Investigating the Role of The Antioxidant N-acetylcarnosine in Attenuating Oxidation-induced Retinal Damage in Retinitis Pigmentosa

Investigating the Role of The Antioxidant N-acetylcarnosine in Attenuating Oxidation-induced Retinal Damage in Retinitis Pigmentosa

Neelaab Nasraty

A Thesis in the Field of Biology

for the Degree of Master of Liberal Arts in Extension Studies

Harvard University

November 2021

Abstract

The goal of this research was to explore the strong antioxidant, n-acetylcarnosine, in its capacity to ameliorate oxidative stress, specifically in the context of slowing disease progression of retinal degeneration. Retinal degeneration affects one out of 1000 individuals globally, with 95% of these retinal diseases sparked by a genetic origin. Since the large majority of genetic-based retinal diseases lead to dysfunction and aberration of rod and cone photoreceptor cells, with a byproduct of this disease progression resulting in oxidative stress that further facilitates disease progression, a novel broad-spectrum therapy that targets this common element, rather than specific genes, is necessary (Haider, 2020).

This study identified both topical as well as injection-based modalities as potential routes of photoreceptor recovery in the rd1 disease model, with a near-doubling of the surmised inner nuclear layer during the injection-based study, as evidenced by histology images. While additional studies are needed to better identify the nature of the cells rescued, these initial studies paint n-acetylcarnosine as a promising prodrug for photoreceptor rescue and as a novel retinal disease therapeutic.

Acknowledgments

This work would not have been possible if not for the incredible support I received from others. First, thank you to Dr. Neena B. Haider, my thesis director, for your mentorship and generously granting me the opportunity to join your research work and explore disease development and treatment in such a multifaceted fashion. I will carry the lessons I have learned from this experience into every other facet of my future research efforts.

I also owe an immense debt of gratitude to my fellow lab members at the Haider Lab, whose collaborative approach, incredible work ethic, and interdisciplinary expertise contributed heavily to this project and to my own efforts. To Zoe Love, Emily Brabbit, Danielle Granata, Saba Khalilpour, Shyamtanu Datta, and Andrew Luo: I can truly say I have been fortunate to meet and work alongside you, and am endlessly appreciative for your assistance, support, and advice over this past year-and-a-half. I dedicate this thesis to you all.

Thank you to Dr. James Morris, my thesis advisor at Harvard, who has been a wonderful advocate from the earliest planning stages of my thesis work.

Thank you to my professors at Harvard who provided the solid foundation in biochemical principles and scientific writing on which I have built all subsequent research and investigative efforts. A special thanks especially to Drs. Margaret Lynch, Alain Viel, and Stephanie Maddox, who not only greatly enhanced the quality of my

education and experience at Harvard, but also provided support and mentorship during my concurrent application cycle to medical school.

And, of course, a final thank you to my mother, Karima, and father, Qudrat, who know the true scope of effort this journey has required, and have provided endlessly patient encouragement through it all. دوستت دارم.

Table of Contents

List of Tables

List of Figures

Chapter I

Introduction

The Research Problem

Retinitis pigmentosa (RP) is the name of a group of inherited retinal dystrophies, the most common form of which is a rod-cone dystrophy, wherein a genetic mutation causes progressive loss of rod photoreceptors and subsequent degradation of cone photoreceptors, eventually leading to blindness. The current working theory behind the long-term progression of the disease is based on oxidative stress; once the genetic mutation causes death of the rod photoreceptors and loss of peripheral vision, a resulting build-up of oxygen levels causes oxidative damage which subsequently causes degradation of the cone photoreceptors and leads to full blindness after several decades. Currently, there is no therapy that is able to cure the disease or restore vision, so therapeutic approaches are limited to attempting to slow down the degeneration (Hamel, 2006). Recent studies have shown promise in vitamin therapies, specifically focusing on antioxidants, with heavy focus on the use of N-acetylcysteine.

N-acetylcysteine's use is predicated on the role of oxidative stress in retinitis pigmentosa disease development. The increased oxidant production caused by the sudden reduction of oxygen-consuming rod photoreceptors leads to a release of free radicals and subsequent cellular degeneration, which is a common feature of many ocular diseases such as cataract development, glaucoma, and age-related macular degeneration

(Babizhayev et al., 2011). While N-acetylcysteine has been shown to be a strong antioxidant in both oral and topical use, it has not shown the ability to halt degradation of cone cell death to a point where the body's natural repair mechanisms are able to prevent further degeneration. Furthermore, N-acetylcysteine is a precursor to glutathione, a master antioxidant in the body, which has been shown to be highly prone to oxidation in the RP disease state (Lee et al., 2011).

These concerns have sparked the search for an alternative antioxidant therapy that can bypass these issues. Carnosine is a naturally-occurring dipeptide of the amino acids alanine and histidine which has shown similar antioxidant capabilities as N-acetylcysteine in studies examining both of their protective effects against oxidation in a mouse model. Therefore, we propose to introduce carnosine as a novel antioxidant treatment in a mouse disease model of retinal degeneration, rd1, to examine its ability to protect against further cone degradation and oxidative stress. However, it is important to note that when administered topically, carnosine is not able to penetrate the ocular surface. Therefore, we utilized the acetylated version, N-acetylcarnosine (NAC). NAC has been shown to penetrate the cornea, and undergo metabolic conversion into L-carnosine, its active form (Dubois & Bastawrous, 2017).

This research is important due to the lack of current treatment options for RP, and due to the exciting prospect of presenting a novel treatment that could help protect against future cone cell degradation, thus preventing against exacerbation of blindness in patients.

Definition of Terms

Cone cell: Photoreceptor cells located in the retina that are primarily responsible for color vision

Histology: provides imaging of microscopic structure of tissues

Immunohistochemistry: immune staining process, in this case used to examine number of oxidative stress markers

Oxidative stress: when production of reactive oxygen species (ROS) is greater than natural endogenous elimination of those same species

Photoreceptor cell: a specialized neuroepithelial cell found in the retina that converts light into neural signals

Post-natal: the stage of development after mouse pups are born, beginning at day zero

rd1: first mutant mouse model for retinal degeneration, which carries a rod-specific cGMP phosphodiesterase-β-subunit (PDEβ) mutation, and exhibits very severe and early degradation of cone cells

Retina: layer of tissue lining the back of the eye which converts light into neural signals

Retinal degeneration: retinopathy with multiple causes leading to deterioration of retina and death of its associated cells

Retinitis pigmentosa: a group of hereditary retinal dystrophies defined by the primary degeneration of rod photoreceptors, leading to secondary degeneration of cone photoreceptors

RNA isolation: extraction of RNA from genes and tissues for use in assessing gene expression levels

Rod cells: Photoreceptor cells located in the peripheral part of the retina and responsible for night vision

Subretinal injection: injecting between the photoreceptor cells of the retina, and the retinal epithelium, with more direct effects on the cells in the subretinal space as compared to intravitreal injection

Background of the Problem

Retinitis Pigmentosa

Retinitis pigmentosa (RP) is a term used to group a variety of hereditary retinal dystrophies defined by the primary degeneration of rod photoreceptors, leading to a subsequent, secondary degeneration of cone photoreceptors (Hamel, 2006). RP has a worldwide prevalence of close to one in 4000, and is a leading cause of visual disability, with at least one million cases worldwide. Its inheritance patterns have been identified as mostly autosomal-recessive (comprised of around 50-60% percent of cases), with autosomal-dominant (30-40% of cases) and X-linked inheritance (5-15%) also identified as the remaining modes of inheritance. Due to the variety of heritable routes of RP, identifying specific mutations causing the disorder has proven difficult; however, researchers have thus far detected at least 45 loci where these disease-causing mutations occur. Current working knowledge traces at least half of all patient cases back to these specific loci (Narayan et al., 2016).

Despite the varying forms of RP, all forms of the disease have mutations that are expressed in the rod photoreceptors, the gradual degeneration of which typically first manifests as night blindness in adolescence, followed by progressive loss of the concentric visual field and peripheral vision (Verbakel et al., 2018). Later progression of the disease causes dysfunction in and loss of the cone photoreceptors, leading to reduced quality of high acuity central vision, and often leading to loss of this central vision and often complete blindness by midlife (Narayan et al., 2016). Imaging conducted at this

stage of the disease, such as optical coherence tomography (OCT) and fundus autofluorescence (FAF) depict a progressive loss of the outer retinal layers, demonstrating the severe structural damage incurred (Verbakel et al., 2018).

