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The Effect of Intravitreal Injection of Atropine on the Proliferation of Scleral Chondrocyte *In Vivo*

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ABSTRACT

Atropine was found to be effective in arresting the progression of myopia. However, the actual mechanism is still unclear. Thus, we tried to investigate the *in vivo* effect of atropine on the proliferation of scleral chondrocytes in chicks of form-deprivation myopia. Twenty chicks were equally divided into 4 groups which included intravitreal injection of normal saline (IVN), IVN with goggling (IVNG), intravitreal injection of atropine (1%) (IVA), and IVA with goggling (IVAG) groups. Intravenous injection of bromodeoxyuridine (BrdU) (30 mg/kg) from subaxillary vein was performed 2 hours before being sacrificed. The eyeballs were then fixed in 10% neutral-buffered formalin at 4°C. Standard BrdU immunohistochemical staining was performed. The BrdU labeling index was obtained from the average of positive labelings of BrdU in scleral chondrocytes for every 100 counting cells in posterior poles and anterior scleral margins by two experienced technicians. The BrdU index on the anterior scleral margin of the IVAG group was less than that of the IVNG group. The index on the anterior scleral margin of the IVNG group was higher than the IVN group. Although the index on the posterior poles of the IVNG group was also higher than the IVN group, it was statistically not different. Also, no statistical difference was found between IVN and IVA on the anterior scleral margins or posterior poles. The index was significantly different on the anterior scleral margins, but not on the posterior pole among each group. Therefore, intravitreal injection of atropine could inhibit the proliferation of chondrocytes on the anterior margins of sclera, but not the posterior poles in form-deprivation myopia.

INTRODUCTION

The prevention and treatment of myopia are important issues of public health in many countries, especially in Taiwan where the prevalence rate of myopia is extremely high (1). Thus, to find out an effective method or a medication to control or prevent the progression of myopia is the most important topic in this area.

A variety of mechanisms of myopia have been postulated before, but most investigators are now convinced that myopia is multifactorial in nature, and that excessive near-work or prolonged reading

is the main cause of myopia. It is believed that excessive or sustained accommodation produces tension in the ciliary muscle and, with it, a tension in the extraocular muscles of convergence (2). The strain, from the accommodation of the lens caused by ciliary muscle, that affects the sclera is thought to be responsible for the progression of myopia. Therefore, cycloplegics have been used to arrest myopic progression for years. Among various cycloplegics, atropine was shown to be effective in controlling the progression of myopia and high myopia in many previous studies (3-13). Its efficacy showed promise in helping to satisfy the clinical demands for prevention and in stopping the progression of myopia.

In the previous study, we used the form-deprivation myopia (FDM) of chick model to study the pathophysiology of myopia and found that the proliferation of scleral chondrocytes is the key point of experimental myopia (14). If there were any treatment modalities or medications that could retard the proliferation of scleral chondrocytes, they also could control the progression of myopia. According to this rationale, we chose atropine as the treatment modality and used it in experimental myopia in chicks for our study. This model of experimental myopia is a good model to study the mechanism of myopia formation and can be used to test the efficacy of various treatment protocols. In the present study, we use an *in vivo* model to investigate the effect of intravitreal injection of atropine on the proliferation of scleral chondrocytes. Using this model, we could confirm the efficacy and pathophysiology of atropine in the control of progression of myopia

MATERIALS AND METHODS

Induction of Myopia in Chicks

All animals were kept in accordance with the ARVO Resolution on the Use of Animals in Research. Twenty White Leghorn chicks were used for this study. They were equally divided into 4 groups that received different treatments: intravitreal injection of normal saline (IVN), intravitreal injection of normal saline and eyes covered with goggles (IVNG), intravitreal injection of atropine (10 μg/100 μl) (IVA), and intravitreal injection of atropine and eyes covered with goggles (IVAG). Calculated concentration of atropine at the retina was around 300-400 μmol/l. The intravitreal injection was performed on the temporal part of the eyeball with a 30 G needle and repeated every 2 days for 6 times. Goggle covering was achieved with a semi-spherical transducent plastic goggle attached to the orbital rim as previously described (14,15). The fellow eyes of all these chicks were left untreated. The chicks were maintained on 12 hours light-dark cycles for 14 days. They were then sacrificed by an overdose of phenobarbital, and both eyes were enucleated on day 14. Intravenous injection of bromodeoxyuridine (BrdU) (30 mg/kg) from the axillary vein was performed 2 hours before being sacrificed. The eyeballs were then fixed in 10% neutral buffered formalin (Sigma, St. Louis, MO).

