly [5, 6, 8, 12, 14]. However, long-term systemic treatment with EPO induces polycythemia and other negative side effects, especially at the doses reported to be effective for neuroprotection [7, 9, 12]. To avoid this complication, intraocular delivery was tested. Intraocular gene delivery of EPO using the high expressing cytomegalovirus (CMV) promoter packaged in an adeno-associated viral (rAAV) vector led to production of up to 1200mU/ml EPO in the eye, but was not protective [7]. In contrast, a single intraocular injection of 10U of EPO led to photoreceptor protection two weeks after injection despite undetectable levels of EPO in the eye after 36 hours [8]. This implies that low levels of EPO in the eye are protective, while high levels are not. This fits with accumulating evidence that indicates EPO, similar to other cytokines, likely has a bell-shaped dose curve in relation to its neuroprotective activities [7, 8, 12]. Repeat injections of up to 8U of EPO into the eye provide short-term protection of the retinal ganglion cells, with 2U being the optimal dose, when delivered every 3 days for the duration of the study [12]. However, repeat intraocular injection of EPO is not ideal since it would likely need to be performed approximately every two weeks [8] for the life of the patient, which is associated with increased risk of damage to the eye and decreased patient compliance.

One of the major advantages of using EPO as a therapeutic agent is that systemic treatment with EPO is already FDA approved for the treatment of anemia. EPO is an endogenous cytokine produced in the kidney and liver, and it crosses the blood brain barrier [15-17]. Under hypoxic conditions, EPO is upregulated and activates the EPO receptor homodimer to stimulate erythropoiesis. With the development of forms of EPO that do not bind the EPO receptor homodimer, it is believed that the neuroprotective function of EPO acts through an EPO receptor heterodimer with the interleukin beta common receptor or glucocorticoid receptor, activating the JAK2/Stat3 pathway [13, 18-21]. EPO has been shown to be neuroprotective in multiple models of neurodegenerative disease (for review see [22]) including glaucoma and retinal degenerations [5-14].

Most importantly, treatment with EPO not only blocks apoptosis and prevents axonal degeneration, but also preserves visual function, thus indicating that it acts earlier in cell death signaling than anti-apoptotic therapies. Long term systemic delivery using rAAV.CMV.EPO or a modified form of EPO that retains the neuroprotective properties and does not induce polycythemia (rAAV.CMV.EPOR76E; [9,10]) resulted in preservation of the electroretinogram in light-damaged rats (rAAV.CMV.EPO, [7]), and preservation of the visual evoked potential in glaucomatous mice (rAAV. CMV.EPO or

rAAV.CMV.EPOR76E, [10]). Both EPO and EPO-R76E work equally well *in vivo*. However, since long-term systemic delivery of EPO is associated with significant negative side effects in patients, we sought to identify therapeutic levels of continuously produced EPO delivered to the eye using gene therapy. These side-effects include development of antibodies against EPO after rAAV-mediated gene delivery into skeletal muscle [23], and pre-existing antibodies against rAAV that so far have prevented translation of this approach to the clinic, for review see [24]. In contrast, rAAV-mediated gene therapy has been very successful in the eye due to ocular immune privilege, for review see [25].

To identify therapeutic levels of continuously delivered EPO in the eye, we employed an inducible promoter system. The tetracycline (tet) inducible system regulates gene expression using the common antibiotic tetracycline or its analog, doxycycline (Dox). Doxycycline is very well characterized, and the dose needed to induce gene expression is well below bactericidal treatment levels [26]. This system is appealing in clinical settings because gene expression is regulated in a dose-dependent manner, and expression can be halted within 10-15 days of withdrawal of Dox [26-28]. In contrast to systemic expression of rtTA, which induces an immune response [29], this system has been utilized in the eye with no negative side effects after 6 months of continuously induced transgene expression and has also been shown to persistently regulate expression out to 5 years [26, 28]. Following a subretinal injection of AAV in non-human primates, Stieger *et al.*, demonstrated that levels of gene expression varied according to the serotype of the AAV and the promoter that was used [28]. Others have developed transgenic mouse lines expressing components of the tetinducible system in the eye and shown successful induction of transgenes after Dox administration [30, 31].