The prevailing theory explaining the connection between rod and cone photoreceptor degradation in RP revolves around oxidative stress. Rod photoreceptors are the major consumers of oxygen in the retina; thus, in RP, the mutations leading to the death of rod photoreceptors also cause a subsequent increase in tissue oxygen level in the outer retina (Lee et al., 2011). This resulting surplus of oxygen activates the enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which produces superoxide radicals and nitric oxide (NO) and generates a highly-destructive free radical, peroxynitrite, which attacks surrounding tissue and causes severe oxidative damage (Komeima et al., 2008). During progression of RP, the natural reparative mechanisms and antioxidant defense systems in cone photoreceptors become overwhelmed by this accrual of oxidative damage, and apoptosis is activated, leading to death of the cone photoreceptors in the central island and subsequent blindness. Therefore, the goal of therapeutic treatment would be to lower the rate of oxidative damage below the rate of natural cellular repair; this would allow time for endogenous repair pathways to utilize antioxidants such as superoxide dismutases (SODs) to slow the rate of degradation of cones (Lee et al., 2011; Narayan et al., 2016).

Oxidation & N-acetylcysteine

Since rod photoreceptor death is caused by a variety of genetic mutations, researchers have explored the possibility of genetic modulation or targeted gene therapies; this has proved to be an elusive effort, however, due to the multitude of mutations in various genes leading to the death of rods in the RP disease state. Recent research efforts have centered on the oxidative damage theory of RP, with major focus placed on identifying treatments to instead preserve cone function, since cone photoreceptor degradation is the factor which leads to complete blindness (Lee et al., 2011). The oxidative stress theory posits that progressive death of cones in RP is caused by accumulated oxidative damage, which has led researchers to investigate exogenous antioxidant therapies as possible therapeutic treatments to stem the onslaught of oxidative damage in the hopes of allowing the body's natural defenses to preserve existing cone function, with the knowledge that any external therapy of this nature would need to be administered as a lifelong treatment. In the past decade, researchers have explored the use of one particular antioxidant, N-acetylcysteine, which is the precursor to cysteine, which is itself the precursor to glutathione, a powerful antioxidant and major component of the endogenous antioxidant defense system. N-acetylcysteine's efficacy and role as a precursor to glutathione has made it useful as a life-saving antioxidant treatment, known for its ability to prevent severe oxidative damage during acetaminophen overdose (Campochiaro et al., 2020).

The use of N-acetylcysteine in retinitis pigmentosa was broached when researchers found that intraocular levels of the reduced, or active, form of glutathione were decreased in the RP disease state, with the oxidized, or non-active, form, glutathione disulphide,

elevated in human patients (Narayan et al., 2016). Based on these findings, it was theorized that use of N-acetylcysteine could replenish the active, reduced form of glutathione and reduce oxidative damage and cone cell death while preserving cone function. A study was conducted to explore both oral and topical administration of N-acetylcysteine in two mouse models of retinal degeneration, *rd1* and *rd10* (more severe and less severe disease states, respectively). It was found that both orally- and topically-administered N-acetylcysteine significantly reduced oxidative damage by increasing the amount of reduced glutathione available, thus preserving cone function in both rodent models (Lee et al., 2011). However, these results come with two main caveats: First, the study was conducted over the period of six months, and found that the cone rescue effect was incomplete due to the continued cell death and exacerbation of disease. This points to the fact that the strategy of increasing endogenous glutathione was not capable of limiting the rate of oxidation in the RP disease state below the natural cellular repair state in either rodent model (Lee et al., 2011). The second issue is that reduced glutathione is highly prone to oxidation in the RP disease state, and its long-term administration may simply exacerbate RP by increasing the presence of oxidized glutathione. However, the study does illustrate the capability of a strong antioxidant in slowing oxidative damage; finding a therapeutic option that is able to further slow the rate of accrual of oxidative damage may allow natural endogenous repair functions to then prevent further cone cell death (Campochiaro et al., 2020).

N-acetylcarnosine

The promising results shown by usage of N-acetylcysteine in RP models has initiated the search for other antioxidants that could prove to be not only possibly more effective, but also bypass the concerns broached above. A study was conducted to compare the efficacy of orally-administered N-acetylcysteine and another antioxidant, carnosine, against sodium nitrite-induced intestinal toxicity in a rodent model. Results of the study showed both antioxidants to have similar protective effects against oxidative damage and nitrite-induced metabolic alterations (Ansari et al., 2018). However, it is important to note that the non-acetylated version of carnosine was used in the study, which may have lessened the bioavailability of the carnosine and, thus, its efficacy.

Carnosine (β-alanyl-l-histidine) is a dipeptide naturally found in mammalian muscle and lens tissue and involved in metabolic processes throughout the body. Its parent compound, N-acetylcarnosine (NAC), is used as an ophthalmic prodrug to facilitate delivery into tissues, since carnosine cannot penetrate the cornea. This particular modality allows for the most effective uptake of carnosine in the aqueous humor, which helps to protect carnosine from hydrolysis (Babizhayev et al., 2008).

Carnosine has been shown to delay aging and subsequently rejuvenate senescent cultured human fibroblasts; its ability to provide protective and anti-glycosylating activity against aging is theorized to be due to ‘carnosinylation’, that is, carnosine’s ability to inhibit the cross-linking of glycoxidized proteins to healthy, normal macromolecules and tissues (Hipkiss et al., 2001). Furthermore, a recent survey was done to assess whole blood amino acids in patients with various subtypes of RP, showing that patients with the

disease showed reduced levels of whole blood histidine, one of the amino acids in carnosine (Arshinoff et al., 1981), which has anti-inflammatory activity and the capability to scavenge toxic oxidative species (Wade & Tucker, 1998).

Carnosine has been examined in the context of ocular disease, particular in the case of cataract development; past studies have shown levels of carnosine naturally found in human lenses to be depleted in mature cataracts. Utilization of 1% NAC drops on cataract development has shown significant improvement, with reduction of opacity and increased transmission of light via the lens (Babizhayev et al., 2009). Since oxidative stress and subsequent oxidative damage to lens proteins is a key causative facet in cataract formation, the ability of carnosine to facilitate a measure of improvement in the disease pathology shows promise for its ability to be utilized as an antioxidant treatment in other ocular oxidative diseases like RP (Braakhuis et al., 2019).

rd1 Mouse Model

While there are a variety of animal models available for studying RP, one of the most widely utilized animal models of retinal degeneration is the FVB-*Pde6ß rd1*/NJ (*rd1*) mouse model. The *rd1* mouse carries a nonsense mutation in the *Pde6b* gene, mapped on chromosome 5. This mutant *Pde6b* gene has a murine leukemia provirus insertion in intron 1, alongside a point mutation, which correlate to the creation of a stop codon (Y347STOP) in exon 7. A second, independent mutation has also been identified in this gene, where a murine leukemia virus is found in intron 1 (Li et al., 2020). Aberration of this gene in this fashion results in rapid rod photoreceptor degradation, with early and intermediate degeneration of the outer nuclear layer (ONL) taking place as early as post-natal day 21 (Li et al., 2020) and leaving only one layer of cone photoreceptors as early as 4 weeks of age, making the *rd1* mouse one of the most severe models of retinal degeneration currently identified (Nishiguchi et al., 2015).

The *rd1* model's mutation of cGMP phosphodiesterase-β-subunit leads to initial rod destruction, and a subsequent cascade of cone cell destruction, mirroring the same pattern of retinal disease development in humans; this makes it a valuable model in which to study disease (Punzo & Cepko, 2007). The mutations in the human *Pde6b* gene are associated with human clinical presentation of RP and autosomal dominant congenital stationary night blindness (Li et al., 2020).

Research Aims, Goals, and Hypothesis

The primary research purpose of this study was to determine whether we are able to slow the progression of retinal degeneration, in the context of oxidative stress causing cone photoreceptor degradation, with the treatment of the antioxidant N-acetylcarnosine (NAC) in the *rd1* mouse model. Based on previous literature, it was hypothesized that the targeted delivery of n-acetylcarnosine to the retina would result in its natural conversion to carnosine, whose antioxidant properties could potentially slow the rate of rod-cell-death-mediated oxidative damage and possibly allow natural repair functions to range from preventing further photoreceptor damage to even facilitating increased cellular repair and rescue. To assess this hypothesis, two specific aims were explored in order to pursue this primary objective.

