

# Short Wavelength (Blue) Light Is Protective for Lens-Induced Myopia in Guinea Pigs Potentially Through a Retinoic Acid–Related Mechanism

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**PURPOSE.** To investigate the effect of short-wavelength light (SL) on guinea pigs with lens-induced myopia (LIM) and the possible retinoic acid (RA)–related mechanisms.

**METHODS.** Two-week-old guinea pigs ( $n = 60$ ) with monocular  $-5D$  lenses were reared under white light (WL, 580 lux) or SL (440 nm, 500 lux). The left eyes were uncovered as control. Refractive error (RE) and axial length (AL) were measured at baseline, one week, two weeks, and four weeks after intervention. Retinal RA was measured from four guinea pigs after two and four weeks of treatment with HPLC. Two-week-old guinea pigs ( $n = 52$ ) with monocular  $-5D$  lens were fed with either RA or its synthesis inhibitor citral every third day in the morning, and half from each group were reared under WL or SL conditions. RE and AL were recorded at baseline and two and four weeks after intervention. Retinal RA was measured after four weeks of intervention.

**RESULTS.** At the end of treatment, guinea pigs exposed to SL were less myopic than to WL ( $2.06 \pm 1.69D$  vs.  $-1.00 \pm 1.88D$ ), accompanied with shorter AL ( $P = 0.01$ ) and less retinal RA ( $P = 0.02$ ). SL reduced retinal RA even after exogenous RA supplementation ( $P = 0.02$ ) and decelerated LIM compared to WL ( $1.66 \pm 1.03D$  vs.  $-3.53 \pm 0.90D$ ). Citral slowed ocular growth, leading to similar RE in W+CI and S+CI groups ( $3.39 \pm 1.65D$  vs.  $5.25 \pm 0.80D$ ).

**CONCLUSIONS.** Overall, SL reduced LIM in guinea pigs, even in those supplemented with oral RA, accompanied by reduced retinal RA levels. Oral RA accelerated eye elongation, but citral equally decelerated eye elongation under SL and WL with no significant retinal RA reduction.

**Keywords:** short-wavelength light, lens-induced myopia, retinoic acid, guinea pig

Eye growth can be altered by lenses placed in front of the eyes, which has been demonstrated in a range of vertebrate species including rhesus monkey,<sup>1</sup> marmoset,<sup>2,3</sup> guinea pig<sup>4,5</sup> and chicks.<sup>6</sup> Similarly, different wavelengths of light can also affect eye growth, as demonstrated in studies on guinea pigs, where short-wavelength light (SL) slowed ocular growth in guinea pigs, even after exposure to negative defocus or middle, and long-wavelength light accelerated eye growth and myopia progression.<sup>7,8</sup> Longitudinal chromatic aberration (LCA) may be one of the possible mechanisms behind SL slowing down lens-induced myopia progression, because light with shorter wavelengths is focused more anteriorly in comparison to light with longer wavelengths.<sup>9</sup> LCA has been calculated to be about 1.23D in chicks between 470 nm and 680 nm and 3.10D in rats.<sup>10</sup> Further evidence that supports this theory can be found in studies on chicks, where those reared under red light were 1.41D more myopic than those reared under SL.<sup>11</sup> However, in other studies, red light slowed eye growth and induced hyperopia in young tree shrews<sup>12</sup>

and rhesus monkeys.<sup>13</sup> Furthermore, it is documented that guinea pig<sup>14,15</sup> and chick<sup>16</sup> eyes can emmetropize but did not match the prediction of LCA in monochromatic light, which suggests that LCA is not the only signal to direct eye growth.

Visual signals reaching the retina require transduction to induce chemical and physical changes in the sclera that drive eye elongation. However, the mechanism behind this process is unclear. One possible mediator is retinoic acid (RA), because retinal RA levels have been shown to be elevated in form-deprived chick eyes.<sup>17</sup> Retinal RA levels also increased in chicks during accelerated eye growth while wearing a  $-15D$  lens and decreased when wearing a  $+15D$  lens that slowed axial elongation.<sup>18</sup> This would allow us to infer that supplementing chicks with RA would increase the rate of eye elongation, and vice versa if they were supplemented with citral, an inhibitor of RA synthesis, as observed by McFadden et al.<sup>19</sup> Similar results have been observed in guinea pigs, in which retinal RA increased in myopic eyes and RA supplementations resulted in rapid eye elongation.<sup>4</sup>



RA is the acid derivative of vitamin A (retinol) and in vertebrate animal models is synthesized locally within the developing eye, with high concentrations ventrally and lesser concentrations dorsally in chicks and mice.<sup>20,21</sup> RA can also modulate cone differentiation by reducing the differentiation of short wavelength-sensitive cones and promoting the differentiation of long-wavelength-sensitive cones in vertebrates.<sup>22</sup> Guinea pig retina is dominated by M-cones dorsally and S-cones ventrally.<sup>23</sup> Exposure to SL over 10 weeks has been shown to increase S-cone density and decrease M-cone density, with accompanying relative hyperopia, which might be driven by a decrease in RA.<sup>24</sup> Because the mechanism behind eye growth deceleration with SL remains unclear, the current study attempted to investigate the effect of SL on guinea pigs with lens-induced myopia and the possible associated role of RA.

