

Increased Choroidal Blood Perfusion Can Inhibit Form Deprivation Myopia in Guinea Pigs

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PURPOSE. In guinea pigs, choroidal thickness (ChT) and choroidal blood perfusion (ChBP) simultaneously decrease in experimental myopia, and both increase during recovery. However, the causal relationship between ChBP and myopia requires further investigation. In this study, we examined the changes of ChBP with three different antimyopia treatments. We also actively increased ChBP to examine the direct effect on myopia development in guinea pigs.

METHODS. Experiment 1: Guinea pigs wore occluders on the right eye for two weeks to induce form-deprivation myopia (FDM). Simultaneously they received daily antimyopia treatments: peribulbar injections of atropine or apomorphine or exposure to intense light. Experiment 2: The vasodilator prazosin was injected daily into the form-deprivation eyes to increase ChBP during the two-week induction of FDM. Other FDM animals received appropriate control treatments. Changes in refraction, axial length, ChBP, ChT, and hypoxia-labeled pimonidazole adducts in the sclera were measured.

RESULTS. The antimyopia treatments atropine, apomorphine, and intense light all significantly inhibited myopia development and the decrease in ChBP. The treatments also reduced scleral hypoxia, as indicated by the decrease in hypoxic signals. Furthermore, actively increasing ChBP with prazosin inhibited the progression of myopia, as well as the increase in axial length and scleral hypoxia.

CONCLUSIONS. Our data strongly indicate that increased ChBP attenuates scleral hypoxia, and thereby inhibits the development of myopia. Thus ChBP may be a promising target for myopia retardation. As such, it can serve as an immediate predictor of myopia development as well as a long-term marker of it.

Keywords: myopia, choroidal blood perfusion, choroidal thickness, guinea pigs

In recent years, the prevalence of myopia has rapidly increased, especially in East and Southeast Asia, where 80% to 90% of young adults have myopia and around 20% have high myopia.^{1,2} High myopia increases the risks of ocular pathologic complications, such as retinal detachment, myopic macular degeneration, and choroidal neovascularization, all of which can cause uncorrectable vision loss.^{3,4} There is no doubt that myopia has become an international public health concern and creates a tremendous global economic burden.^{5,6} Despite the global myopia epidemic, the exact mechanisms underlying the development of myopia are still not fully understood.

Myopia is produced by excessive axial elongation and is characterized by scleral thinning due to a loss of collagen from the extracellular matrix (ECM).^{7,8} Studies of various myopia models have shown that the increase in axial elongation is driven by the remodeling of the scleral ECM.⁹⁻¹² More specifically, our previous study demonstrated that the restructuring of the sclera in myopia was accompanied by large-scale transdifferentiation of scleral fibroblasts into

myofibroblasts.¹³ Importantly, hypoxia-inducible factor-1 α (HIF-1 α) in the sclera has a prominent role in signaling this restructuring, suggesting that a scleral hypoxia-dependent mechanism plays an important role in the underlying myopic development.¹³ However, the mechanism by which scleral hypoxia occurs in the development of myopia still remains unclear.

The choroid, a highly vascularized structure, is positioned between the retina and sclera and provides oxygen, as well as nutrients, to the adjacent structures.^{14,15} It is plausible that scleral hypoxia in experimental myopia could be caused by decreases in choroidal thickness (ChT) and choroidal blood flow, which in turn would cause a decrease in oxygen supply to the sclera.

Previous studies showed that the choroid becomes thinner in form-deprivation myopia (FDM) and in lens-induced myopia, in animal models of myopia such as chicks^{16,17} and guinea pigs.^{18,19} In both models, the thinned choroid becomes thicker after removal of the myopia-inducing stimulus.^{16,20,21} Similarly, many clinical studies have found that

reductions of choroidal blood flow^{22,23} and ChT^{24,25} are associated with high myopia. The close correlation between choroidal blood perfusion (ChBP) and ChT, and their bidirectional association with the development of myopia and the recovery from it, were clearly demonstrated in our previous studies with the guinea pig model.²¹ These correlations provided strong evidence for the involvement of the choroid in myopia development. However, the inference of a causal relationship between ChBP and myopia requires further investigation.

Here, we studied more closely the direct effect of ChBP on the promoting and retardation of experimental myopia in guinea pigs. Form-deprivation is a well-established method of inducing myopia in guinea pigs^{18,21,26} and other experimental models of myopia research.^{27,28} FDM in guinea pigs is accompanied by decreases in ChT and ChBP.²¹ In the current experiments, we first investigated the levels of correlation of ChBP with myopia development and scleral hypoxia, in various experimental settings, including those with antimyopia treatments such as the administration of atropine,²⁹ apomorphine,²⁶ and the exposure to intense light.³⁰ We then explored the impact of increasing ChBP on the development of myopia, by administering the vasodilator prazosin in the form-deprivation guinea pig eyes.

MATERIAL AND METHODS

Animals

This study was approved by the Animal Care and Ethics Committee at the Wenzhou Medical University (Wenzhou, China), and the treatment and care of animals were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Three-week-old pigmented guinea pigs (*Cavia porcellus*, English short-hair stock, tricolor strain, $n = 121$) were randomly assigned to different experimental groups. The animals were reared under a daily 12-hour light/12-hour dark cycle, with lights on at 8:00 AM, in the animal facilities where the room temperature was kept at 25°C. The animals had free access to standard food and were provided with fresh vegetables twice a day.

Experimental Design

The right eye of each guinea pig was treated as described below, whereas the left eye served as the untreated fellow control. The right eye of each animal was covered with a facemask for two weeks to induce monocular FDM.³¹ In certain experiments (described below), during the two weeks of form-deprivation treatment the FDM eyes were treated once per day with specific drugs. In other experiments both the FDM eyes and the corresponding fellow eyes were exposed to intense light treatment. The animals were assigned to groups, and the treatments were administered, as described below.

Experiment 1: To Determine Whether the Underlying Mechanisms of the Known Myopia-controlling Interventions Atropine, Apomorphine, and Intense Light Are Accompanied by Increases in ChBP and the Attenuation of Scleral Hypoxia. FDM was treated with atropine, a nonselective antagonist at muscarinic acetylcholine receptors²⁹ (Experiments 1.1.1 and 1.1.2; $n = 14$), apomorphine, a non-selective antagonist at dopamine receptors²⁶ (Experiments 1.2.1 and 1.2.2;

$n = 18$), or intense light (Experiments 1.3.1 and 1.3.2; $n = 18$). The respective control treatments were normal saline solution ($n = 16$), vitamin C ($n = 18$), and normal light ($n = 13$). Details regarding dose, vehicles, and route of administration are provided below. Baseline refraction and axial length (AL) of all eyes were measured as described below. After two weeks of FDM and simultaneous antimyopia treatment with atropine, apomorphine, or intense light, or the respective control conditions, we measured refraction, AL, ChBP, and ChT (described below). Additionally, the level of induced scleral hypoxia was assessed by immunodetection of injected pimonidazole hydrochloride—a hypoxia-sensitive imidazole that undergoes reduction in hypoxic cells, forming immunodetectable adducts with thiols in proteins, peptides, and amino acids.³²

Experiment 2: To Assess the Impact of Increasing ChBP on the Development of Myopia.

The purpose of Experiment 2 (Experiments 2.1.1 and 2.1.2) was to determine whether increasing ChBP could inhibit the FDM that is associated with scleral hypoxia. For this, the α_1 -adrenergic blocker prazosin ($n = 12$), a vasodilator, was administered (as described below) in only the form-deprivation eyes, to increase choroidal perfusion during the two weeks of form deprivation. The control group received normal saline solution injections ($n = 12$) in only the form-deprivation eyes during the two weeks of form deprivation. For Experiment 2, key parameters, including refraction and AL, were measured in all eyes at baseline and after two weeks of FDM paired with injections. In addition, after the two weeks of treatment, ChBP, ChT, and the hypoxia-labeled pimonidazole adducts in the sclera were measured.

