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Retinoic Acid Reduces Ocular Elongation in Chicks with Form-Deprivation Myopia

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ABSTRACT

Purpose. Retinoic acid (RA) has been proposed to be of value in treating myopia, as it affects scleral growth in chicks with form-deprivation myopia. The present study aimed to test the effects of RA injection on a form-deprivation myopia (FDM) model in chicks. *Methods.* The chicks were form-deprived by having a black goggle sutured over one eye with the contralateral eye as the control. Daily intraperitoneal injection of all-trans RA was performed for 12 days. The eye weight (EW), ocular length (OL), equatorial diameter (ED), and the thickness of retina and sclera were measured to reflect the effects of RA on ocular development under form-deprivation myopia. *Results.* The effect of RA was biphasic with alleviating effect on FDM limited to a daily dose of up to 10 µg/day with relatively less myopic development than the controls. Significant differences of EW and OL, but not ED, at 10 µg/day RA

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($P < 0.05$) were observed. The thinning of retina and the thickening of sclera as a result of FDM were reversed, albeit not significantly, in the presence of RA up to a daily dose of 10 µg/day. *Conclusions.* The results show that exogenously applied RA has the potential to inhibit scleral growth in form-deprived chicks. Such findings are in line with the understanding that regulation of scleral growth is one of the key factors in myopia, and RA may be of value in its treatment.

Key Words: Retinoic acid; Form-deprivation myopia; Retina; Sclera; Chicks.

INTRODUCTION

Myopia remains one of the most common eye disorders worldwide, and epidemiological data suggest that the prevalence of myopia in America is about 25% (1), whereas in the Far Eastern countries such as Singapore, Taiwan, Hong Kong, and Japan, the prevalence is up to 70% (2,3). As such, the understanding of any possible treatments that could alleviate myopia are obviously important. In experimental endeavors, much effort has been placed in evaluating the factors or mediators that may be responsible for causing, as well as alleviating, myopia. One such important factor is retinoic acid (RA), a retinoid metabolite widely distributed in different organs such as the liver, kidney, retina, and brain. It has been demonstrated that RA affects the growth of sclera in chicks with form-deprivation myopia (FDM) (4–8). The distribution and localization of retinoid binding proteins, which are transport proteins for retinal and vitamin A; retinoid receptors, which are expressed in a variety of growth of tissues and development of vertebrate; and retinaldehyde dehydrogenase, which is required for the formation of RA, have been characterized in chicks (9,10). The evidence indicates that RA is crucial to normal ocular functions and development, which might be related to effects of RA from binding to nuclear receptors that activate transcription factors (11). Indeed, the loss of retinol-binding proteins in serum and the disruption of retinoic receptors (RAR_α , RAR_β , and RAR_γ) can adversely affect the growth of the eye and development of the retina (8,12,13). Under normal conditions in the retina, RA is produced as a by-product of the dark regeneration process. It has been shown that diurnal rhythm acts as cues to ocular growth and development (14,15) such that, when chicks wearing black goggles were raised in constant dark, myopia did not occur (16). It suggests that just the absence of images alone is not sufficient to induce myopia. A regular circadian setting is just as important.

The process of emmetropization can be altered by depriving the eye of formed images, which leads to ocular elongation and thus myopia (17). A variety of animal species, particularly newly born chicks, are often used as a model of myopia (17). Chicks are particularly useful in that they are available from the day of hatching, and in sufficient numbers to provide controls and test subjects. Most importantly, myopia can occur in the chicks' eyes within a few weeks of form deprivation (18). Since RA is such an important factor in normal ocular growth, and development and synthesis of RA is activated by light adaptation (19), the relationship between RA and light, and thus images, is obviously crucial in the regulation of ocular growth. Since FDM is often associated with changes in the growth of sclera (20–22) as well as that of the retina (23), and RA seems to have an effect on the proliferation of scleral tissues (4), it

is therefore possible that there might be a significant effect of RA on myopia development. The present study aims to induce myopia in newly born chicks by depriving them of image and light by covering one eye with a black goggle, which would reduce endogenous RA production (16,19), and to evaluate possible protective effects of different concentrations of RA on FDM.