## MATERIALS AND METHODS

### Animals and Housing

All research procedures were approved by the Institutional Animal Care and Ethics Committee at the Eye and ENT Hospital of Fudan University and were in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The indoor temperature of the animal facility was maintained at 22°C to 26°C, and relative humidity was at 55% to 65%. The peripheral walls and ceiling were all installed with LED light bars, either SL (peak value 440 nm, half bandwidth 10 nm; Ruiliu Light & Electronic Co., Shanghai, China) or WL (color temperature 5000K). The light illumination was set to be comparable in each group, which was 500 lux and 580 lux (LX-101 lux meter; Lutron Electronics Enterprise Co., Taiwan) in SL and WL, respectively. The intensity of illumination in each group was controlled and adjusted by a voltage regulator. All groups underwent a 12-hour/12-hour light/dark cycle (light: 6 AM to 6 PM).

### Lens Wearing

A -5D lens (PMMA, diameter 20 mm, optical zone 12 mm), mounted onto a piece of Velcro, was glued around the right eye of the guinea pig, aligned with the center of the pupil, as described by Howlett and McFadden.<sup>5</sup> The lenses were worn continuously during the experiments except when they were removed for cleaning once a day or during ocular measurements. In addition, the lens would be replaced whenever visible scratches were found at its optical zone.

### Experiment 1. Effect of SL on Lens-Induced Myopia and Retinal Retinoic Acid

Sixty pigmented guinea pigs (*Cavia porcellus*, two weeks old) were randomly divided into a WL (n = 30) group and a SL (n = 30) group. Guinea pigs in both groups wore a -5D lens on the right eye. The left eyes were uncovered and set as control. Refractive error (RE), axial length (AL) and corneal curvature were measured before and at one, two, and four weeks after intervention; four guinea pigs in each group were sacrificed at two weeks and four weeks after treatment, respectively, for retinal RA measurement.

### Experiment 2. Effect of Supplementing Retinoic Acid or Its Inhibitor

**Preparation and Administration of Retinoic Acid and Citral.** Fifty-two pigmented guinea pigs (two weeks old) were divided into the WL group and the SL group, half of them in each group were supplemented with RA (W+RA group, n = 11, S+RA group, n = 15) or its synthesis inhibitor, citral (W+CI group, n = 16, S+CI group, n = 10). The animals were raised under WL or SL with a -5D lens on the right eye. RE, AL, and corneal curvature were measured before and two and four weeks after intervention; retinal RA level was measured four weeks after lens treatment.

Guinea pigs from the RA groups were fed 24 mg/kg (guinea pigs were weighed before each RA administration) all-trans retinoic acid (atRA; Sigma, St. Louis, MO, USA) mixed with 0.4 mL peanut oil; the method we used to prepare the RA-peanut oil suspension has been reported in previous studies.<sup>4,19</sup> Guinea pigs in the citral group were fed 445 mg/kg citral (mixture of cis and trans; Sigma) dissolved in 0.4 mL peanut oil (using the chick model as a reference<sup>19</sup>). Guinea pigs were administered the appropriate substance by gavage orally every three days at approximately 11 AM while they were awake.

### Biometric Measurements

RE was measured while the guinea pigs were awake using retinoscopy (66 Vision-Tech Co., Ltd., Suzhou, Jiangsu Province, China) 15 minutes after cycloplegia. Guinea pigs were given a total of three drops of cycloplegic agent (1% cyclopentolate hydrochloride; Alcon Ophthalmika GmbH, Austria) in five-minute intervals to dilate pupils and inhibit ocular accommodation. RE was recorded after averaging the results from two independent experienced technicians, who measured RE in the two meridians and calculated the spherical equivalent power (spherical power +1/2 cylindrical power). No correction was made for the artefact of retinoscopy, which is relatively small in guinea pigs.<sup>25</sup>

AL, including anterior chamber depth (ACD), lens thickness and vitreous chamber depth (VCD), was measured using A-scan ultrasonography with a 10-MHz probe (KN-1800; Kangning Medical Device Co., Ltd., Wuxi, Jiangsu Province, China) with one drop of 0.5% proparacaine hydrochloride (Alcaine, Alcon). Results from 10 readings were averaged for each eye.

Corneal radius of curvature (CRC) was measured using a keratometer (OM-4; Topcon, Tokyo, Japan) with a +8D lens attached to the anterior surface. A set of stainless-steel ball bearings with diameters ranging from 5.5 to 11.0 mm was used for calibration, and CRC was deduced by averaging three readings on the balls with known radii.<sup>26</sup> The mean of the horizontal and vertical measurements was recorded as the final data.

