• Basic Research •

Effects of endogenous dopamine induced by low concentration atropine eye drops on choroidal neovascularization in high myopia mice

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Abstract

● **AIM:** To evaluate effects of endogenous dopamine induced by low concentration atropine eye drops on choroidal neovascularization (CNV) in high myopia mice.

● **METHODS:** The C57BL/6J mice were deprived of the right eye for 4wk, and the high myopia was diagnosed by optometry, the diopter was less than -6.00 D, and CNV was induced by 532 nm laser. The changes of dopamine D1 receptor (DRD1), dopamine D2 receptor (DRD2), and vascular endothelial growth factor A (VEGFA) were detected by Western blot technology at 0.5, 1, 2h, and 7d after 0.01%, 0.05%, and 0.1% atropine eye drops, respectively, the area of CNV was measured.

● **RESULTS:** Significant increases were observed on the expression of DRD2 in mouse high myopia model at 0.5, 1, 2h, 7d with 0.05% and 0.1% atropine eye drops (P<0.05). Significant decreases were observed on the expression of DRD1 and VEGFA in mouse high myopia model at 0.5, 1, 2h, 7d with 0.05% and 0.1% atropine eye drops (P<0.05). The area of CNV induced by laser in the drug-treated group was significantly smaller than that in the control group, and the higher the concentration, the more significant the inhibitory effect (P<0.05).

● **CONCLUSION:** The 0.01%, 0.05%, 0.1% atropine eye drops can decrease the level of VEGFA and inhibit high myopia CNV indirectly by up-regulating the level of DRD2 and down-regulating the level of DRD1, and the effect of 0.05% and 0.1% atropine eye drops is more significant.

● **KEYWORDS:** high myopia; choroidal neovascularization; low concentration atropine eye drops; dopamine D1 receptor; dopamine D2 receptor

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INTRODUCTION

The risk of choroidal neovascularization (CNV) caused by myopia increased gradually with the increase of dioptre's myopia, which increased 2-fold from 1.00 to 2.00 D, 4-fold from 3.00 to 4.00 D, and 9-fold from 5.00 to 6.00 D[1]. Bleeding and edema caused by CNV are the common complications leading to visual function loss in patients with high myopia, which greatly reduces the vision-related quality of life of patients. Without treatment, the vision of most patients with high myopic CNV will further deteriorate and may lead to blindness[2]. The vascular endothelial growth factor A (VEGFA) secreted by retinal pigment epithelium cells (RPE), an important subtype of VEGF, is involved in the genesis and development of CNV[3]. CNV secondary to high myopia are myopic choroidal neovascularization (mCNV), which mainly belong to type II CNV, and present in fundus a grey round or oval lesion. In other words, CNV cells break into RPE and grow in the retinal neuroepithelium[4]. CNV is one of the important causes of vision loss and even blindness in patients with high myopia. Currently, anti-vascular endothelial growth factor (VEGF) is the first-line drug for mCNV[5-6], but there are many postoperative injections required, expensive injections, and side effects of anti-VEGF therapy, *etc*. There is still a need to develop new drugs with anti-VEGF effects as alternative or supportive treatments for mCNV disease.

Atropine stimulates the release of endogenous dopamine, which also inhibits the progression of form deprivation

myopia[7]. Dopamine functions through its corresponding membrane receptors, a family of seven transmembrane regions of G-protein-coupled receptors that dopamine receptor into five major subtypes, they were D1-class receptors (D1, D5) and D2-class receptors (D2, D3, D4)[8]. D2 dopaminergic receptor agonists can downregulate VEGF overexpression and should be taken under consideration as new anti-VEGF drug used in ophthalmological vasculature diseases, such as age-related macular degeneration and proliferative form of diabetic retinopathy and diabetic macular edema[9]. Therefore, low concentrations of atropine may indirectly affect pathological neovascularization of the ocular fundus.

It is well known that atropine eye drops at low concentration has a significant advantage in the prevention and control of myopia[10-11], but the study in pathological myopia is still blank, so this study is intended to investigate the role of endogenous dopamine produced by low concentration atropine in CNV model of high myopia mice, to investigate the relationship between endogenous dopamine and VEGF and its effect on CNV induced by low concentration atropine.