MATERIALS AND METHODS

Study Animals

White Leghorn chicks (*Gallus gallus*) were obtained from the Agriculture and Fisheries Department, Government of the Hong Kong SAR. The chicks were kept at $\sim 30\text{--}35^\circ\text{C}$ in a 12-hour light/dark cycle (light at 07:00) with good air ventilation. They were housed in groups of two or three in spacious containers for 6–7 days prior to the commencement of the experiment and eventually housed as singlets during the experiment. Chicks received water and chick basic starter ad libitum. The use of experimental animals was in accordance with the The Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. The study protocol has obtained approval from the Ethics Committee on Animal Research, the Chinese University of Hong Kong.

Preparations and Goggle Suturing

To deprive eyes of light and form image, custom-made blackened plastic goggles, 1.5 cm in diameter with a centrally elevated region of volume $0.5\text{ cm (height)} \times \pi 0.25\text{ cm}^2$ (area) to accommodate the anticipated excess ocular growth, were used. The chicks (up to about 8–9 days postnatal) were anesthetized using a mixture ($\sim 0.03\text{ mL}$) containing 1 mg ketamine (10% v/v) and 0.4 mg xylazine (2% v/v). The goggles were sutured over the right eye of the chicks for 12 days with the left eye as the control. Lignocaine (0.5% v/v) was applied to the sutured points immediately post suture. The animals did not appear in any state of discomfort. The chicks were weight-registered at the beginning and the end of experiments. Intraperitoneal injection of all-trans-retinoic acid began 18–24 hours following goggle suturing.

Determination of the Effects of All-Trans Retinoic Acid on Eye Development

A stock solution of 1 mg/mL all-trans-retinoic acid (RA, SIGMA, St. Louis, MO, USA) was made up using absolute alcohol and was kept at -4°C in the dark until use. The required quantities of RA for daily injections were made just prior to administration and protected from light. The serial dilution of the stock was done using sterile 0.9% saline. A total of 250 μL of prewarmed RA (1, 10, 30, or 100 μg) or 0.9% saline containing the equivalent maximal concentration of alcohol (vehicle control) was consistently injected into the right iliac region of the chicks intraperitoneally. This

injection was performed every morning for 12 days. The systemic parenteral route of administration was chosen over the topical intravitreal route, because the latter method may cause more complications due to repeated RA injections such as retinal detachment or intravitreal infections. In addition, the effect of solvents such as high concentrations of alcohol or Dimethyl sulfoxide (DMSO) (another possible choice of solvent) in the eye may affect the results or even damage the eye. Furthermore, the systemic route would resemble the intake of vitamin A more closely than the topical route, as vitamin A or related retinoids tends to be taken in via everyday diet.

Measurements and Data Analysis

After 12 days of RA or saline-vehicle injections, the chicks were sacrificed with a terminal dose of anesthetic followed by cervical dislocation. The eyes were immediately enucleated. The ocular length (cm) and equatorial diameter (cm) were measured using calipers, and the eye weight (g) was determined using a digital meter balance. In order to show differences of the above parameters together, the results are expressed as relative percentage difference between the left and right eyes (i.e., measurements of the right eye – measurements of the left eye/measurements of the left eye $\times 100\% \pm$ standard error of the mean (s.e.m.). Percentage differences between the above measurements were compared using single factor analysis of variance (ANOVA). The obtained eyeballs were fixed and paraffin-embedded for histological examinations (hematoxylin and eosin). Histological sections were collected by cutting the eye sagittally at three predetermined positions (i.e., midsagittal and equidistant from either side of it). Five sections were taken at each of these regions, and the total of 15 sections of each eye were used to determine the relative thickness changes of the retina and sclera before and after form deprivation as well as with or without RA administration. In the present study, only the cartilaginous scleral layer could be measured, as the fibrous scleral layer was often too thin and detached from the prepared section. Thickness changes of the retina and cartilaginous scleral layers were determined by first capturing the image of the prepared histological sections with a digital camera and then measuring the sections using a precalibrated scale bar. Image Pro-PlusTM, Media-Cybernetics, Inc, MD, USA was used to measure the actual widths of retinal (right up to and on the edge of the retinal pigment epithelium) and scleral layers in each section, and the values were exported to Microsoft Excel for analysis. The difference in retinal and scleral layer thickness (μm) with or without treatment between different quantities of RA injected in form-deprived chicks was analyzed using ANOVA. The tissue thickness is expressed as mean \pm s.e.m.