### Retinoic Acid Level Measurement

The retinal retinoic acid level at weeks 2 and 4 was detected after corresponding biometric measurements were completed. The level of RA in the retina was detected using high performance liquid chromatography (HPLC, LC-8A, SHIMADZU.co, Japan) with an electrochemical detector (Coulochem111, ESA, USA). After the guinea pigs were sacrificed by cervical dislocation, eyes were removed and hemisected on the line of the ora serrata. The retinal layer

was carefully dissected from the RPE, and all pigmented cells were removed to ensure RPE was not collected. Procedures were carried out on ice and under dim red light. The atRA standard sample was purchased from Sigma ( $C_{20}H_{28}O_2$ ; molecular mass, 300.44). Concentration of retinal RA ( $\mu g$ ) in each tissue was determined using methods reported in previous studies.<sup>27</sup>

### Statistical Analysis

All statistical analyses were performed using SPSS 20.0 software (SPSS, Inc., Armonk, NY, USA), and the results are presented as mean  $\pm$  standard deviation (SD) unless otherwise stated. Two-way repeated measures analysis of variance (ANOVA) was used to compare the mean RE, VCD, and AL and intraocular difference (IOD) among the guinea pigs reared under SL or WL conditions with different diets subgroups across time. Least significant difference (LSD) correction was applied for post hoc analysis to find differences of refraction and ocular biometric parameters among different diet groups (routine diet, added RA, or citral) at each timepoint. Pearson's correlation analyses was performed to characterize the relationship between the change in RE and AL. Mann-Whitney U test was used to analyze retinal RA results among different groups. A  $P$  value  $< 0.05$  was considered to indicate a statistically significant difference.

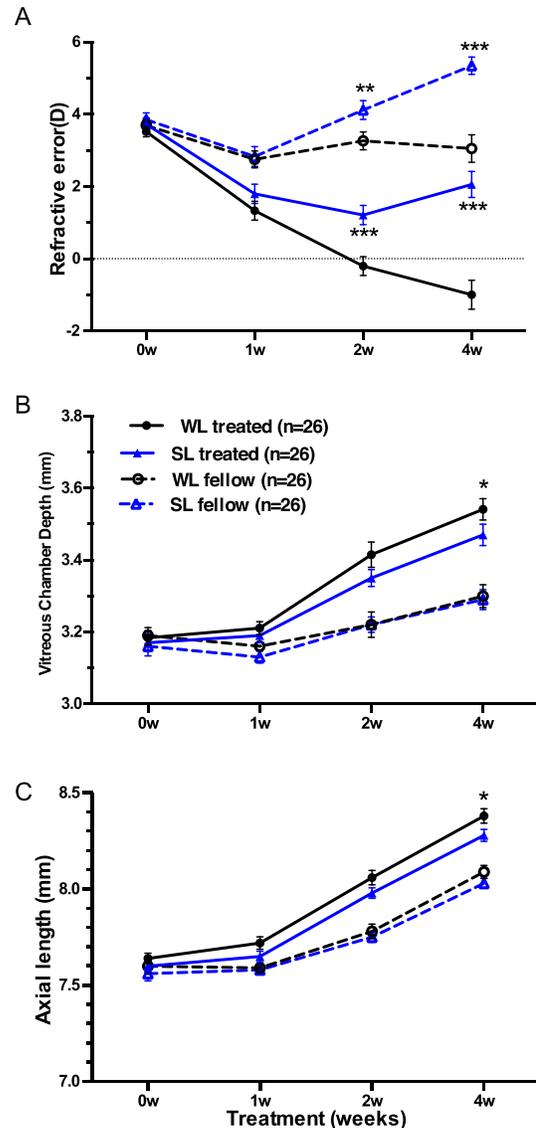
## RESULTS

### Experiment 1. SL Slowed Myopia Progression and Reduced Endogenous Retinal Retinoic Acid

**The Effect of SL on Lens-Induced Myopia.** At the end of the experiment, both defocus and lighting characteristics together influenced the refractive error outcome ( $F[1, 50] = 6.13, P = 0.004$ , two-way repeated measures ANOVA), as well as AL ( $F[1, 50] = 6.37, P = 0.015$ ) and VCD ( $F[1, 50] = 7.76, P = 0.008$ ), but the effect did not change across ages (RE:  $F[2.28, 113.97] = 2.46, P = 0.08$ , AL:  $F[2.58, 128.95] = 0.25, P = 0.86$ , VCD:  $F[2.50, 124.75] = 1.77, P = 0.17$ ). Eyes wearing a  $-5D$  lens for four weeks under WL were on average  $-1.00 \pm 1.88D$  more myopic than eyes exposed to SL, which was  $2.06 \pm 1.69D$  (post hoc LSD,  $P < 0.001$ , see Supplementary Table S1 for detailed data, Fig. 1A), accompanied by a longer VCD (post hoc,  $P = 0.046$ , Supplementary Table S1, Fig. 1B) and longer AL (post hoc,  $P = 0.01$ , Supplementary Table S1, Fig. 1C). Moreover, treated eyes, as well as fellow eyes in SL, were relatively more hyperopic than WL from the second week of lens treatment (post hoc, treated:  $P < 0.001$ ; fellow:  $P < 0.01$ ), as shown in Figure 1A.

The interaction between different lighting conditions and lens treatment did not change significantly between the two- and four-week time points for IOD in RE ( $F[2.28, 113.97] = 2.46, P = 0.08$ , two-way ANOVA); however, SL reduced lens-induced myopia significantly at the end of the experiment (post hoc,  $P = 0.004$ , Supplementary Fig. S1A). However, IOD of AL and IOD of VCD growth was not influenced by interaction of time and color signals (AL:  $F[2.58, 128.97] = 0.244, P = 0.84$ , VCD:  $F[2.50, 124.75] = 1.77, P = 0.17$ ; Supplementary Figs. S1B, S1C).