Both atropine and α_{2A} adrenoreceptor antagonists stimulate dopamine release whereas α_{2A} adrenoreceptor agonists strongly suppress its release. Atropine at a dose of 250 μg can stimulate the synthesis and release of dopamine of the retina in chicks developing form deprivation myopia or undergoing normal ocular development[12]. In short, a very small dose of atropine can cause the synthesis and release of retinal dopamine. In this study, dopamine D1 receptor (DRD1) and dopamine D2 receptor (DRD2) were detected.

The amount of dopamine in the eyeball is the highest in the retina, and the amount of dopamine in the vitreous, choroid, and sclera is one-tenth, one-third, and one-twentieth of that in the retina, respectively[13]. So the dopamine level in the retina is measured.

According to literature, younger animals are more sensitive to the induction of form deprivation, and the earlier the onset of deprivation, the faster myopia is induced[14-15]. Liu *et al*[16] carried out monocular form deprivation for 16d in 5-day-old guinea pigs. On day 6, myopia occurred in the eyes with form deprivation, and on day 11, myopia was higher than that in the contralateral eyes for 60d, and on day 16, myopia in the eyes with individual deprivation was higher than that in the contralateral eyes for 50d. Therefore, in this experiment, form-deprived newborn mice were induced to high myopia, and optometry.

At present, the application of low concentration atropine in pathological myopia is still blank. Therefore, this part of the experiment firstly applied low concentration atropine eye drops in the mouse model of high myopia CNV to explore the influence on pathological myopia CNV and related protein expression, and explored whether low concentration atropine in the animal model increases the risk of choroidal bleeding. In pathological myopia, choroidal atrophy is extreme, and deep retinal capillaries initiate compensatory blood supply to the outer retina[17]. Therefore, this part of the experiment also tested the changes of low-concentration atropine eye drops on normal vascular tissue and deep capillary density.

SUBJECTS AND METHODS

Ethical Approval The use of laboratory animals in this study were performed following the Statement on the Use of Animals in Ophthalmic and Vision Research of Association for Research in Vision and Ophthalmology (ARVO 2013). Institutional Animal Care and Use Committee of Tianjin Medical University approved the experimental procedures of the study.

Experimental Design The animals were reared adaptively in specific pathogen free (SPF) level feeding room, and the birth time was recorded to reduce disturbance and avoid feeding. Twenty-four C57BL/6J mice were obtained from Beijing Sibafu Biotechnology Co., Ltd and were randomly divided into four groups: control group (n=6), 0.01% atropine group (n=6), 0.05% atropine group (n=6), and 0.1% atropine group (n=6).

The day of birth of the mice was recorded as day p0. The newborn mice were raised in normal environment, and high myopia was induced by right eye form deprivation for 4wk, the high myopia was diagnosed by optometry, the diopter was less than -6.00 D. Then the mice in each group were anesthetized by intraperitoneal injection of pentobarbital sodium 40 mg/kg, compound tropicamide eye drops were used to dilate the pupils, and sodium hyaluronate was applied to the corneal surface to avoid corneal damage. Krypton laser was used for retinal photocoagulation. Laser parameters[18]: laser wavelength is 532 nm, power is 120 mW, blasting time is 120ms, spot diameter is 100 μm. One laser spot was made at 3:00, 6:00, 9:00, and 12:00 respectively around the optic nerve.

After stimulating the eyeground to produce new blood vessels, after 0.5h, 1h, 2h of atropine eye drops (0.01%, 0.05%, 0.1%), the levels of DRD1, DRD2, and VEGF in retina were measured, are processed between 9–11 *a.m.* The remaining 3 rats in each group were treated with atropine eye drops of different concentrations. After one week, the contents of DRD1, DRD2, and VEGFA in retina and the changes of retinal and CNV were measured.

