Scleral changes induced by atropine in chicks as an experimental model of myopia

Patricia Gallego, Carmen Martínez-García, Pablo Pérez-Merino, Lucía Ibares-Frías, Agustín Mayo-Iscar and Jesús Merayo-Lloves

1Department of Cell Biology, Histology and Pharmacology, School of Medicine, University of Valladolid, 2Department of Statistics, School of Medicine, University of Valladolid, Valladolid, and 3Fernandez Vega Ophthalmic Research Foundation, Oviedo, Spain


Keywords: atropine, chicks, myopia, sclera

Correspondence: Patricia Gallego Muñoz
E-mail address: patricia.gallego.munoz@uva.es

Received: 11 January 2012; Accepted: 2 August 2012

Abstract

Purpose: To determine the effects of intravitreal atropine on scleral growth in the form-deprived chick as an experimental model of myopia.

Methods: Five groups of five chicks were studied from day 0–12 post-hatching. One group remained untreated (C), and four were form-deprived by monocular light diffusers to induce myopia. Two groups (RL and A) wore diffusers for 9 days, and the other two groups (D and D+A) wore diffusers throughout the study. Group D received no further treatment (myopia positive control). Groups A and D+A received intravitreal injections of atropine for days 9–12. Measurements of refractive error and axial length were performed on days 0, 9, and 12. Sclera changes were assessed in cartilaginous and fibrous layers by histological analysis.

Results: All form-deprived eyes had a myopic refractive error on day 9. All atropine-treated groups were hyperopic on day 12. The effect of atropine was most evident in Group D+A in which diffusers were maintained throughout treatment and changes in refractive error were statistically significant. The observed changes in axial length were in line with the changes in refractive error. The scleral fibrous layer thickness increased, and the scleral cartilaginous layer underwent a slight thinning compared to Group D, the myopia positive control.

Conclusions: If the signals that induce growth remain during atropine treatment, morphological changes in sclera are produced: the scleral fibrous layer thickened, and the scleral cartilaginous layer thinned. These changes resulted in refractive error recovery, and the ocular growth was stopped. The data suggested the atropine was acting throughout the scleral fibrous layer.

Introduction

The process of emmetropisation is based on an active and visually-guided mechanism in the growing eye that achieves a close match of the focal length of cornea and lens to the axial length. When the retinal image is degraded by the absence of a normal visual experience in the early stages of development, the normal course of emmetropisation is compromised, and the eye continues to grow in the axial dimension, resulting in myopia. Myopia is a very common condition in humans, and it is related to environmental and genetic factors. Since the shape of the eye is defined by the mechanical properties of the sclera, alterations in this layer may be linked to the progression of myopia. Furthermore, it has been suggested that myopia is associated mainly with a longer axial length due to enlargement of the vitreous chamber. During the development of myopia, a retinal-scleral photomechanotransduction pathway mediates the remodelling of the scleral matrix. This leads to a reduced stiffness in the scleral layer associated with sclera thinning and the formation of posterior sclera ectasias.
The requirement of a clear retinal image for proper emmetropisation has been extensively studied using different mammalian and avian models. Animal models based on form deprivation and optical defocus have been used in the early developmental stages to alter emmetropisation and to induce myopia.\textsuperscript{3,12–16} The chick has been the most common animal model for studying the progression of myopia due to its low cost and fast response to alterations in the visual environment. While there are similarities between the fibrous layer of the chick and that of mammals, the chick sclera also has a cartilaginous layer that is not present in human eyes.\textsuperscript{4,17,18} During the progression of myopia by visual manipulation in the chick model, the cartilaginous and the fibrous layers have opposite effects on eye growth. The posterior cartilaginous layer increases in thickness, while, the fibrous layer thins, as it does in mammals.\textsuperscript{4}

Recently, considerable efforts have been made to develop drugs that prevent myopia. Two drugs have been clinically tested for their myopia-preventing effect, atropine and pirenzepine. Atropine is a non-selective muscarinic receptor antagonist\textsuperscript{19–23} and pirenzepine preferentially blocks mammalian M1 and M4 receptors.\textsuperscript{24–26} However, both drugs have ocular and systemic side effects, and the preventative effects on the development of myopia are lost after extended application.\textsuperscript{19,27}

Previous studies have explored the effects of atropine in preventing the development of myopia in the chick animal model.\textsuperscript{20,22,24,26,28} The high potency of intravitreal atropine suggests an intraocular target, e.g., the sclera.\textsuperscript{20,22,25} However, the mechanism by which atropine produces its action is still unclear. This is a critical question, as atropine is currently used as an off-label treatment for myopia in children, especially in Southeast Asia.