In this study, mice that express the reverse tetracycline transactivators (rtTA) from the retinal pigment epithelium (RPE) specific vitelliform macular dystrophy 2 (VMD2) promoter were crossed with homozygous *retinal degeneration slow (rds/rds)* mice. The *rds/rds* mouse has a 10kb insertion in the peripherin/rds gene [32]. The characteristic and first noticeable phenotype is the absence of photoreceptor cell outer segments, which is apparent by three weeks of age [33]. The outer nuclear layer (ONL) thickness is also slightly decreased at three weeks, and at two months, many of the photoreceptor cells have died, reducing the ONL thickness by half. Only one to two rows of photoreceptor cell nuclei are present by nine months, and there are no photoreceptors remaining after one year [33]. The *rds/rds* mouse phenotype is very similar to that of autosomal dominant retinitis pigmentosa in humans caused by mutations in the peripherin/rds gene. Mutations in the human RDS gene are also associated with macular dystrophy, retinitis punctata albescens, and Stargardt disease [4]. These mice were then given a single subretinal injection of rAAV2/1.tet.eGFP.EPOR76E. Serotype 2/1 transduces the RPE efficiently, but does not transduce the photoreceptors, thus providing an additional level of cellular specificity. The injected mice were treated with Dox to induce expression of EPOR76E from the RPE. We correlated EPO-R76E levels with outer nuclear layer (ONL) thickness as a measure of therapeutic efficacy.

MATERIALS AND METHODS

Mice

Wildtype Balb/c mice (Normal) and *rds/rds* mice were acquired from Jackson Laboratories (Bar Harbor, ME). The *VMD2.rtTA* mice were a gift from Dr. Don Zack (Johns Hopkins University, Baltimore, MD; [30]). The *VMD2.rtTA* mice were crossed to the *rds/rds* mice for 8 generations. Mice were genotyped by PCR for the presence of *VMD2.rtTA* and the 10kb insertion into the peripherin/*rds* gene. At the end of the experiments tail blood was collected for microcapillary centrifugation to determine the hematocrit just prior to euthanasia. All animal experiments were conducted under the approval of the Vanderbilt University Animal Care and Use Committee and according to the standards stated in the eighth edition of the Guide for the Care and Use of Laboratory Animals.

Generation of rAAV2/1.tet.eGFP.EPOR76E

Briefly, the pAAV backbone was obtained as a gift from UPenn Vector Core (Dr. James Wilson). The bidirectional tet-inducible promoter (Clontech, Mountain View, CA) and EPOR76E were subcloned into pAAV containing eGFP. The CMV promoter was removed during cloning. Viral vectors were generated by triple transfection into HEK-293 cells and purified by cesium chloride gradient as described in Hildinger *et al.* [34]. The titer was obtained by QPCR using primers and probes specific to BGH polyA (Integrated DNA Technologies, Coralville, IA).

Subretinal Injections

Bilateral subretinal injections were performed in five to seven-day-old *VMD2.rtTA: rds/rds* mice according to previously published methods [8]. Mice were injected with 2 μ L of 1.4×10^9 gc/ml rAAV2/1.tet.eGFP.EPOR76E using a blunt 10 μ L, 30 gauge Hamilton syringe (Reno, NV).

Doxycycline Treatment

VMD2.rtTA:rds/rds mice were weaned at PD21 and given an intraperitoneal (IP) injection of 200 μ l of 4 mg/ml Dox (IP only and Dox treated groups) or 0.1M sodium phosphate buffer (Buffer control group). Mice were then provided drinking water containing 0mg/ml Dox (Buffer control and IP only groups) or 0.5 or 5mg/ml Dox and 5% sucrose continuously from PD22 to PD60 (Dox treated group). Water was changed every 2-3 days. N values refer to the number of mice.