Pharmaceutical Preparation and Administration

Atropine sulfate monohydrate ($\geq 97\%$) (Stanford Chemicals, Eugene, OR, USA) and prazosin (prazosin hydrochloride; Sigma-Aldrich, Corp., St. Louis, MO, USA) were dissolved in normal saline solution. Apomorphine (Tocris, Glasgow, UK) was dissolved in sterilized injection water with 0.01% ascorbic acid (Sigma-Aldrich Corp.) added as an antioxidant. Before peribulbar injection, topical anesthesia was administered with one drop of 0.5% proparacaine hydrochloride (Alcon, Puurs, Belgium) after removal of the facemask. The injection took about 10 seconds, and the facemask used for form deprivation was then reset over the eye. Each drug (100 μ L solution with 1 mg atropine, 750 ng apomorphine, or 383 ng prazosin) was injected using a 26-gauge needle (0.45 \times 16 mm), connected to a 1-mL syringe cannula (Shanghai Kindly Medical Devices Co., Ltd, Shanghai, China), that was gently inserted into the inferior peribulbar space. Form-deprivation right eyes of animals in the control groups were injected with the respective solvent (100 μ L). The right eyes of all animals were injected while the left eyes remained untreated. All injections were performed daily (about 9:00 AM) under dim red light during the two weeks of form deprivation.

Lighting Conditions

The illuminance of normal and intense lighting at the cage floor was approximately 300 lux and 10,000 lux, respectively, provided by fluorescent lamps (Osram Dulux L, 55W/954; Osram Dulux, Munich, Germany). During the 12 hours of light-on, the guinea pigs in the intense light group were

exposed to normal light for three hours, followed by six hours of intense light,³³ and then another three hours of normal light. The animals in the normal light group were raised under normal light for 12 hours.

Optical Coherence Tomography Angiography (OCTA)

OCTA is a novel noninvasive technology that provides depth-resolved visualization of the retinal and choroidal microvasculature without the need for dye injection to identify the motion contrast of blood flow.³⁴ In this study, we acquired B-scans from the Spectralis HRA+OCT (Heidelberg Engineering, Heidelberg, Germany) for analysis of ChBP and ChT. The operational approaches and conditions of the machine were similar to those described previously.²¹ After the standard B-scans were acquired, they were exported in three different formats: a structural OCT image, an OCTA image, and the overlay of structural OCT and OCTA images. On the OCTA images, the yellow signal points indicated the moving red blood cells. From yellow ChBP signal points in the choroid, we calculated the total numbers of movement-positive pixels in the regions of interest, which provided a semiquantitative measure of blood perfusion.²¹ The images were processed by a custom program (MATLAB R2017a; MathWorks, Natick, MA, USA) that simultaneously determined both ChBP and ChT.²¹ Importantly, the guinea pig is a nearly ideal animal model for studying ChBP, as the avascularity of its retina¹⁴ ensures minimal interference from the blood flow signals that are generated by the retinal blood vessels in many other animals.²¹

Biometric Measurements

Refraction in the vertical pupil meridian was measured without cycloplegia using an eccentric infrared photorefractor, as described previously,³⁵ in a dark room. Three readings were recorded for each eye, and the mean was used for statistical analysis.

AL was measured by A-scan ultrasonography (AVISIO Echograph Class I-Type Bat; Quantel Medical, Clermont-Ferrand, France).³⁵ Before the measurement, the cornea was anesthetized with a drop of 0.5% proparacaine hydrochloride. Each eye was measured 10 times, and the average was taken as the final value for the statistical analysis.

Immunofluorescent Labeling

Pimonidazole hydrochloride (Hypoxyprobe, 600 µg, HP3-100 Kit; HPI, Burlington, MA, USA) was injected into the inferior peribulbar space of each eye. About 45 minutes later, the eye was enucleated, and the posterior eyecup was cut, then fixed in 4% paraformaldehyde buffered with 0.1M phosphate buffer at room temperature for 30 minutes and dehydrated in 30% sucrose at 4°C for 24 hours. For cryosectioning, a mounting medium (Neg-50; Thermo Fisher Scientific, Waltham, MA, USA) was used to embed the eyecup, which was then submerged in liquid nitrogen. Sections (12 µm thick) were cut on a cryostat at -20°C, followed with blocking by 10% normal donkey serum, primary antibody rabbit anti-pimonidazole antibody incubation (1:100; PAb2627AP, HPI), and secondary antibody incubation (FITC conjugated donkey anti-rabbit IgG,

1:400; Invitrogen, Waltham, MA, USA), with buffer washes in PBS between steps as needed.

The sections were examined with a confocal microscope (LSM880 META; Carl Zeiss Meditec, Göttingen, Germany), and the intensities of the hypoxia signals, indicated by pimonidazole staining, were analyzed by means of Zeiss ZEN 2.3 software (Carl Zeiss Meditec). The signals were estimated in the sclera at the nasal and temporal sides in each section. These locations were chosen in the second 20 × imaging field areas from either side of the optic nerve (shown red boxes in Fig. 2A). The arithmetic mean intensity of the two scleral locations was taken as the sample value.

Statistical Analysis

All data were verified to be normally distributed, and the descriptive statistics were calculated as means ± standard deviations. Interocular differences (the value for the treated eye minus the fellow eye) of refraction and AL in different groups were compared by repeated measures ANOVA, with groups as factors and with time as the repeated measures. Repeated measures ANOVAs were also used in comparisons of intergroup ChT and ChBP, with groups as factors and with eyes as the repeated measures. For immunofluorescence experiments, paired *t*-tests were used to compare the effects on treated and fellow eyes, and independent *t*-tests were used to compare the effects in the various treatment groups. Bonferroni corrections were applied in post hoc analyses. Values of *P* < 0.05 were considered to be significant. SPSS software (SPSS version 16.0, Chicago, IL, USA) was used for the statistical analysis.

RESULTS

At the beginning of each experiment, there were no significant differences in refraction or AL between the right and left eyes of individual animals, nor between the different groups of drug-treated or intense light-treated eyes. After two weeks of form deprivation treatment, all of the right eyes that simultaneously received either control normal saline solution, vitamin C, or normal light treatments, developed myopia that was associated with longer ALs, thinner ChTs, lower ChBPs, and greater scleral hypoxic signals. In contrast, compared with the corresponding control groups, the drug-treated (atropine, apomorphine, or prazosin) or intense light-treated eyes developed less myopia and had correspondingly shorter ALs, thicker ChTs, higher ChBPs, and lower scleral hypoxic signals.

Experiment 1.1.1: Atropine Inhibited FDM and Attenuated the FDM-Induced Reductions of ChT and ChBP

As determined by repeated measures ANOVA, the main effects on refraction and AL were significant in both treatment groups (FDM+atropine and FDM+normal saline solution groups) and times (baseline and two weeks of FDM paired with injections) (Table 1A). Additionally, the interaction effects of groups and times on refraction and AL were also significant (Table 1A). The interocular difference in refraction between the atropine-injected FDM eye and the fellow eye, -2.30 ± 1.31 diopter (D), was smaller than the difference between the normal saline solution-injected FDM and fellow eyes, -5.86 ± 2.73 D (*P* < 0.001, Fig. 1A).

