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Retina

Retinal Function in Young Adults Following Topical Application of Levodopa to the Eye

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Purpose: Levodopa has been investigated as a therapeutic solution for ocular disorders involving dysregulation of the dopaminergic system, especially in the context of myopia. However, given the critical role dopamine plays in normal vision, this phase I trial examined whether levodopa/carbidopa eye drops induce any regional changes in retinal structure and function.

Methods: Twenty-nine healthy male subjects 18 to 30 years of age were randomly assigned to receive either a low (1.4/0.34 µmoles/day, $n = 14$) or high (2.7/0.68 µmoles/day, $n = 15$) dose of levodopa/carbidopa eye drops in 1 eye for 28 consecutive days. A placebo solution was applied to all fellow eyes. Measures included visual acuity, regional frequency doubling perimetry, regional multifocal electroretinogram (mfERG) and optical coherence tomography (retinal thickness). Outcome measures were undertaken at baseline, end-of-treatment (4 weeks), and at a follow-up (4 months post-treatment).

Results: For low dose treated eyes, regional analysis showed a small, statistically significant change in mfERG recordings (increase in ring 5 amplitude in low dose treated eyes, $P < 0.05$) and the retinal thickness map (localized retinal thinning in low dose treated eyes, $P < 0.05$). These changes were not clinically significant. No significant changes were observed in high dose treated eyes. Pharmacokinetic analysis (rabbits) demonstrated that levodopa was not detectable within blood and peaked within the eye at 15 to 30 minutes (and eliminated within 4 hours).

Conclusions: No clinically significant effects of levodopa/carbidopa eye drops were found with regard to normal retinal structure and function following short-term use.

Translational Relevance: This study further demonstrates the safety of topical levodopa, which may support its use in the treatment of ocular disorders in which the dopamine system is dysregulated.

Introduction

Dopamine, the primary catecholamine of the retina, has been shown in animal models to play a critical role in the normal functioning of the visual system, including enhancing signal flow through photoreceptor cone circuits, light adaptation, circadian entrainment, cell survival, and ocular growth regulation.[1–5] Dysregulation of this system has been implicated in several visual disorders, including myopia,[6–9] amblyopia,[10] diabetic retinopathy,[11] and, to a lesser extent, retinitis pigmentosa.[12] Thus, the administration of levodopa, a drug widely used for the treatment of neurological disorders involving dopaminergic dysfunction,[13] has been investigated as a potential therapeutic solution for several of these visual conditions. In a clinical setting, levodopa is commonly co-administered with carbidopa to prevent

its premature conversion to dopamine before reaching the target tissue (i.e. the brain), enhancing treatment outcomes.[14]

As systemic administration of levodopa/carbidopa has the potential to induce off-target changes in dopamine levels within the brain, localized administration of levodopa/carbidopa to the eye is preferred for the treatment of visual disorders. Therefore, with a view toward its use in the treatment of myopia progression, recent preclinical and clinical trials have investigated the direct application of levodopa to the eyes as topical drops.[6–9]

In chick models, topical levodopa/carbidopa application significantly inhibits the development of experimental myopia in a dose-dependent manner, with no detectable systemic distribution.[6,8,9] Topical application was not associated with any adverse ocular effects in preclinical studies.[6] In agreement with these findings, we have recently reported that levodopa/carbidopa eye drops are safe and tolerable during a 1-month safety and tolerability trial in healthy young adults.[7] Based on dopamine's critical role in the retina, using additional data collected during this study, we wished to investigate in more depth what effect levodopa/carbidopa treatment has on the retinal structure and function in healthy young adults.

Previous studies in both animals and humans have indicated that increased dopamine levels associated with systemic (oral) levodopa treatment may improve visual function.[10,11,15–19] In humans, this has been observed as improved contrast sensitivity at medium to high spatial frequencies, as well as reduced pattern-electroretinogram (ERG) and visually evoked potential implicit times.[12,15–19] However, many of these studies have taken place in association with a disease state, such as amblyopia, diabetic retinopathy, or Parkinson's disease. This has made it difficult to isolate whether the improvements in visual function observed are solely due to levodopa administration, or due to a drug-disease interaction. Interestingly, these visual improvements are often not seen in non-symptomatic eyes.[15,16,20,21] It is also difficult to ascertain whether these effects would occur during topical treatment, as systemic levodopa may also affect higher visual centers within the brain. Accordingly, we have previously reported that topical levodopa/carbidopa treatment does not affect central retinal thickness or overall visual function, as measured by visual acuity, average frequency doubling perimetry, and central multifocal ERG (mfERG) responses.[7] However, oral levodopa has previously been reported to affect contrast sensitivity differently across low to high spatial frequencies in healthy volunteers,[22] which may indicate that increases in dopamine could affect the retina differently at a regional level. Therefore, we wished to further analyze our safety trial data by studying how topical levodopa/carbidopa eye drops affect visual function and retinal structure at a regional level.

To assess the effect of topical levodopa/carbidopa administration on retinal function, measures of visual acuity, as well as regional matrix perimetry and mfERG were undertaken. Structural changes (regional retinal thickness) were assessed using optical coherence tomography (OCT). Data were generated from a phase I, first-in-human, safety and tolerability trial of levodopa/carbidopa topical eye drops carried out in 29 healthy male adults (aged 18–30 years old), detailed in full below.

In an additional set of animal experiments, we examined the pharmacokinetics and metabolism of levodopa/carbidopa when applied as topical eye drops in rabbits. The pharmacokinetics and metabolism of levodopa/carbidopa has been heavily characterized in humans when administered systemically in both immediate and sustained release formulations. In short, when levodopa is given systemically in an immediate release tablet form, plasma levels peak within 30 minutes to 2 hours of administration and remain measurable for 4 to 6 hours (for review, see Yeh et al. Neurology 1989[23]). Carbidopa plasma levels peak within 2 to 4 hours of administration and remain measurable until approximately 7 hours following administration (for review, see Yeh et al. Neurology 1989[23]). However, the ocular pharmacokinetics and, importantly in the context of topical application, systemic distribution of levodopa/carbidopa eye drops has not yet been characterized. Therefore, to establish the distribution of levodopa/carbidopa within the different ocular layers/compartments, and to assess whether systemic distribution occurs, pharmacokinetic analysis of this topical formulation was undertaken in rabbits.

Methods

Human data were generated from a placebo-controlled, paired-eye, double-blind, monocular phase 1b safety and tolerability trial for which the full protocol details have been published.[7] In short, participants were randomized to receive either a low concentration (1.4 levodopa:0.34 carbidopa µmoles/day) or high concentration (2.7 levodopa:0.68 carbidopa µmoles/day) of levodopa/carbidopa eye drops and were instructed to instill two drops per day in one eye upon awakening. These doses were chosen based on their effectiveness against experimental myopia in animal

models.[9] The fellow eye received a placebo in the form of a vehicle solution (consisting of 0.1% w/v ascorbic acid and 0.001% w/v benzalkonium chloride [BAK] dissolved in 1 × phosphate-buffered saline [PBS]) to serve as a control for age, ethnicity, and environmental and genetic factors. The eye which received the active levodopa/carbidopa solution was also randomized so there was an even distribution of left and right eyes in each treatment group.

Twenty-nine male participants aged from 18 to 30 years were recruited at the University of Canberra, Australia, following a promotional campaign on campus. Participants received a baseline eye examination and follow-up visits were scheduled weekly for 4 weeks followed by a final review 4 months after cessation of treatment. The data presented in this paper were collected at the primary measurement points (0 weeks [baseline], 4 weeks [end-of-treatment], and 4 months post-cessation of treatment [follow-up]). As discussed earlier, this paper reports a subanalysis of the data collected in this clinical trial.

All participants provided written consent and the clinical study was approved by the Human Ethics Committee of the University of Canberra (HREC-0406) with Australian New Zealand Clinical Trial registration (ANZCTR; ACTRN12620001259932) and observed the tenants of the Declaration of Helsinki.