Optical Coherence Tomography

Ultra-High Resolution Spectral Domain Optical Coherence Tomography (OCT; Bioptigen, Durham, NC) of the retina was performed on *VMD2.rtTA:rds/rds* mice at PD60. Mice were anesthetized with ketamine/xylazine/urethane (25/10/20mg/kg body weight), eyes were dilated with 1% tropicamide and moistened with Systane Ultra. Each mouse was wrapped in gauze, placed in a mouse holder and imaged with a mouse retina bore (Bioptigen).

ELISA

Mice were euthanized, and the eyes were enucleated. The anterior half of the eye, including the lens, was removed. Eye cups were weighed and then homogenized and sonicated in 100 μ L of provided specimen diluent and then run in duplicate on a high sensitivity Human Erythropoietin Platinum ELISA kit according to manufacturer protocol (eBioscience, San Diego, CA). The plate was read on a MicroQuant plate reader (BioTek, Winooski, VT) at 450nm with a 620nm reference. N values refer to the number of eyes.

Histology

Mice were euthanized by overdose of ketamine/xylazine and cervical dislocation. Before enucleation, a small area of the cornea on the nasal side was cauterized for orientation of the eye during embedding. Eyes were preserved in 4% paraformaldehyde overnight at 4°C then cryo-protected in 30% sucrose solution overnight at 4°C. Eyes were embedded in Tissue Freezing Medium (Triangle Biomedical Sciences, Inc., Durham, NC) and stored at -80°C. Ten micron thick cross sections of the eye were collected in round so that each slide contained representative sections through the entire eye. Sections were stained with hematoxylin and eosin and imaged on a Nikon Eclipse 80i microscope (Tokyo, Japan). The NIS Elements Version 3.0 (Nikon, Tokyo, Japan) program was used to measure the outer nuclear layer thickness every 0.5mm on both sides of the retina starting from the optic nerve head. N values refer to the number of eyes.

Immunohistochemistry

A Mouse on Mouse immunodetection kit (M.O.M Kit, Vector Laboratories, Burlingame, CA) was applied to cryo-sections. Sections were rinsed in PBS, followed by Avidin/Biotin blocking according to the manufacturer protocol (Vector Laboratories, Burlingame, CA). Sections were then blocked with M.O.M Mouse IgG Block containing 5% normal donkey serum at room temperature for 1 hour. Mouse monoclonal, anti-green fluorescent protein (1:100, Life Technologies, Carlsbad, CA) and Cy-3 conjugated streptavidin (1:150, Jackson ImmunoResearch Labs, Inc., West Grove, PA) antibodies were applied according to the manufacturer M.O.M. Kit staining procedure. Sections were mounted in Vectashild Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA) and were imaged using a Nikon Eclipse 80i microscope and NIS Elements Version 3.0 software (Tokyo, Japan).

RESULTS

The hematocrit was measured at PD 60 in mice of all groups. The hematocrit level in the Buffer control, IP only and Dox treated mice was approximately 46%, which is in the normal range for a mouse (Table 1; [7, 9-11]).

As Dox doses increased, the volume of Dox water consumed decreased, presumably because of the bitter taste of the water despite the addition of sucrose (Fig. 1). Mice that received 0.5mg/ml Dox water (n=15) consumed an average of 7.6 ± 0.3 (SEM) ml water per day. Mice that received 5mg/ml Dox water (n=16) consumed an average of 4.6 ± 0.2 (SEM) ml water per day. Although water consumption in the 5mg/ml Dox dose group was lower, they

consumed 6 times more Dox than did the mice that were treated with 0.5mg/ml Dox water. The 0.5mg/ml Dox group consumed approximately 4mg Dox per day, and the 5mg/ml Dox group consumed approximately 23mg Dox per day. Despite the increase in Dox consumption in the 5mg/ml group as compared to the 0.5mg/ml group, there was not a correlative rise in intraocular EPO-R76E levels. In fact, the intraocular EPOR76E levels were identical in both groups. Therefore, all Dox water treatment groups were combined into a single group (Dox treated) for the rest of the analyses. The Buffer control (n=8) and IP only (n=8) treatment groups were not provided Dox-containing water and therefore consumed 0mg Dox per day.