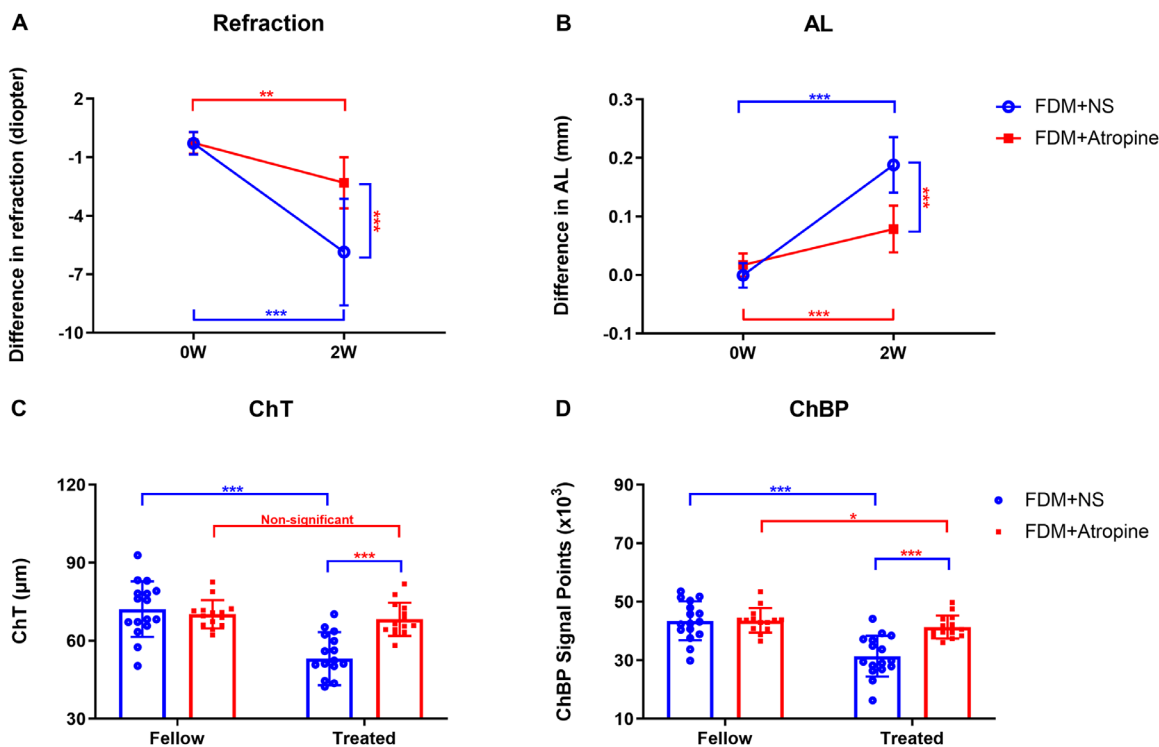


FIGURE 1. Refraction, AL, ChT, and ChBP for the FDM+NS and FDM+Atropine groups. Comparison of the interocular differences in (A) refraction and (B) AL, in the FDM+NS and FDM+Atropine groups, at the beginning (0W) and end (2W) of the treatment period. Comparison of (C) ChT and (D) ChBP, in the FDM+NS and FDM+Atropine groups, at the end of two weeks' treatment. W, weeks; Fellow, eye contralateral to the FDM eye and not treated with either normal saline solution or atropine; Treated, FDM eye treated with either normal saline solution or atropine; NS, normal saline solution. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, repeated-measures ANOVA with Bonferroni correction (A–D).

TABLE 1A. Main and Interaction Effects of FDM+Atropine on Refraction and AL as Determined by Repeated Measures ANOVA

Source	Refraction		AL	
	F _{1,28}	P	F _{1,28}	P
Group	19.721	<0.001	24.113	<0.001
Time	79.828	<0.001	225.041	<0.001
Time * Group	17.280	<0.001	58.286	<0.001

Group, FDM+NS and FDM+Atropine groups; Time, baseline and after two weeks of FDM with simultaneous injections of NS or atropine; NS, normal saline solution.

TABLE 1B. Main and Interaction Effects of FDM+Atropine on ChT and ChBP as Determined by Repeated Measures ANOVA

Source	ChT		ChBP	
	F _{1,28}	P	F _{1,28}	P
Group	4.845	0.036	6.313	0.018
Eye	110.883	<0.001	149.792	<0.001
Eye * Group	73.406	<0.001	70.335	<0.001

Group, FDM+NS and FDM+Atropine groups; Eye, fellow and treated eyes; NS, normal saline solution.

Similarly, the interocular difference in AL between the atropine-injected FDM eye and the fellow eye, 0.08 ± 0.04 mm, was smaller than that between the normal saline solution-injected FDM and fellow eyes, 0.19 ± 0.05 mm ($P < 0.001$, Fig. 1B).

As determined by repeated measures ANOVA, the main effects on ChT and ChBP were significant in both treatment groups (FDM+atropine and FDM+normal saline solution groups) and eyes (the fellow eye and the treated eye) (Table 1B). Additionally, the interaction effects of groups and eyes on both ChT and ChBP were also significant (Table 1B). On the other hand, the ChT of normal saline solution-treated FDM eyes, 53.13 ± 10.18 µm, was thinner than that in untreated fellow eyes, 72.14 ± 10.67 µm ($P < 0.001$, Fig. 1C). Furthermore, like ChT, ChBP in normal saline solution-treated FDM eyes, $31.38 \pm 6.98 \times 10^3$, was lower than that in untreated fellow eyes,

$43.51 \pm 6.67 \times 10^3$ ($P < 0.001$, Fig. 1D). However, the ChT of atropine-treated FDM eyes, 68.27 ± 6.43 µm, was greater than that of normal saline solution-treated FDM eyes, 53.13 ± 10.18 µm ($P < 0.001$, Fig. 1C), essentially rescuing them from the FDM-induced thinning. Similarly, the ChBP of atropine-treated FDM eyes, $41.35 \pm 3.95 \times 10^3$, was higher than that of normal saline solution-treated FDM eyes, $31.38 \pm 6.98 \times 10^3$ ($P < 0.001$, Fig. 1D).

Experiment 1.1.2: Atropine Attenuated the Scleral Hypoxia Induced by FDM

The hypoxic responses in the sclera, indicated by pimonidazole staining, were estimated at the nasal and temporal sides in each section (shown as red boxes in Fig. 2A). Scleral pimonidazole intensities in the nasal and temporal regions were significantly correlated ($R = 0.87$, $P < 0.001$; Fig. 2B),