Western blot analysis was conducted as previously described[19]. After sonication in lysis buffer (AR0101, Wuhan Boshide Bioengineering Co. Ltd.), samples were centrifuged at 10 000g at 4°C for 10min, and total protein concentrations were measured. The extracted protein supernatants were boiled with 5× protein loading buffer at 100°C for 10min, and denatured proteins were separated by electrophoresis

on separation gels and transferred to polyvinylidene fluoride (PVDF) membranes. The first antibody was diluted with the first antibody diluent, and the PVDF membrane was immersed in the first antibody incubation solution and incubated at 4°C overnight. The second antibody was labeled with horseradish peroxidase (HRP) and diluted with Tris buffered saline and Tween-20 Tris-HCl (TBST; blocking solution) at 1:5000. The PVDF membrane was immersed in the second antibody incubation solution and incubated in 37°C shaker for 2h. The protein signal was detected by chemiluminescence kit (P0010, Shanghai Biyuntian Biotechnology Co. Ltd., China). The protein expression levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein level, respectively.

The eyes were removed and fixed in 4% paraformaldehyde solution (G1101, Wuhan Seville Biotechnology Co. Ltd., China) for 10min, the RPE-choroid-sclera mixture was fixed in 4% paraformaldehyde solution for another 50min. The retina and the RPE-choroid-sclera mixture were carefully separated and rinsed with PBS containing 0.5% Tritonx-100 by volume. After 5min of PBS washing twice, it was moved to a clean 48-well plate and the configured Isolectin GS-IB4 solution (I21413 ThermoFisher) was added to the well plate containing the sample at 200 μL per well. Then dark operation, 4°C incubation overnight. Wash the incubated sample three times with PBS for 5min, then spread the clover sample on the slide with a brush. Cover the slide with a coverslip and nail polish to hold the coverslip in place. Keep the fluorescence microscope in the dark. The RPE-choroid-sclera complex was observed and fluorescence microscope. The discoid neovascular plexus was observed under the microscope as an effective CNV, the effective CNV number (*n*) was 4–6 per RPE-choroid-sclera complex specimen. The CNV area was measured by Image J software.

Statistical Analysis SPSS25.0 (IBM, Armonk, New York, USA) software was used for statistical analysis. Data were presented as by mean±standard error (SE), one-way ANOVA was used for data comparison between multiple groups, and LSD-*t* test was used for pairwise comparison between multiple groups. $P<0.05$ was considered as significant difference.

RESULTS

Western blot analysis showed that DRD2 expression in retina of high myopia mice was significantly increased (0.51±0.04, 0.58±0.05) after 0.5h of 0.05% and 0.1% atropine eye drops, compared with the control group (0.42±0.02), the difference was statistically significant ($P<0.05$, <0.01). After 0.5h of 0.05% and 0.1% atropine eye drops, the expression of DRD1 in retina of high myopia mice was significantly decreased (0.49±0.04, 0.46±0.03), compared with the control group (0.59±0.03), the difference was statistically significant ($P<0.05$, <0.01). After 0.5h of 0.05% and 0.1% atropine eye drops, the expression level of VEGFA in retina of high myopia mice was significantly decreased (0.49±0.02, 0.45±0.03), compared with the control group (0.55±0.05), the difference was statistically significant (all $P<0.05$). The expression of DRD2 in retina of high myopia mice was significantly increased (0.50±0.04, 0.53±0.02) after 0.05% and 0.1% atropine eye drops for 1h, and the difference was statistically significant compared with the control group (0.38±0.02), respectively ($P<0.01$, <0.01). The expression of DRD1 in retina of high myopia mice was significantly decreased (0.45±0.03, 0.35±0.02) after 0.05% and 0.1% atropine eye drops for 1h, and the difference was statistically significant compared with the control group (0.54±0.05, $P<0.05$, <0.001). The expression level of VEGFA in retina of high myopia mice was significantly decreased (0.37±0.02) after 0.1% atropine eye drops for 1h, and the difference was statistically significant compared with the control group (0.45±0.03, $P<0.05$). The expression of DRD2 in retina of high myopia mice was significantly increased after 0.01%,0.05% and 0.1% atropine eye drops for 2h (0.54±0.03, 0.63±0.05, 0.70±0.04), compared with the control group (0.44±0.03), the difference was statistically significant ($P<0.05$, <0.001, <0.001). Drop by 0.01%, 0.05% and 0.1% atropine eye drops after 2h high myopia mouse retina DRD1 expression significantly reduce the amount of (0.49±0.05, 0.38±0.04, 0.26±0.02), the difference was statistically significant compared with the control group (0.69±0.03; $P<0.001$, <0.001, <0.001); After 0.01%, 0.05%, and 0.1% atropine eye drops for 2h, the expression of VEGFA in retina of high myopia mice was significantly decreased (0.59±0.02, 0.44±0.01, 0.35±0.03). Compared with the control group (0.77±0.03), the difference was statistically significant ($P<0.001$, <0.001, <0.001). After 7d of 0.05% and 0.1% atropine eye drops, the expression of DRD2 in retina of high myopia mice was significantly increased (0.57±0.03, 0.65±0.05), and the difference was statistically significant compared with the control group (0.49±0.02; $P<0.05$, <0.001). After 7d of 0.01%, 0.05% and 0.1% atropine eye drops, the expression of DRD1 in retina of high myopia mice was significantly decreased (0.43±0.02, 0.38±0.02, 0.32±0.03), compared with the control group (0.58±0.06) difference was statistically significant ($P<0.01$, <0.001, <0.001), drops by 0.01%, 0.05%, 0.1% atropine eye drops after 7d high myopia mouse retina VEGFA expression quantity significantly reduced (0.46±0.04, 0.38±0.03, 0.31±0.02), compared with the control group (0.64±0.03), the difference was statistically significant ($P<0.001$, <0.001, <0.001; Figure 1).