As it is difficult to guarantee the same angle of sectioning, the thickness differences between retina and sclera are also expressed as retina vs. sclera ratio. The relative difference between the left and the right eye is expressed as myopic index \pm s.e.m, which is calculated by dividing the retina–sclera ratio of the left eye by the retina–sclera ratio of the right eye. A myopic index of close to or equal to 1 indicates less myopia-related ocular development (i.e., the thickness ratios of these cell layers are almost identical between the left and the right eye). Myopic index significantly greater than 1 indicates an increase in scleral growth, and/or a reduction in retinal thickness of the right goggled eye and, thus, a smaller retina–sclera ratio of the right eye as a result of an increase in FDM-induced ocular changes.

RESULTS

The Rate of Ocular Growth of Chicks Under Form-Deprived Conditions

Among the 34 chicks that were goggled for the right eye, 27 survived through the whole experimental period. They appeared healthy, with body weight increasing from 45.2 ± 1.1 g on the first day following goggle suturing (5–7 days after birth) to 82.5 ± 4.0 g ($n = 27$) on day 12. There was a pronounced difference between the goggle-wearing and the open eyes, and the difference in size was clearly visible (Fig. 1). Among the 27 chicks, the left eyes were used as controls. Seven chicks were given 0.9% alcohol/saline and no RA, and their goggled right eyes were thus FDM eyes. For the remaining 20 chicks, five had 1 $\mu\text{g}/\text{day}$, five had 10 $\mu\text{g}/\text{day}$, six had 30 $\mu\text{g}/\text{day}$, and four had 100 $\mu\text{g}/\text{day}$ of RA.

The eye weights, ocular lengths, and equatorial diameters (only the maximum diameter was measured as the chick's eye is not a perfect sphere) of the control ($n = 27$) were significantly different from those of the FDM eyes ($n = 7$) (Table 1). In general, the relative change in equatorial diameter measurements upon FDM eyes, but without RA administration, was comparably smaller (difference of $7.70 \pm 1.46\%$ between the open and goggled eyes) than changes in eye weights ($28.91 \pm 2.81\%$) and