The first specific aim focuses on the capability of carnosine in ameliorating oxidation. The second aim focuses on exploring a secondary question, specifically whether n-acetylcarnosine administered topically can reach the retina. Previous literature has explored n-acetylcarnosine's ability to penetrate the cornea of the rabbit eye, and subsequently penetrate into the aqueous humor (Babizhayev et al., 1996). These studies posited the need for follow-up studies to explore the possibility of carnosine subsequently penetrating the lens and entering the vitreous humor, to eventually travel to the retina (Figure 4 (Aslan et al., 2013) illustrates this potential ocular pathway). Recent literature elucidated specific routes of travel toward the retina for general topical agents after penetrating the cornea, including horizontal diffusion and systemic circulation, illustrated in Figure 5 (Tsai et al., 2018). Thus, our study has included our secondary aim to bridge

these two research elements to serve as an initial examination into the possibility of viewing photoreceptor rescue via usage of carnosine in a mouse model of retinal degeneration.

Details of the three-part study design are found in Table 1.

Primary objective: Show efficacy of carnosine as an antioxidant and potential broad-spectrum therapy for retinal degeneration.

Specific Aim 1: Assess whether n-acetylcarnosine delivered directly to the retina (via subretinal injection) ameliorates oxidation.

Methods: Perform subretinal injection of 1% NAC, utilize histological analysis to visualize photoreceptor cell layers in the retina, and visually examine thickness of cell layers.

Expected results: There will be a potential reduction in oxidation and/or rescue represented by thickened cell layers.

Specific Aim 2: Assess whether n-acetylcarnosine administered topically can reach the retina in a mouse model, and subsequently ameliorate oxidation.

Methods: Administer 1% NAC formulation via drops to mouse eyes, utilize histological analysis to visualize photoreceptor cell layers in the retina, and visually examine thickness of cell layers.

Expected results: There will be rescue represented by thickened cell layers, suggesting both effective delivery of carnosine to the retina as well as reduction in oxidation.

Chapter II

Materials and Methods

Animals

A total of 4 C57BL6/J (B6) mice (gender unknown), and 19 (11 females and 8 males) FVB-*Pde6ϐ* rd1/NJ (rd1) mice were used during the course of this study. B6 is a control strain, as well as the genetic background control for the mutant strain, rd1, used in this study. The rd1 strain is homozygous for the *Pde6ϐ* gene, and was selected due to rd1 mice exhibiting severe and early-onset retinal degeneration (Haider, 2020). The B6 mice used in the study were euthanized with their tissue collected for histology at post-natal day 30. Previous studies have outlined the aging alignment of a control B6 mouse at a young (post-natal day 0 until 3 months) age (as corresponds in this study) to a human individual from birth to 20 years of age (Iannaccone et al., 2021).

Animal Maintenance

All mice used during the course of this study were bred and housed in the vivarium at the Schepens Eye Research Institute, under standard conditions. C57BL6/J (Jax stock #000664) and FVB-*Pde6ß* rd1/NJ (rd1; Jax stock #001800) mice were obtained from Jackson Laboratories, Bar Harbor, ME (Li et al., 2020). Prior to the study, all mice-users and technicians underwent full animal training through the Animal Facility office to learn all associated animal husbandry procedures.

So as to minimize animal usage, contralateral eyes were utilized for the subretinal injections or mock injections/controls, rather than additional injection controls.

All mice were ear-punched as means of identification. After separation by sex and coat color (if applicable), mice with identical coat color were punched as follows: 1 - no ear punch; 2 - one right ear punch; 3 - one left ear punch; 4 - one R and one L ear punch; 5 - 2 right ear punches; 6 - 2 left ear punches, 7 - 2 right ear punches and 1 left punch; 8 - 1 right ear punch and 2 left ear punches; and 9 - 2 right ear punches and 2 left ear punches. For the purposes of our study, no mice were administered ear punches past "2 right ear punches and 1 left punch" as there were a maximum of seven mice used per individual trial. Each mouse was assigned a specific identification number according to its ear punch, specific strain, and cage number. Animals were monitored throughout the study to confirm that the administered ear punches did not cause major structural damage to the mouse ear (Haider, 2020).

Animals with premature unintended death, as well as samples (specifically, paraffin blocks used for histology) which were manually damaged during the course of study analyses, were excluded from the study. One (female) mouse which died during the initial round of topical administration was excluded based on these criteria. Additionally, one paraffin block was destroyed or damaged during the histology analyses of both the second trial of topical drops as well as the injections.

NAC 1% Solution Preparation

Although 1% NAC eye drops are commercially available, for the purposes of the study NAC powder was reconstituted into the highest non-pharmaceutical grade of Hank's Balanced Salt Solution (HBSS) to create a 1% NAC solution for both topical drops and injections. This was done in order to minimize variability in quality and concentration in available N-acetylcarnosine drops, owing to usage of extraneous constituents and additives. The NAC solution was freshly prepared by weighing 0.01 g of pure NAC powder (which is stable for 2 years with proper storage, and has no expiration date listed on the bottle) and was subsequently added to 1 mL of HBSS (pH 6-7). This solution was filtered via sterile syringe filtration, to create individual dosages (Haider, 2020).

NAC 1% Topical Dosage Administration

Two drops of 1% NAC eye drops were administered twice a day (6 hours apart, typically at 10 AM and 4 PM) continuously for two weeks after mice reached age P15 (post-natal day 15), which is when the mouse eye typically opens after birth. This total dosage amounted to 0.1 ml, with one drop per eye per time point, and a total of two drops per eye per day.

Two separate trials were performed: an initial pilot study, and a follow-up study. During the pilot study, 1% NAC eye drops were administered twice a day to a group of 7 mice over a two-week period starting from post-natal day 15, after the eye opens.

During the study, one female mouse died during the 15-day administration period, prior to completion of the study, and thus was removed from subsequent analyses.

A second trial was performed, under the same parameters, and again to a group of 7 mice over a two-week period. All mice survived the second trial, though a paraffin block was destroyed prior to H&E analyses, and its associated sample number was removed from subsequent analyses. In total, 14 mice were involved in the topical trials, with 12 mice analysed and imaged.

Animals were manually restrained during the administration of the drops, and all topical administration was conducted in the Animal Facility Procedure Room 085C.

NAC 1% Injection Administration

Injections were performed on neonatal mice, at P0 (day of birth). Prior to performing subretinal injections, neonatal mice were anesthetized by being placed on ice for 2-3 minutes. The eye area was cleaned, with debris removed by using a sterile cotton swab dipped in autoclaved sterile water. The neonate mice's skin was protected by folded paper towels prior to placing them on a bed of crushed ice. The eyelid of the neonate was gently cut open with the bevel of a 30G-needle along their natural lid line. After creating a hole in the sclera, adjacent to the cornea, with a 30G needle, 0.5 ul of the NAC 1% solution was administered into the subretinal space of the eye with a blunt Hamilton syringe. Experiencing a slight resistance to the needle indicated the Bruch's membrane had been reached (Li et al., 2020). Between injections, the syringe tip was

cleaned with 70% ethanol. Post-injection, mice were placed onto a heating pad/warming blanket for recovery (approximately 20 minutes, with a maximum of 40 minutes), and then subsequently placed in a new cage alongside their mother and littermates.

Throughout the procedure and recovery time, mice were monitored every 15 minutes to ensure continuous breathing and a comfortable environment, with assessments examining their position and demeanor. All findings were documented in the Cage Clinical Record area, located on the back of their respective cage card (Haider, 2020). All injections were administered in the Animal Facility Procedure Room 085E.

A total of 5 mice were utilized in the injection trial, with the left eye (OS) uninjected and serving as a contralateral control, and the right eye (OD) injected with 0.5 ul of 1% NAC. All mice were euthanized at P23.