C57BL/6J mice developed high myopia after the right eye was covered for 4wk, laser induced CNV was treated with 0.01%, 0.05%, and 0.1% atropine eye drops, respectively. The

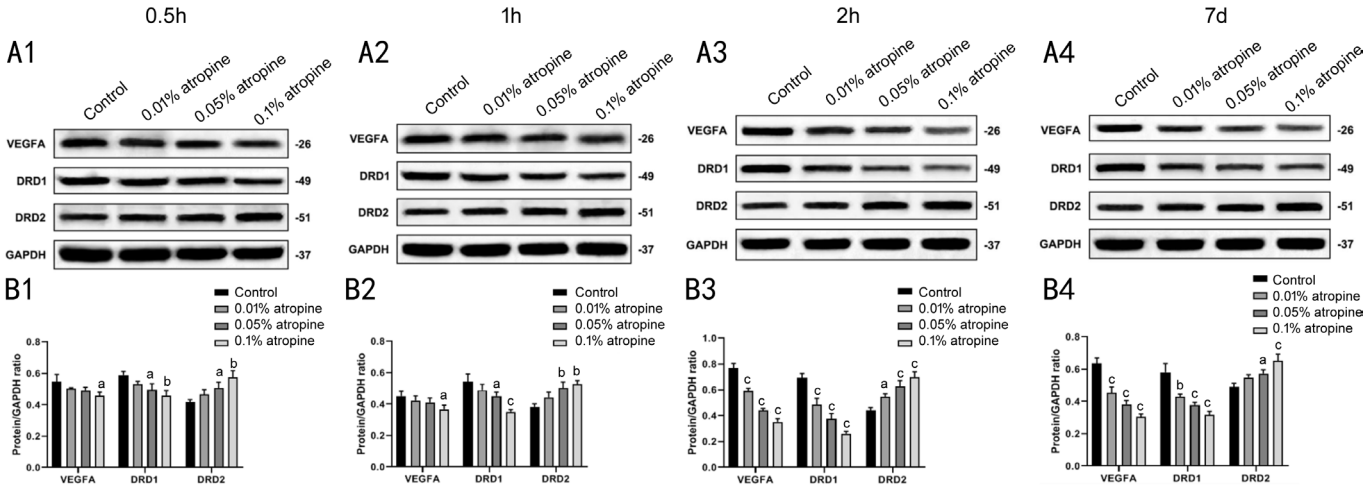

Figure 1 The effect of atropine eye drops with different low concentrations on the expression of DRD1, DRD2, and VEGFA in mouse high myopia model at 0.5, 1, 2h, and 7d A1–A4: The grey-scale graphs of Western blot; B1–B4: The statistical graphs. Low concentration atropine eye drops could decrease the expression of DRD1 and VEGFA, but increase the expression of DRD2, which was still high on the 7th day. $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$. DRD1: Dopamine D1 receptor; DRD2: Dopamine D2 receptor; VEGFA: Vascular endothelial growth factor A.