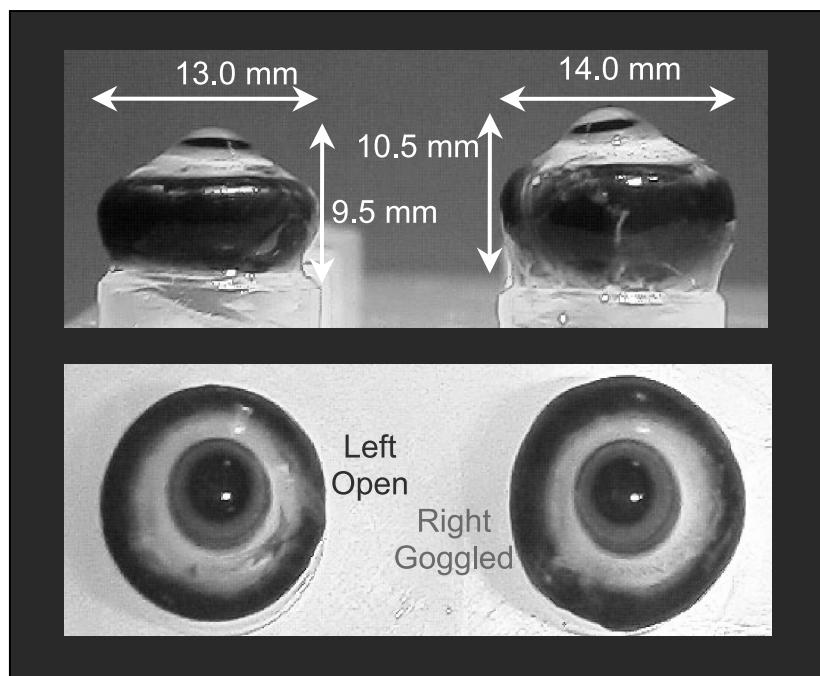


Figure 1. Enucleated eyes (side and bird's-eye views) of the same chick after 12 days of form deprivation. The weight of the right form-deprived eye was noticeably bigger than the left by approximately 28%. (View this art in color at www.dekker.com.)

Table 1. Comparison of physical measurements of the left and right eyes in terms of eye weight, ocular length, and equatorial diameter.

Dimensions	Eye weight	Ocular length	Equatorial diameter
Left (open)	0.62 ± 0.01 g	0.92 ± 0.01 cm	1.23 ± 0.01 cm
Right (goggled)	0.79 ± 0.03 g ^b	1.04 ± 0.03 cm ^a	1.31 ± 0.01 cm ^a

Student's paired t-test, n = 7.

^aP < 0.01.

^bP < 0.001.

ocular lengths ($13.18 \pm 1.98\%$). In chicks that were treated with RA, the difference in eye weight between the right (goggled) and left (open) eyes lessened with increasing concentrations of RA (1–10 µg/day). As shown in Fig. 2, although there was still a relative increase (bigger than zero relative left and right eye measurements) in the goggled right eye weights of the RA-treated chicks (only up to 10 µg/day), this increase was less than that of the control animals (goggled but without RA treatment) with significant ($P < 0.05$) reduction (closer to zero) observed at 10 µg/day RA. This is because the relative difference would have been zero if the two eyes were identical.

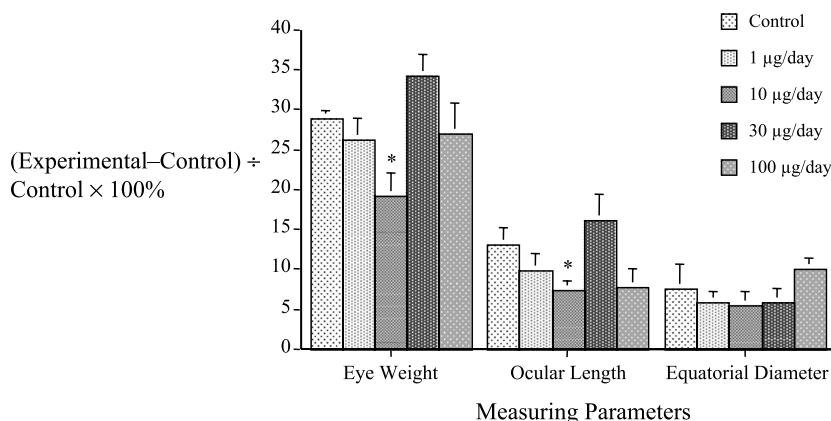


Figure 2. The degree of ocular growth, as indicated by the relative changes of eye weights, ocular lengths, and equatorial diameters, between right and left eyes, is biphasic. In the presence of retinoic acid, the extent of increased growth as a direct result of form deprivation decreased as the daily injection quantity increased from 1 to 10 µg/day. However, form-deprived induced growth became more severe when the daily retinoic acid injection increased above 30 µg/day. Each bar represents the difference in relative changes between the right and left eyes [$(\text{Experimental} - \text{Control}) \div \text{Control} \times 100\%$]. Differences between the left and right eyes of the controls vs. differences observed in the RA-treated groups were compared using one factor ANOVA (* $P < 0.05$). Number of chicks: seven controls given no RA, five given 1 µg/day RA, five given 10 µg/day RA, six given 30 µg/day RA, and four given 4 µg/day RA. (View this art in color at www.dekker.com.)