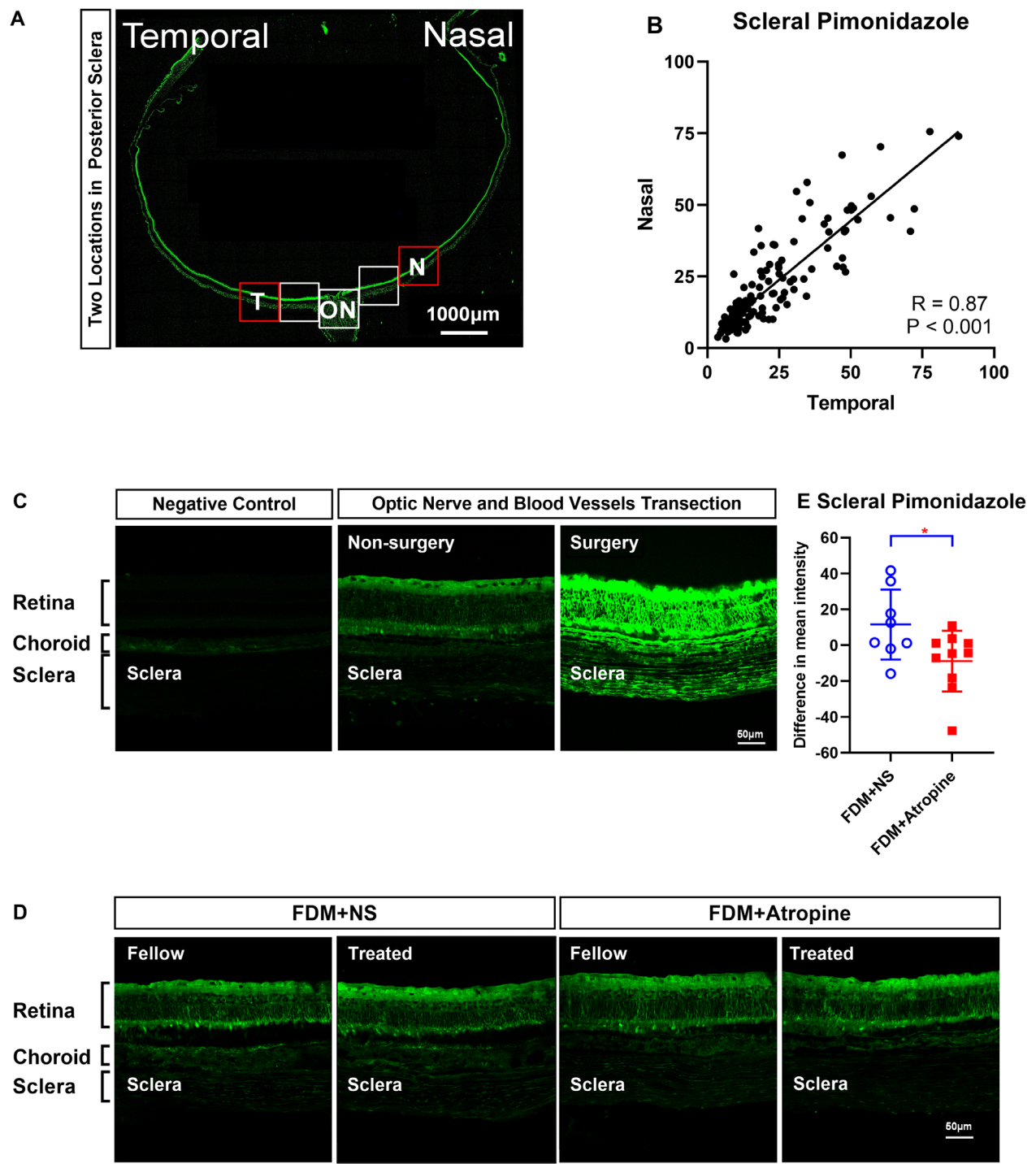


FIGURE 2. Hypoxia-dependent scleral binding of pimonidazole for the FDM+NS and FDM+Atropine groups. **(A)** Frozen sections were assayed for scleral pimonidazole content. The two boxed areas outlined in red show the nasal and temporal locations that were assayed. **(B)** Correlation of scleral pimonidazole content in the nasal and temporal regions. **(C)** The negative and positive controls for pimonidazole labeling. The negative control was a section incubated with only antibody diluent as replacement for the primary antibody, whereas the positive control (the surgery eye) showed the maximum intensity of pimonidazole labeling for hypoxia after transection of the optic nerve and its surrounding blood vessels. The nonsurgery eye was contralateral to the surgery eye. **(D, E)** Comparison of pimonidazole content, in the scleras of the FDM+NS and FDM+Atropine groups, at the end of two weeks' treatment. N, nasal; T, temporal; ON, optic nerve; Fellow, eye contralateral to the FDM eye and not treated with either normal saline solution or atropine; Treated, FDM eye treated with either normal saline solution or atropine; NS, normal saline solution. * $P < 0.05$, independent t -test (E).

and there were no significant differences in fluorescence intensity between the nasal and temporal locations (Nasal: 20.47 ± 15.85 , vs. Temporal: 20.87 ± 16.78 , $P = 0.45$). Addi-

tionally, there was very little pimonidazole staining in the sclera of the negative control, which was incubated with only antibody diluent as its primary antibody. Compared

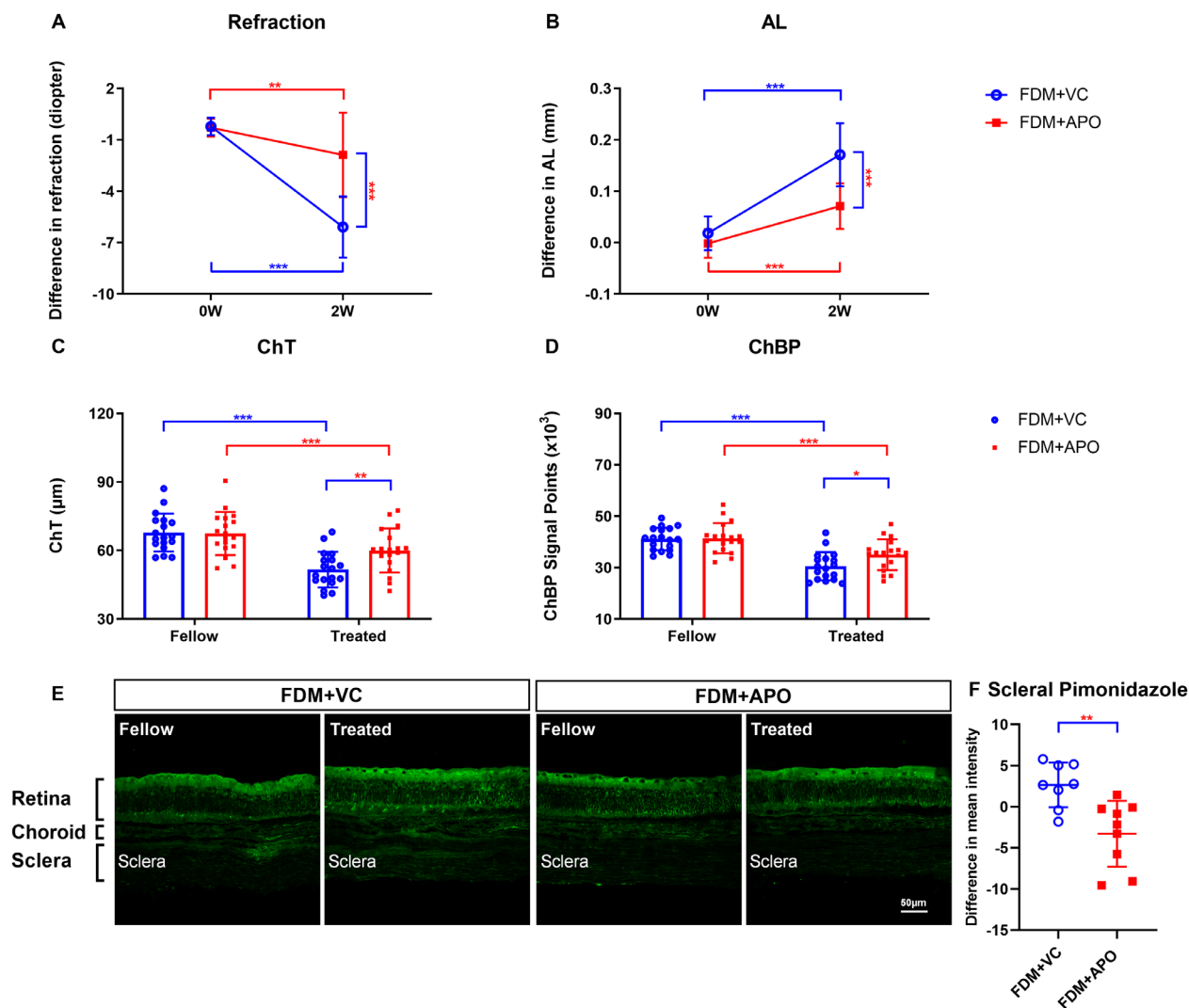


FIGURE 3. Refraction, AL, ChT, ChBP, and hypoxia-dependent scleral labeling with pimonidazole for the FDM+VC and FDM+APO groups. Comparison of the interocular differences in (A) refraction and (B) AL in the FDM+VC and FDM+APO groups, at the beginning (0W) and end (2W) of the treatment period. Comparison of (C) ChT and (D) ChBP, in the FDM+VC and FDM+APO groups, at the end of two weeks' treatment. (E, F) Comparison of pimonidazole labeling in the scleras of the FDM+VC and FDM+APO groups, at the end of two weeks' treatment. W, weeks; Fellow, eye contralateral to the FDM eye and not treated with either vitamin C or apomorphine; Treated, FDM-eye treated with either vitamin C or apomorphine; APO, apomorphine; VC, vitamin C. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, repeated-measures ANOVA with Bonferroni correction (A–D) and independent t -test (F).

with the negative control, the positive control (the surgery eye) showed the maximum intensity of the hypoxic signals after transection of the optic nerve and its surrounding blood vessels.

