

# Latanoprost eye drops induce conjunctival lymphatic vessel development

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## Abstract

• **AIM:** To investigate the effect of latanoprost eye drops on the conjunctival lymphatics.

• **METHODS:** Twenty-four healthy New Zealand White rabbits weighing 1.5 to 2.0 kg were randomly divided into three groups: latanoprost group ( $n=8$ ) administered with latanoprost eye drops once a day for 2mo, carteolol group ( $n=8$ ) administered with carteolol eye drops once a day for 2mo, and control group ( $n=8$ ) without any treatment. The conjunctival tissues in the three groups were extracted to investigate the expression levels of 5'-nucleotidase (5'-Nase) by Western blot, reverse transcription-polymerase chain reaction (RT-PCR), and immunofluorescence staining, respectively.

• **RESULTS:** The protein expression level of 5'-Nase was significantly higher in latanoprost group than carteolol group ( $F=231.175$ ,  $P<0.001$ ) and control group ( $P<0.001$ ), while there was no significant difference between the carteolol group and the control group ( $P>0.05$ ). The mRNA expression level of 5'-Nase in the latanoprost group was also significantly higher than carteolol group ( $F=71.169$ ,  $P<0.005$ ) and control group ( $P<0.005$ ). The conjunctival lymphatics were positive immunofluorescence stained with the 5'-Nase antibodies in the latanoprost group and not stained in the control group.

• **CONCLUSION:** Latanoprost eye drops can induce conjunctival lymphangiogenesis which may be concerned in clinical implications.

• **KEYWORDS:** conjunctival lymphatic vessels; lymphangiogenesis; latanoprost

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## INTRODUCTION

Glaucoma featured by chronic and degenerative optic neuropathy with resultant loss of visual field is the leading cause of irreversible blindness worldwide<sup>[1-2]</sup>. Vision loss caused by ganglion cell dysfunction and death is most often related to the elevated intraocular pressure (IOP) and it can be preserved by lowering IOP. To reduce IOP is the only method that has been proven to treat glaucoma<sup>[3-5]</sup>. And, reducing IOP to the target levels is recommended as the current guideline for glaucoma management<sup>[6]</sup>. Prostaglandin analogs (PGAs) has become the first-line treatment modality for open angle glaucoma<sup>[7-9]</sup>. However, when medical treatments have unacceptable adverse effects or target IOP cannot be achieved, incisional surgeries are indicated. The most commonly performed incisional surgical procedure lowering IOP is known as the trabeculectomy that consists of an excision of a small part of the trabecular meshwork and or adjacent corneoscleral tissue to provide a drainage route for aqueous humor from within the eye to underneath the conjunctiva where it is absorbed. In this process, it seems not that a full endothelium lining pathway is established to drain the aqueous humor from the anterior chamber into the blood vascular system, but that trans-epithelium pathways and/or passive diffusion as well as lymphatic system have been involved as conjunctival lymphatic vessels have been confirmed to maintain pathways for long term humor drainage after initial conjunctival bleb formation, thereby playing a vital role in glaucoma filtration surgery<sup>[10-12]</sup>. And, bleb histology from failed trabeculectomy blebs has demonstrated that it completely lacks lymphatics compared to normal conjunctiva<sup>[13]</sup>. Clinically, it would be of great importance to assess whether conjunctival lymphatic vessels could be influenced by glaucoma medications. Endogenous PGAs have been confirmed to induce lymphangiogenesis<sup>[14]</sup>. In recent years, several functional molecules specifically expressed and localized in lymphatic endothelial cells, such as 5'-nucleotidase (5'-Nase), lymphatic vessel endothelial receptor-1, vascular

endothelial growth factor receptor-3, podoplanin, and Prox-1, have been identified<sup>[15]</sup>. Histochemical methods for detecting 5'-Nase, among others, are effective in identifying lymphatic vessels. This study herein is to explore the effect of latanoprost on the conjunctival lymphatics by investigating the change in the expression levels of 5'-Nase.