The amount of EPO-R76E in the eye was measured at PD 60 (Table 1). An average of 6.1 ± 0.1 (SEM) mU/ml EPO-R76E was detected in the eyes (n=3) of Buffer control mice. Eyes from the IP only group (n=8) contained 11.8 ± 2.0 (SEM) mU/ml intraocular EPO-R76E. There was no statistically significant difference in the amount of intraocular EPO-R76E levels between the IP only and Buffer control groups. In contrast, the eyes of mice from the Dox treated group contained an average of 35.9 ± 2.4 (SEM) mU/ml EPO-R76E. To determine the amount of EPO in the eye in terms of mg wet weight, the eyecup wet weights were measured to be an average of 7.15 ± 0.2 (SEM) mg, and a calculated vitreous volume of the mouse eye of 5.3 l [35] was used. Therefore, we detected approximately 4.5mU/g, 8.7mU/g, and 26.6mU/g EPO-R76E in the eyes of the Buffer control, IP only, and Dox treated groups, respectively. The amount of EPO in terms of mU EPO in the eye is also shown, again using a volume of 5.3 μ l.

To assure the subretinal injection was successful in the eyes collected for histology, we performed immunohistochemistry to detect the EGFP co-expressed from the bidirectional tet- inducible promoter in the rAAV. No signal was detectable by direct imaging of the EGFP fluorescence, likely due to the low levels of gene expression. Amplification of the signal by the use of immunohistochemistry allowed visualization of the EGFP (Fig. 2). As expected, EGFP immunolabeling was only found in the RPE due to the restriction of the rAAV serotype and the VMD2 promoter driving expression of the rtTA. It is important to note that the area of RPE that was transduced was very limited. (Fig. 2) shows positive and negative areas from the same eye. Only those eyes that contained EGFP fluorescence were used for measuring ONL thickness.

The thickness of the ONL was measured both *in vivo* and by standard histological analysis (Fig. 3). The OCT images show that the ONL is similar in thickness in the *VMD2.rtTA:rd5/rd5* Buffer Control and IP only mice (Fig. 3A, B). In contrast the retina is thicker in the Dox treated mice (Fig. 3C). These findings were confirmed by histological analysis (Fig. 3D-F). A normal retina is shown in Fig. 3D with a thick ONL in contrast to the significantly thinned ONL in the IP only group (Fig. 3E), which looked similar to the Buffer Control (data not shown). The ONL of the Dox treated *VMD2.rtTA:rd5/rd5* mice was much thicker than the Buffer or IP only controls, but not as thick as an ONL from a normal Balb/c mouse (Fig. 3F). As expected, the retinas still lacked outer segments. The thickness of the ONL was quantified at 0.5mm increments from the optic nerve head in nasal – temporal sections of PD 60 retinas from Normal mice (Fig. 3G) as well as Buffer Control, IP only, and Dox treated *VMD2.rtTA:rd5/rd5* mice (Fig. 3H). The ONL thickness in

VMD2.rtTA:rd5/rd5 control mice was comparable to the ONL thickness in *rd5/rd5* mice that were not crossed to the *VMD2.rtTA* line (Fig. 3; [29]). The average ONL thickness in the normal mice at 1.0mm from the optic nerve head was 60.8 ± 2.2 (SEM) μm (Fig. 3G). The ONL thickness was 16.6 ± 0.9 (SEM) μm in mice in the IP only group and 24.4 ± 1.2 (SEM) μm in the Dox treated mice (Fig. 3H). Please note the difference in the y-axis scale. With Dox treatment, the ONL thickness was still approximately 36 μm less than age-matched wildtype mice, indicating partial preservation. Measurements from both sides of the retina at 1mm from the ONH were combined for each treatment group and analyzed using one-way analysis of variance and Bonferroni multiple comparison test. ANOVA was significant ($p < 0.0001$), and Bonferroni post hoc test revealed that the Dox treated group showed significant protection of the photoreceptors compared to the Buffer control ($p < 0.05$) and IP only ($p < 0.01$) groups.