ACD and lens thickness had no significant difference between the two groups at all-time points ( $P > 0.05$ , Supplementary Table S1). CRC in all groups increased gradually with treatment time compared to pretreatment ( $P < 0.001$ ,



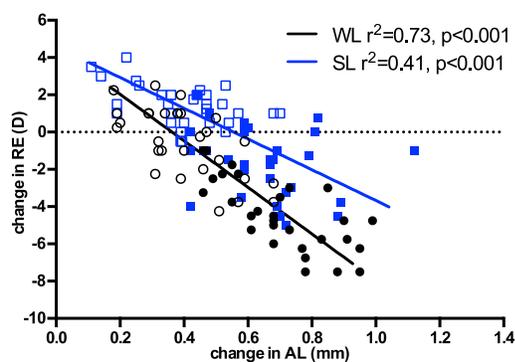
**FIGURE 1.** Biometric parameters (Mean  $\pm$  SEM), including (A) refractive error (Diopter, D), (B) vitreous chamber depth (mm), and (C) axial length (mm) of treated (solid lines) and fellow (dashed lines) eyes plotted against treatment time (weeks) in guinea pigs wearing monocular  $-5D$  lenses (treated eyes) reared under white light (WL, black lines) or short-wavelength light (SL, blue lines) conditions. Asterisks refer to the significant difference between guinea pigs under WL or SL conditions. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .  $n = 26$  across all treatment time.

but no intergroup differences were found at each time-point ( $P > 0.05$ ).

The correlation analysis showed that the change in RE after 4 weeks of treatment significantly correlated with the change in AL in both WL ( $R^2 = 0.73, P < 0.0001$ , Fig. 2) and SL ( $R^2 = 0.41, P < 0.0001$ ) conditions, and the slopes were not significantly different between WL and SL groups ( $-10.92$  vs.  $-7.22$  in WL and SL, respectively;  $P = 0.36$ ).

### Endogenous Retinal Retinoic Acid Level Change.

Eyes which had experienced hyperopic defocus developed a myopic RE, a longer AL and had an increased level of retinal RA relative to their fellow eye in WL group from the second



**FIGURE 2.** Linear regression of change in refractive error (change in RE) and change in axial length (change in AL) after four-week treatment period, in guinea pigs reared under white light (WL, black circle/line) or short wavelength light (SL, blue box/line). Open circles/boxes represent fellow eyes, closed circles/boxes represent treated (wearing  $-5D$  lens) eyes. The line is fitted to both lens defocus and fellow eyes. AL changed in parallel with a change in RE in both groups ( $P < 0.001$ ).

week to the end of the treatment (Figs. 3A, 3B). Guinea pigs exposed to SL had lower retinal RA level in both eyes compared to those under WL illumination at the end of the intervention (Fig. 3B).

Retinal RA levels decreased from two to four weeks in both eyes under SL (OD:  $13.24 \pm 11.68 \mu\text{g/g}$  to  $2.26 \pm 2.37 \mu\text{g/g}$ ; OS:  $5.85 \pm 3.07 \mu\text{g/g}$  to  $2.07 \pm 1.63 \mu\text{g/g}$ , respectively), whose retinal RA levels were three to six times higher at two weeks than at four weeks. This phenomenon suggested that SL could reduce RA levels by inhibiting the synthesis or increasing the degradation of RA in the retina.

To analyze the data further, eyes with two weeks of lens-wearing under SL were relatively hyperopic, accompanied with a slightly lower retinal RA level compared to under WL. By the time the difference in retinal RA levels between WL and SL groups reached significance (i.e., after four weeks of treatment), the difference in RE between these two groups were greater. In addition, IOD of retinal RA level (OD-OS) under SL was smaller than WL after four weeks of intervention (M-W test,  $P = 0.02$ ), which is concordant with lower myopia progression in SL compared to WL.

## Experiment 2. Effect of Feeding Retinoic Acid or Its Inhibitor

At the end of the experiment, the interaction of defocus, lighting characteristics, drug administration, and treatment time all together influenced the refractive error outcome (two-way ANOVA,  $F[8.81, 172.73] = 10.88$ ,  $P < 0.001$ ). Correspondingly, the interaction effect influenced axial elongation ( $F[10, 196] = 3.25$ ,  $P = 0.001$ ) and VCD ( $F[10, 196] = 2.84$ ,  $P = 0.003$ ).

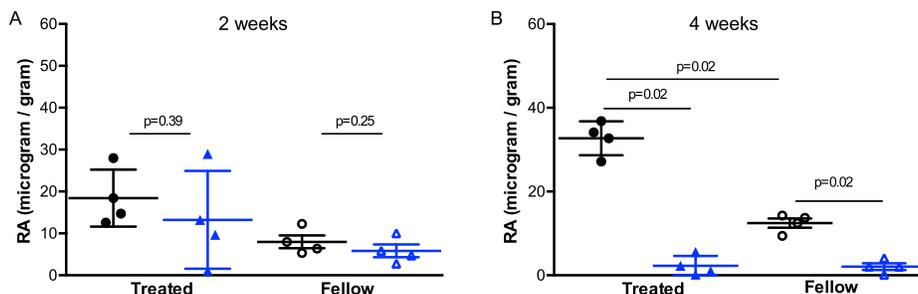
**The Effect of SL on Guinea Pigs Fed With Retinoic Acid.** For guinea pigs fed with RA, post hoc analysis showed that SL slowed down the myopic shift of the lens-treated eye compared with WL ( $P < 0.001$ , Supplementary Table S2, Fig. 4A), as well as reduced VCD or AL significantly ( $P < 0.001$  for VCD,  $P < 0.01$  for AL, Figs. 4B, 4C).