**MATERIALS AND METHODS**

**Ethical Approval** All animals used in this study were treated in accordance with the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and this study was approved by the Ethics Committee of Putuo Hospital, Shanghai University of Traditional Chinese Medicine.

**Animals and Treatments** Twenty-four 13- to 15-week-old New Zealand White rabbits weighing 1.5 to 2.0 kg were included in this study. All animals were healthy and had no clinically observable ocular disease. The included animals were randomly divided into latanoprost group, carteolol group, and control group, with each group having 8 rabbits and were all raised under standard laboratory conditions.

Latanoprost eye drop (50 mg/mL, Xalatan; Pfizer Manufacturing Belgium NV, Belgium) was applied to each rabbit once a day in the latanoprost group and carteolol eye drop (2%, carteolol, Otsuka Pharmaceutical Co., Ltd., China) was administered once a day in the carteolol group, both of which lasted two months. Then, the rabbits in both groups as well as in the control group that had no intervention were euthanized by injection of 2% sodium pentobarbital (1 mL/kg) into the marginal ear vein.

Half of the enucleated eyes were fixed in 4% paraformaldehyde (PFA) buffered at pH 7.2 and left overnight in 4°C for immunofluorescence microscope examinations. The other half of the rabbit eyes were preserved in a -80°C refrigerator for reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis.

**Western Blot** Western blot analysis was performed to detect the changes in the expression levels of 5'-Nase in conjunctival lymphatic vessels using specific antibodies. Total protein was extracted from conjunctival tissues in each group. Tissue extracts were incubated for 10min in ice and clarified by centrifugation at 12 000 rpm for 15min at 4°C, and then the supernatants were collected. Total protein in conjunctival extracts was measured by BCA protein assay kit, then boiled for 10min. Twenty micrograms of protein per lane were separated on a 10% to 15% linear gradient SDS-PAGE gel (Bio-Rad). Protein was transferred onto a nitrocellulose membrane, then blocked for 70min with 5% bovine serum albumin (BSA; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) at room temperature. The membrane was incubated with mouse anti-CD73 antibody (Abcam,

**Table 1 Primers used for real-time RT-PCR**

Gene	Primer sequence (5'-3')	Product size (bp)
β-actin	F: ATCATGAAGTGCACGTGGA	113
	R: GCGGTGATCTCCTTCTGCAT	
5'-Nase	F: GGGTCGGATCAAGTTTCTGC	132
	R: ATGGTGGCTTCTCCAGACC	

Cambridge, MA, UK) at 4°C overnight, then probed with HRP-conjugated goat anti-mouse IgG(H+L) secondary antibody (1:1000, Beyotime, China) at room temperature for 1h. Densities of the bands were measured using Image J software. All experiments were performed in triplicate.

**Reverse Transcription-Polymerase Chain Reaction** RT-PCR was performed to investigate the mRNA expression level of 5'-Nase in conjunctival lymphatic vessels. Total RNA was isolated from conjunctival tissues with an RNA extraction kit (TRIzol; Sigma-Aldrich, Munich, Germany) following the manufacturer's instructions. The PCR primers were designed and synthesized by Sangon Biotech (Shang Hai, China), as shown in Table 1. The endogenous control, β-actin, was used to normalize target genes. The real-time PCR profile consisted of 15min of initial denaturation at 95°C followed by 40 cycles of 20s denaturation at 95°C and annealing at 54°C for 30s, and extension at 72°C for 40s. To confirm amplification specificity, the PCR products from each primer pair were subjected to a melting curve analysis and subsequent gene expression. All experiments were performed in triplicate. A specific cDNA sample was included in each run and served as a reference for the comparison between runs. Results are expressed as the relative expression level of the gene in the experimental groups compared with that of the control group.