Exclusion criteria consisted of evidence of spherical equivalent refraction (sphere power + [cylindrical power/2]) of hyperopia > +1.00 diopters (D) or high myopia of < −6.00 D. Participants with ocular pathologies that affect the anterior segment (ocular surface disease) and/or macular function, amblyopia, and strabismus were excluded. Furthermore, participants with any systemic pathologies that affect vision, including diabetes, and previous use of atropine or topical medication or systemic medications that interfere with dopamine metabolism (i.e. anti-depressants) were also excluded.

Baseline and follow-up examinations at week 4 (end-of-treatment) and 4 months following the cessation of treatment (final follow-up) included the following measures reported in this manuscript: high and low contrast habitual visual acuity (Hi-Low contrast LogMAR chart, National Vision Research Institute), matrix perimetry (Frequency Doubling Technology), spectral domain OCT and autofluorescence imaging of the retinal posterior pole and optic disc, and mfERG recordings. Following measurement of visual acuity and matrix perimetry, temporary dilation and cycloplegia was achieved by instillation of 1 drop of phenylephrine 2.5% and 1 drop of cyclopentolate 1.0% before undertaking OCT and mfERG measures. Figure 1 presents the regional stimulus layout of mfERG and the corresponding locations for OCT 8 × 8 retinal thickness grids overlapping the boundaries of the Matrix 10-2 stimulus array.

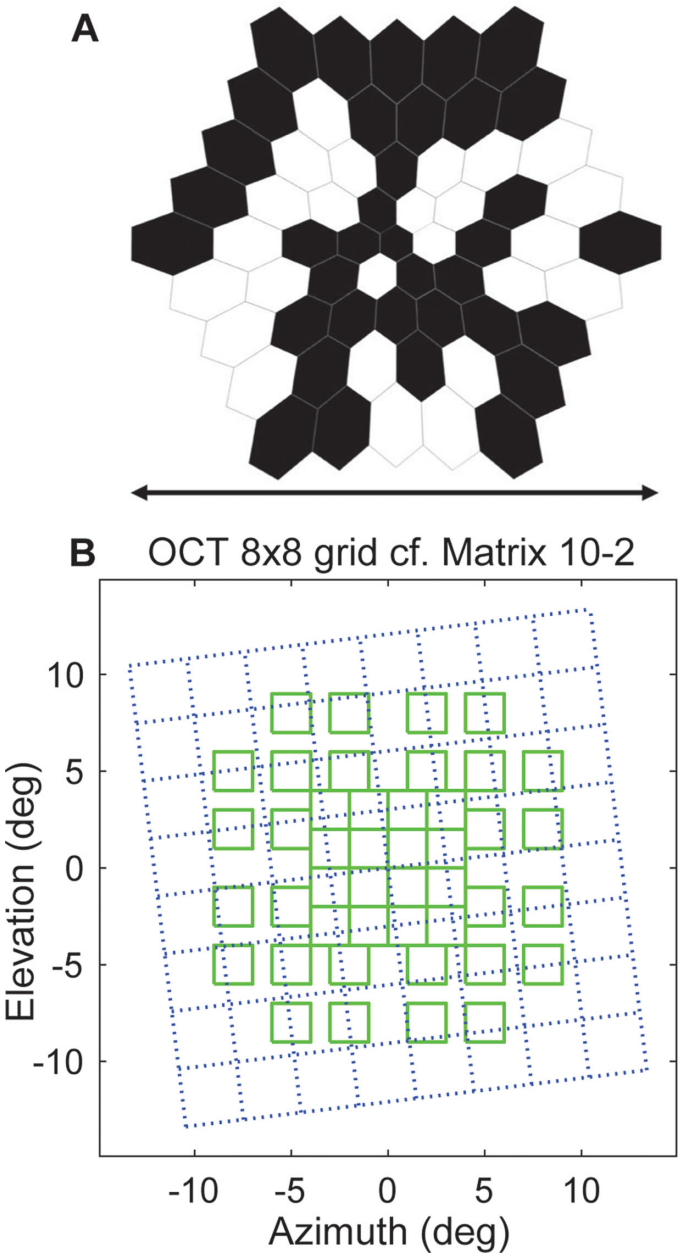

Figure 1. Corresponding regional locations for structural and functional measures presented for (**A**) multifocal ERG, and (**B**) overlapping regions of Spectralis OCT 8 × 8 posterior pole grids in *dashed blue* with overlapping 44 stimulus regions of the Matrix 10-2 array in *solid green*.

Visual Acuity and Matrix Perimetry Assessment

Visual acuity letter measures were recorded with the National Vision Research Institute (NVRI) charts

presented at 3 m.[24] The logMAR letter score was equal to the total number of letters read correctly plus 30 and measurements were terminated following 4 or more errors on a single line.[25] Prior to dilation, matrix perimetry (Frequency Doubling Technology) was conducted with the 10-2 test paradigm with fast threshold strategy (Carl Zeiss Meditec, Dublin, CA, USA) to use its high resolution of sampling in the macular visual field. The Matrix perimeter presents stimuli as low spatial frequency sinusoidal gratings (0.5 cycles/deg) at high temporal frequencies (12 hertz [Hz]). The 10-2 testing stimulus layout presents 2 degrees stimuli across 44 test locations in the central 10 degrees eccentricity.

OCT Retinal and Optic Nerve Imaging

OCT and autofluorescence imaging of the retinal posterior pole and optic disc were obtained from each eye (Spectralis, HRA + OCT; Heidelberg Engineering, Heidelberg, Germany, software version 6.9.5.0). We obtained two OCT scans on each participant. The first was a posterior pole scan represented by the dense volume scan centered on the fovea (12 degrees × 12 degrees), 40 B-scans each spaced 60 µm apart, with an automatic real-time (ART) mean of 12 repeats of the 768 A-scans utilizing enhanced depth imaging. The subsequent scan was imaging the peripapillary retinal nerve fiber layer (pRNFL) centered on the optic disc 12 degrees in diameter and consisted of 768 A-scans and ART mean of 100. Automated retinal segmentation was performed by Heidelberg Heyex software. The 8 × 8 grid is a standard way of reporting thickness data on higher resolution posterior pole scans on the Heidelberg. These present the mean full retinal thickness measures for each cell of an 8 × 8 array. These data were extracted from the "ThicknessGrid" fields of the .xml files recorded by the Spectralis for the posterior pole volume scans. Retinal thickness was measured from the retinal pigment epithelium to the inner limiting membrane.

Multifocal ERG Recording and Analysis

The mfERG recordings were taken for each eye (VERIS 5.1.5x refractor/camera system; Electro-Diagnostic Imaging Inc., Redwood City, CA, USA). The mfERG measures were performed according to the International Society for Clinical Electrophysiology of Vision (ISCEV) standard using the Visual Evoked Response Imaging System (VERIS) under mesopic room conditions (30 lux). Pupils were dilated to at least 7 mm and the participant was in ordinary room illumination for at least 15 minutes prior to mfERG testing. A disposable Dawson-Trick-Litzkow (DTL) thread electrode was used as the recording electrode, with gold-plated electrodes for the reference (attached to the temple) and ground (attached to the forehead) electrodes. The multifocal stimulus array was presented on a 22-inch Liquid Crystal display monitor with a display rate of 67 frames per second that consisted of 61 hexagons scaled with retinal eccentricity to achieve a balanced response amplitude across all locations, spanning 25 degrees on either side of fixation when viewed at a 30 cm distance. The presentation sequence was temporally modulated for each region according to a pseudorandom *m*-sequence, with an ON-region luminance of 1000 cd/m^2 and an OFF luminance of 2 cd/m^2 at a frequency of 75 Hz (see Fig. 1). Trial frames and lenses with the participant's cycloplegic autorefraction result, corrected for the accommodative demand at 30 cm, were used. Participants were asked to maintain fixation on the central red cross and to minimize blinks during testing segments. Artifacts due to blinks and eye movements were detected online and discarded. Segments with missing data were repeated. Each recording session consisted of 8 segments of 30 seconds of duration for each eye, with 10 second rest breaks between sessions. On average, including occasional repeats, the testing duration was approximately 15 minutes. The mfERG responses were band-pass filtered (300 Hz), sampled every 1.87 milliseconds and amplified by 30K times. This test was performed monocularly, with the right eyes tested first, followed by the left eyes.