Histology

Immediately following euthanasia of mice, left eyes (OS) were collected and immersed in a 3:1 solution of methanol and acetic acid (for hematoxylin/eosin staining), and right eyes (OD) in freshly-prepared 4% paraformaldehyde (PFA) in 1X phosphate buffered solution (PBS) (for future immunohistochemical staining), and placed in a 4-degree refrigerator overnight. The next day, the collected eyes were embedded in paraffin blocks with dorsal/ventral orientation. These blocks were subsequently cut down to size in a pyramid-shape for ease of further cutting, and were then cut on a standard microtome, with 5 micrometer (μm) sections collected over 100 μm of retinal depth onto glass slides, with 2-3 slides collected per animal per procedure.

The slides were subsequently processed for hematoxylin/eosin (H&E) staining. Through this staining procedure, the retina sections were deparaffinated in xylene and washed in ethanol, and then stained with hematoxylin and eosin Y. The slides were then mounted with Permount mounting medium (Vector Laboratories) (Li et al., 2020).

Sections were then imaged with the Leica DMI6000 microscope, with images taken at 5x, 10x, and 20x magnification. The 20x images were then analyzed and compared to the B6 control images (also imaged at 20x magnification), with 100μm retinal area examined per image for potential photoreceptor rescue. Due to the nature of disease progression and presentation, most notably the oxidation-based destruction of the outer nuclear layer (ONL), and subsequent progressed-disease state collapse of the outer plexiform layer (OPL) it is difficult to discern a clear or defined boundary between inner nuclear layer (INL), ONL, and OPL (Iannaccone et al., 2021), whose structures are shown in Figure 3. Therefore, all histology images have the examined area, designated by black arrows, labeled as INL (Figures 6, 7, and 8).

Use of Human Subjects

No human subjects or trials on human subjects were utilized or conducted during the course of this study.

Chapter III

Results

Topical Administration (Pilot Study)

Two representative histological images of 100 um central retina sections from experimental identification numbers, 379 and 380, were chosen to illustrate photoreceptor layer thickness after the pilot topical administration trial (Figure 6). These images were selected from the larger pool of histological images of central retina sections collected from the six subjects in this study (2-3 images per subject). Overall cell layer thickness was comparable to the rd1 uninjected control, suggesting lack of rescue. However, while the disease control (B) demonstrated a significant space above the retinal pigment epithelium (RPE), correlated with the exacerbated disease phenotype, the experimental images show a marked increase in photoreceptor cells just above the RPE.

Topical Administration (Second Trial)

Two representative histological images of 100 um central retina sections from experimental identification numbers, 434 and 432, were chosen to illustrate photoreceptor layer thickness after the second topical administration trial (Figure 7). These images were selected from the larger pool of histological images of central retina

sections collected from the six subjects in this study (2-3 images per subject). The results of the second topical trial are noticeably different from the images of the pilot study (Figure 6). The cell layer is noticeably thicker, and comparable to cell thickness demonstrated in the B6 control (A).

Subretinal Injections

Two representative histological images of 100 um central retina sections from experimental identification numbers, 428 and 430, were chosen to illustrate photoreceptor layer thickness after the subretinal injection trial (Figure 8). These images were selected from the larger pool of histological images of central retina sections collected from the five subjects in this study (2-3 images per subject). A marked near-doubling of the cell layer is observed at the injection site in both images C and D.

Chapter IV

Discussion

Significance of Results

Gauging potential rescue (and potentially correlated reduction in oxidation levels and association photoreceptor degradation) was done through visual assessment of cell layer thickness over 100 um retinal sections for each trial through representative images.

The non-disease (indicated here by the B6 control) mouse retina is structurally comprised of 10-12 layers of rod and cone photoreceptor nuclei in the outer nuclear layer and 5-6 inner retinal cells in the inner nuclear layer (Li et al., 2021). In each of the three trials, the treated rd1 disease model had an attenuated ONL layer, in accordance with the expected rd1 mouse model, wherein the INL does not change in nuclei number, while the ONL typically presents with significantly reduced cell layers. However, each of the trials presented with a visually different presentation of photoreceptor cells, whether in location or cell layer thickness.

Because retinal degeneration and the procession of photoreceptor degeneration often leads to morphological changes in retinal structure, it is difficult to discern specific boundaries between the outer and inner nuclear layers (Li et al., 2021). Thus, the difference in localization of photoreceptor cells demonstrated in the topical trials (Figures 6 and 7) is difficult to extrapolate upon without further (immunohistochemical) analyses to identify cell types. Specifically, late-stage retinitis pigmentosa not only

affects the photoreceptor cells of the ONL, but also multiple individual cell classes and their structural and functional connections to one another. This is because in retinitis pigmentosa (as well as other diseases where photoreceptor degradation is present), photoreceptor loss triggers subsequent retinal remodeling, where neural retina is reconfigured and affects changes to the overall topology of the retina, further complicating the picture of what we see when we see increased cell numbers, regardless of the region in which they appear (Jones et al., 2016; Li et al., 2021).

Regardless, the significant increase (near-doubling) of the cell layer in the injection trial holds promise for carnosine as a potential therapy for retinitis pigmentosa. Previous studies have indicated that even partial rescue (i.e. even retaining a single layer of photoreceptor cells) can facilitate maintenance of at least low-level visual function (Li et al., 2021). Thus, injection-based carnosine administration in our recent trial demonstrates strong possibility to ameliorate oxidation-based retinal cell degeneration.

Research Limitations

There are a number of issues which may have presented during the course of this experiment. The first experiment relies on delivery of the NAC topical drops to the retina; while NAC drops have been shown in other studies to penetrate the cornea, there is no guarantee they will reach the retina at the back of the eye.

Another issue is that the source of oxidation involved in the RP disease state may be mitochondrially-based; prior studies have shown that mitochondria-targeted antioxidants may be more effective in preventing oxidation of lipids and proteins in the inner mitochondrial membrane in age-related ocular pathologies and retinopathies (Babizhayev et al, 2016).

Furthermore, there is a marked difference in results and location of photoreceptor cells between the first and second topically-treated trials. This may be due to improvement in administration technique achieved in the time between the first and second trials.

Future Research Directions

The difference in results between the two topical trials supports the need to repeat the experiment through subsequent trials, with the same dosage (1%) as well as increased dosages.

Two major areas of interest result from examination of the histological images: 1) whether topically-administered carnosine does indeed reach the retina through the ocular pathway (with specific focus on whether it is metabolized and broken down to its constituents in the vitreous humor); and 2) identifying the specific cell types making up the composition of the increased cell layers, specifically seen in the injection trial. A useful follow-up experiment would be to perform immunohistochemistry with a carnosine antibody, as well as cell-specific antibodies to identify the nature of the cells which have been rescued.

Conclusion

I hypothesized that the examination of the strong antioxidant carnosine, administered through its precursor n-acetylcarnosine both topically and via injection, could serve as a tandem force: a potential broad-spectrum therapy for genetically-based retinal degeneration, as well as a new entry to contribute to the dearth of studies examining carnosine as a method of ameliorating oxidative stress and its potential as an antioxidant in disease therapy.

The topical administration of NAC suggests at least an interesting pilot study on which to build future studies examining whether carnosine affects retinal remodeling.

The injection modality of NAC provides evidence for carnosine as a promoter of photoreceptor rescue, and warrants further repeated trials and studies.

Appendix

Trial Type + Dosage	Number of Mice	Gender	Age at Administration	Age at Sacrifice	Additional Study	Fixative + Eye	Additional Notes
B6 (Control)	4	unknown	N/A	P30	H&E	2 PFA, 2 Met	N/A
1% Drops (Pilot Study)	6 (started with 7)	2F, 5 M	P15-P30	P30	H&E	Met (OS)	377 (F) died during study; no collection or imaging
1% Drops (2nd trial)	6 (started with 7)	6F, 1M	P15-P30	P30	H&E	Met (OS)	431 (F) block was destroyed
Uninjected controls	4 (started with 5)	3F, 2M	N/A	P23	H&E	Met (OS)	430 (M) block missing
Injected 1% NAC	5	3F, 2M	P0	P23	H&E	PFA (OD)	N/A

Table 1. Study Design and Summary of Trials.