Similarly, IOD of RE was significantly affected by the interaction of color signals, drug administration and treatment time ( $F[8.81, 172.73] = 10.88$ ,  $P < 0.001$ ), as well as IOD of AL ( $F[10, 196] = 3.25$ ,  $P = 0.001$ ) and IOD of VCD ( $F[10, 196] = 2.84$ ,  $P = 0.003$ ). Lens-induced myopia was significantly decreased by SL ( $-2.94D \pm 1.01D$  vs.  $-6.78 \pm 1.11D$ , post hoc,  $P < 0.001$ , Supplementary Fig. S1D), but AL or VCD had no significant difference between W+RA and S+RA groups (Supplementary Figs. S1E, S1F).

The correlation analysis showed that the change in RE after four weeks of treatment significantly correlated with the change in AL in guinea pigs fed with RA in both WL ( $R^2 = 0.86$ ,  $P < 0.001$ ) and SL ( $R^2 = 0.54$ ,  $P < 0.001$ , Fig. 5A) conditions, and the slopes were significantly different between W+RA and S+RA groups ( $-16.92$  vs.  $-5.15$  in W+RA and S+RA, respectively;  $P = 0.008$ ).

**Biometric Changes After Feeding Citral.** For guinea pigs fed with citral, hyperopic shift was significantly enhanced by SL compared with WL (post hoc,  $P = 0.002$ , Fig. 4D), and significant VCD reduction was observed under SL (post hoc, VCD:  $P = 0.01$ , Fig. 4E). However, lens-induced myopia (IOD of RE) was not significantly different between S+CI and W+CI groups at the end of treatment (post hoc,  $P = 0.27$ , Supplementary Fig. S1G), and no significant difference in IOD of AL was observed (post hoc,  $P = 0.06$ , Supplementary Fig. S1I).

Similar to guinea pigs fed with RA, guinea pigs fed with citral also showed a significant association between change in RE and change in AL under both WL ( $R^2 = 0.33$ ,  $P < 0.001$ ) and SL ( $R^2 = 0.15$ ,  $P = 0.04$ ; Fig. 5B), but the slopes were



**FIGURE 3.** Retinal retinoic acid level (RA concentration/weight of retina,  $\mu\text{g/g}$ ) in guinea pigs reared under short wavelength or white light conditions at two weeks (A) and four weeks (B) after monocular lens treatment. Each black circle represent RA level from each guinea pig under white light; each blue triangle represents RA level from each guinea pig under short wavelength light. Closed symbols represent RA level from lens treated eyes, open symbols represent RA level from fellow eyes. Error bars denote SD. Treated: eyes with  $-5D$  lens wearing; fellow: left eyes set as control.  $P$  values calculated by Mann-Whitney U test are presented above the corresponding bars.