In this study, we used the form-deprived chick experimental model of myopia to analyse ocular biometric, refractive and morphological changes in the scleral layers. Form deprivation was achieved by a light diffuser, and the effect of atropine was tested in myopic eyes after the diffuser was removed and in eyes that continued to develop myopia.

**Materials and methods**

**Animals**

Twenty-five male White Leghorn chicks (\textit{Gallus gallus domesticus}) obtained from a local hatchery (Ibertec, [http://www.ibertec.es](http://www.ibertec.es)) on the same day they hatched, day 0, were used in this study. The animals were cared for following the guidelines of the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. The chicks were kept in a large incubator on a 12-h light/12-h dark cycle in the animal facilities of the university. The room temperature was kept at 30°C during the first post-hatching week and at 28°C thereafter. Water and food were supplied \textit{ad libitum}.

On day 0, light diffusers, handmade from frosted plastic foil, were attached to the left eyes as previously described.\textsuperscript{20} The light diffusers had little effect on retinal illumination, but prevented ‘form vision’. The right eyes remained untouched as a control.

We conducted a preliminary study to select the lowest dose of atropine that completely blocked the development of myopia in the form-deprived eyes. Atropine sulfate monohydrate (1%, Colircusi, Alcon Cusi, S.A., www.alcon.es) was dissolved in ringer lactate (RL, B. Braun, www.bbraun.es) to yield final doses of 0, 2.5, 250, 750, and 2500 μg. The lowest dose that completely suppressed the development of form deprivation myopia was 750 μg. That dose was selected for the experiments reported here.

From days 9 to 12, a monocular intravitreal injection of the fresh atropine solution (12.5 μL) was administered daily with a 0.03 mm (30 G) × 8 mm needle. The chicks were anaesthetised by intramuscular injection of ketamine hydrochloride (37.5 mg kg\textsuperscript{−1}, Ketolar; Pfizer, [http://www.pfizer.com](http://www.pfizer.com)) and xylazine hydrochloride (5 mg kg\textsuperscript{−1}, Rompun; Bayer AG, www.bayer.com). Additionally, topical anaesthesia was provided by application of 0.5% tetracaine hydrochloride and 1 mg of oxybuprocaine (Colircusi Anestésico Doble; Alcon Cusi, S.A., www.alcon.es).

The chicks were divided into five different groups of five animals (Table 1). The control group, Group C, received no treatment. The second group, Group D, wore light diffusers for days 0–12 and had no other treatment. The third group, Group RL, wore the light diffusers for days 0–9, and received intravitreal injections of Ringer’s lactate solution (12.5 μL) for days 9–12. The fourth group, Group A, wore light diffusers for days 0–9 and were treated with atropine for days 9–12. The fifth group, (Group D + A) wore the diffusers for days 0–12 and were treated with atropine from...

**Table 1. Groups and treatment protocols**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Duration of diffuser wear (days)</th>
<th>Treatment (days 9–12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>D</td>
<td>12</td>
<td>–</td>
</tr>
<tr>
<td>RL</td>
<td>9</td>
<td>Ringer lactate</td>
</tr>
<tr>
<td>A</td>
<td>9</td>
<td>Atropine</td>
</tr>
<tr>
<td>A + D</td>
<td>12</td>
<td>Atropine</td>
</tr>
</tbody>
</table>

C: Untreated animals. D: Group treated with diffusers throughout the study and untreated. RL: Group treated with diffusers until day 9 and treated with ringer lactate from day 9 to 12. A: Group treated with diffusers until day 9 and treated with atropine from day 9 to 12. A + D: Group treated with diffusers throughout the study and treated with atropine from day 9 to 12. n = 5 for each group.
days 9–12. The aim of injecting atropine after removal of the diffusers on day 9 (Group A) was to determine if atropine enhanced the rate of the normal recovery process. The RL group, in which Ringer’s lactate was injected from day 9, served as a control to determine if the vehicle itself had an effect on the rate of recovery.