results showed that the laser-induced CNV area of 0.01%, 0.05%, and 0.1% atropine eye drops treatment groups was significantly decreased compared with the control group, which was (0.69±0.08, 0.57±0.02, 0.48±0.09), and the higher the concentration, the more significant the inhibition effect was (P<0.05, <0.01, <0.01). The results showed that low concentration atropine eye drops significantly inhibited angiogenesis in mouse high myopia CNV models (Figure 2).

After 7d of treatment with 0.01%, 0.05%, and 0.1% atropine eye drops, the deep blood vessel count of retina was (193±44, 125±36, 53±27) in 0.01%, 0.05%, and 0.1% atropine eye drops, respectively, which was significantly lower than the control group (314±26), the difference was statistically significant (P<0.001), and the higher the concentration, the more obvious the reduction. At that time, the shallow middle blood vessels were not found. It may be that the shallow middle blood vessels were destroyed after laser instrument modeling, so only the deep blood vessel counts were analyzed (Figure 3).

DISCUSSION

VEGF is a cytokine that promotes angiogenesis and increases vascular permeability, and is closely related to the occurrence and development of CNV[20]. In pathological myopia, the absence of choroidal vessels or capillaries may cause hypoxia of pigment epithelial cells and glial cells, leading to up-regulation of VEGF expression, induction of proliferation of choroidal endothelial cells, and ultimately the formation of CNV[21]. Some studies have found[22] that the occurrence and development of CNV is closely related to VEGF, and excessive release of VEGF and down-regulation of epithelial-derived factors in macular area lead to leakage of CNV and granulation tissue through retina. Although the levels of VEGF were lower in myopic CNV eyes than in normal eyes, they were still significantly higher than in highly myopic eyes. Therefore, elevated VEGF levels may play an important role in the pathogenesis of myopic CNV[21]. Therefore, any drug or method that reduces the overexpression of VEGF may be an effective means to prevent the progression of highly myopic CNV.

We used laser to evoke hypoxia leading to rapid pathological CNV in high myopia mice. This model of chemical hypoxia is quite well-known and used in mice[23-24]. In high myopia, the choroid of macula is affected by the traction force of axial extension, which results in the obvious thinning of choroid, the thinning of blood vessels, and the change of its fine tissue structure, the microvascular perfusion of the choroid is affected, the microcirculation is disturbed, the choroidal vessels are further atrophied and thinned, and then maculopathy occurs. The ischemia of the eyeground can also stimulate the expression of VEGFA up-regulated, increases the risk of CNV, aggravating visual impairment. The macula area is the cone cell concentration area, its metabolism speed is fast, the oxygen consumption is high, therefore is extremely sensitive to the ischemia hypoxia, and the blood flow perfusion insufficient will affect the retina inner and outer layer blood supply[25].

Our study indicates that 0.01%, 0.05%, 0.1% atropine eye drops could decrease the level of VEGFA and inhibit abnormal CNV indirectly by up-regulating the level of DRD2 and down-regulating the level of DRD1, and the effect of 0.05% and 0.1% atropine eye drops was more significant.