The first order kernel of each regional waveform was determined providing both amplitude and implicit time. The amplitude was measured for the N1 wave (from the isoelectric to first negative trough), for the P1 wave (from the N1 trough to the peak of the positive wave), and N2 wave (from the P1 to the second negative trough). Implicit times were measured for each of these waveforms. Here, we report the values for P1 Latency (ms) and P1 Amplitude (nV), with the responses averaged in a dartboard layout across five concentric rings with the central ring representing the foveal response. The visual angles were: ring 1 = 1 degree, ring 2 = 2 degrees to 7.6 degrees, ring 3 = 7.6 degrees to 14.8 degrees, ring 4 = 14.8 degrees to 23 degrees, and ring 5 = 23 degrees to 30 degrees.

Pharmacokinetic Testing Methods – Animal Studies

Following the guidelines (International Council for Harmonisation of Technical Requirements for

Pharmaceuticals for Human Use guideline M3(R2) on non-clinical safety studies for the conduct of human clinical trials and marketing authorisation for pharmaceuticals) of the US Food and Drug Administration (FDA) and the Therapeutic Goods Administrator (TGA, Australia), pharmacokinetic testing was undertaken in the non-rodent mammalian model of the rabbit at the University of Canberra. Eleven-week-old weaned New Zealand white rabbits were obtained from Konijnen Farm (Baldivis, WA, USA) and housed at the University of Canberra until experiments began at 12 weeks of age. Rabbits were housed individually in 3 tier rabbit research cages (Techniplast) in 19°C to 23°C temperature-controlled rooms under a 12:12 hour light/dark cycle (lights on at 8 AM and off at 8 PM). All rabbits had constant access to food (Jack Rabbit Premium) and water, with daily additions of hay, leafy vegetables, or carrots. Authorization to conduct experiments was approved by the University of Canberra Animal Ethics Committee under the Australia Capital Territory Animal Welfare Act 1992 (project number: CEAE 11515) and conformed to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

To undertake pharmacokinetic studies in rabbits, ocular and systemic samples were collected at six time points following topical drug administration.[26] Following standard practice, to reduce the number of animals required, both eyes from each animal were used. Due to the natural diurnal cycling of dopamine and levodopa, in which its levels increase over the day, samples from a placebo treated control group were also collected at all six time points, rather than comparing back to a T0 value.

Biological samples were taken at six different time points (15 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, and 6 hours following treatment) after treatment with levodopa/carbidopa eye drops (1.4 µmoles levodopa:0.34 µmoles carbidopa, administered 2 hours after light onset). For the control group, a topical placebo solution (0.1% ascorbic acid, 0.001% BAK dissolved in 1 × PBS) was given. For each time point, biological samples (blood, tears, cornea, aqueous humor, iris/lens/ciliary body, vitreous humor, retina, and choroid) were collected from two treated and two control animals. Following deep anesthesia with isoflurane (5% in 1 L of medical grade oxygen per minute), blood samples were collected from the marginal ear vein or central ear artery, whereas tear samples were collected using Shirmer strips. Following this, injection of sodium pentobarbitone (325 mg/mL at a dose rate of 0.5 mL/kg) was undertaken on all animals for euthanasia, after which the eyes were enucleated and all remaining ocular samples were collected. All samples were snap-frozen on dry ice and stored at −80°C until analysis. Levodopa and carbidopa concentration in blood, tears, and each of the ocular tissue samples was measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS) following a previously described method[6] adapted to include carbidopa (see Appendix for full methods).

Statistical Analysis

Data from each instrument (Matrix, mfERG, and OCT) were exported as .xml files for analysis. Regional differences were then analyzed with custom code in MATLAB (R2020b; The MathWorks, Natick, MA, USA). All data types were found to be normally distributed.

For OCT analysis, to account for the equal distribution of left and right eyes within the different treatment groups, the OCT data were flipped for right eye regions to be correctly aligned with left eye regions. For comparison of the OCT data between groups, the number of data $8 \times 8 = 64$ thickness grid regions between treated eyes were slightly different in participant numbers (low dose, 14*64 OCT regions = 896 and high dose, 15*64 = 960). Bland-Altman analysis with linear regression was used to evaluate variation in retinal thickness over time.

For all regional analyses, we applied linear mixed effect (LME) models to account for the repeated measures in each eye. Such LME models are required due to treatment groups (levodopa versus placebo) being nested within individuals due to the paired-eye nature of this trial. Thus, for the retinal thickness studies, there were random effects for doses within eyes. For the mfERG models, there were random effects for rings within an eye, and eyes within subject. This allowed us to determine the effects that treatment had on the structural and functional factors measured. In all LME analyses, we used the fellow placebo eye at baseline as the reference group to represent an untreated control group.

For pharmacokinetic analysis using LC-MS/MS, data are presented as the means ± the standard error of the means of the amount of levodopa or carbidopa detected (ng) per milligram or microliter of tissue. Drug concentration was calculated using a standard curve and deuterated internal standard (see Appendix for full details). Before analyzing the effect of treatment, data were first tested for normality and homogeneity of variance (Shapiro-Wilk test). Following this, the effect of treatment was analyzed via a repeated measures analysis of variance (ANOVA). To analyze specific between group effects, ANOVA

testing was followed by a student's unpaired *t*-test, with Bonferroni correction for multiple testing, when statistical significance was reached.

Results

Overview

In total, 29 young men (mean 24.9 ± 2.7 years) commenced and completed at least 2 weeks of treatment and their data are presented in this study (Table 1). Two participants (one from the low dose treated group and one from the high dose treated group) withdrew from the study due to inability to attend clinic visits and did not complete the week 4 measures. As previously described,[7] when looking at average measures across the retina, no levodopa/carbidopa-induced changes were observed in visual acuity, visual function (as measured by average matrix perimetry or mfERG recordings), or retinal structure and thickness (Table 2) at the end of 4 weeks of treatment or at follow-up (4 months). Although treatment did not induce significant changes at an average or whole-retina level, this study looked to examine whether levodopa/carbidopa treatment was associated with regional changes in visual function or retinal structure that may have been missed by such a global analysis. As will be described in detail below, for the most part, there were no significant regional changes with regard to visual structure and function. The caveat to this was a small number of significant changes in mfERG and retinal thickness measures within distinct regions in response to low dose levodopa/carbidopa treatment. However, these effects were small in size and did not represent a clinically significant change in visual function or retinal thickness.

Visual Function – Matrix Perimetry

As shown in Figure 2, and as described previously,[7] there were no significant differences over time or between cohorts with regard to the standard matrix perimetry measures of mean deviation and pattern standard deviation (see Table 2). To explore whether regional changes were missed in this initial analysis, the Matrix total deviation data for each region was compared between placebo, low dose and high dose levodopa/carbidopa treatment groups (see Fig. 3, Table 2). For both concentrations of levodopa tested, there were no significant differences in regional matrix total deviation between treated and placebo eyes at the cessation of treatment or

Table 1. Participant Characteristics

	Baseline			End-of-Treatment (Week 4)			Follow-Up (4 Mo)		
Participant Characteristics	Placebo	Low Dose	High Dose	Placebo	Low Dose	High Dose	Placebo	Low Dose	High Dose
No. of eyes	29	14	15	27	13	14	23	10	13
Age, y	25.0 ± 2.6	24.7 ± 3.0	25.1 ± 2.3						
HCVA	101.1 ± 6.5	91.7 ± 2.9	97.6 ± 3.0	93.0 ± 5.9	88.1 ± 1.6	91.8 ± 3.2	93.07 ± 5.8	94.92 ± 1.6	91.35 ± 3.2
LCVA	71.3 ± 6.3	86.5 ± 4.0	91.0 ± 2.9	87.6 ± 4.5	82.4 ± 2.1	85.2 ± 5.0	87.70 ± 4.5	88.70 ± 2.0	85.21 ± 5.0
Perimetry, mean deviation, dB	0.33 ± 2.15	0.33 ± 2.04	0.32 ± 2.68	0.70 ± 2.60	0.17 ± 3.30	0.40 ± 1.57	0.02 ± 2.60	0.38 ± 2.88	−0.46 ± 2.48
Perimetry pattern standard deviation, dB	2.65 ± 0.45	2.68 ± 0.46	2.71 ± 1.43	2.63 ± 0.45	2.82 ± 0.75	2.64 ± 0.36	2.87 ± 0.55	2.57 ± 0.48	2.86 ± 0.34
mfERG amplitude, nV	18.19 ± 2.34	16.53 ± 2.77	17.07 ± 2.11	18.76 ± 6.93	17.53 ± 1.57	17.56 ± 2.42	17.46 ± 2.13	19.32 ± 11.49	15.91 ± 2.70
mfERG implicit time, ms	31.09 ± 2.99	31.96 ± 2.73	32.03 ± 1.62	33.13 ± 6.19	31.59 ± 1.29	32.17 ± 2.86	34.36 ± 10.25	37.27 ± 13.05	33.75 ± 9.83
Central retinal thickness, µm	281 ± 21	283 ± 17	279 ± 20	280 ± 22	282 ± 18	278 ± 23	279 ± 21	279 ± 17	277 ± 20

Global structure and function measures for participants at each measurement visit. Data represent the means ± standard deviation. Levodopa/carbidopa treatment was not associated with any significant changes at a global level. HCVA and LCVA data represent habitual high and low contrast visual acuity (mean ± SD), respectively. Multifocal electroretinogram (mfERG) data represent the P1 latency and amplitude.