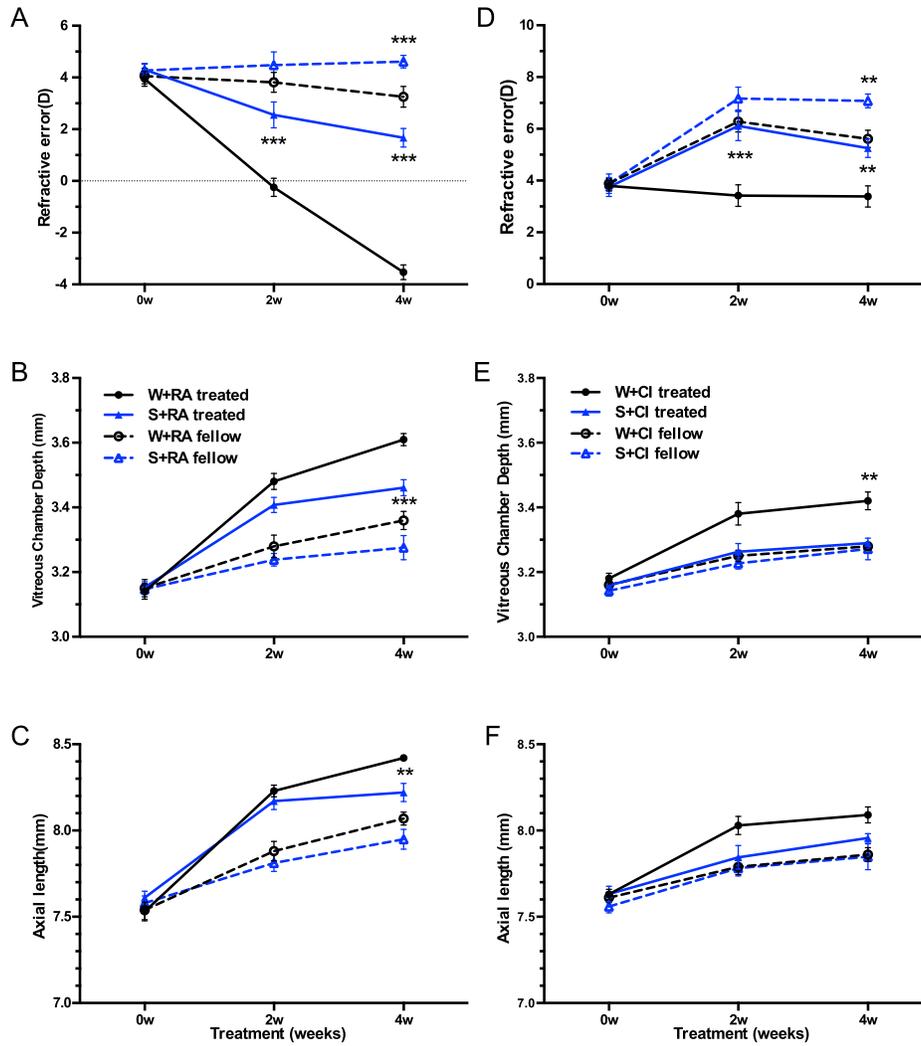


FIGURE 4. Biometric parameters between guinea pigs fed with retinoic acid (Left column, A, B, C) or fed with citral (Right column, D, E, F) under white and short wavelength light conditions: Mean  $\pm$  SEM values in A and D, refractive error in D, vitreous chamber depth (mm) in B and E, and axial length (mm) in C and F of treated (solid lines) and fellow (dashed lines) eyes plotted against treatment time (weeks) in guinea pigs wearing monocular  $-5D$  lenses (treated) reared under WL (black lines) or SL (blue lines) conditions. Asterisks refer to the significant difference between guinea pigs under WL or SL conditions. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .

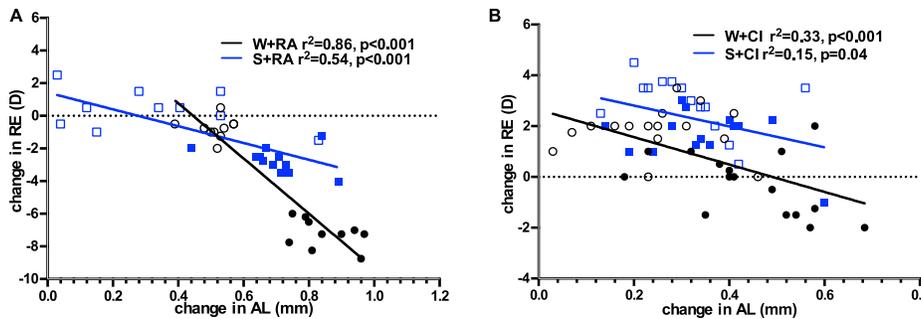
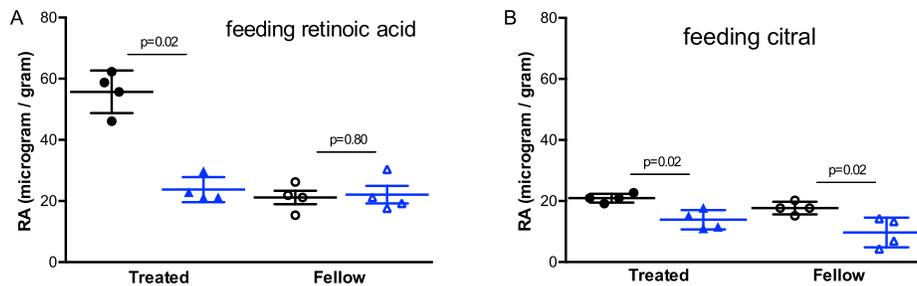


FIGURE 5. Linear regression of change in refractive error (change in RE) and change in axial length (change in AL) during the four-week treatment period, in guinea pigs fed with RA (A) or citral (B) and reared under white light (black circle/line) or short-wavelength light (blue box/line). The line is fitted to both the lens defocus and fellow eye. Closed circles/boxes represent treated eyes, open circles/boxes represent fellow eyes. AL changed in parallel with a change in RE in all groups ( $P < 0.05$ ).



**FIGURE 6.** Retinal retinoic acid level (RA concentration/weight of retina, ug/g) between guinea pigs fed with retinoic acid (A) or fed with citral (B) reared under white (A, W+RA; B, W+CI) or short-wavelength light (A, S+RA; B, S+CI) conditions four weeks after monocular lens treatment. Error bars denote SD. Each white circle represent RA levels from each guinea pig under white light; each blue triangle represent RA levels from each guinea pig under SL. Closed symbols: treated eyes with  $-5D$  lens wearing; open symbols: fellow eyes set as control. *P* values calculated by Mann-Whitney U test were distributed above the corresponding bars.

not significantly different between W+CI and S+CI groups ( $-5.39$  vs.  $-4.11$  in W+CI and S+CI, respectively;  $P = 0.32$ ).