Preparation of Tissue Section and BrdU Labeling Assay

Paraffin blocks of formalin-fixed scleral coats were cut into 4 μm thicknesses of tissue sections. They were then mounted on poly-L-lysine-coated slides and allowed to dry overnight at room temperature. A commercial BrdU (5-bromo-2'deoxyuridine) kit (Boehringer Mannheim Biochemica, Germany) was used to evaluate cell proliferation following the protocol from the manufacturer. Briefly, glass slides were deparaffinized with xylene for 3 x 10 minutes. They were then rehydrated with graded alcohol (100%, 95%, 85%, 75%, 50%) for 1 min at each step. After being washed with PBS for 2 x 5 minutes, they were incubated with anti-BrdU monoclonal antibody in 1/10 concentration of anti-BrdU working solution (Tris buffer, 66 mM; $MgCl_2$, 0.66 mM; 2-mercaptoethanol, 1 mM) in humidified atmosphere at 37°C overnight. The secondary antibody anti-mouse-Ig-alkaline phosphatase was added, and the sections were incubated in a humidified atmosphere at 37°C over-

night. After being washed with PBS for 3 x 5 minutes, they were labeled with a freshly prepared chromogenic solution [13 µl of NBT (nitroblue tetrazolium salt, 75 mg/ml in 70% dimethylformamide), 10 µl of x-phosphates (5-bromo-4-chloro-3-indolyl phosphate, tolidinium salt, 50 mg/ml in dimethylformamide), and 3 ml of substrate solution (Tris-HCl buffer, 100 mM; NaCl 100 mM; MgCl2, 50 mM; pH 9.5)] and incubated at room temperature for 15-30 minutes. They were not counterstained by hematoxylin. After dehydration, tissue sections were covered with permount (Fisher, Fair Lawn, NJ) and evaluated under a light microscope. The BrdU labeling index was obtained from the average of positive labeling of BrdU in sclera chondrocytes for every 100 counted cells in 4 tissue sections of posterior poles and anterior scleral margins by two experienced technicians.

The difference of BrdU indexes in each group was checked by one-way *ANOVA,* and the difference between the two groups was tested by multiple range tests, Tukey-B test. Independent Student's t-test was used to compare the difference of BrdU indexes between anterior scleral margins and posterior poles. The p-value less than 0.05 was considered statistically significant.

RESULTS

The BrdU labeled cells are shown in Figure 1. The positively stained cell revealed a densely stained nucleus. In normal and treated groups, the anterior margins of cartilaginous layers of scleral coats in tissue sections showed relatively thicker than other areas of scleral coats, including the posterior pole. Also, the densities of chondrocytes showed heavier than other areas of scleral coats. However, because there were many dehydration, rehydration, and washing steps during the preparation of tissue sections and immunohistochemical staining, the actual thickness was difficult to determine in these tissue sections.

FIGURE 1. **A**: BrdU Labeling of Anterior Margins of Cartilaginous Layer of Scleral Coats in Chicks (100 x); **B**: BrdU Labeling of Posterior Poles of Cartilaginous Layer of Scleral Coats in Chicks (100 x). Arrow heads show positively stained nuclei of scleral chondrocytes.

BrdU labeling indexes in each group are shown in Table 1. In the anterior margin, the difference of BrdU labeling index of anterior scleral margins among these four groups was statistically significant (one-way *ANOVA*, $p<0.05$). There was also a statistically significant difference in labeling between IVNG and other groups, i.e. IVN, IVA, and IVAG (Multiple Range Tests: Tukey-B test with significance level 0.05). However, there was no statistical difference in the posterior poles of the scleral coats among these four groups (one-way *ANOVA*, $p=0.3588$). In the anterior margins and the posterior poles of the scleral coats, there was also no statistical significance between IVNG and IVA groups in these areas (Multiple Range Tests: Tukey-B test with significance level 0.05).

TABLE 1.
The BrdU Labeling Index of Each Group

	Treatment			
	IVN (n=5)	IVNG (n=5)	IVA (n=5)	IVAG (n=5)
Anterior	29.2 ± 5.7	51.46 ± 6.5	29.8 ± 4 9	30.4 ± 2.6
Posterior	10.4 ± 11.5	24.4 ± 10.8	22.4 ± 1(.2	22.0 ± 13.6

IVN = intravitreal injection of normal saline; IVNG = intravitreal injection of normal saline and goggle covering; IVA = intravitreal injection of atropine; IVAG = intravitreal injection of atropine with goggle covering

When the BrdU indexes between the anterior scleral margins and posterior poles were compared, we found that BrdU indexes of the anterior margins were higher than those of the posterior poles in both IVN and IVNG groups (p=0.018 and 0.002, respectively, by independent Student's t-test). However, there was no difference between IVA groups and between IVAG groups (p=0.376 and 0.241, respectively, by independent Student's t-test).