**Immunofluorescence** The conjunctival tissues were fixed in 4% PFA overnight at 4°C. and then embedded in optimum cutting temperature compound (Tissue-Tek®, Tokyo, Japan) and stored at -80°C. Frozen sections were cut in 6-μm sections, then pretreated with H<sub>2</sub>O at 37°C for 3min. The sections were washed three times with phosphate buffer saline (PBS; Hyclone, USA) for 10min at room temperature and then were incubated with 5% BSA for 1h to block nonspecific binding activity. The slides were incubated with mouse anti-CD73 antibody (Abcam, Cambridge, MA, UK) overnight at 4°C, and subsequently incubated with secondary antibodies Cy3-AffiniPure goat anti-mouse IgG+IgM(H+L) (Jackson, Pennsylvania, USA) in dilution buffer for 2h at room temperature, after washing three times with PBS, the sections were incubated with 4',6-diamidino-2-phenylindole (DAPI, 1:1000; Vectashield®, Vector Laboratories, Burlingame, USA) diluted in PBS for 30min, after immunostaining, then were examined with a confocal laser scanning microscope.

**Statistical Analysis** Data analysis was performed using SPSS V.21.0 Software (SPSS Inc.; Chicago, IL, USA). Data were recorded as mean±standard deviation. Differences in the expression level of 5'-Nase among the three groups were analyzed with one-way ANOVA. *P*-value of less than 0.05 was considered statistically significant.

**RESULTS**

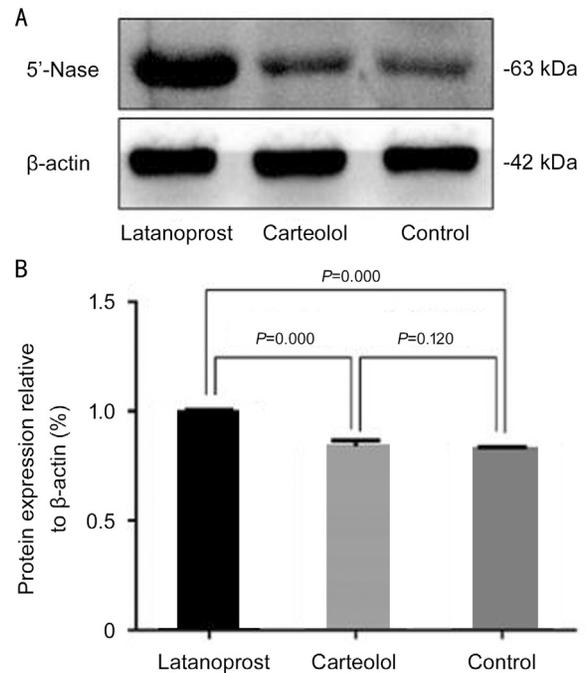
**Western Blot** Western blot analysis was performed with protein extracted from the rabbit conjunctiva. Quantitative analysis of the band is shown in Figure 1. The levels of 5'-Nase were analyzed. The latanoprost group (0.99±0.010) showed significant increases in the levels of 5'-Nase compared with the carteolol group (0.84±0.028, *F*=231.175, *P*<0.001) and the control group (0.83±0.010, *P*<0.001; Figure 1).

**RT-PCR** The mRNA levels of 5'-Nase expression are shown in Figure 2. The 5'-Nase appeared to be expressed at significantly higher levels in latanoprost group (2.64±0.398) than in carteolol group (1.19±0.224, *F*=71.169, *P*<0.005) and control group (1.00±0.010, *P*<0.005).

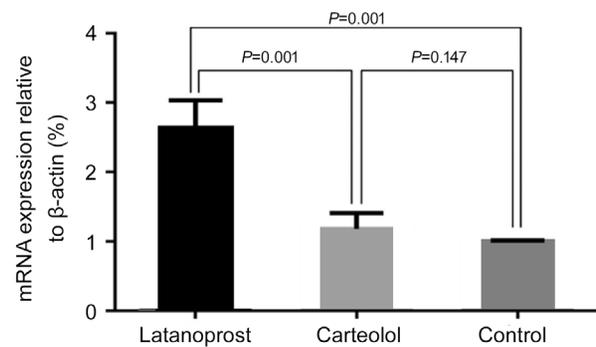
**Immunofluorescence** By immunofluorescence microscopy, obvious differences in the labeling intensity of 5'-Nase in the conjunctival lymphatic vessels were observed between the latanoprost group and control group, histologically confirming the results from Western blot and RT-PCR (Figure 3).