Table 2. Linear Mixed Effects Model Summary for Visual Function Variables

HCVA	Estimate	SE	*t*-Stat	*P* Value
Reference	2.3	0.77	2.98	0.004
Low dose	1.16	1.39	0.86	0.39
High dose	−1.51	1.36	−1.14	0.26
LCVA				
Reference	2.41	1.23	1.96	0.05
Low dose	−0.02	2.16	−0.01	0.99
High dose	−2.55	2.1	−1.21	0.231
Matrix mean deviation, dB				
Reference	0.4	0.29	1.36	0.18
Low dose	−0.2	0.51	−0.4	0.69
High dose	0.2	0.5	0.39	0.7
Matrix pattern standard deviation, dB				
Reference	−0.01	0.17	−0.07	0.94
Low dose	0.11	0.3	0.36	0.72
High dose	−0.1	0.3	−0.32	0.75
Linear mixed effects model summary for retinal structure variables				
Central thickness, µm				
Reference	−0.33	0.77	−0.43	0.67
Low dose	0.41	1.36	0.3	0.76
High dose	0.12	1.32	0.09	0.93
Total volume				
Reference	<0.01	0.02	0.37	0.71
Low dose	<0.01	0.03	0.1	0.92
High dose	−0.02	0.03	−0.68	0.5
pRNFL thickness, µm				
Reference	−0.88	0.58	−1.52	0.13
Low dose	0.89	1.02	0.88	0.38
High dose	0.93	1.00	0.93	0.36

t-Stat, *t*-statistics.

at follow-up (see Table 2, Supplementary Fig. S1). There were also no regions in either treated or placebo eyes that showed a statistically significant change between baseline and end-of-treatment (or follow-up) tests.

When compared to baseline values, there was evidence of hypersensitivity (improved performance) at both end-of-treatment and follow-up measures for the low dose treated group (with 59% of regions; e.g. 26/44; showing positive total deviations) and toward hyposensitivity for the high dose treated group and the placebo group (59% and 52% of regions, respectively). However, these regional changes failed to reach statistical significance and did not represent a clinically significant deviation.

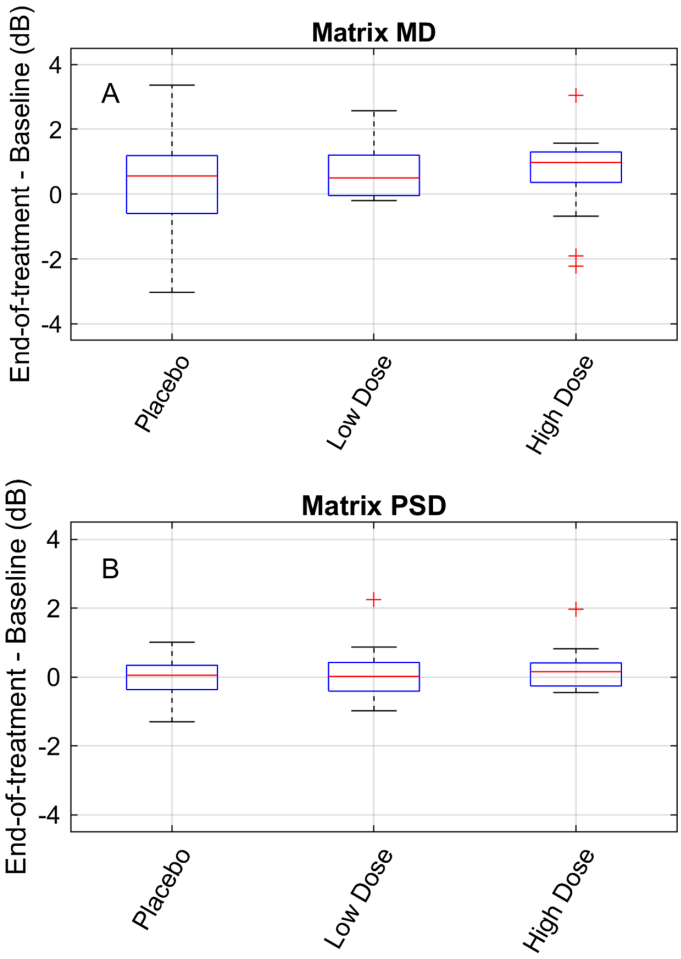

Figure 2. Boxplot of differences between baseline and end-of-treatment visits in (**A**) mean defect (MD), and (**B**) pattern standard deviation (PSD) for the Matrix 10-2 test. None of the differences reached statistical significance (see Table 2).

Retinal Function – mfERG Recordings

To study regional differences in mfERG recordings, the amplitude and latency of the P1 waveform were examined following 4 weeks of levodopa/carbidopa treatment (Fig. 4, Table 3). Although the LME models outlined in Table 3 found no effect of time or treatment on mfERG responses, individual comparisons found a small but statistically significant difference between baseline and end-of-treatment for ring 5 amplitude in low dose treated eyes. Similar to end-of-treatment, there were no significant differences between treated and placebo eyes at follow-up relative to baseline (Supplementary Fig. S2).

Several small time-dependent changes were observed in the mfERG data, although none reached clinical significance. For example, mfERG amplitudes showed an increase in responses between baseline and end-of-treatment for low and high dose treated eyes (see Figs. 4B, 4C) with a bias toward the central