DISCUSSION

The ONL of *rd5/rd5* mice expressing rtTA specifically from the RPE thinned to the same level as *rd5/rd5* mice at PD60, showing that the *VMD2.rtTA* cassette had no effect on the retinal degeneration (positive or negative). Our use of the bidirectional tet-inducible promoter allowed us to confirm a successful subretinal injection of vector based on the presence of EGFP fluorescence in the RPE. While delivery of rAAV.CMV.eGFP yields levels of EGFP that are easily detected in retinal sections by direct fluorescence microscopy [7, 36], this was not the case in the current study using the inducible promoter system. Indirect immunohistochemistry was required in this study to detect EGFP, likely due to the low level of gene expression induced in the eye. The presence of EGFP specifically in the RPE demonstrates that the subretinal injections were successful, that all components of the inducible system were present and functional, and that gene expression was restricted to the RPE. The low level of EGFP expression correlated with the low level of EPO that we measured in the eye after systemic treatment with Dox. The lack of ability to induce higher levels of transgene expression despite a ten-fold increase in Dox dose is likely due to the limited ability of Dox to cross the blood retina barrier.

While others have reported increased transgene expression in the eye using the tet-inducible system and systemic treatment of increasing concentrations of Dox, we did not detect a dose-dependent response. We used previously published doses and tested a ten-fold range in Dox dose [26, 27]. Our results fit with other published studies that demonstrate that Dox does not cross the blood brain barrier efficiently. In 1976, Andersson *et al.* found that only fourteen percent of the concentration of Dox found in the serum was present in the cerebral spinal fluid [37]. Also, in 2001 Dejneka *et al.* observed dose-dependent expression of human growth hormone in the eye using the tet-inducible promoter system that appeared to saturate after administration of 2mg/ml Dox containing water regardless of the starting serum levels of human growth hormone [27].

The current study demonstrates quantification of therapeutic levels of continuously produced EPO in the eye, which has significant clinical relevance for two reasons. First, since most retinal degenerative diseases are long-term and slowly progressing, treatment also needs to be long-term. Repeat intraocular injections of EPO introduce a high risk of

injury, including cataracts and hemorrhage, and the increased likelihood of low patient compliance. Repeat systemic injections of high dose EPO causes a dangerous rise in hematocrit and increases the risk of other adverse events. Therefore, the most clinically applicable approach is likely continuous release/production of EPO in the eye via gene therapy, cell encapsulation, or nanotechnology. Second, we and others show that EPO, like other cytokines, has a bell-shaped dose curve in terms of neuroprotection [7, 8, 12, this study]. High levels of certain cytokines in the eye can be deleterious (i.e. CNTF; [38]), and at the very least, ineffective [7]. Therefore, identification of the therapeutic dose range of EPO is necessary for safe and effective intraocular delivery of EPO for the long-term treatment of blinding diseases.

It was recently shown using oligochitosan based nanoparticle delivery of EPO that systemic delivery of 50U/kg of EPO nanoparticles was as effective as injections of 5000U/kg rhEPO [39]. This agrees with our findings that continuous delivery of low doses of EPO is as effective as repeat bolus injections of high doses of EPO, with the added benefit that the low dose avoids negative side-effects associated with high dose EPO treatment [8, 39, this study]. Another group assessed the *in vivo* pharmacokinetic profile of EPO release from microspheres into the bloodstream after intramuscular injection [40]. They detected approximately 25mU/ml EPO in the serum of rats one day after injection of microspheres loaded with 30,000U EPO. This dropped to 13mU/ml at about 5 days post-injection, and was maintained at approximately 9mU/ml from 8-28 days. While this level of release was sufficient to induce a rise in red blood cell count, it may be below the level needed in the blood for neuroprotection based on our past studies [9-11].