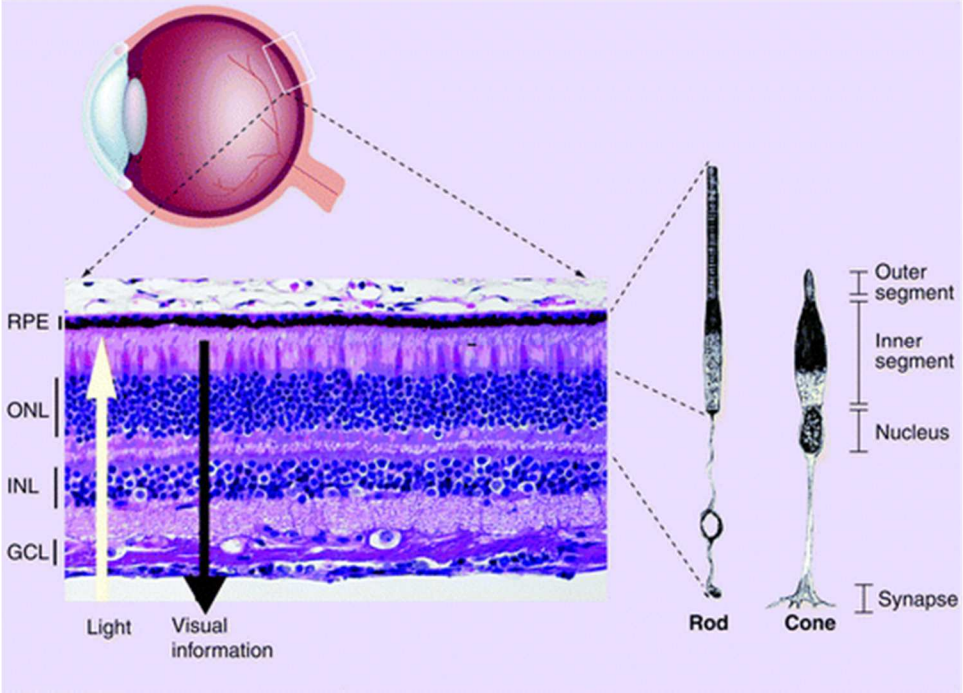


Figure 1. A section of the human retina, displayed in fine detail via a hematoxylin and eosin-stained (H&E) section. The processing pathway of light-to-visual signals follows a transduction route from the ganglion cell layer (GCL), to the inner nuclear layer (INL), to the outermost cell layer of the retina, known as the outer nuclear layer (ONL), which houses the photoreceptors (rods and cone cells) (Montana & Corbo, 2008).

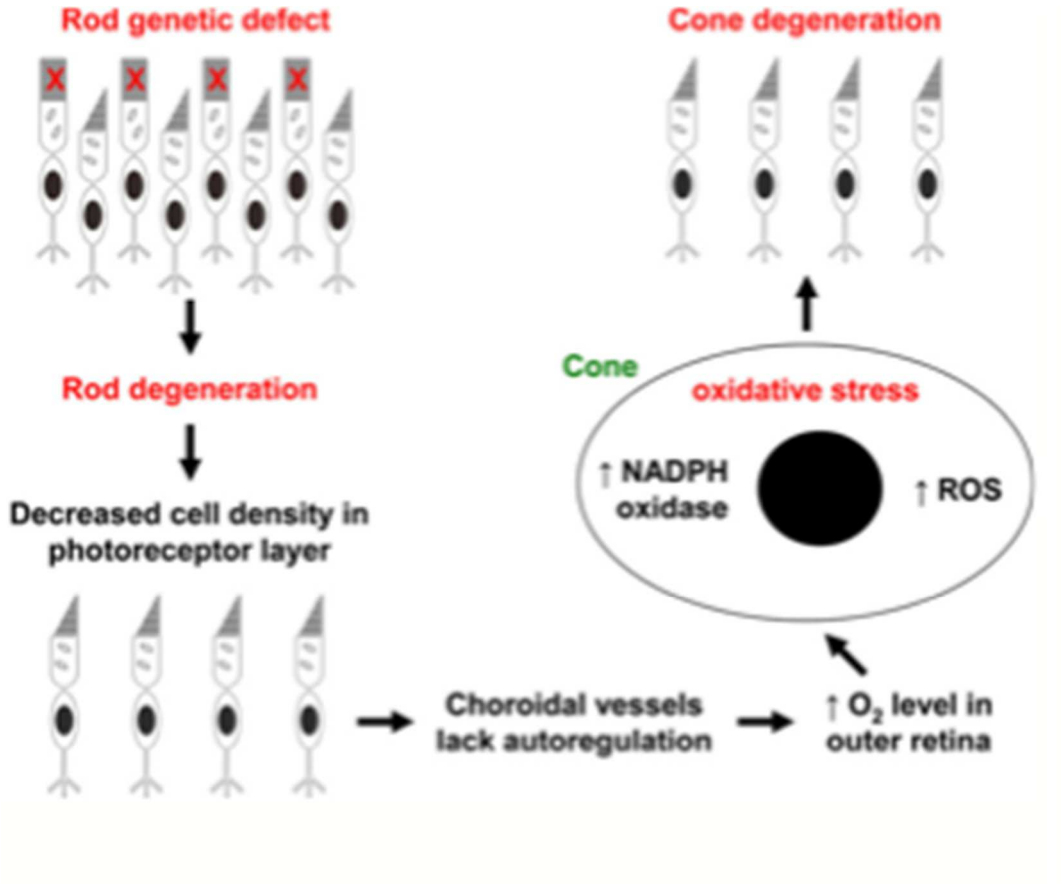


Figure 2. An illustration of the oxidative stress theory, demonstrating how genetic-based rod cell destruction leads to the increase in oxidative stress, and culminates in cone cell degeneration, and subsequent disease exacerbation (Narayan, et al., 2016).

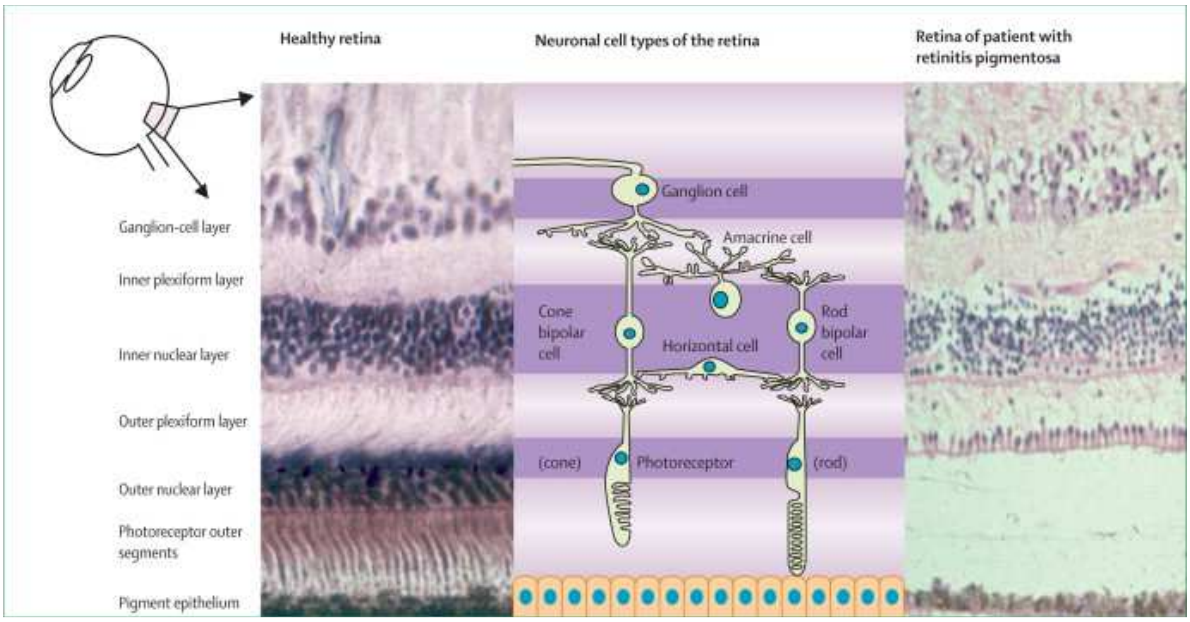


Figure 3. Two H&E-stained retinal sections displayed side-by-side: on the left, a depiction of a healthy human retina; on the right, the retinal section of a patient with retinitis pigmentosa at the mid-stage of disease. Most notably, the outer nuclear layer of the retinal image on the right is severely attenuated, correlating with the loss of rod and cone photoreceptor cells housed in the layer. The loss of these cells is depicted in the histological image as a large space between the retinal pigment epithelium (RPE), and outer plexiform layer (OPL) (Hartong, et al., 2006).