In Table 2, we show that there was no difference of statistical significance in equatorial diameters among each group (one-way *ANOVA*, p>0.05), but there was a difference of statistical significance in axial lengths among each group (one-way *ANOVA*, p<0.05). Also, the mean axial length in IVNG groups was longer than those in the other groups (Multiple Range Tests: Tukey-B test with significance level 0.05).

TABLE 2.
The Axial Length and Equatorial Diameters of Each Group

	Treatment			
	IVN (n=5)	IVNG (n=5)	IVA (n=5)	IVAG (n=5)
Equatorial diameter (mm)	1.280 ± 0.052	1.291 ± 0.050	1.247 ± 0.037	1.277 ± 0.064
Axial length (mm)	0.942 ± 0.036	1.011 ± 0.043	0.923 ± 0.047	0.930 ± 0.053

IVN = intravitreal injection of normal saline; IVNG = intravitreal injection of normal saline and goggle covering; IVA = intravitreal injection of atropine; IVAG = intravitreal injection of atropine with goggle covering

DISCUSSION

Many previous studies have demonstrated the effect of atropine on the prevention of myopia and control of the progression of myopia by blocking accommodation (15-17). McBrien *et al.* showed that chronic intravitreal atropine administration could prevent experimental myopia in chicks *via* a nonaccommodation mechanism. They observed no difference in carbachol-induced accommodation and light-induced pupillary constriction between this procedure and saline injection (18). Chew et al. suggested that both muscarinic agonists and antagonists may act directly on scleral fibroblasts to modulate the development of myopia (19,20). They also found growth-promoting effects of M1 agonists in scleral fibroblasts and inhibitory effects of M2 agonists and muscarinic inhibitors

(20). These results suggested a direct action of the muscarinic agents in controlling human scleral fibroblast growth.

Our results showed that the proliferation of scleral chondrocytes in FDM was retarded by intravitreal injection of atropine. However, this phenomenon focused on the anterior scleral margins rather than on the posterior poles in FDM of chicks. The sclera of the chick is composed of two components: a thick cartilaginous layer and a fibrous outer layer (21). The cartilaginous layer becomes thicker during FDM, but the fibrous layer does not (22). The components of sclera, such as proteoglycan and protein contents, are altered as well; for example, accumulation of extracellular matrix proteoglycans (23-25). This increased cell proliferation and increased synthesis of the extracellular matrix suggested that active growth of the sclera was associated with myopia (14,26). If there were any treatment modalities or medications that can retard the proliferation of scleral chondrocytes, they should also be able to control the progression of myopia. The BrdU index between the anterior and posterior sclera of the IVN and IVNG groups was not different. As for axial lengths and equatorial diameters, we found that axial lengths and equatorial diameters in the IVNG group were longer than those in the other groups, but only the axial lengths showed differences of statistical significance. Our results are different from the previous results; first, we found that the anterior scleral margin of chicken sclera is the area which was rarely discussed before; second, the proliferation of scleral chondrocytes in anterior scleral margins was retarded after chronic intravitreal atropine injection; third, atropine had no effect on the scleral chondrocytes in normal chicks. The anterior scleral margin was thicker than the other areas of scleral coats, but its anatomical significance has not been noted.

The discovery of active scleral growth as the primary event in the axial elongation of the globe has focused the attention of investigators on the regulation of ocular growth and the development in the causation of myopia (26). Retina and retinal pigment epithelium complex was shown to regulate the growth of sclera in experimental myopia and human myopia in many previous studies. Neurotransmitters from the retina or retinal pigment epithelium, such as vasoactive intestinal polypeptide, dopamine and acetylcholine, may act directly on the sclera or trigger the release of growth factors, thereby indirectly leading to scleral growth (27-29). Therefore, we may be able to prevent the progression of myopia through these neurotransmitters and molecules. In this study, we first found that the anterior scleral margin is another important locus that has never been discussed. The area of the anterior scleral margin is close to the ciliary muscle. Atropine can block the accommodation of lens and relax ciliary muscle. We postulated that there might be some relationship between the two anatomical locations. From our results, we suggest a nonaccommodation mechanism by atropine in FDM of chicks. We need further studies to see whether such a mechanism can be extended to human eyes.

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