**Refraction and axial length**

On days 0, 9, and 12, refraction measurements were made on the unanaesthetised chicks. The refractive state was evaluated with an automated eccentric infrared (IR) photorefractor.\(^3,29–31\) Afterwards, axial length was measured under topical anaesthesia with a modified ultrasound biometer (Allergan Humphrey Mod. 826, www.allergan.com) as previously described.\(^32,33\)

**Tissue processing and light microscopy**

Under anaesthesia, the chicks were killed on day 12 with an overdose of intraperitoneal sodium pentobarbital (Dolethal, register number: 0737-ESP, www.vetoquinol.com), and the ocular globes were enucleated. All of the eyes were dissected, and the external orbital tissue was removed. Each eye was divided into anterior and posterior hemispheres. The posterior hemispheres were fixed with buffered formalin 10% for 24 h, washed in 0.1 M phosphate buffered solution, and embedded in paraffin wax. Sections (7 μm) were stained with haematoxylin-eosin (H-E). The sections were evaluated under a light microscope (BX41; Olympus, www.olympus.com), and photomicrographs were obtained with an Olympus DP20 Digital Camera (Olympus, www.olympus.com) in three different areas per sample.

**Cell proliferation**

One hour before euthanasia, we injected 5-bromo-2′-deoxyuridine (intramuscular, 5 ml kg\(^{-1}\) of BrdU-BS002; Sigma, www.sigmaaldrich.com) to label newly synthesised DNA. Sections of labelled tissue, prepared as described above, were deparaffinised and treated with 2 N HCL for 1 h at 37°C. After rinsing with tris-buffered saline (TBS), the samples were incubated with mouse monoclonal IgG anti-BrdU (1:20 dilution in TBS, M0744; Dako, www.dako.com) for 30 min at room temperature. The secondary antibody was fluorescein goat anti-mouse IgG (F2761; Molecular Probes, www.invitrogen.com). Sections were examined under an Axiophot fluorescence-incorporated microscope (426 510-9901-000; Carl Zeiss, www.zeiss.com) and the photomicrographs were captured using the Axio CAM HRc 10–33 VDC Zeiss (Carl Zeiss, www.zeiss.com). Labelled cells were counted at × 20 magnification.

**Statistics**

Means ± standard deviations were calculated for each of the variables. T-tests were used to determine the statistical significance of changes in each group on days 9 and 12. The differences between groups were evaluated with the Scheffe multiple comparison procedure. This test allowed for the control of type I error in a global way. The level of statistical significance was set at \(p < 0.05\).

**Results**

There were no significant differences in refraction or axial length between the untreated contralateral right eyes and untreated left eyes of Group C. For the purpose of this study, only the left eyes of Group C animals were used as controls.

**Refractive error**

Before the light diffusers were placed, all chicks exhibited moderate hyperopic errors (Table 2). On day 9, animals with light diffusers (Groups D, A, D + A, and RL) had a collective myopic refractive error of \(-2.69 \pm 0.32\) dioptres (D). On day 12, the treated eyes of Groups A and D + A were hyperopic (+1.11 ± 0.21 D and +1.46 ± 0.41 respectively). At the same time, the treated eyes of Group RL had a level of hyperopia similar to that of Group C (Table 2).

**Axial length**

Control eyes (Group C) increased in axial length from 7.41 ± 0.47 mm on day 0 to 7.92 ± 0.26 mm on day 9 (Table 2). For all of the groups with light diffusers, the axial length on day 9 was greater than for the controls. By day 12, the axial lengths were similar for all groups except Group D, 9.14 ± 0.31 mm, which was slightly greater than the others. The differences found between days 9 and 12 in each group were significant only for Group A, which changed from 8.53 ± 0.06 to 8.08 ± 0.10 mm (\(p = 0.02\)).