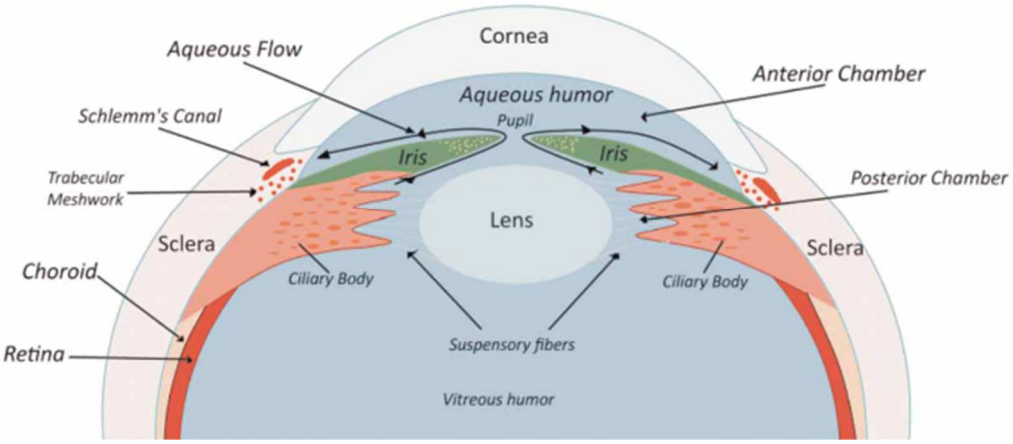


Figure 4. The structures of the eye, illustrating passage of substances from cornea, to aqueous humor, and potentially through the lens and into the vitreous humor (Aslan et al., 2013)

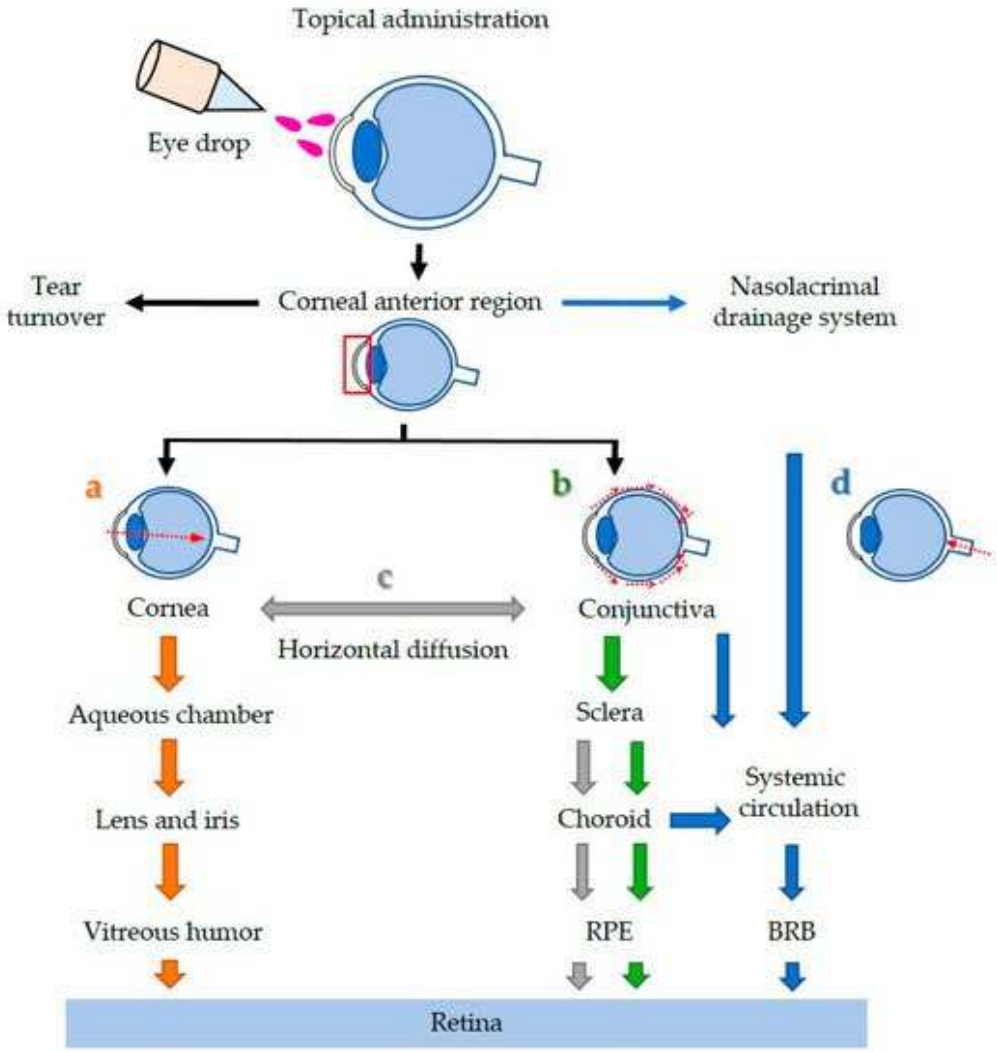


Figure 5. Theorized potential modalities of topical solutions in reaching the retina (Tsai et al., 2018)