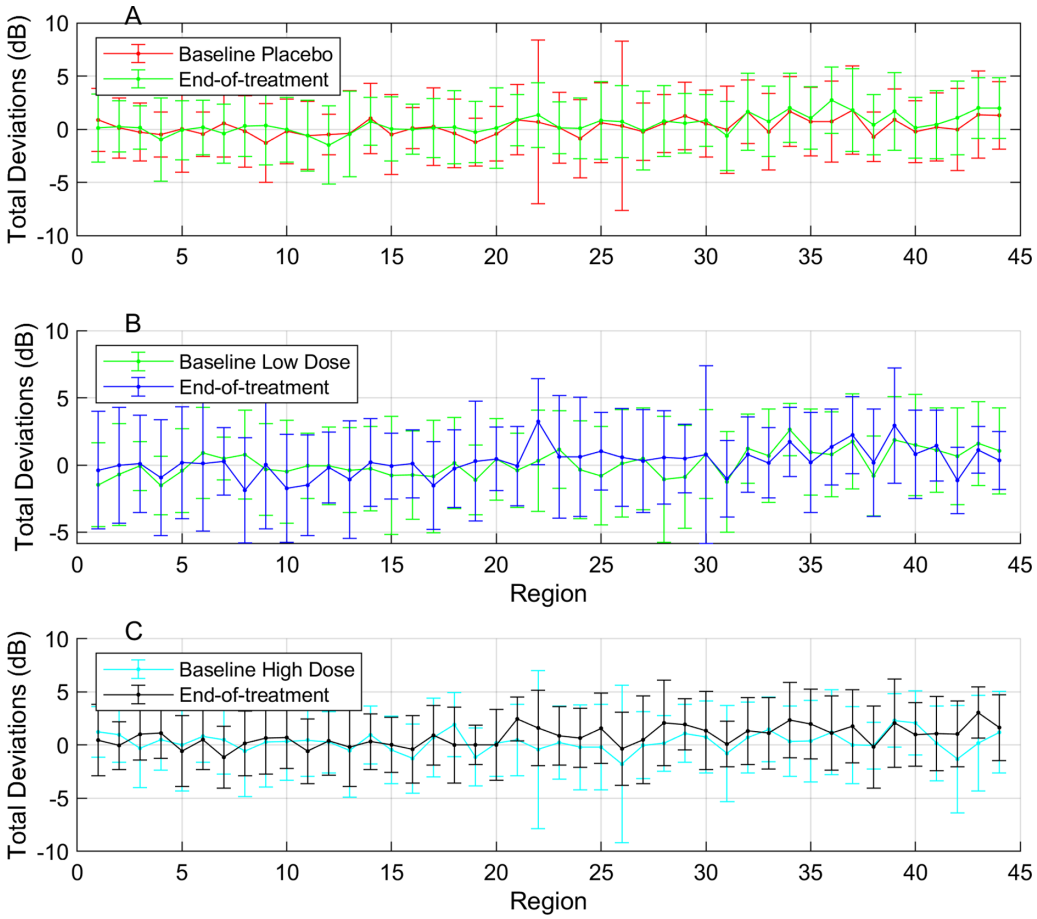

Figure 3. Total deviations recorded for Matrix 10-2 perimetry across 44 regions for (**A**) placebo treated, (**B**) low dose treated, and (**C**) high dose treated eyes at baseline and end-of-treatment visits. There was no significant difference between regional sensitivity values at end-of-treatment. Increased sensitivity from baseline for low dose 1 (**B**) and decreased sensitivity from baseline for high dose 2 (**C**) eyes were noted, but these did not reach statistical significance and did not represent a clinically significant change.

rings. However, this only reached significance for ring 5 in low dose treated eyes ($P = 0.0456$). Given that ring 5 sits beyond the borders of the macula, it is unlikely that this small change would be of clinical significance. Low dose treated eyes showed quicker responses (see Fig. 4E) in the central two rings and delayed responses in the outer three rings, however, this failed to reach significance. Placebo and high dose treated eyes showed a nonsignificant change toward increased implicit times across the entire central field (see Figs. 4D, 4F). Although similar results were observed at follow-up, these were also not significant (see Supplementary Fig. S2).

As would be expected, analysis of the differences among rings 1 to 5 showed that relative to the central ring (ring 1) mfERG responses became smaller and faster toward the peripheral rings (see Table 3). This effect was observed in both levodopa/carbidopa and placebo treated eyes.

Retinal Structure – OCT Measures

To study regional differences in retinal structure, retinal thickness was mapped across an 8 × 8 macular grid representing a 12 degrees × 12 degrees square of the visual field (corresponding to the matrix perimetry measures reported above). Neither individual comparisons nor linear mixed effects model results found a significant effect of treatment or time on regional retinal thickness and pRNFL by end-of treatment (see Fig. 5; Table 2) or at follow-up (Supplementary Fig. S3).

The change in retinal thickness among baseline, end-of-treatment, and follow-up are illustrated in Figure 6 and Supplementary Figure S4, respectively. Bland-Altman analysis allowed us to evaluate the agreement of the thickness data between the end of treatment and baseline measures in terms of coefficients of reproducibility (CoRs). All the CoRs were

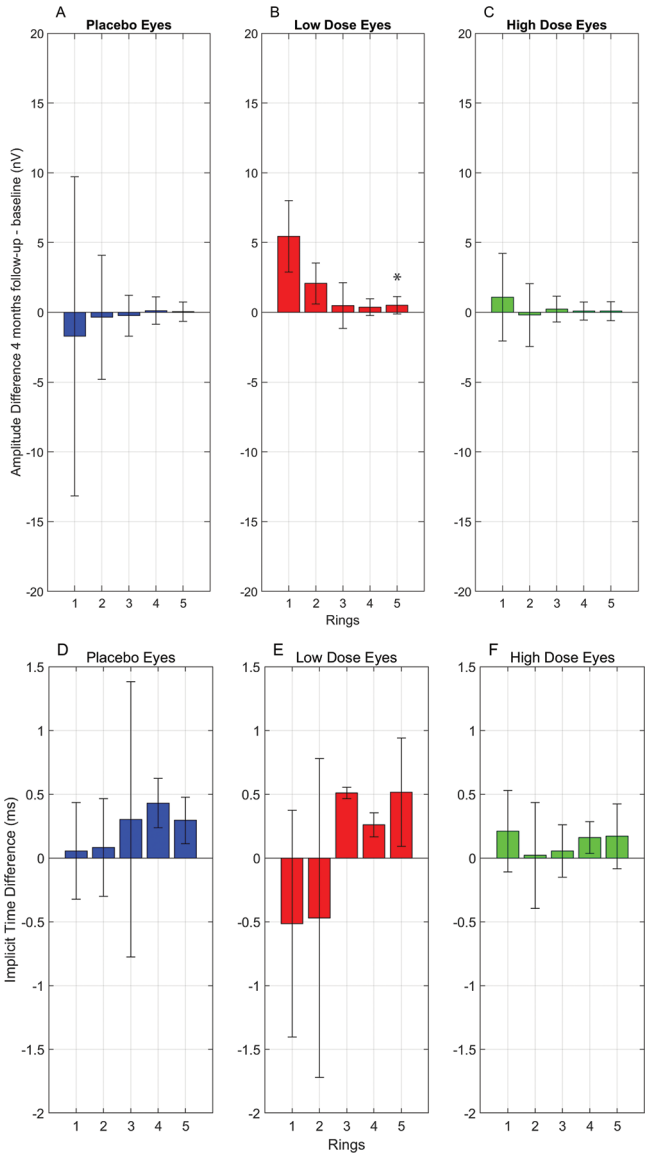

Figure 4. Mean multifocal ERG amplitude (**A**, **B**, **C**) and implicit time (**D**, **E**, **F**) differences (mean ± SD) between end-of-treatment and baseline for placebo treated, low dose treated, and high dose treated eyes, respectively. Implicit times showed increased delays across most rings for all groups, although this did not reach statistical significance. Response amplitudes increased for low and high dose treated eyes but only reached significance for ring 5 in low dose treated eyes (**B**). * Statistical significance from baseline at $P < 0.05$.

very similar at 10.23 ± 3.35 µm (mean ± standard deviation [SD]) against a mean thickness of 301.6 ± 1.29 µm. To investigate if any significant differences in variability were present in the data sets, we applied a linear regression which found no significant change over time or between treatment groups (low dose treated group $P = 0.91$ and high dose treated group $P = 0.74$) relative to placebo treated control eyes. Of note, however, an analysis of regression did suggest a small and statistically significant thinning of the retina

Table 3. Mixed Effects Model Summary for mfERG Response Amplitudes and Delays

Variable	Amplitudes			Implicit Times		
Estimate	(nV)	SE	*t*-Stat	(ms)	SE	*t*-Stat
Reference	44.7	0.84	53.45*	17.8	0.28	62.46*
Right eye	1.8	0.59	2.97*	−0.1	0.20	−0.37
Low dose	0.7	0.78	0.87	0.1	0.27	0.21
High dose	0.2	0.67	0.25	−0.1	0.22	−0.50
Ring 2	−20.6	0.89	−23.01*	−0.9	0.30	−3.10*
Ring 3	−31.1	0.89	−34.78*	−1.3	0.30	−4.22*
Ring 4	−35.7	0.89	−39.94*	−1.3	0.30	−4.24*
Ring 5	−38.0	0.89	−42.49*	−1.1	0.30	−3.52*
End-of-treatment	0.0	0.69	0.03	0.4	0.24	1.52
Follow-up	−0.2	0.69	−0.28	−0.4	0.24	−1.65

Regression coefficients (nV or ms), standard errors (SE), and *t*-statistics (*t*-Stat) for mean reference response and contrast variables. The reference condition represents the mean response of left placebo eyes in ring 1 at the baseline visit. Positive coefficients represent additional multiplicative increases in mfERG amplitude or implicit times.