The intensity of hypoxic signals in the scleras of form-deprivation eyes, as indicated by pimonidazole labeling, was significantly greater in the scleras of eyes treated with either control normal saline, vitamin C, or normal light treatments ($P = 0.038$, $P = 0.028$, $P = 0.048$, respectively, Figs. 2D, 3E, 4E, 5E) than in the scleras of the corresponding fellow eyes. The increased expression of hypoxic signals was completely suppressed by atropine injection. In FDM eyes, atropine induced a large difference in scleral hypoxic signal expression between the treated and fellow eyes, -8.91 ± 16.99 , compared to normal saline solution treatment, 11.58 ± 19.53 ($P < 0.05$, Fig. 2E).

Experiment 1.2.1: Apomorphine Blocked FDM and Attenuated the Reductions of ChT and ChBP

As determined by repeated measures ANOVA, the main effects on refraction and AL were significant in both treatment groups (FDM+apomorphine and FDM+vitamin C groups) and times (baseline and two weeks of FDM paired with injections) (Table 2A). Additionally, the interaction effects of groups and times on both refraction and AL were also significant (Table 2A). In FDM eyes, the interocular difference in refraction following two weeks of daily peribulbar apomorphine injections, -1.88 ± 2.46 D, were smaller compared to those in vehicle-vitamin C-treated FDM eyes, -6.09 ± 1.78 D ($P < 0.001$, Fig. 3A). In parallel with refractive changes, the interocular difference in AL after apomorphine injections, 0.07 ± 0.04 mm, was smaller than

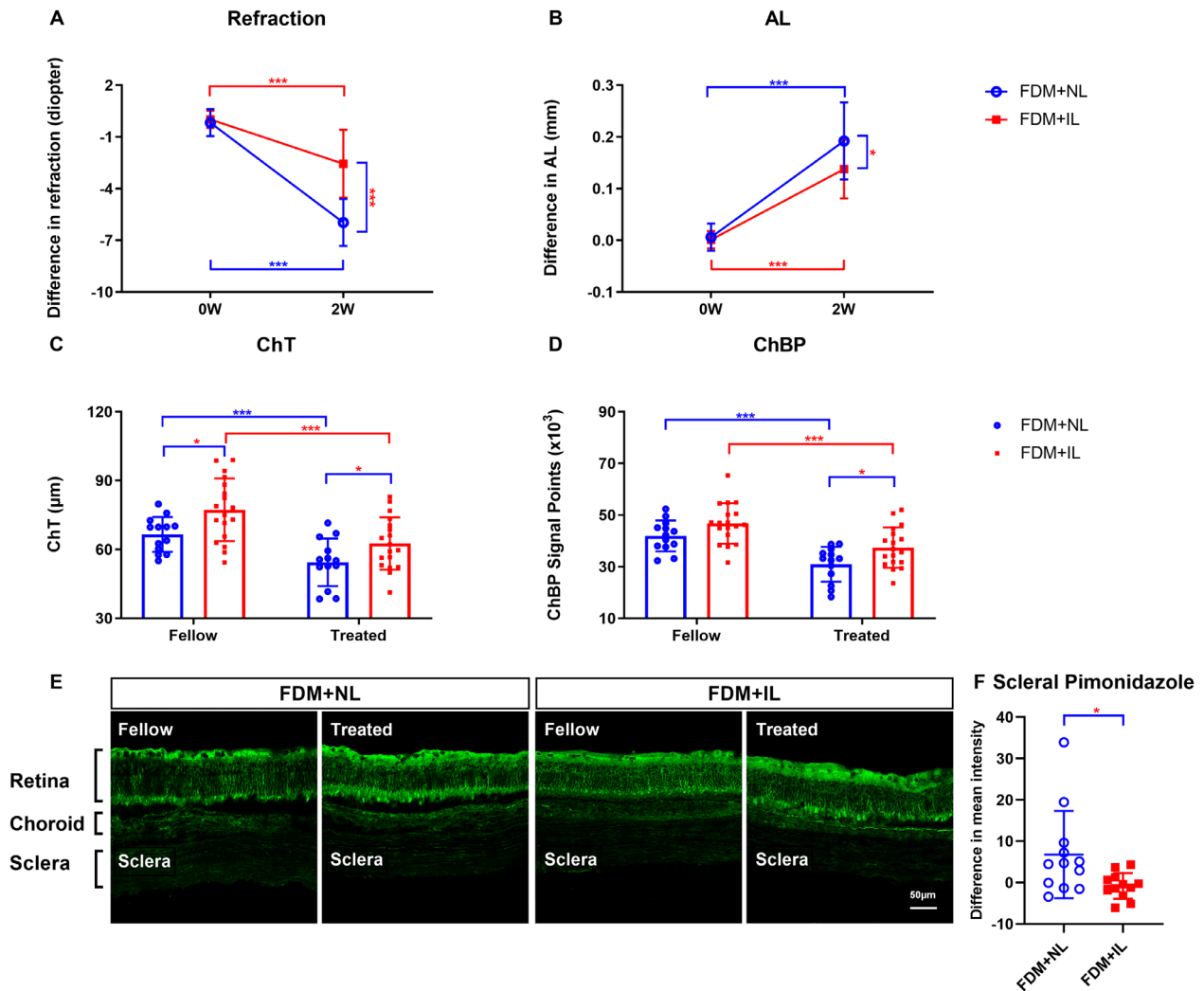


FIGURE 4. Refraction, AL, ChT, ChBP, and hypoxia-dependent scleral labeling with pimonidazole, in the FDM+NL and FDM+IL groups. Comparison of the interocular differences in (A) refraction and (B) AL in the FDM+NL and FDM+IL groups, at the beginning (0W) and end (2W) of the treatment period. Comparison of the changes of (C) ChT and (D) ChBP in the FDM+NL and FDM+IL groups, at the end of two weeks' treatment. (E, F) Comparison of labeling with pimonidazole in scleras in the FDM+NL and FDM+IL groups, at the end of two weeks' treatment. W, weeks; Fellow, eye contralateral to the FDM eye and treated with either normal light or intense light; Treated, FDM-eye treated with either normal light or intense light; IL, intense light; NL, normal light. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, repeated-measures ANOVA with Bonferroni correction (A–D) and independent t -test (F).

TABLE 2A. Main and Interaction Effects of FDM+APO on Refraction and AL as Determined by Repeated Measures ANOVA

Source	Refraction		AL	
	F _{1,34}	P	F _{1,34}	P
Group	28.467	<0.001	39.277	<0.001
Time	114.974	<0.001	106.979	<0.001
Time * Group	37.696	<0.001	13.458	<0.001

Group, FDM+VC and FDM+APO groups; Time, baseline and after two weeks of FDM with simultaneous injections of VC or APO; APO, apomorphine; VC, vitamin C.