**The Effect of Different Diets on Guinea Pigs Reared Under White Light.** Under WL, citral decelerated myopia progression at the end of the experiment compared to routine diet or oral RA (post hoc, WL vs. W+CI and W+RA vs. W+CI; both  $P < 0.001$ ; Supplementary Fig. S2A). Correspondingly, citral slowed VCD extension (post hoc, WL vs. W+CI,  $P = 0.004$ ; W+RA vs. W+CI,  $P = 0.007$ ; Supplementary Fig. S2B) and axial elongation (post hoc, WL vs. W+CI,  $P = 0.002$ ; W+RA vs. W+CI,  $P = 0.02$ ; Supplementary Fig. S2C) as well.

Lens-induced myopia (IOD of RE) was significantly increased by oral RA and decreased by oral citral (post hoc, W+RA vs. WL, WL vs. W+CI; both  $P < 0.001$ ; Supplementary Fig. S2G), and IOD of VCD was correspondingly lessened with citral supplement (post hoc, WL vs. W+CI and W+RA vs. W+CI; both  $P < 0.001$ ; Supplementary Fig. S2H).

**The Effect of Different Diets on Guinea Pigs Reared Under SL.** Under SL, for guinea pigs wearing a monocular  $-5D$  lens and fed with a different diet, oral RA did not accelerate myopia development compared to a normal diet (SL vs. S+RA;  $P = 0.62$ , post hoc). However, citral induced a hyperopic shift at the end of the treatment compared to RA or routine diet (post hoc, S+CI vs. S+RA and S+CI vs. SL; both  $P < 0.001$ ; Supplementary Fig. S2D). Correspondingly, AL elongation in S+CI group was less than that in S+RA or SL groups (post hoc, S+CI vs. S+RA,  $P = 0.021$ ; S+CI vs. SL,  $P = 0.021$ ; Supplementary Fig. S2F).

Exogenous citral could reduce IOD of RE (post hoc, S+RA vs. S+CI,  $P = 0.04$ ; SL vs. S+CI,  $P < 0.001$ ; Supplementary Fig. S2G), while RA could induce and citral could reduce IOD of VCD (post hoc, S+RA vs. SL,  $P = 0.04$ ; SL vs. S+CI,  $P = 0.02$ ; Supplementary Fig. S2H) and IOD of AL (post hoc, S+RA vs. SL,  $P = 0.001$ ; SL vs. S+CI,  $P = 0.005$ ; Supplementary Fig. S2I).

**Endogenous Retinal Retinoic Acid Level After Feeding Retinoic Acid or Citral.** Similar to Experiment 1, SL reduced retinal RA level in the lens-wearing eye even after guinea pigs were fed with RA (S+RA vs. W+RA: M-W test,  $Z = 2.31$ ,  $P = 0.02$ ; Fig. 6A), and for guinea pigs fed with citral (lower RA levels), SL also affected RA concentration significantly (S+CI vs. W+CI:  $Z = 2.32$ ,  $P = 0.02$ ; Fig. 6B). As expected, retinal RA level in the lens-wearing eye increased 72% with oral RA but decreased almost 40% with feeding citral in WL groups (W+RA:  $55.72 \pm 6.96$  vs. W+CI:  $20.94 \pm 1.43$ ). Also, in SL groups, retinal RA increased ten times

after RA supplement in the lens-wearing eyes (S+RA:  $23.75 \pm 4.09$  µg/g vs. SL:  $2.26 \pm 2.37$  µg/g). An unexpected finding was that citral did not decrease retinal RA levels under SL ( $13.87 \pm 3.18$  µg/g).

IOD of retinal RA level (OD-OS) in guinea pigs fed with RA under SL was much smaller than WL after four weeks of intervention (M-W test,  $P = 0.002$ ), which is concordant with lower myopia progression in S+RA compared to W+RA. However, for guinea pigs fed with citral, IOD of retinal RA did not show a difference between WL and SL groups ( $P = 0.68$ ), and IOD of RE did not show a significant difference between S+CI and W+CI groups simultaneously.

## DISCUSSION

The principal finding in the current study is that guinea pigs exposed to negative lens defocus and reared under SL experienced a lower myopia progression and a reduced retinal RA level compared to their counterparts under WL, even after exogenous RA supplementation. Furthermore, oral RA administration induced accelerated myopia progression under WL, but not under SL; on the contrary, feeding citral stopped myopia progression and slowed down eye elongation under WL, and the inhibition was almost equally strong when combined with SL.

The effect of SL in emmetropization has remained controversial for a long time. Chickens<sup>28,29</sup> and guinea pigs<sup>7,30</sup> became more hyperopic in SL; however, the effects of SL on tree shrew refraction is more complicated because flickering SL drove the eye to significant myopia whereas steady SL had little effect.<sup>31</sup> In the current study, we observed a hyperopic shift after exposure to SL in guinea pigs and confirmed the inhibitory effect of SL on lens-induced myopia development compared to WL, as previous studies have demonstrated.<sup>7,30</sup> In addition, the reduction of myopia progression under SL was also accompanied with a shorter AL.