A recent publication demonstrated protection of retinal ganglion cells using intraocular delivery of nanoparticles carrying EPO for up to 60 days post-injection [41]. Unfortunately, they did not perform quantification of the amount of EPO delivered to the eye by the nanoparticles. It would be interesting to know how much EPO they detected in the eye at various time-points after injection of the nanoparticles. Ranchon-Cole *et al.* showed that IP injection of 5000U/kg EPO in the rat resulted in approximately 15mU EPO/mg total protein in the eye at 8-16 hours after injection [42]. Considering the average protein content of a rat retina is 1mg, they detected approximately 15mU EPO in the eye and this level was neuroprotective. This is comparable to the levels we detected in the eye 14 hours after a single bolus injection of 10U EPO, i.e. 10.5mU EPO [8]. This decreased to 1.2mU EPO at 36 hours post-injection and protection was detected two weeks after injection [8]. In the current study we provided continuous delivery of 0.2mU EPO in the eye and detected photoreceptor preservation. In contrast, when we delivered 32mU EPO continuously, in the eye photoreceptor protection was not achieved [7]. In addition, a recent paper failed to demonstrate protection after a single injection of 2U EPO in the eye and analysis 9 days later [43]. They did not quantify levels of EPO in the eye at the end of the experiment, but it is likely that they were below therapeutic levels. In combination, these data indicate that continuous delivery of 32mU EPO in the eye is above the therapeutic dose range, 0.2-15mU EPO are within the therapeutic range, and 0.06mU EPO is below the therapeutic dose range (Fig. 4).

CONCLUSION

It is important to note that preservation was not complete. The ONL was still thinner than in the normal Balb/c control retina. This is likely due to the delay of onset of gene expression from the viral vector. Although the subretinal injection of vector was performed at PD 5, Dox treatment to induce gene expression was not initiated until weaning at PD 21. It then takes another week before significant levels of gene expression are detectable. By one month of age, the ONL of the *rds/rds* retina is known to have thinned by approximately 15 μm [33]. Therefore, cell death occurred prior to the onset of EPO production in the eye, thus limiting the amount of protection that could be achieved with this experimental paradigm. Future studies utilizing alternative delivery tools will likely provide earlier treatment and, therefore, improved preservation of the photoreceptors.

In future studies, we will test methods such as cell encapsulation of EPO or nanotechnology to continuously deliver EPO into the eye. These studies will further delineate the therapeutic dose curve for EPO in the eye and identify the optimal delivery system. Future studies will also include tests of visual function to assure that the surviving cells are active, and therefore, will include heterozygous *rds* mice or other models of retinal degenerative disease.

Acknowledgments

The authors thank Dr. Don Zack for the kind gift of the VMD2.rTA mice. This project was supported by an Unrestricted Grant from Research to Prevent Blindness (Sternberg), a Career Development Award from Research to Prevent Blindness (Rex), a DoD Vision Research Program grant W81XWH-10-1-0528 (Rex), and a NEI grant EY022349 (Rex). Department of Defense Non-endorsement Disclaimer: The views, opinions and/or findings contained in this research presentation are those of the authors and do not necessarily reflect the views of the Department of Defense and should not be construed as an official DoD/Army position, policy or decision unless so designated by other documentation. No official endorsement should be made.

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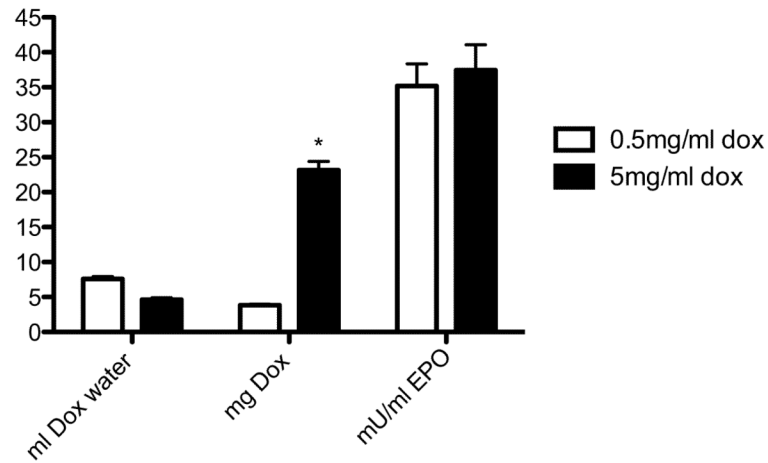


Fig. (1).