TABLE 2B. Main and Interaction Effects of FDM+APO on ChT and ChBP as Determined by Repeated Measures ANOVA

Source	ChT		ChBP	
	F _{1,34}	P	F _{1,34}	P
Group	2.009	0.165	1.993	0.167
Eye	180.404	<0.001	167.693	<0.001
Eye * Group	24.214	<0.001	10.338	0.003

Group, FDM+VC and FDM+APO groups; Eye, fellow and treated eyes; APO, apomorphine; VC, vitamin C.

with the vitamin C injections, 0.17 ± 0.06 mm ($P < 0.001$, Fig. 3B).

As determined by repeated measures ANOVA, the main effects on ChT and ChBP were significant in eyes (fellow and treated eyes) (Table 2B). Additionally, the interaction effects

of group and eye on both ChT and ChBP were also significant (Table 2B). On the other hand, in the FDM+vitamin C group, ChT in the fellow eye, 67.85 ± 8.28 μ m, was greater than in the vitamin C–treated FDM eye, 51.64 ± 7.79 μ m ($P < 0.001$, Fig. 3C). Similarly, ChBP in the fellow

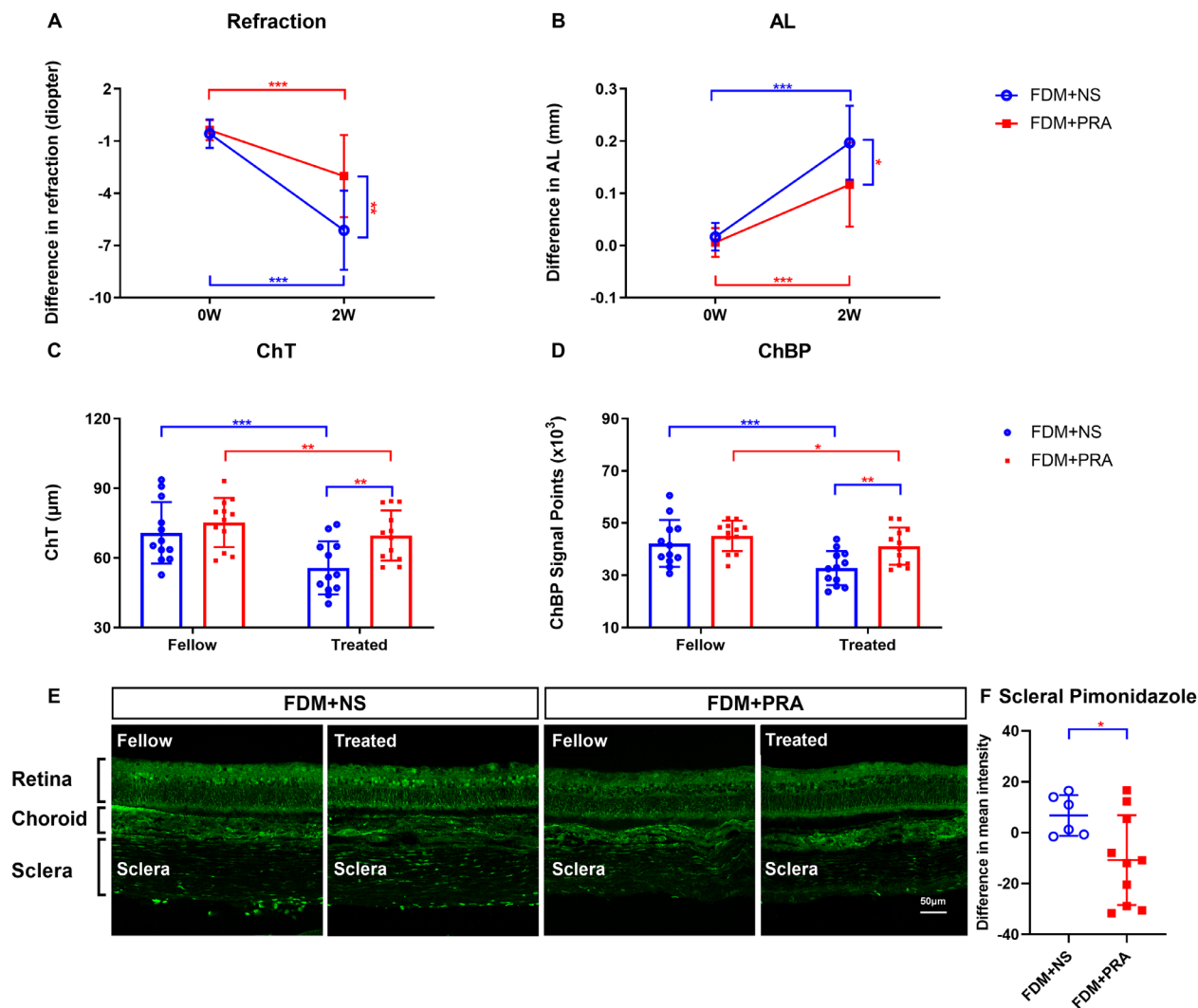


FIGURE 5. Refraction, AL, ChT, ChBP, and hypoxia-dependent scleral labeling with pimonidazole in the FDM+NS and FDM+PRA groups. Comparison of the interocular differences in refraction (**A**) and AL (**B**) in the FDM+NS group and FDM+PRA group, at the beginning (0W) and end (2W) of the treatment period. Comparison of the changes of (**C**) ChT and (**D**) ChBP in FDM+NS and FDM+PRA groups, at the end of two weeks' treatment. (**E, F**) Comparison of pimonidazole labeling in the FDM+NS and FDM+PRA groups, at the end of two weeks' treatment. W, weeks; Fellow, eye contralateral to the FDM eye and not treated with either normal saline solution or prazosin; Treated, FDM-eye treated with either normal saline solution or prazosin; PRA, prazosin; NS, normal saline solution. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, repeated-measures ANOVA with Bonferroni correction (**A–D**) and independent *t*-test (**F**).

eye, $41.15 \pm 4.38 \times 10^3$, was greater than in the vitamin C-treated FDM eye, $30.51 \pm 5.50 \times 10^3$ ($P < 0.001$, Fig. 3D). However, ChT of FDM eyes treated with apomorphine, $59.96 \pm 9.64 \mu\text{m}$, was greater than that in vitamin C-treated FDM eyes, $51.64 \pm 7.79 \mu\text{m}$ ($P < 0.01$, Fig. 3C), and ChBP of FDM eyes treated with apomorphine, $35.03 \pm 5.99 \times 10^3$, was greater than that of vitamin C-treated FDM eyes, $30.51 \pm 5.50 \times 10^3$ ($P < 0.05$, Fig. 3D).

Experiment 1.2.2: Apomorphine Attenuated the Scleral Hypoxia Induced by FDM

There was a significant increase in intensity of the hypoxic signals as shown by pimonidazole labeling in the scleras of form-deprivation eyes (Fig. 3E), compared to that in fellow eyes, and this increase was strongly inhibited by two weeks of apomorphine injections. For the FDM+vitamin C group,

the difference between hypoxia-labeling in the vitamin C-treated FDM eyes and the fellow eyes, 2.65 ± 2.71 , was greater than that between the FDM+apomorphine eyes and the fellow eyes, -3.29 ± 3.99 ($P < 0.01$, Fig. 3F).