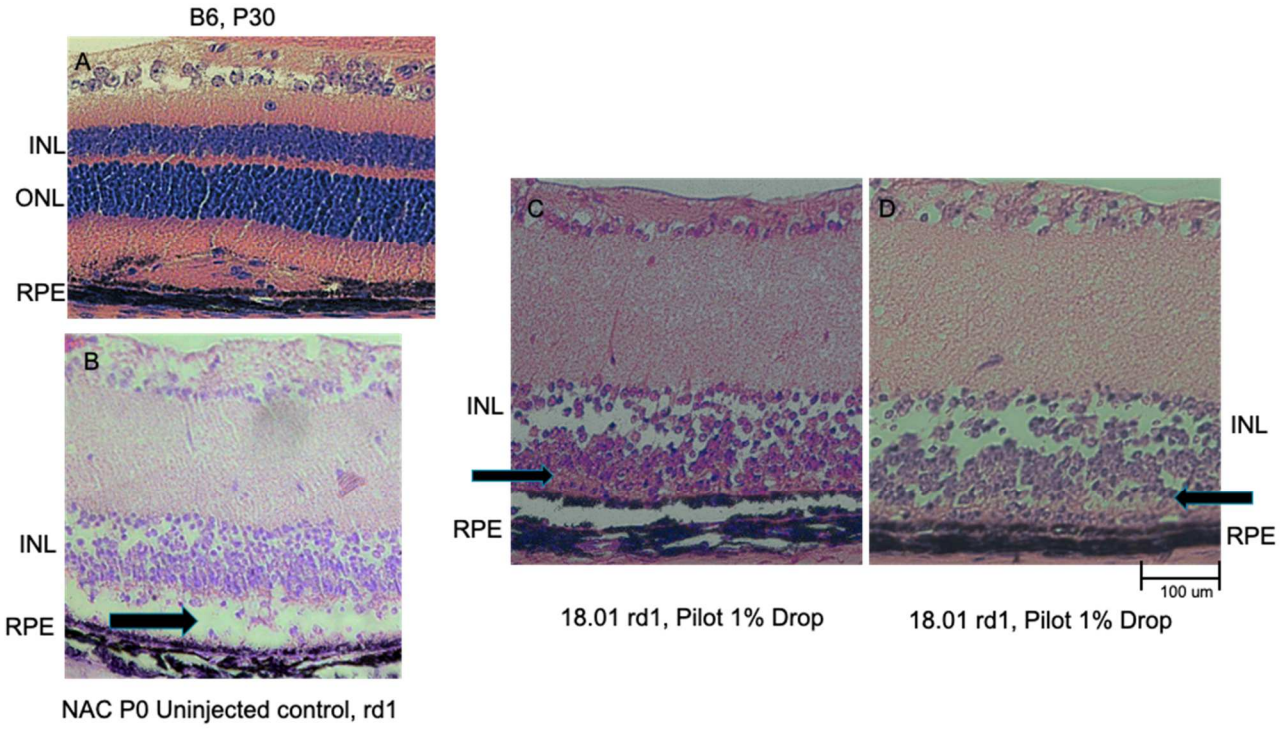
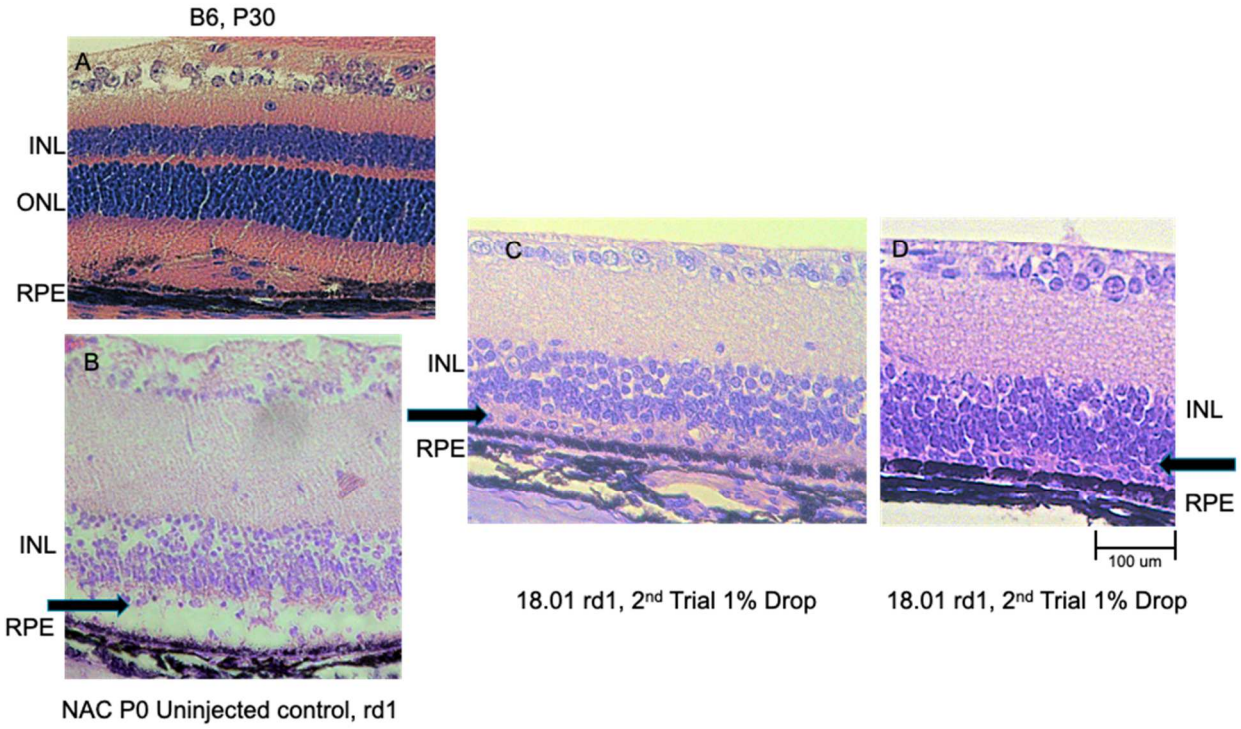


Figure 6. Two representative images of the topical administration pilot study, showing comparable results between (C) and (D) (complete disappearance of outer nuclear layer), and similar cell layer thickness as compared to the rd1 uninjected control. However, the area above the retinal pigment epithelium (RPE) in the rd1 disease model (B) has a large space (indicated by black arrow), whereas the space above the RPE (again indicated by black arrows) in the experimental images (C and D) has noticeable potential rescue.



, Met OS, 20X: 434, slide 3, retina 1

Figure 7. Two representative images of the second topical administration trial, showing comparable results (complete disappearance of outer nuclear layer), but with a thicker cell layer as compared to the pilot study results, as well as the disease control (B). Similarly, the area above the retinal pigment epithelium (RPE) in the rd1 disease model (B) has a large space (indicated by black arrow), whereas the space above the RPE (again indicated by black arrows) in the experimental images (C and D) has noticeable (and uniform) potential rescue.

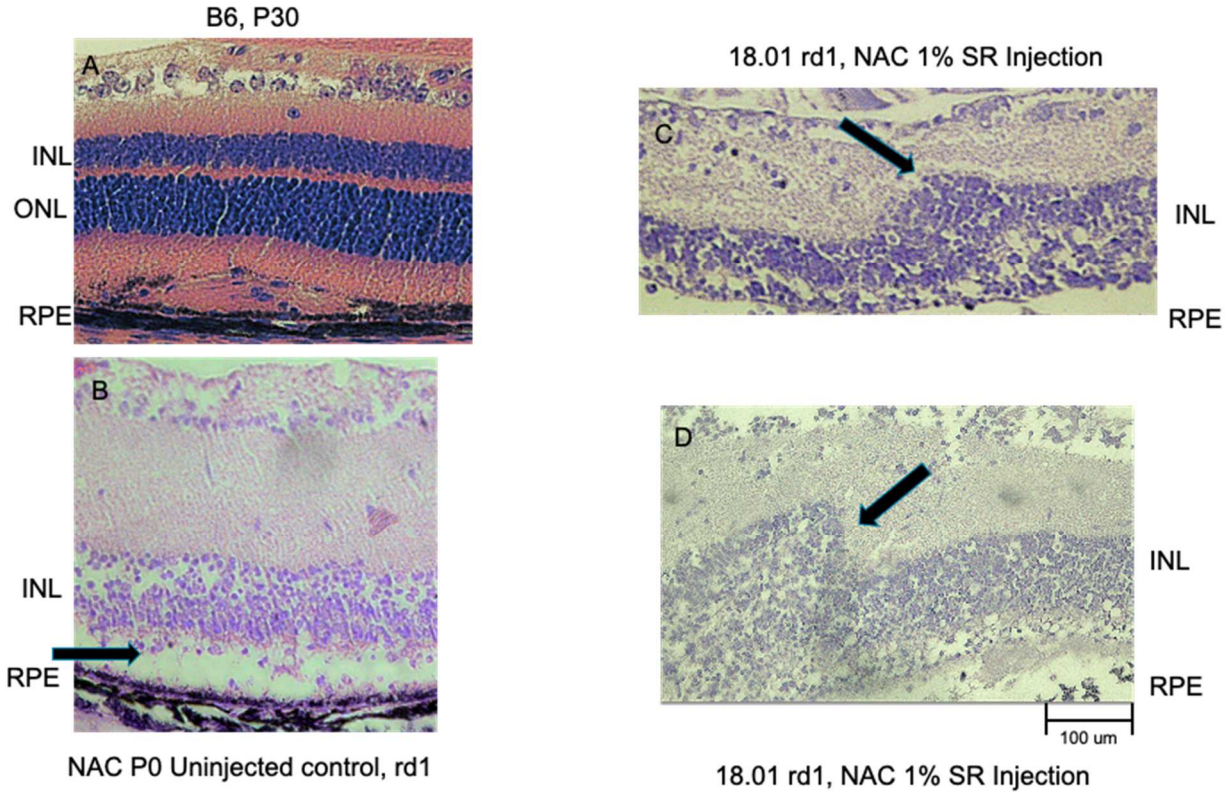


Figure 8. Two representative images of the injection study, showing comparable results (complete disappearance of outer nuclear layer), but with a "staircase"-shaped mass, indicative of photoreceptor rescue. The area above the retinal pigment epithelium (RPE) in the rd1 disease model (B) has a large space (indicated by black arrow), whereas the space above the RPE (again indicated by black arrows) in the experimental images (C and D) has noticeable potential rescue, with a near-doubling of the entire cell layer.