Bar graph comparing Dox water consumption, mg Dox consumed, and mU/ml EPO-R76E in the eye after either 0.5mg/ml or 5mg/ml Dox in the drinking water from PD21 to PD60. Although the mice received the 5mg/ml Dox water drank less, they consumed approximately 6 times more Dox. But, both groups produced the same amount of EPO in the eye, therefore, the two groups were combined into one treatment group for the rest of the study. All values represent average \pm SEM. Asterisk indicates statistical significance.

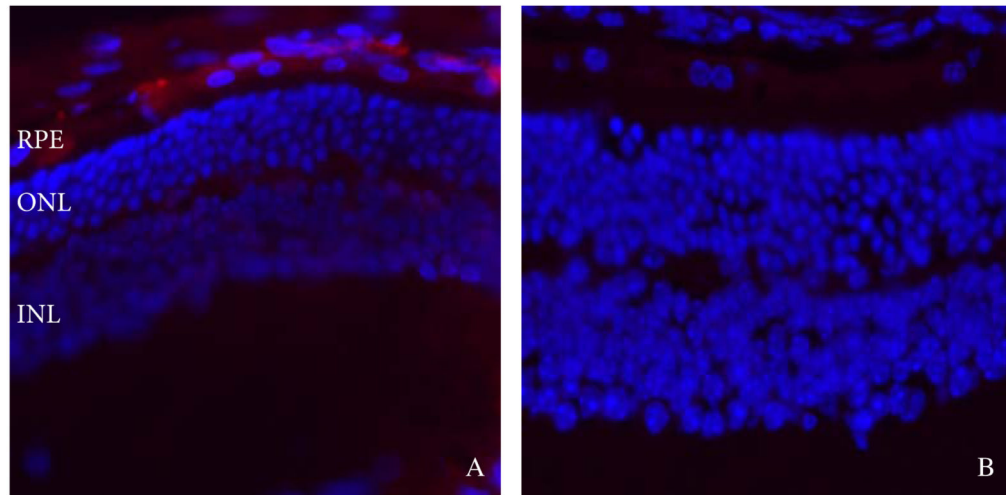
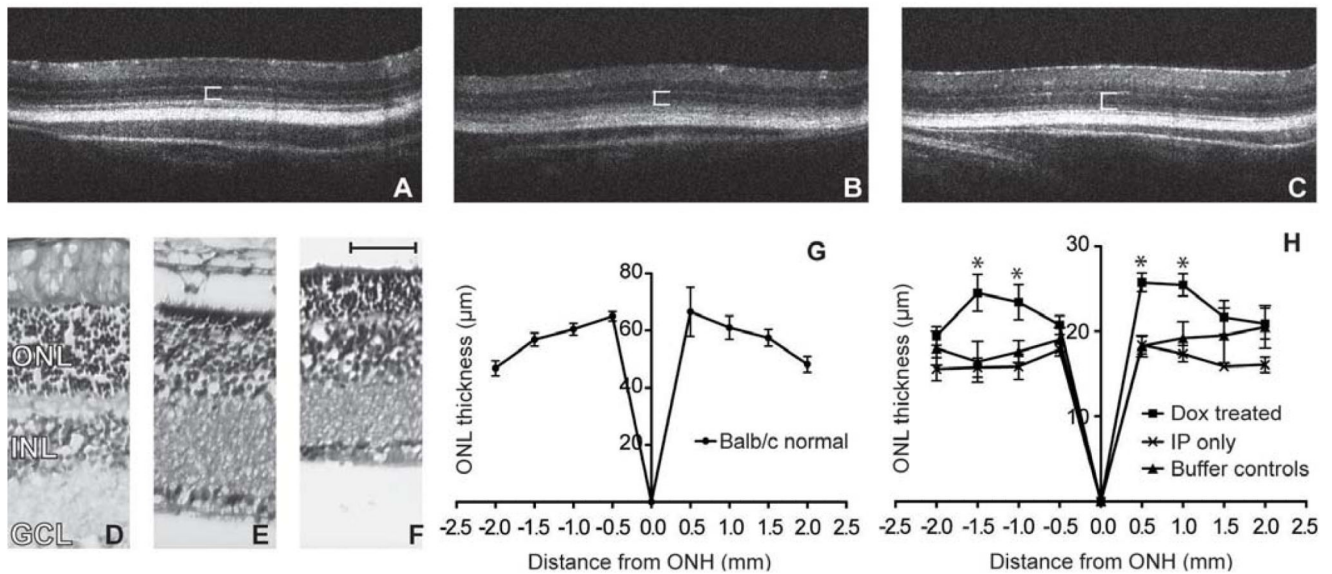