Experiment 1.3.1: Intense Light Suppressed FDM and Attenuated the Thinning of ChT and Reduction of ChBP Induced by FDM

As determined by repeated measures ANOVA, the main effects on refraction and AL were significant in both treatment groups (FDM+intense light and FDM+normal light groups) and time (baseline and two weeks of FDM with simultaneous treatment with intense light or normal light) (Table 3A). Additionally, the interaction effects of group and time were also significant for refraction (Table 3A). The interocular refractive difference induced by FDM,

TABLE 3A. Main and Interaction Effects of FDM+IL on Refraction and AL as Determined by Repeated Measures ANOVA

Source	Refraction		AL	
	F _{1,29}	P	F _{1,29}	P
Group	28.228	<0.001	6.735	0.015
Time	154.137	<0.001	149.387	<0.001
Time * Group	22.864	<0.001	3.511	0.071

Group, FDM+NL and FDM+IL groups; Time, baseline and after two weeks of FDM with simultaneous treatment with NL or IL; NL, normal light; IL, intense light.

TABLE 3B. Main and Interaction Effects of FDM+IL on ChT and ChBP as Determined by Repeated Measures ANOVA

Source	ChT		ChBP	
	F _{1,29}	P	F _{1,29}	P
Group	6.309	0.018	5.484	0.026
Eye	69.688	<0.001	89.708	<0.001
Eye * Group	0.610	0.441	0.594	0.447

Group, FDM+NL and FDM+IL groups; Eye, fellow and treated eyes; NL, normal light; IL, intense light.

-5.96 ± 1.36 D, was suppressed by two weeks of intense (10,000 lux) light treatment, -2.56 ± 1.97 D ($P < 0.001$, Fig. 4A). The changes in AL elongation induced by intense light were consistent with those in refraction; thus, the interocular difference in elongation induced by FDM, 0.19 ± 0.07 mm, was suppressed by the intense light, 0.14 ± 0.06 mm ($P < 0.05$, Fig. 4B).

As determined by repeated measures ANOVA, the main effects on ChT and ChBP were significant in both treatment groups (FDM+intense light and FDM+normal light groups) and eyes (the fellow eye and the treated eye) (Table 3B). However, there were no significant differences in interaction effects of groups and eyes on either ChT or ChBP (Table 3B). In the FDM+normal light group, ChT in the normal light-treated FDM eyes, 54.39 ± 10.34 μ m, was less than in the fellow eyes, 66.54 ± 7.59 μ m ($P < 0.001$, Fig. 4C). Similarly, ChBP in the normal light-treated FDM eyes, $30.97 \pm 6.74 \times 10^3$, was lower than in the fellow eyes, $41.96 \pm 5.96 \times 10^3$ ($P < 0.001$, Fig. 4D). In contrast, the ChT of FDM eyes exposed to intense light, 62.61 ± 11.39 μ m, was greater than that in normal light-treated FDM eyes, 54.39 ± 10.34 μ m ($P < 0.05$, Fig. 4C). Similarly, the ChBP of FDM eyes exposed to intense light, $37.44 \pm 7.78 \times 10^3$, was greater than that of normal light-treated FDM eyes, $30.97 \pm 6.74 \times 10^3$ ($P < 0.05$, Fig. 4D). Furthermore, intense light affected ChT and ChBP in the fellow eyes; ChT in the fellow eyes of intense light-treated FDM animals, 77.26 ± 13.65 μ m, was greater than that in fellow eyes of normal-light FDM animals, 66.54 ± 7.59 μ m ($P < 0.05$, Fig. 4C). In addition, ChBP was significantly higher in the fellow eyes of the intense-light group than in those of the normal-light group.

Experiment 1.3.2: Intense Light Inhibited the Scleral Hypoxia Induced by FDM

Intense light for two weeks reduced the amplitude of the hypoxia signals indicated by pimonidazole labeling in form-deprivation scleras, more than did normal light (Fig. 4E). Furthermore, the difference in hypoxia-labeling between the FDM+normal light eyes and their fellow eyes 6.76 ± 10.52 ,

TABLE 4A. Main and Interaction Effects of FDM+PRA on Refraction and AL as Determined by Repeated Measures ANOVA

Source	Refraction		AL	
	F _{1,22}	P	F _{1,22}	P
Group	10.580	0.004	5.676	0.026
Time	73.141	<0.001	120.454	<0.001
Time * Group	9.219	0.006	6.813	0.016

Group, FDM+NS and FDM+PRA groups; Time, baseline and after two weeks of FDM with simultaneous injections of PRA or NS; PRA, prazosin; NS, normal saline solution.

TABLE 4B. Main and Interaction Effects of FDM+PRA on ChT and ChBP as Determined by Repeated Measures ANOVA

Source	ChT		ChBP	
	F _{1,22}	P	F _{1,22}	P
Group	4.130	0.054	4.178	0.053
Eye	58.764	<0.001	45.949	<0.001
Eye * Group	12.246	0.002	7.867	0.010

Group, FDM+NS and FDM+PRA groups; Eye, fellow and treated eyes; PRA, prazosin; NS, normal saline solution.

was greater than that between the FDM+intense light eyes and their fellow eyes, -0.80 ± 3.10 ($P < 0.05$, Fig. 4F).

Experiment 2.1.1: Prazosin Increased ChBP and ChT and Inhibited FDM

As determined by repeated measures ANOVA, the main effects on refraction and AL were significant in both treatment groups (FDM+prazosin and FDM+normal saline solution groups) and time (baseline and two weeks of FDM paired with injections) (Table 4A). Additionally, the interaction effects of group and time on refraction and AL were also significant (Table 4A). In unilaterally form-deprivation animals, the interocular difference in refractive error after two weeks of daily prazosin treatment, -3.02 ± 2.36 D, was smaller than in form-deprivation animals treated with normal saline solution, -6.13 ± 2.27 D ($P < 0.01$, Fig. 5A). The declines in myopic refractive error were consistent with suppression of the induced increase in AL by prazosin. The interocular difference in AL in form-deprivation animals treated with prazosin, 0.12 ± 0.08 mm, was less than that in normal saline solution-treated eyes, 0.20 ± 0.07 mm ($P < 0.05$, Fig. 5B).

As determined by repeated measures ANOVA, the main effects of eyes (fellow and treated eyes) on both ChT and ChBP were significant, as were the interaction effects of groups and eyes on both ChT and ChBP (Table 4B). In contrast, ChT in prazosin-treated FDM eyes, 69.63 ± 10.78 μ m, was greater than ChT in normal saline solution-treated FDM eyes, 55.71 ± 11.45 μ m ($P < 0.01$, Fig. 5C). Similarly, ChBP in prazosin-treated FDM eyes, $41.18 \pm 7.13 \times 10^3$, was greater than ChBP in normal saline solution-treated FDM eyes, $32.75 \pm 6.53 \times 10^3$ ($P < 0.01$, Fig. 5D). Comparisons between fellow eyes and FDM eyes that received either normal saline solution or prazosin injections again showed the effectiveness of prazosin. For normal saline solution-injected FDM eyes, ChT was significantly thicker in the fellow eyes, 70.79 ± 13.25 μ m, than in the normal saline solution-injected FDM eyes, 55.71 ± 11.45 μ m ($P < 0.001$, Fig. 5C). In agreement with changes in ChT,

the ChBP in fellow eyes, $42.18 \pm 8.98 \times 10^3$, was greater than in normal saline solution-treated FDM eyes, $32.75 \pm 6.53 \times 10^3$, ($P < 0.001$, Fig. 5D). Findings in prazosin-treated eyes were similar; ChT was greater in fellow eyes, $75.26 \pm 10.58 \mu\text{m}$, than in prazosin-injected FDM eyes, $69.63 \pm 10.78 \mu\text{m}$ ($P < 0.01$, Fig. 5C), and ChBP in the fellow eyes of prazosin-treated FDM animals, $45.09 \pm 5.80 \times 10^3$, was greater than in the prazosin-treated FDM eyes, $41.18 \pm 7.13 \times 10^3$ ($P < 0.05$, Fig. 5D).