Atropine stimulates the release of endogenous dopamine[7], dopamine has a pronounced enhancing effect on the retinal perfusion[26]. A loss in endogenous dopamine inhibitory tone is concomitant with tumor progression and is associated

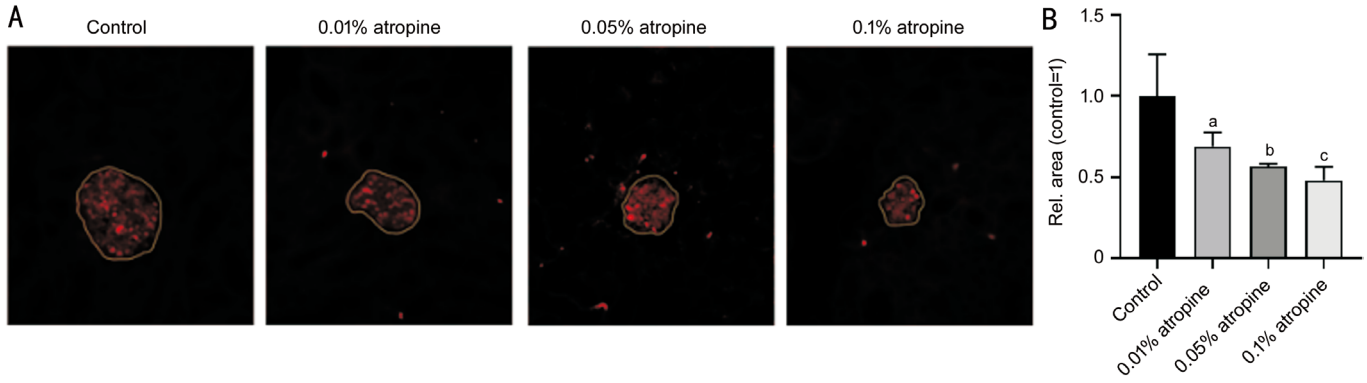

Figure 2 The effect of atropine eye drops of different low concentrations on neovascularization in high myopia mice model A: The map of CNV, yellow coils show the extent of choroidal vessels; B: An area chart of choroidal vessels in A. The area of CNV was significantly decreased in atropine eye drops of low concentration compared with the control group, the higher the concentration, the more significant the decrease. $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$. CNV: Choroidal neovascularization.

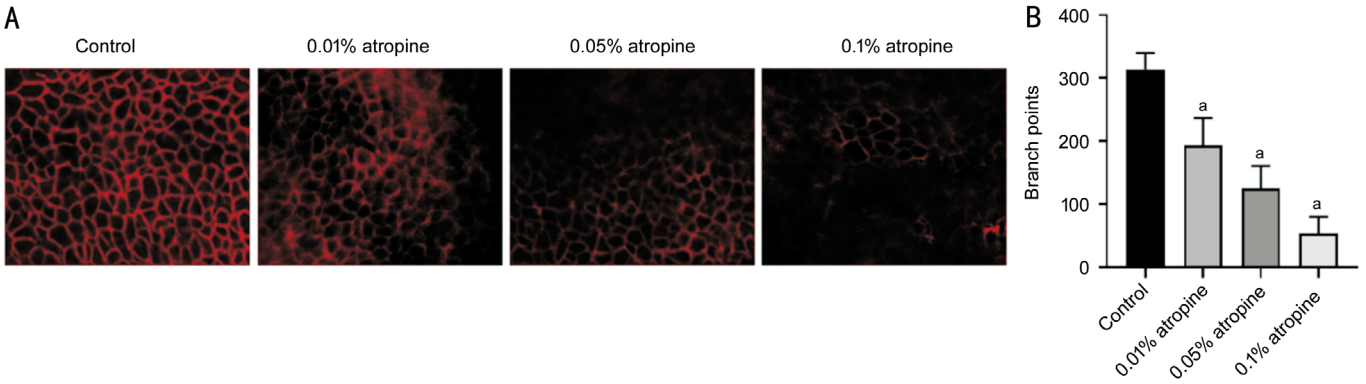

Figure 3 Effects of 0.01%, 0.05%, and 0.1% atropine eye drops on the development of normal blood vessels in the deep retina A: The deep vascular patch of high myopia mice model after 7d with different concentrations of atropine eye drops; B: The corresponding statistical diagram of the number of vascular branches. $^{a}P<0.001$.