*Indicates a significant difference to the reference condition at $P < 0.05$. Significant differences in amplitude were observed between the right and left eyes and between rings 2 to 5 and ring 1. Significant differences in implicit times were observed between rings 2 to 5 and ring 1. All other comparisons were not significant.

in low dose treated eyes at the end-of-treatment ($P < 0.05$). A similar response was for low dose treated eyes at 4 months follow-up, but this did not reach statistical significance. Importantly, the above changes were extremely small (<3 microns) and are not classed as clinically significant (i.e. the change is less than 20%[27]).

Pharmacokinetic Results From Rabbits

With regard to systemic distribution, in the 6 hours following topical treatment, levodopa was detectable and quantifiable in all blood samples (treated and control), whereas carbidopa remained undetected (see Table 4). Fifteen minutes following levodopa/carbidopa treatment, there was a small increase in levodopa levels in the blood (1.57% of the total levodopa administered), but this was not reflected in a statistically significant change in blood levodopa levels ($P = 0.101$). At all subsequent time points there was no statistical or discernible change in blood levodopa levels. Despite this small shift in levodopa levels at 15 minutes, there was also no significant change in dopamine levels within the blood in

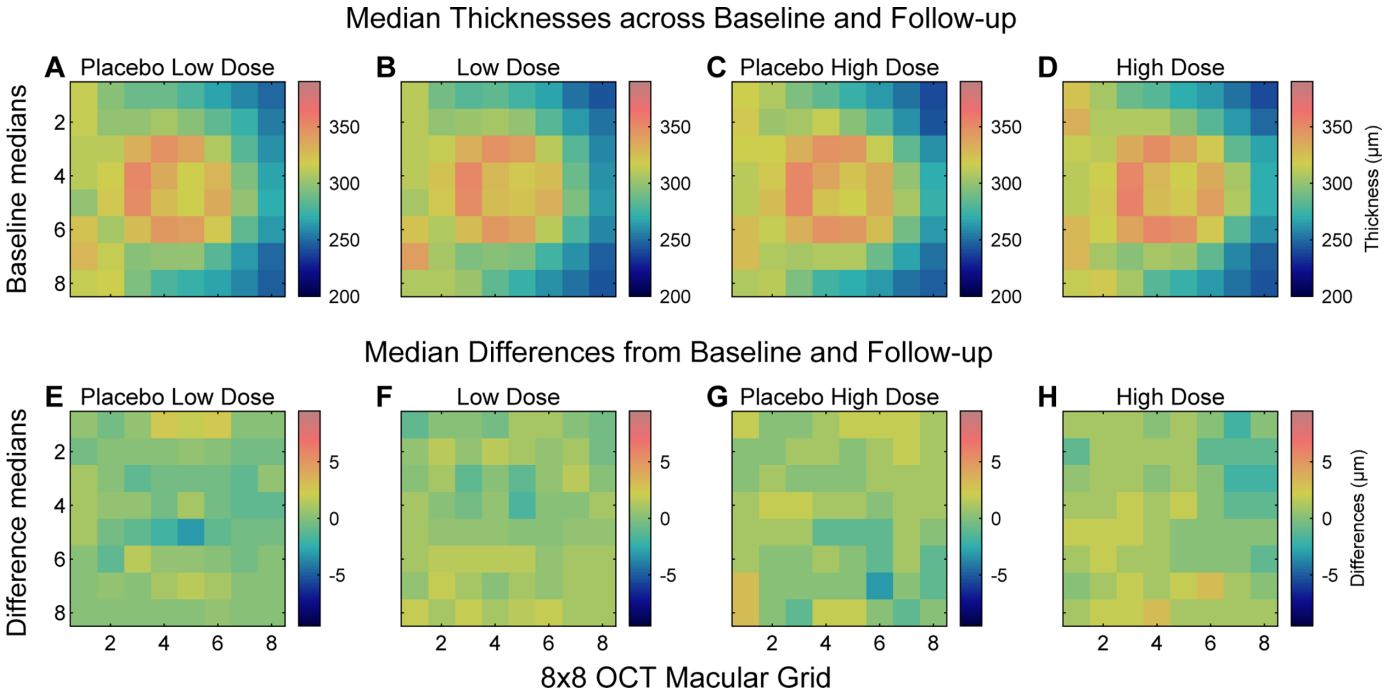

Figure 5. Median regional retinal thickness for placebo (low dose (**A**) and high dose (**C**)) and levodopa/carbidopa (low dose (**B**) and high dose (**D**)) treated eyes. Also displayed are differences between baseline and end-of-treatment, with regard to retinal thickness, for placebo (low dose (**E**) and high dose (**G**)) and levodopa/carbidopa (low dose (**F**) and high dose (**H**)) treated eyes. For all thickness data, right eye grids were flipped to be anatomically equivalent to the left eye so the right eye data could be included. Placebo and treated eyes showed negligible change between visits with no regions reaching statistical significance.

treated animals compared to untreated controls ($P = 0.955$; Table 4). In support of these findings, no detectable change in levodopa or dopamine levels were previously observed within the blood following topical application of levodopa in chickens.[6,9]

With regard to ocular distribution, levodopa/carbidopa eye drops lead to a significant increase in levodopa levels in all ocular tissues measured (Fig. 7). This peak in levodopa concentration was commonly observed 15 to 30 minutes following topical treatment. In all samples, levodopa levels quickly declined following this peak, becoming no different to that seen in untreated control samples by 6 hours after topical treatment. Carbidopa remained undetectable in all samples except for tears (Supplementary Fig. S5), where its concentration peaked 15 minutes following treatment before declining.

Discussion

Based on the reported association between dysregulation in the dopaminergic system and a number of visual disorders,[6–12,28] the administration of levodopa (the precursor molecule to dopamine) has been proposed as a potential pharmacological treatment for these conditions.[6–12,28] Given the critical role dopamine plays in normal vision, this study addressed whether levodopa, when applied as a therapeutic topical eye drop, affects retinal structure or function in healthy young adult men. Our current data showed no significant mean changes in regional measures of retinal function (measured by matrix perimetry and mfERG recordings) or regional retinal thickness (measured by OCT) in healthy young adults. This study did, however, observe a number of small, but statistically significant, changes in mfERG recordings and retinal thickness at a regional level for participants treated with low dose levodopa/carbidopa. It should be noted, however, this small number of changes did not reach clinical significance.

Short-Term Levodopa Treatment Does Not Alter Retinal Activity or Health

Our previous analysis indicated that foveal structure (health) and function (sensitivity) was not affected by short-term (1 month) topical levodopa treatment.[7] However, a reported change in spatial contrast sensitivity in adults following systemic levodopa administration[22] may suggest that non-foveal regions are