References

Ansari, F. A., Khan, A. A., & Mahmood, R. (2018). Protective effect of carnosine and N-acetylcysteine against sodium nitrite-induced oxidative stress and DNA damage in rat intestine. *Environmental Science and Pollution Research*, *25*(20), 19380–19392. doi: 10.1007/s11356-018-2133-9

Arshinoff, S. A., Mcculloch, J. C., Macrae, W., Stein, A. N., & Marliss, E. B. (1981). Amino acids in retinitis pigmentosa. *British Journal of Ophthalmology*, *65*(9), 626–630. doi: 10.1136/bjo.65.9.626

Aslan, M., Dogan, S., & Kucuksayan, E. (2013). Oxidative stress and potential applications of free radical scavengers in glaucoma. *Redox Report*, *18*(2), 76–87. https://doi.org/10.1179/1351000212y.0000000033

Babizhayev, M. A., Yermakova, V. N., Sakina, N. L., Evstigneeva, R. P., Rozhkova, E. A., & Zheltukhina, G. A. (1996). Nα-Acetylcarnosine is a prodrug of L-carnosine in ophthalmic application as antioxidant. *Clinica Chimica Acta, 254*(1), 1-21. doi:10.1016/0009-8981(96)06356-5

Babizhayev, M.A. (2008). N-Acetylcarnosine sustained drug delivery eye drops to control the signs of ageless vision: Glare sensitivity, cataract amelioration and quality of vision currently available treatment for the challenging 50,000-patient population. *Clinical Interventions in Aging*, 31. doi: 10.2147/cia.s4090

Babizhayev, M., & Kasus-Jacobi, A. (2009). State of the Art Clinical Efficacy and Safety Evaluation of N-Acetylcarnosine Dipeptide Ophthalmic Prodrug. Principles for the Delivery, Self-Bioactivation, Molecular Targets and Interaction with a Highly Evolved Histidyl-Hydrazide Structure in the Treatment and Therapeutic Management of a Group of Sight-Threatening Eye Diseases. *Current Clinical Pharmacology*, *4*(1), 4–37. doi: 10.2174/157488409787236074

Babizhayev, M. A. (2011). Biomarkers and special features of oxidative stress in the anterior segment of the eye linked to lens cataract and the trabecular meshwork injury in primary open-angle glaucoma: challenges of dual combination therapy with N-acetylcarnosine lubricant eye drops and oral formulation of nonhydrolyzed carnosine. *Fundamental & Clinical Pharmacology*, *26*(1), 86–117. doi: 10.1111/j.1472-8206.2011.00969.x

Babizhayev, M. A. (2016). Generation of reactive oxygen species in the anterior eye segment. Synergistic codrugs of N-acetylcarnosine lubricant eye drops and mitochondria-targeted antioxidant act as a powerful therapeutic platform for the treatment of cataracts and primary open-angle glaucoma. *BBA Clinical*, *6*, 49–68. doi: 10.1016/j.bbacli.2016.04.004

Braakhuis, A. J., Donaldson, C. I., Lim, J. C., & Donaldson, P. J. (2019). Nutritional Strategies to Prevent Lens Cataract: Current Status and Future Strategies. *Nutrients*, *11*(5), 1186. https://doi.org/10.3390/nu11051186

Campochiaro, P. A., Iftikhar, M., Hafiz, G., Akhlaq, A., Tsai, G., Wehling, D., … Kong, X. (2020). Oral N-acetylcysteine improves cone function in retinitis pigmentosa patients in phase I trial. *Journal of Clinical Investigation*, *130*(3), 1527–1541. doi: 10.1172/jci132990

Dubois, V. D.-P., & Bastawrous, A. (2017). N-acetylcarnosine (NAC) drops for age-related cataract. *Cochrane Database of Systematic Reviews*. doi: 10.1002/14651858.cd009493.pub2

Haider, Neena. (2020). *Institutional Animal Care And Use Committee Animal Studies Protocol.* Unpublished internal document, Schepens Eye Research Institute.

Hamel, C. (2006). Retinitis pigmentosa. *Orphanet Journal of Rare Diseases*, *1*(1). https://doi.org/10.1186/1750-1172-1-40

Hartong, D. T., Berson, E. L., & Dryja, T. P. (2006). Retinitis pigmentosa. *The Lancet*, *368*(9549), 1795–1809. https://doi.org/10.1016/s0140-6736(06)69740-7

Hipkiss, A. R., Brownson, C., & Carrier, M. J. (2001). Carnosine, the anti-ageing, anti-oxidant dipeptide, may react with protein carbonyl groups. *Mechanisms of Ageing and Development*, *122*(13), 1431–1445. doi: 10.1016/s0047-6374(01)00272-x

Iannaccone, A., Brabbit, E., Lopez-Miro, C., Love, Z., Griffiths, V., Kedrov, M., & Haider, N. B. (2021). Interspecies Correlations between Human and Mouse NR2E3-Associated Recessive Disease. *Journal of Clinical Medicine*, *10*(3), 475. https://doi.org/10.3390/jcm10030475

Jones, B. W., Pfeiffer, R. L., Ferrell, W. D., Watt, C. B., Marmor, M., & Marc, R. E. (2016). Retinal remodeling in human retinitis pigmentosa. *Experimental eye research*, *150*, 149–165. https://doi.org/10.1016/j.exer.2016.03.018

Koehler, C. C., Hall, L. M., Hellmer, C. B., & Ichinose, T. (2019). Using Looming Visual Stimuli to Evaluate Mouse Vision. *Journal of Visualized Experiments*, (148). https://doi.org/10.3791/59766

Komeima, K., Usui, S., Shen, J., Rogers, B. S., & Campochiaro, P. A. (2008). Blockade of neuronal nitric oxide synthase reduces cone cell death in a model of retinitis pigmentosa. *Free Radical Biology and Medicine*, *45*(6), 905–912. https://doi.org/10.1016/j.freeradbiomed.2008.06.020

Lee, S. Y., Usui, S., Zafar, A.-B., Oveson, B. C., Jo, Y.-J., Lu, L., … Campochiaro, P. A. (2011). N-acetylcysteine promotes long-term survival of cones in a model of retinitis pigmentosa. *Journal of Cellular Physiology*, *226*(7), 1843–1849. doi: 10.1002/jcp.22508

Li, S., Datta, S., Brabbit, E., Love, Z., Woytowicz, V., Flattery, K., Capri, J., Yao, K., Wu, S., Imboden, M., Upadhyay, A., Arumugham, R., Thoreson, W. B., DeAngelis, M. M., & Haider, N. B. (2020). Nr2e3 is a genetic modifier that rescues retinal degeneration and

promotes homeostasis in multiple models of retinitis pigmentosa. *Gene Therapy*, *28*(5), 223–241. https://doi.org/10.1038/s41434-020-0134-z

Montana, C. L., & Corbo, J. C. (2008). Inherited diseases of photoreceptors and prospects for gene therapy. *Pharmacogenomics*, *9*(3), 335–347. https://doi.org/10.2217/14622416.9.3.335

Narayan, D. S., Wood, J. P. M., Chidlow, G., & Casson, R. J. (2016). A review of the mechanisms of cone degeneration in retinitis pigmentosa. *Acta Ophthalmologica*, *94*(8), 748–754. https://doi.org/10.1111/aos.13141

Nishiguchi, K. M., Carvalho, L. S., Rizzi, M., Powell, K., Holthaus, S.-M. K., Azam, S. A., … Ali, R. R. (2015). Gene therapy restores vision in rd1 mice after removal of a confounding mutation in Gpr179. *Nature Communications*, *6*(1). https://doi.org/10.1038/ncomms7006

Punzo, C., & Cepko, C. (2007). Cellular Responses to Photoreceptor Death in the *rd1* Mouse Model of Retinal Degeneration. *Investigative Opthalmology & Visual Science*, *48*(2), 849. https://doi.org/10.1167/iovs.05-1555

Tsai, C-H., Wang, P-Y., Lin, I-C., Huang, H., Liu, G-S., Tseng, C-L. (2018). Ocular Drug Delivery: Role of Degradable Polymeric Nanocarriers for Ophthalmic Application. *International Journal of Molecular Sciences*, 19(9):2830. https://doi.org/10.3390/ijms19092830

Verbakel, S. K., Huet, R. A. V., Boon, C. J., Hollander, A. I. D., Collin, R. W., Klaver, C. C., … Klevering, B. J. (2018). Non-syndromic retinitis pigmentosa. *Progress in Retinal and Eye Research*, *66*, 157–186. https://doi.org/10.1016/j.preteyeres.2018.03.005

Wade, A. M., & Tucker, H. N. (1998). Antioxidant characteristics of L-histidine. *The Journal of Nutritional Biochemistry*, *9*(6), 308–315. doi: 10.1016/s0955-2863(98)00022-9