**DISCUSSION**

The lymphatic system plays pivotal roles in body fluid macromolecular homeostasis and immune function. Little research has been reported on the conjunctival lymphatics, which may be partially because of the lack of specific lymphatic endothelial markers. The insights into the lymphatics have been improved and the scientific literature has thus been enriched after the specific lymphatic endothelial markers are available<sup>[16-17]</sup>. It has been demonstrated that, similarly in other organs, initial and collecting lymphatics are both present in the conjunctiva<sup>[10]</sup>, whereby the interstitial tissue fluid including solutes, cells, and particulate matter can be drained from the conjunctival tissue through the lymphatic system into the blood vascular system. Despite the lymphatics' integral role in preserving tissue fluid homeostasis in conjunctiva, a connection between the conjunctival lymphatics and the aqueous humor drainage system is not found in a normal eye<sup>[10]</sup>. Conventional outflow *via* the trabecular meshwork and unconventional uveoscleral outflow through the ciliary body are the main two pathways for aqueous humor to leave the eye<sup>[18]</sup>. In the so-called unconventional uveoscleral outflow, aqueous humor moves through interstitial spaces of the ciliary muscle into the suprachoroidal space and then flows through the sclera. A rich lymphatic network has been found in the ciliary body and possibly contributes to the outflow of aqueous humor *via* this route that is named uveolymphatic pathway<sup>[19]</sup>.



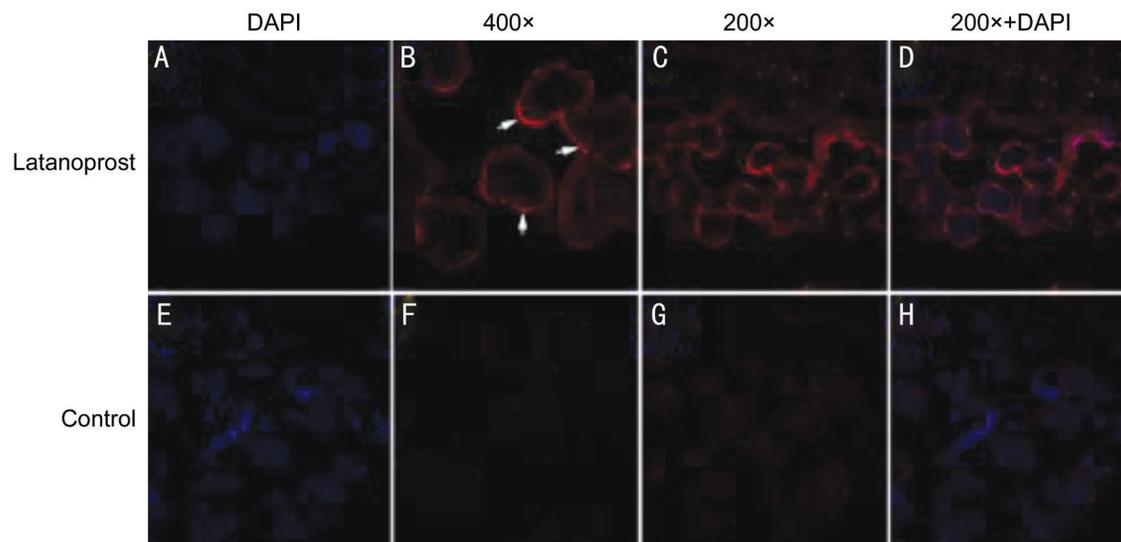
**Figure 1** The conjunctiva exhibited significantly more 5'-Nase labeling in the latanoprost group than in the carteolol group and control group.



**Figure 2** The mRNA expression levels of 5'-Nase expression in the three groups.

And, PGAs are believed to lower IOP through actions on the uveolymphatics<sup>[20]</sup>.