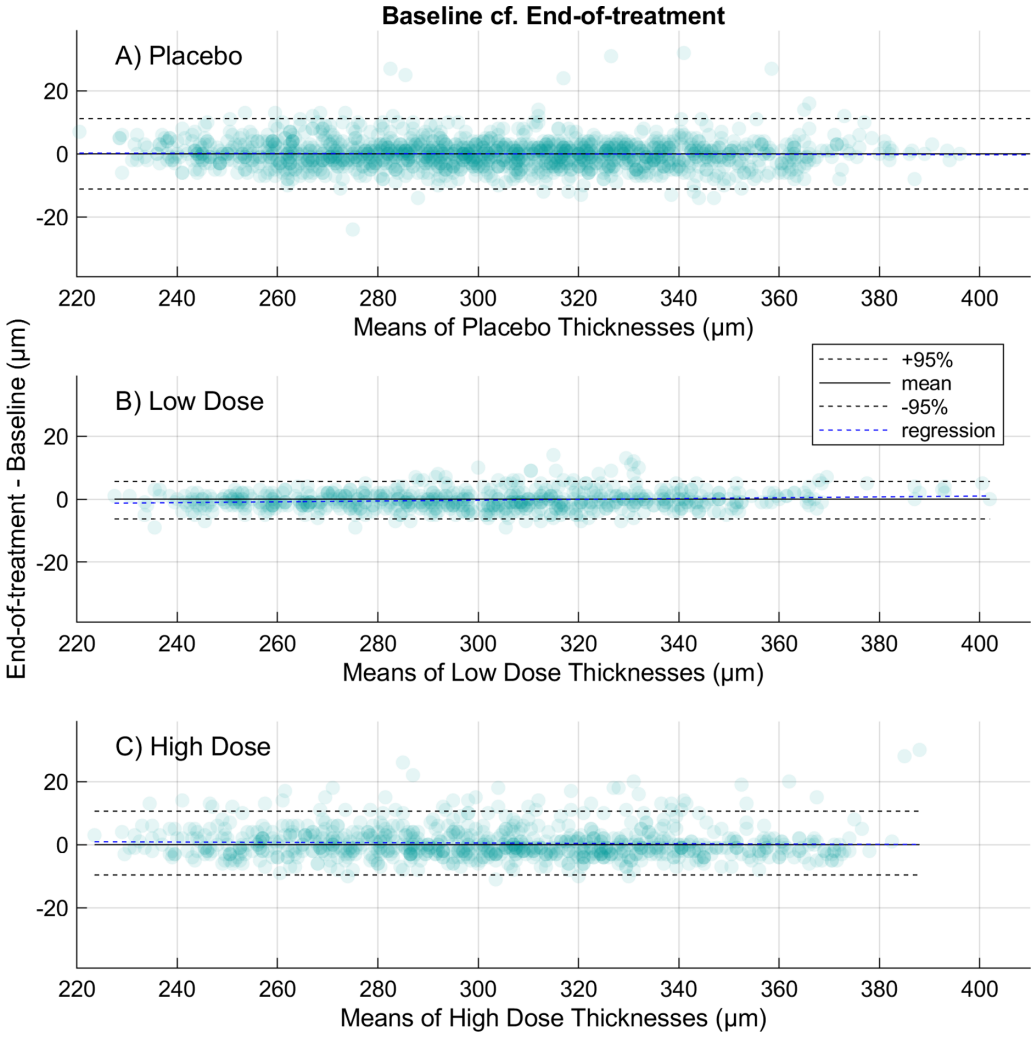

Figure 6. Bland-Altman plots estimate the changes in overall retinal thickness between baseline and end-of-treatment. (**A**) Placebo treated eyes showed positive outliers and a similar variability to high dose treated eyes (**C**). (**B**) Eyes receiving low dose treatment showed the least thickness change from baseline to follow-up with a significant decrease in thickness of approximately 1% of average thickness at follow-up. However, the changes were not clinically significant.

Table 4. Levodopa and Carbidopa Concentrations in Blood Following Eye Drop Treatment

Time After Treatment	Levodopa Detected, Treated-Control; µg/mL	Percent of Initially Applied Drug	Carbidopa Detected, Treated-Control; µg/mL	Dopamine Detected, Treated-Control; µg/mL	Percent of Initially Applied Drug if Converted
15 min	0.045 ± 0.014	1.57%	ND	0.002 ± 0.020	0.09%
30 min	0.015 ± 0.025	0.52%	ND	−0.019 ± 0.0004	−0.86%
1 h	0.020 ± 0.007	0.70%	ND	0.006 ± 0.018	0.27%
2 h	0.005 ± 0.023	0.17%	ND	0.009 ± 0.001	0.41%
4 h	0.010 ± 0.007	0.35%	ND	−0.003 ± 0.028	−0.14%
6 h	0.015 ± 0.011	0.52%	ND	0.0003 ± 0.008	0.01%

ND, not detected.
Data are presented as the means ± standard error of the means.

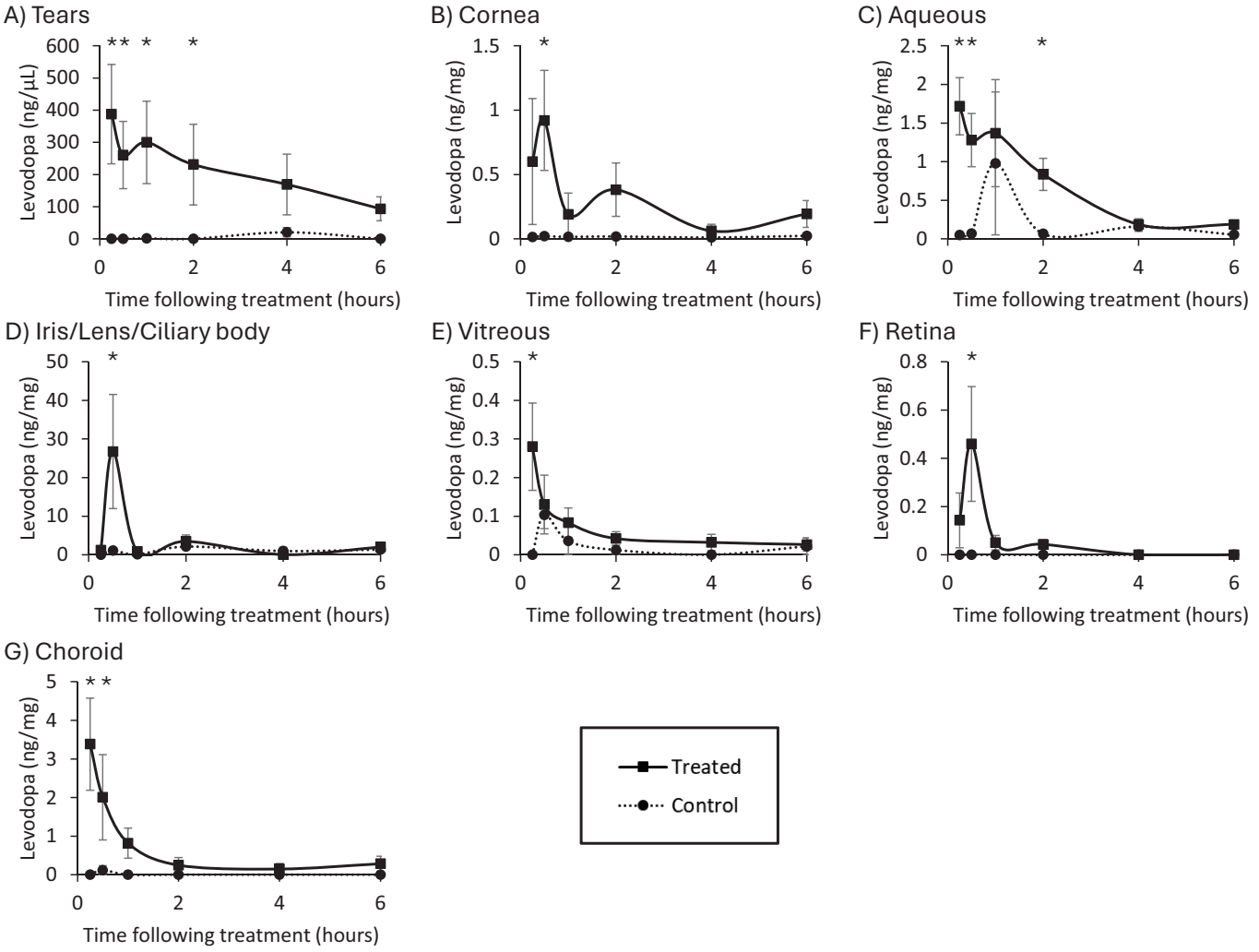

Figure 7. Distribution of levodopa for topical application in (**A**) tears, (**B**) cornea, (**C**) aqueous humor, (**D**) iris/lens/ciliary body, (**E**) vitreous humor, (**F**) retina, and (**G**) choroid samples. New Zealand white rabbits were treated with topical eye drops of levodopa/carbidopa (*solid line*) or a placebo solution (*dotted line*) prior to sample collection and drug quantification using liquid chromatography-tandem mass spectrometry. All data are presented as the mean nanograms of levodopa per milligram of sample ± the standard error of the means. Statistics (*) represent a significant difference ($P < 0.05$) between levodopa/carbidopa treated animals and placebo treated animals.