**Morphological changes in the scleral layers**

*Figure 1* shows representative changes in the thickness of the scleral layers in each group. For Group RL, the thickness of the cartilaginous layer, 44.10 ± 5.31 μm, was significantly thinner than the same layer in Group D (Table 3). The fibrous layer of Group RL eyes was slightly, but not significantly, thicker than the fibrous layer of the Group D eyes. The cartilaginous layer of Groups A and D + A,
42.61 ± 2.53 μm and 47.95 ± 5.78 μm respectively, were both significantly thinner than in the Group D eyes. In contrast, the fibrous layers of Groups A and D + A, 30.76 ± 3.66 and 26.34 ± 3.69 μm respectively, were thicker than in the Group D eyes.

Cell proliferation in the scleral layers

The number of BrdU-labelled cells in the cartilaginous layer of Group C was 18.60 ± 5.9 (Table 3). For Groups D and RL, the number of labelled cells was slightly higher than the controls, and for Groups A and D + A, the atropine-injected groups, the number of labelled cells was slightly lower. However, none of the differences in labelling were significant. For the fibrous layer, in Group C the number of BrdU-labeled cells was 11.40 ± 2.30 (Table 3). For all other groups, there were slightly fewer labelled cells, but again, the differences were not statistically significant.

Discussion

Previous studies have described the anatomical changes in chick eyes during the development of form deprivation-induced myopia, as well as the inhibitory effects of

<table>
<thead>
<tr>
<th>Groups</th>
<th>Measurements</th>
<th>Day 0</th>
<th>Day 9</th>
<th>Day 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>RE (D)</td>
<td>+7.64 ± 1.35</td>
<td>+2.27 ± 0.30*</td>
<td>+2.85 ± 0.45</td>
</tr>
<tr>
<td></td>
<td>AL (mm)</td>
<td>7.41 ± 0.47</td>
<td>7.92 ± 0.26</td>
<td>8.03 ± 0.34</td>
</tr>
<tr>
<td>D</td>
<td>RE (D)</td>
<td>+7.54 ± 1.84</td>
<td>−2.24 ± 0.51</td>
<td>−3.78 ± 0.86</td>
</tr>
<tr>
<td></td>
<td>AL (mm)</td>
<td>7.70 ± 0.26</td>
<td>8.61 ± 0.43</td>
<td>9.14 ± 0.31</td>
</tr>
<tr>
<td>RL</td>
<td>RE (D)</td>
<td>+6.13 ± 0.24</td>
<td>−2.73 ± 0.07*</td>
<td>+2.18 ± 0.52</td>
</tr>
<tr>
<td></td>
<td>AL (mm)</td>
<td>6.92 ± 0.24</td>
<td>8.60 ± 0.44</td>
<td>8.56 ± 0.62</td>
</tr>
<tr>
<td>A</td>
<td>RE (D)</td>
<td>+6.98 ± 1.80</td>
<td>−3.02 ± 0.28*</td>
<td>+1.11 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>AL (mm)</td>
<td>7.88 ± 0.70</td>
<td>8.53 ± 0.06*</td>
<td>8.08 ± 0.10</td>
</tr>
<tr>
<td>D + A</td>
<td>RE (D)</td>
<td>+7.21 ± 1.09</td>
<td>−2.80 ± 0.99*</td>
<td>+1.46 ± 0.41</td>
</tr>
<tr>
<td></td>
<td>AL (mm)</td>
<td>7.37 ± 0.42</td>
<td>8.80 ± 0.14</td>
<td>8.75 ± 0.17</td>
</tr>
</tbody>
</table>

*Statistically significant differences (p < 0.05) found in the changes between days 9 and 12 in the same group. C: Untreated animals. D: Group treated with diffusers throughout the study and untreated. RL: Group treated with diffusers until day 9 and treated with ringer lactate from day 9 to 12. A: Group treated with diffusers until day 9 and treated with atropine from day 9 to 12. D + A: Group treated with diffusers throughout the study and treated with atropine from day 9 to 12.