Experiment 2.1.2: Prazosin Inhibited Scleral Hypoxia Induced by FDM

The intensity of scleral hypoxia signals, as shown by pimonidazole staining, was greater in normal saline solution-treated form-deprivation eyes than in corresponding fellow eyes. In contrast, prazosin completely blocked the FDM-induced increase in hypoxia signals (Fig. 5E); the interocular difference in intensity of scleral hypoxia signals in the FDM+normal saline solution group was 6.73 ± 8.01 , but in the prazosin-treated group it was -10.80 ± 17.63 ($P < 0.05$, Fig. 5F).

DISCUSSION

The Inhibition of FDM in Guinea Pigs Could Be Mediated, at Least in Part, by Increased ChBP

In this study, we found that three different antimyopia treatments, that is, the nonselective muscarinic antagonist atropine, the nonselective dopamine receptor agonist apomorphine, and intense light, all significantly inhibited the reduction of ChBP and intensity of hypoxia-dependent labeling of the sclera by pimonidazole, which were induced by FDM. The consistent vasodilating effects of muscarinic antagonists and dopamine agonists were also reported in rats,³⁶ rabbits,³⁷ and humans.³⁸

Our key finding is that, after administration of the α_1 -adrenergic blocker prazosin (a vasodilator) to increase ChBP, the myopic shift in refraction and excessive axial elongation in form-deprivation guinea pig eyes were inhibited, and scleral hypoxia was attenuated. Previous work also has found that nitric oxide (NO, a vasodilator) prevented FDM in chicks³⁹ and NO has been shown to increase choroidal blood flow in cats.⁴⁰ The co-occurrence of increases in ChT and ChBP, with reduction in FDM, is consistent with a causal linkage between the two factors. However, the evidence for this connection is not decisive. The α_1 -adrenoceptors were also expressed in retinal tissues.⁴¹ Some animal studies found that prazosin decreased burst firing of dopamine neurons⁴² and inhibited dopamine release.⁴³ Based on these results, prazosin might also act in the retina and decrease dopamine release. However, previous work found that retinal dopamine release²⁶ and ChBP²¹ are decreased in form-deprivation eyes. Therefore, if prazosin were to affect FDM by decreasing retinal dopamine release, it should cause myopia to develop; but this prediction was contradicted by our experimental results. We found that prazosin (the α_1 -adrenergic blocker) increased ChBP and inhibited form-deprivation myopia, indicating that inhibition of dopamine release is not responsible for the main effects of prazosin in preventing myopia development. We also found that the changes of ChBP were closely and positively associated with changes of ChT. This is consistent with previous studies showing that both ChBP and ChT

increased in response to systemic sildenafil,⁴⁴ a vasodilator, and decreased after intravitreal bevacizumab injection,⁴⁵ an antiangiogenic drug. These findings clearly indicate that the changes in ChBP could lead to the changes in ChT. Further experiments remain to determine the cause-and-effect relationship between ChBP and ChT and whether either one of them is responsible for inhibiting scleral enlargement in FDM.

The oxygen tension of the choroidal circulation is very high, with an arteriovenous oxygen difference of only 3%, which is critical in meeting the oxygen demands of the underlying retina and overlying sclera.¹⁵ As a result, both the retina and the sclera are exposed to a relatively hypoxic environment when the choroidal blood flow is significantly decreased. Consistent with this, we previously found that there was a bidirectional relationship between the scleral HIF-1 α expression and the development of myopia, that is, scleral hypoxia was increased in myopia development and was reduced when FDM was inhibited. It is plausible that the HIF-1 α signaling pathway in the sclera played an essential role in myopia development.¹³

We previously found, in guinea pigs, that ChBP and ChT simultaneously decreased in experimental myopia and increased during recovery.²¹ Consistent with our findings, others have reported that changes of ChBP^{22,23} and ChT^{24,25} were strongly correlated with the development of myopia in clinical settings. Additionally, previous studies showed that in humans, a 6-D accommodation stimulus that produced a myopia shift was associated with a reduced ChT.^{46,47}

All of these results are consistent with the hypothesis that increased ChBP attenuates scleral hypoxia and thereby inhibits the development of myopia. Further experiments are needed to clarify the exact mechanisms of interaction between the choroid and the sclera.

ChBP Could Be a Common Pathway for Myopia Control Underlying Apomorphine and Atropine Treatments as well as Intense Light Exposure

Consistent with our study, many animal experiments found that apomorphine, a nonselective dopamine receptor agonist, and atropine, a non-selective muscarinic receptor antagonist, inhibit FDM in (e.g.) guinea pigs,^{26,29} mice,^{48,49} and chickens.^{28,50} Similarly, intense light contributes to myopia inhibition in chicks,⁵¹ mice,²⁷ and rhesus monkeys.⁵² In agreement with animal experiments, clinical studies also found that outdoor exposure is a strong protective factor against myopia.^{53,54} Although atropine can slow myopia progression in humans,⁵⁵ the exact mechanisms by which it, apomorphine, or intense light suppress myopia progression are unknown.

Previous studies suggested a retinal mechanism through which atropine and apomorphine worked as myopia-inhibiting treatments. More specifically, dopamine plays a critical role in mediating control of myopia development, and dopamine receptors are expressed in the retina.⁵⁶ Additionally, the inhibition of myopia by apomorphine is mediated through dopamine signaling in the retina.^{26,48,56} Likewise, acetylcholine receptors are expressed in both the retina and the choroid. It has been proposed that the antimyopia effects of atropine are mediated through cholinergic-, α_{2a} -adrenergic-, and gamma aminobutyric acid signaling in the retina,⁵⁷⁻⁵⁹ but the site and mechanism of this action remain uncertain and open to debate.

In this study, it is worth noting that apomorphine and atropine both attenuated the reduction of ChBP by FDM while blocking myopia progression. Reitsamer et al.⁵⁷ showed that dopamine can cause choroidal vasodilation in rabbits, and atropine increases choroidal thickness in chickens⁶⁰ and humans.⁶¹ Based on these results, a plausible common pathway for inhibition of myopia by apomorphine and atropine could be that scleral hypoxia is attenuated by the choroidal vasodilation caused by both agents.

Similarly, we found that intense light increased both choroidal thickness and blood perfusion while preventing myopia in the form-deprivation eyes, and that it also increased ChT in the fellow eyes. Consistent with our findings, intense light also induces choroidal thickening in chickens.⁶² Here we propose two possible pathways by which intense light increases ChBP. One is through retinal dopamine signaling. Cohen et al. found that light-stimulation increased the release of dopamine,⁶³ and Reitsamer et al.⁵⁷ found that dopamine caused choroidal vasodilation in cats. Another possibility is through pupillary constriction, which can increase the depth of focus and weaken the hyperopic defocus,⁵¹ leading to a thicker choroid.⁶⁴ Therefore it is plausible that intense light suppresses myopic development through increasing ChBP.

CONCLUSIONS

In summary, we found that all of the three proven interventions for myopia control in guinea pigs—that is, atropine, apomorphine, and intense light—increased ChBP and reduced scleral hypoxia. Furthermore, we found that actively increasing ChBP with prazosin inhibited scleral hypoxia and myopia development. These results are consistent with an increasing body of evidence, that increased ChBP may attenuate scleral hypoxia and thereby inhibit myopia development. We conclude that treatments that increase ChBP may be promising therapies for retarding the development of myopia, and that ChBP may serve as an immediate predictor of myopia development, as well as a long-term marker of it.

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