differently affected. Therefore, using data generated from our phase Ib clinical trial of levodopa eye drops in young healthy adults, the current study assessed whether any localized changes in retinal structure or function were observed out to 30 degrees eccentricity. Like that observed for the fovea, no clinically significant regional changes were observed for measures of perimetry (visual field sensitivity), mfERG (neural communication [electrical activity]) or OCT (retinal thickness [retinal health]) during this expanded analysis. Whereas we did not directly assess spatial or temporal contrast sensitivity, a suggested outcome of dopamine pathway stimulation, one could expect that a change in contrast sensitivity would also lead to a change in perimetry measures (which was not observed). Further, in our previous analysis, no change in high and low contrast visual acuity was seen in response to levodopa treatment,[7] which concurs with a number of other studies.[15,16,20,21]

Although a consistent finding in the literature, it is somewhat surprising that levodopa-induced elevations in retinal dopamine levels do not alter visual acuity and contrast sensitivity in healthy participants. Several factors could explain this lack of effect in our current study. First, topical levodopa treatment may not have led to a change in retinal dopamine levels in our participants. However, animal models (chickens and, in this study, rabbits) have demonstrated that topically applied levodopa penetrates the eye and significantly enhances retinal dopamine levels.[6,9] Second, at each

measure, participants were instructed to administer their eye drops before attending the clinic. Thus, any levodopa induced visual enhancements may have been lost (washed out) at the time of assessment (up to 24 hours following their last administered dose). This is possible, as improvements in visual function have previously been reported to persist for varying periods of time, from 5 hours[29] to up to 1 week following the cessation of oral levodopa treatment.[30] Alternatively, as discussed earlier, increased dopamine levels may only enhance visual function when a disease state is underway, indicating that a "healthy retina" is less susceptible to pharmacological manipulation. Finally, as this trial was short in duration, it could be proposed that an accumulation of levodopa in the system, or an accumulation of its effects, over a chronic treatment period may be required to observe these changes. However, in several studies,[18,20] levodopa induced changes in retinal function have been observed after a single treatment, suggesting an accumulation effect may not be required.

Topical Levodopa May Be Applicable to the Treatment of a Number of Ocular Conditions

As noted, levodopa has been proposed as a treatment for diabetic retinopathy,[11] amblyopia,[10] myopia,[6–9] age-related macular degeneration (AMD), and, to a lesser extent, retinitis pigmentosa.[12] With respect to diabetic retinopathy and amblyopia, levodopa's therapeutic effects appear to be associated with dopaminergic improvements in visual function. Specifically, in animal (mouse) models of diabetic retinopathy, systemic levodopa treatment significantly enhanced retinal function as measured by ERG recordings.[31] In agreement with these findings, a significant increase in visual acuity and retinal function (as assessed by spatial contrast sensitivity and ERG recordings) is observed during systemic levodopa administration in clinical studies of patients showing early signs of diabetic retinopathy.[32] Similarly, oral levodopa treatment has been reported to significantly improve visual acuity, visually evoked potentials, contrast sensitivity, and visual fields in affected eyes of patients with amblyopia.[10,16,30,33–37] In myopia, chicks, mice, and guinea pigs show a strong anti-myopic effect of both topically and systemically administered levodopa, which is associated with a significant increase in retinal dopamine synthesis.[6,8,9,38,39]

Whereas levodopa has also been proposed for the treatment of AMD,[40] this appears to be through a secondary, non-dopaminergic mechanism. Specifically, oral levodopa has been shown to decrease the expression of vascular endothelial growth factor (VEGF) in the retinal pigment epithelium by targeting levodopa specific G-protein coupled receptors.[41] Previous studies[42] have postulated that this decreased expression of VEGF plays a protective role against the pathogenesis of neovascular AMD. Accordingly, patients with AMD undergoing systemic levodopa treatment have demonstrated improved visual outcomes and have been found to require less frequent anti-VEGF treatment, indicating a reduction in disease progression.[40] Similarly, retrospective analyses have found that oral levodopa treatment can delay AMD onset by an average of 8 years in patient populations.[42] This inhibition of VEGF expression could also provide a neuroprotective mechanism for levodopa in the treatment of diabetic retinopathy in addition to its role in enhancing visual function. Such a mechanism could also be postulated to underly the small, nonsignificant statistical change toward a reduction in retinal thickness in the temporal parafovea observed in this study.

Finally, although it has not yet been put forward directly, topical levodopa may also be applicable for the management of retinitis pigmentosa. Specifically, a loss of retinal dopaminergic activity has previously been observed in mouse models of retinal degeneration that resemble retinitis pigmentosa,[43] whereas levodopa may be able to induce similar visual improvements to that seen in the treatment of diabetic retinopathy. Future phase II studies will therefore be well placed to study both efficacy and whether long-term topical levodopa administration would induce changes in retinal structure and function.

Distribution

As discussed earlier, systemic administration of levodopa, as undertaken for neurological disorders, is not appropriate for the treatment of myopia as it would lead to changes in dopaminergic activity not only within the eye, but within the brain and the rest of the body in an otherwise healthy developing child. Topical application of levodopa would maximize the amount of levodopa reaching the presumed target tissue of the retina while minimizing systemic distribution. Accordingly, this study found that, in a non-rodent mammalian model (rabbit), topical eye drops did not induce a detectable change in levodopa, carbidopa, or dopamine levels within the blood at 15 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, or 6 hours post-treatment. This would indicate that what systemic distribution of levodopa/carbidopa (following topical application) does occur does not alter the naturally occurring levels of levodopa or dopamine within the blood.

Within the eye, levodopa was found to peak 15 to 30 minutes following treatment before falling back to control levels in all ocular tissues by 4 hours. This rate of levodopa clearance is in accordance with that previously observed in pharmacokinetic studies in children[29] and adults following systemic distribution.[44] Levodopa was detectable in all ocular samples (tears, cornea, aqueous humor, iris/lens/ciliary body, vitreous humor, retina, and choroid). At its highest penetration point of 15 to 30 minutes, levodopa was most prominent in tears by a factor of 20 relative to the other ocular tissues. Within the eye, levodopa accumulated at its highest amounts within the iris/ciliary body/lens, followed by the choroid, aqueous humor, cornea, retina, and vitreous humor (see Fig. 7). Scleral levels of levodopa or carbidopa were not measured in this study (a limitation that will be addressed in later work).

Interestingly, carbidopa was found not to penetrate the eye and was only detectable in tears. This was somewhat unexpected, given the significant enhancement carbidopa provides with regards to levodopa's anti-myopic efficacy in animals.[6,9] This finding would suggest therefore, that carbidopa enhances levodopa's bioavailability by protecting it from degradation by aromatic l-amino acid decarboxylase within the tears or drainage canal of the eye, but not at its suggested target tissues within the eye. A caveat to this statement was again that carbidopa levels within the sclera were not investigated. However, it would be unlikely for carbidopa to penetrate to the sclera but not be detectable in any other internal ocular sample. The other possibility is that carbidopa was present at levels below the limits of detection of our system, but for this to occur carbidopa would have to be present below 0.3 ng/μL (which would represent less than 0.2% of the drug applied).

Limitations

As discussed previously,[7] due to the short duration of this trial, we are unable to comment on whether long-term levodopa administration could induce changes in retinal structure and function. However, it should be noted that chronic treatment with levodopa eye drops was not associated with retinal changes in chicks or mice.[6] This study was also limited to healthy young adult men, rather than the target populations for myopia and amblyopia treatment (pediatric/adolescents) or AMD and diabetic retinopathy treatment (older adults), which are comprised of both male and female patients. Female participants were not enrolled due to unknown risk of systemic absorption of levodopa on fetal health. We were also limited in our ability to perform additional measures, such as formal testing for potential systemic effects and spatial and temporal contrast sensitivity. This was due to clinical visits already being at their maximum duration owing to the comprehensive list of measures undertaken in this trial.[7] As this study followed a paired-eye design to control for age, ethnicity, environmental, and genetic factors, we are also limited in our ability to comment on potential between eye effects, although comparisons to baseline measures can address this somewhat. As noted previously,[7] planned phase II efficacy studies will address each of these limitations.

Conclusions

In support of our previous work,[7] the expanded analysis in this study observed no clinically significant changes in regional measures of retinal function (measured by matrix perimetry and mfERG recordings) or regional retinal thickness (measured by OCT) in healthy young adult men over a 4-week period or 4 months following treatment cessation.

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* FS and KT contributed equally to this work and should be considered joint first authors.

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