A similar trend was observed with the ocular length but not with equatorial diameter measurements. However, this reduced increase in form-deprived eye weights and ocular lengths of animals under FDM diminished when the RA concentrations exceeded 30 µg/day.

The Relative Change of Retina and Sclera

Histological examinations of the two ocular layers, retina and sclera, showed that chicks treated with RA had measurements that were consistent with a reduction in myopia development. Under FDM, the right retina (goggled eye) was thinner than the left (control) by $24.3 \pm 30.5\%$. In contrast, the right sclera, from the chick's eye that was subjected to form-deprived conditions, became thicker than the left, with the right sclera thickened by $24.6 \pm 17.5\%$. Upon RA administration of up to 10 µg/day, reduction in retinal thinning and inhibition of scleral thickening were observed (Fig. 3). However, the difference between the control and test eyes was not significant. This is probably due to a relatively small sample size and the fact that it is difficult to guarantee the angle of sectioning, leading to large standard deviations. This was overcome by dividing the thickness of the retina–sclera ratio of the left to the right eye in order to obtain the myopic index. This effectively removed the inherent errors in the angle of sectioning between eyes. As such, the myopic index shows that chicks treated with RA became less myopic (closer to 1) and had a lower myopic index than the control animals. The retina–sclera ratio between the left and right eyes was progressively restored (Fig. 4A) with the myopic index of 1.43 ± 0.23 in the control group

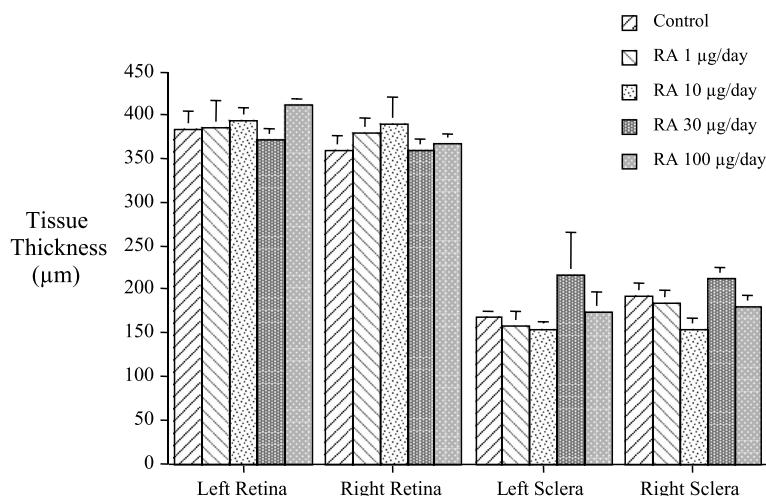


Figure 3. The retina and sclera tissue thickness in the absence and presence of retinoic acid (RA, 1–100 µg/day). The goggled eyes (right eye) had thinner retina than the controls (left eye), and this was reversed in the presence of RA. Thickening of the sclera (right goggled eye) was observed under form-deprived conditions, and this was inhibited by the presence of RA. (View this art in color at www.dekker.com.)

reduced to 1.05 ± 0.13 in animals having up to $10 \mu\text{g}/\text{day}$ of RA treatment ($P < 0.05$ vs. control, Fig. 4B), which was comparable to the normal control ungoggled eye. Analogous to the physical measurements obtained above, the dose-related effect of RA diminished when the dosage equaled to or exceeded $30 \mu\text{g}/\text{day}$. It appeared that a further increase in RA daily dosage caused an increase in scleral growth, whereas the effect of this dose of RA on the growth of retina appeared to be inhibitory. It is thus apparent that the concentration used is critical to the potential benefits of RA in alleviating myopia.