Figure 1. Photomicrographs of the scleral sections stained with Hematoxylin-Eosin (H&E) in different experimental groups on day 12. C: untreated animals; D: group treated with diffusers throughout the study and untreated; RL: group treated with diffusers until day 9 and treated with ringer lactate from day 9 to 12; A: group treated with diffusers until day 9 and treated with atropine from day 9 to 12. D + A: Group treated with diffusers throughout the study and treated with atropine from day 9 to 12. The numbers in each layer denote the thickness in micrometers (μm). Scale bar is 20 μm. Magnification × 400.
intravitreal atropine treatment. However, none of these studies have reported the morphological changes in the sclera due to the atropine treatment.

Myopia induced by wearing diffusers is highly variable among individual chicks, ranging from −20.3 ± 1.0 to −6.1 ± 0.3 D. In the present study, control eyes wore the diffuser for 12 days and received the atropine treatment, consistent with other reports.

Because the scleral layer shows ultrastructural and biomechanical changes in high myopia, this layer might represent one of the targets for therapeutic manipulation of myopia. Several studies showed opposite modulation of growth in the scleral fibrous and cartilaginous layers.

In the present study, the scleral thickness of both layers was analysed to evaluate the morphological changes of different groups. Consistent with previous studies, the study found that the cartilaginous layer was thicker in Group D animals and the fibrous layer was thinner than the normal eyes in Group C. In addition, the scleral changes after atropine treatment were the opposite of the changes in Group D. The thickness of both scleral layers in Group A, which had the diffuser for 9 days and then atropine treatment until day 12, was similar to those in Group C on day 12. For Group D + A, the signal that induced eye growth remained constant throughout the study because the chicks wore light diffusers. Thus, the real effect of atropine treatment may be best observed in this group.

The thickness of the cartilaginous layer was similar in Groups D and D + A. However, treatment with atropine in Groups A and D + A restored the thickness of the posterior fibrous layer to the control level in Group C. This suggests that the effect of atropine treatment is located in this area of the sclera.

There is a significant volume of literature regarding changes in scleral structure during the development of experimental myopia, the localisation of muscarinic cholinergic receptors within the eye, the effects of muscarinic agents on scleral remodelling and on the possible sites of action of such compounds. Lind et al. demonstrated that cellular proliferation and extracellular matrix production in the cartilaginous sclera are inhibited by muscarinic antagonists, including atropine, through what appears to be either an M1 or M4 receptor pathway. This work was further supported by the studies of Wang et al. and Troung et al. that demonstrated pirenzepine-induced reductions of glycosaminoglycan levels in the cartilaginous sclera, but not in the fibrous sclera. Such work suggests that atropine inhibits cellular proliferation and extracellular matrix production within the cartilaginous layer, but not within the fibrous layer of the chick sclera.

The biometric and refractive measurements carried out in our study and the histological results demonstrated morphological changes in the posterior scleral layers after atropine treatment, specifically in the fibrous layer, in which the thickness increased. These changes resulted in the recovery of refractive error and the cessation of ocular growth. This work suggests that the effects of atropine on myopia are largely due to its effect on the sclera, and...
therefore, the changes produced by atropine treatment in the fibrous layer may delay the progression of myopia.

Despite these results, more studies are necessary to understand how and where atropine affects the development of myopia. The present study has some limitations. First, it was carried out in chicks as the experimental model; thus comparable studies in mammals are required to determine if they behave similarly with respect to the development of myopia. Another limitation is the fast rate at which myopia occurs in post-hatched animals. It would be useful to analyse the effects of atropine in advanced stages to mimic the development of myopia in humans.

Acknowledgements

The authors acknowledge funding in part from the PROFIT CIT-300 100-2007-50 grant, Spanish Ministry of Science and Innovation. We would also like to thank Roberto Cantalapiedra Rodríguez for his technical assistance with the histopathology. This work was presented in part as a poster at the annual meeting of the Association for Research in Vision and Ophthalmology, ARVO, Fort Lauderdale, FL, USA, May 2009.

Conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References