Fig. (2). Fluorescence micrographs of EGFP immunolabeling in the RPE following a subretinal injection of rAAV2/ 1.tet.eGFP.EPOR76E showing a positive area (A) and negative area (B) of the same retina.

**Fig. (3).**

OCT images, brightfield micrographs, and ONL thickness quantification showing partial preservation of photoreceptors in Dox treated mice. A-C. OCT images of retinas from mice that received a subretinal injection of vector: **A)** Buffer control; **B)** IP only; **C)** Dox treated. Brackets indicate the ONL. D-F. Light micrographs of hematoxylin and eosin stained sections of PD60 retinas: **D)** Normal, and *VMD2.rTA:rds/rds* mice treated **E)** IP only or **F)** with Dox water. ONL = outer nuclear layer; INL = inner nuclear layer; GCL = ganglion cell layer. Scale bar for D-F is shown in F. G, H. Graphs of average ONL thickness measurements at 500 micron increments from the optic nerve head, ONH of **G)** Normal mice or **H)** *VMD2.rTA:rds/rds* mice. All values represent average \pm SEM. Scale bar represents 50 microns. Asterisks indicate statistical significance.

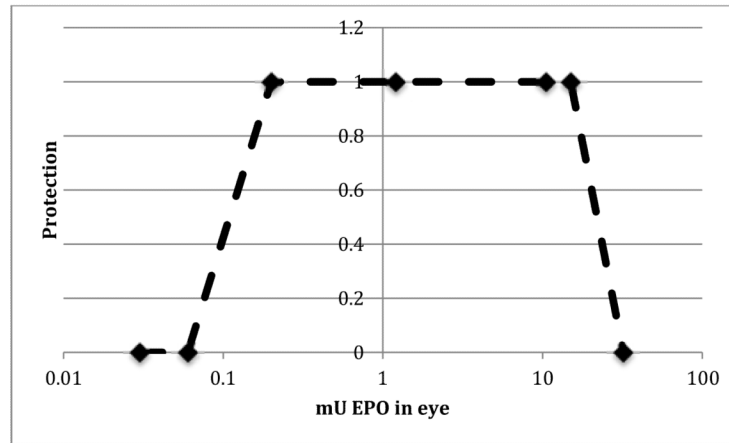


Fig. (4).

Graph of estimated therapeutic dose range by treatment with EPO. This is a compilation of data from this and other studies [7, 8, 42]. No protection was achieved with intraocular EPO levels of 0.03, 0.06, or 32mU (this study, [8]). Therefore, these values are outside the therapeutic range. Protection was reached by treatment of 0.2, 1.2, 10.5, or 15mU EPO in the eye (this study, [7, 42]). Therefore, the peak therapeutic dose is likely within the range and will be identified in future studies.

Table 1
Quantification of Intraocular EPO and Hematocrit. EPO Levels were Highest in the Dox Treated Group. There was no Rise in Hematocrit in Any Group

Treatment Group	mU/ml EPO	mU EPO/g Wet Weight	mU EPO	Hematocrit
Buffer controls	6.1±0.1	4.5	0.03	46.5±0.8
IP only	11.8±2.0	8.7	0.06	NA
Dox treated	35.9±2.4	26.6	0.19	46.0±2.0

* All values represent average ± SEM

Conversion to mU was performed using 5.3 µl as the volume of the mouse vitreous.