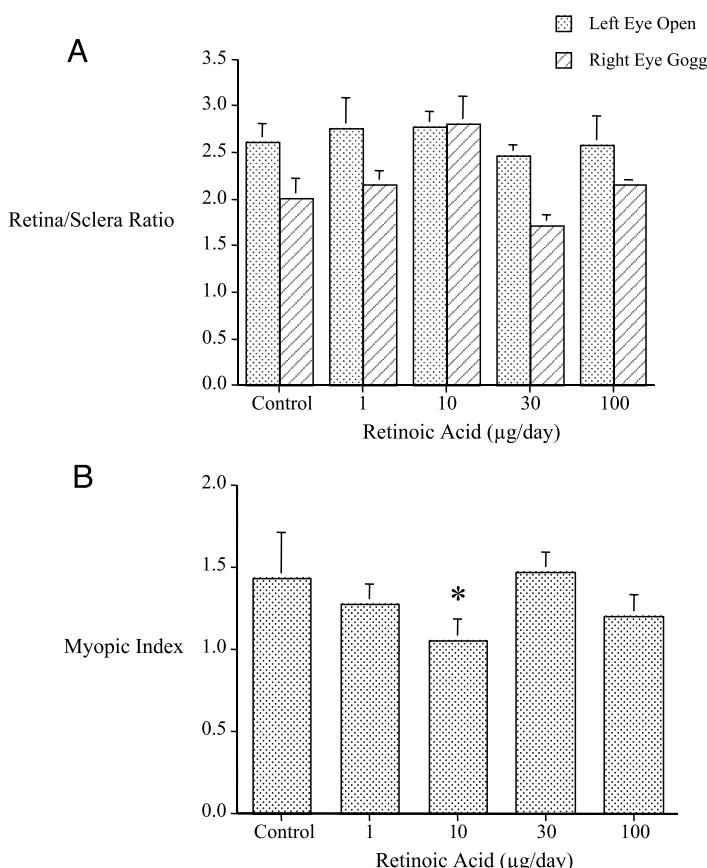


Figure 4. The thickness changes of the left and right eyes are calculated as Retina vs. Sclera Ratio (A). The relative difference between the right (goggled) and left (open) eyes is expressed as the myopic index, which is calculated by dividing the retina-sclera ratio of the left eye by the right eye (B). An index close or equal to 1 indicates no difference between the two eyes and thus, less myopia, and an index significantly greater than 1 indicates thickening of the scleral layer and thinning of the retinal layer in response to form deprivation. The difference between the left and right eyes lessened as the quantity of daily all-trans retinoic acid injection was increased from $1-10 \mu\text{g}/\text{day}$ (ANOVA, $P < 0.05$ vs. Control). However, this retinoic-acid-related protective effect diminished when the daily injection exceeded $30 \mu\text{g}/\text{day}$. (View this art in color at www.dekker.com.)

DISCUSSION

The present study demonstrates the possible use of retinoic acid (in the form of all-trans RA) for the first time in alleviating form-deprivation myopia (FDM) in a chick model. The effect of RA on the FDM-related development, albeit small, appeared to be dose-related up to 10 µg/day but not when the dose equaled or exceeded 30 µg/day. The FDM-induced changes were reduced in chicks treated with RA (1 and 10 µg/day) as indicated by the eye weights, ocular lengths, equatorial diameters, and the difference of the ocular tissue thickness between the left and right eyes.