Inhibited eye growth under SL may be mediated by reduced retinal RA levels observed during SL exposure. RA may be one of the possible mediators that translates the visual signals from the retina to sclera to modulate eye growth, as multiple studies have shown elevated retinal RA levels during myopia development in defocus induced guinea pigs and these RA changes preceded ocular elongation.<sup>32-34</sup> Only one previous study examining monochromatic light in animal models of myopia included RA testing.<sup>35</sup> The results demonstrated that guinea pig eyes elongated faster after four weeks' exposure to green light in

comparison to SL, along with a higher retinal RA level. In the current study, lens-wearing eyes under WL expressed more RA in the retina at four weeks than at two weeks (Fig. 3), and the difference between lens-wearing eyes and the fellow eyes was greater at four weeks than two weeks. These results are consistent with the above-mentioned lens-induced myopia studies. Additionally, retinal RA level in eyes exposed to SL with or without lens-wearing were lower than those exposed to WL at two weeks, and further reduced to reach statistical significance at four weeks. This observation suggests that SL might reduce the synthesis or increase the degradation of retinal RA to slow down myopia development, but it needs further research to confirm this hypothesis.

RA is known to affect extracellular matrix metabolism in many systems and is involved in the expression of hundreds of genes and transcription factors influencing eye development.<sup>36,37</sup> RA has been shown to inhibit the differentiation of blue sensitive cones and enhance red sensitive cones.<sup>22,38</sup> Also, ventral/dorsal gradient retinal RA level determines the dorsal-ventral gradient of middle-wavelength sensitive and short-wavelength sensitive opsin expression via specific receptors.<sup>39,40</sup> In guinea pigs, s-opsin in the retina was upregulated, and m-opsin was downregulated when exposed to SL, with a shorter AL compared to WL exposure, suggesting the involvement of RA.<sup>24,41</sup> In addition, RA has been demonstrated to directly inhibit scleral proteoglycan synthesis in chicks and primates.<sup>27,42,43</sup>

In the second experiment, we observed that oral RA administration in guinea pigs under WL significantly increased retinal RA level and accelerated myopia progression in the lens-wearing eye, but not the fellow eye (Fig. 4A). Similar to a previous study,<sup>4</sup> feeding RA had no significant effect on RE in eyes not exposed to negative lens defocus. However, in lens-wearing eyes, supplementing with RA showed longer AL, as well as increased magnitude of myopia, suggesting that RA could enhance the myopia-genic effect of negative lenses. Contrary to these results, McFadden et al.<sup>19</sup> observed that chicks wearing  $-6$  D lenses compensated normally for the lenses despite the enhanced ocular elongation caused by feeding RA, which may indicate species differences. In guinea pigs reared under SL, although retinal RA increased after oral RA administration, their levels were lower than those seen in guinea pigs exposed to WL (Fig. 6A). Correspondingly, guinea pigs from the S+RA group developed less lens-induced myopia compared to guinea pigs from the W+RA group, but was similar with their counterparts under a routine diet (S+RA vs. SL:  $-2.94$ D vs.  $-3.28$ D).

Feeding guinea pigs with RA synthesis inhibitor (citral) resulted in significant reduction of retinal RA and inhibition of myopia development in the lens-wearing eye of guinea pigs reared under WL. In addition, citral inhibited myopia progression starting at the second week of treatment, stabilizing the RE in both eyes, especially the lens-wearing eye, resulting in more hyperopia and shorter VCD and AL than WL and W+RA groups. However, retinal RA level in the fellow eye did not decrease in W+CI group as expected, despite eye growth inhibition, suggesting that citral may work through inhibiting RA synthesis, as well as affecting RA receptors and subsequently decelerate eye growth. Citral not only inhibits the mitochondrial isozyme of rat aldehyde dehydrogenase to reduce RA synthesis, it could also work as an antagonist to alter a variety of genes expression through retinoid receptors because it bears a structural resemblance

to RA.<sup>44,45</sup> Therefore we speculate that both the above two mechanisms may come into play in eye growth inhibition by citral. Further research is needed to determine the mechanisms of citral.

An unexpected and interesting finding in this study was that retinal RA did not decrease after citral intake in all eyes under SL, although shorter axial elongation was observed. This could be due to a feedback mechanism that attempted to compensate for the inhibitory effect of citral, because citral not only reduces RA levels but also combines retinoid receptors as an antagonist.<sup>44,45</sup>

The limitations of this study include the following: first, no choroidal thickness or retinal thickness data was collected during the experiment, which could provide us with more information to understand their relationship with changes in RE and AL. Second, detecting the downstream molecules of RA may give us more clues to understand how SL and RA works to influence eye growth. Third, the lack of a vehicle control group fed only with peanut oil in Experiment 2, however, based on the effect of RA and citral on eye growth, it is reasonable to deduce that the vehicle would not have affected eye growth.

## CONCLUSION

In summary, although the specific mechanisms of our observations regarding the influence of SL in modulating ocular elongation remain obscure, this preliminary study led us to the conclusion that in the guinea pig, retinal RA levels are affected by visual conditions and can enhance ocular elongation in a negative defocus model. Such changes are inhibited in the presence of SL, even with exogenous RA supplement. Furthermore, feeding RA to young guinea pigs rapidly enhanced ocular elongation, with significant changes in refraction observed in the lens-wearing eye, but not in the fellow eye. On the contrary, citral stopped myopia progression and slowed eye elongation, but it may act via pathways other than those altering RA levels.

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