with aberrant growth of blood vessels[27]. In animal models, the researchers found that activation of DRD1 promoted the formation of new blood vessels at the site of the lesion. The activity of DRD1 is an important regulatory point of angiogenesis: activation of DRD1 can promote angiogenesis, while antagonism of DRD1 can inhibit angiogenesis[28]. It has also been shown in our study that low concentrations of atropine eye drops cause a decrease in DRD1 and indirectly a decrease in CNV in high myopia. Regarding the regulatory role of the dopamine system on angiotensin II type 1 receptor (AT1R) in renin-angiotensin system, Sarkar *et al*[29] also noted that increased peripheral dopamine can suppress AT1R expression, thereby reducing angiogenesis. DRD1 activation has been shown to inhibit the progression of glioblastoma tumor cells[30]. The mechanism of the diverging effects of DRD1 on neovascularization needs further study. However, our study showed that low concentration of atropine eye drops decreased DRD1 expression in highly myopia, indirectly antagonizing DRD1 and reducing CNV area.

In the ischemic model of DRD2 knockout mice, the healing rate was significantly increased compared with the control group, and the new blood vessels were also significantly increased in the ischemic DRD2 knockout mice compared with the control group[29]. In subcutaneous tumor models of breast cancer, DRD2 can inhibit the proliferation of breast cancer both *in vitro* and *in vivo*[31]. The DRD2 agonists bromocriptine and quinapirol significantly inhibited angiogenesis of ovarian cancer tumors in mice[32]. In conclusion, DRD2 in all models, dopamine can inhibit angiogenesis by activating DRD2, and antagonistic DRD2 can significantly promote the formation of neovascularization. This study also showed that DRD2 expression was increased in the low concentration atropine group compared with the control group, and the indirect activation of DRD2 resulted in the decrease of CNV area and VEGFA expression. Recently published a study on fundus neovascularization found that D2 dopaminergic receptor agonists can downregulate VEGF overexpression and should be taken under consideration as new anti-VEGF drug used in ophthalmological vasculature diseases, such as age-related macular degeneration and proliferative form of diabetic retinopathy and diabetic macular edema[9]. Although the level of VEGF in myopia CNV eyes was lower than that in normal

eyes, it was still significantly higher than that in high myopia eyes. Therefore, the increased level of VEGF may play an important role in the pathogenesis of myopia CNV[21]. Our study indicates that 0.01%, 0.05%, 0.1% atropine eye drops could decrease the level of VEGFA and inhibit abnormal CNV, it was more pronounced in 2h and 7d.

Low concentrations of atropine may have a bidirectional regulatory mechanism. Atropine can increase the blood flow density of the normal blood vessels in the eyeground and inhibit the proliferation of abnormal blood vessels. Low concentrations of atropine may block M1 and M4 receptors in the retina and sclera, thereby inhibiting axial growth by affecting scleral remodeling and reducing vitreous cavity growth[33]. In this study, low concentrations of atropine may inhibit abnormal CNV indirectly by up-regulating the level of DRD2 and down-regulating the level of DRD1. Although atropine could cause an increase in blood flow perfusion[34], in the mouse high myopia CNV model, it did not cause a risk of CNV bleeding, but instead, it reduced the area of CNV.

In this experiment, low concentration atropine eye drops can not only inhibit pathological neovascularization, but also affect the repair of deep normal retinal capillaries. However, previous studies have shown that low concentration atropine eye drops can cause an increase in retinal blood density[34-35], which may be because animal models and human eyes have different effects on normal retinal blood density. It may also be speculated in this study that the microstructure of deep retinal blood vessels also changed correspondingly after the occurrence of pathological neovascularization or that laser affected the microstructure of deep retinal blood vessels during modeling. In the experiment, it was detected that the shallow middle blood vessels were destroyed, which failed to reflect the influence of low concentration atropine eye drops on the shallow middle blood vessels. However, in this experiment, the deep blood vessels were reduced after the use of low concentration atropine eye drops. In future clinical studies, it is necessary to further verify the influence of low concentration atropine eye drops on high myopic CNV and on normal blood vessel density. The lowest concentration and least side effects of CNV treatment for high ocular myopia were also sought.

In summary, low concentrations of atropine may be an effective and feasible treatment for pathological high myopia. Long-term follow-up is needed to determine whether low concentrations of atropine can delay the progression of high myopia to pathological myopia.

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