It is of no surprise that RA is important for ocular growth and development since there is a wide distribution of retinoid-binding proteins, retinoid receptors, and retinaldehyde dehydrogenase across all layers in the posterior part of the eye (8). The retinoid receptor gene transcripts were found in the neural retina of the chicks throughout development (8). It is known that eye development requires external input (24) and that myopia still occurs in chicks having their optic nerves severed (25). Furthermore, form deprivation (or image degradation) will cause changes in ocular tissue thickness, such as choroidal expansion due to increase in choroidal proteoglycan synthesis (26,27) and loss or replacement of scleral tissues (21,28). As eyes in the dark, such as in this case with chicks wearing black goggles, the endogenous RA production is reduced (6,19). This serves as a convenient way to investigate the changes that occur when the RA levels are decreased and if exogenously applied RA can reverse these changes. In chicks, FDM leads to an increased growth of sclera and thus ocular elongation (29,30), which was also observed in this study. Under form-deprived conditions, there was a global increase in eye size and an increase in scleral thickness and thinning of retina as seen in other studies (23,31). The increase in eye size is due to the growth of posterior cartilaginous sclera but not the fibrous sclera as shown in a study by Kusakari, Sato, and Tokoro (20). In this study, only the cartilaginous layer was measured, with the assumption that there was no change to the fibrous sclera.

The FDM-related changes measured in this study are reflected by the increase in eye weights, ocular lengths, and equatorial diameters, and by the thinning of the retina and thickening of sclera. The equatorial diameter difference between controls and the form-deprived eyes was less than 8%, whereas the differences in eye weights and ocular lengths were up to 28% and 13%, respectively (Fig. 2). These data suggest that under the present form-deprived conditions, the increase in ocular length is greater than the width of the eye, and this ocular growth is probably due to that of sclera at the posterior end causing ocular elongation. This growth was inhibited by a daily injection of RA of up to 10 µg/day. Also, figures obtained for retinal/scleral tissue thicknesses and the subsequently calculated myopic index suggest that the goggled-eye measurements were returning to values close to those of the nongoggled eyes upon RA treatment, with significant reversal occurring at 10 µg/day. The inhibition of scleral growth may be due to RA as indicated in a previous study, where the proliferation of scleral chondrocytes and scleral fibroblasts was inhibited by the treatment with RA in a dose-dependent manner (4), or through the inhibition of proteoglycan production (6). Although the endogenous RA levels in the retina have been found to increase following a few days of form deprivation or with negative lens treatment (32), RA levels in the choroid and sclera actually declined (32,33). This may be an important fact as it has

been shown, albeit in cultures, that RA applied to sclera produced an inhibition of proteoglycan production (6). Furthermore, visual conditions that cause increased rates of ocular elongation (such as wearing occluders) can sharply decrease in all-trans RA synthesis in chicks (6). It seems, therefore, reasonable to assume that the main effects of RA on FDM occurs in the chick's cartilaginous sclera by means of inhibiting its growth, leading to less ocular elongation and general growth and thus, less myopia.

The diminishing protective effect on myopia was somewhat puzzling. The data suggest that there is an upper limit of RA beyond which, the presence of high levels of RA has no beneficial effect on FDM. It is well known that vitamin A can cause problems in the eye, such as microphthalmia and anophthalmia, when taken in excess during the fetal stage of development (34,35). Although it has been shown that both vitamin A and RA stimulate scleral fibroblast proliferation (36), there is no direct evidence showing that vitamin A or RA stimulates ocular growth when given in excess. Further experiments are required to elucidate the effects of high RA concentrations on the eye.

As indicated in the present study, the normal eye size and tissue thickness of retina and sclera under form-deprived conditions were partially restored in chicks having a daily RA injection. Although it is known that the morphology and physiology of chickens are quite different from those of humans and other mammals, it is possible that RA may still have an effect on myopia in humans, possibly through proteoglycan synthesis inhibition. The disappearance of the protective effect of RA on FDM at high concentrations seems to be consistent with the established fact that excess as well as deficiency of vitamin A during development is often associated with ocular pathology (37). The current plan of our laboratories is to investigate whether RA affects the growth of individual ocular layers in any beneficial way with regards to myopia development in higher mammals. Nonetheless, the data in the present study suggest that daily, systemic administration/injection of RA to chicks may be sufficient to prevent the decline of RA levels under form-deprived conditions, thus enabling the inhibition of excess scleral growth and retinal thinning.

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