The greater importance the conjunctival lymphatics would have assumed may lie in its potential influence on the outflow of aqueous humor after glaucoma filtration surgery, which has not received special attention. The filtration surgery for treating glaucoma is aimed to redirect the aqueous humor to the subconjunctival space where it is drained to lower IOP. The aqueous humor presenting in the subconjunctival space following filtration surgery is analogous to extravasated or interstitial tissue fluid and the conjunctival lymphatics are therefore anticipated to participate in the drainage process, as evidenced by an experimental study in which the draining lymphatic vessels appearance were connected to the conjunctival lymphatic system and they are believed to be lymphatic vessels<sup>[10]</sup>. Clinically, XEN blebs with



**Figure 3** Obvious differences in the labeling intensity of 5'-Nase in the vessels were observed between the latanoprost group and control group. The conjunctival lymphatics were positively stained with the antibody against 5'-Nase in the latanoprost group (A-D, white arrows) while they were not stained in the control group (E-H).

luminal openings and a look of lymphatics usually have a greater IOP reduction<sup>[21]</sup> and better IOP reduction is also seen in trabeculectomy cases when conjunctival blebs show lymphatic-like outflow pathways during tracer studies<sup>[11]</sup>. Thus, conjunctival lymphatics are thought to be associated with bleb outflow.

Under normal circumstance, the aqueous humor leaves the eye mainly through the pathway consisting of trabecular meshwork, Schlemm's canal, intrascleral channels, and episcleral and conjunctival veins. This stable normal aqueous flow process is maintained by notable immune privilege conditions in the intraocular environment<sup>[22]</sup>. However, when the aqueous humor enters the highly immune-reactive conjunctival tissue as interstitial fluid, it may have altered properties. The conjunctival lymphatics appearing here are believed to drain antigens into the blood stream, where the antigens can be degraded. So, conjunctival lymphatics not only can function to drain extravasated tissue fluid back to the blood circulation but also possibly enhance immune surveillance, whereby longterm drainage pathway that is significantly different from the initial bleb forming immediately after surgery can be efficiently established and maintained.

In this study, we have first demonstrated that long term administration of topical latanoprost can up-regulate the expression of 5'-Nase, the marker for lymphatic endothelial cell, in the rabbit conjunctiva by Western blot and RT-PCR along with the immunofluorescence staining examination, which implies that topical latanoprost have the potential to induce the lymphangiogenesis in conjunctiva. It seems that patients having received long-term topical latanoprost treatment will stand a better chance of achieving a successful

bleb if they require a filtration surgery. Of note, the lymphatics in conjunctiva are the initial lymphatics consisting of blind-ended tubes. And there exists a gap between the exit site of the scleral channel and the lumen of the initial lymphatics. Therefore, a selected drainage site both rich in and having little damage to lymphatic vessels and closer proximity of the initial lymphatics to the site of origin of the subconjunctival aqueous can theoretically facilitate the drainage and removal of the aqueous humor within the bleb. However, we are not aware how the conjunctival lymphatic vessels distribute dimensionally in the normal conjunctiva and in the conjunctiva having long-term intervention of topical latanoprost. And this scenario may be complicated by another finding that long-term use of latanoprost eye drops can induce conjunctiva thinning in glaucoma patients<sup>[23]</sup>. PGAs are now the widely prescribed anti-glaucoma drugs and patients who undergo filtration surgery have often been administered PGAs. Therefore, efforts to understand whether other PGAs have the same influence on the conjunctival lymphatics as the latanoprost and whether these influences have positive or negative effect on glaucoma patients who need filtration surgery may have clinical importance. These are the interesting topics worth further clinical investigation.

As far as we are aware, there are no studies dealing with the effect of PGAs on conjunctival lymphatics or the mechanism by which they induce lymphangiogenesis in conjunctiva. Lymphangiogenesis is noted in many cancers with lymphatic metastasis and tumor or host-derived prostaglandin E2 or exogenous prostaglandin E2 have been shown to directly stimulate lymphatic endothelial cells growth by activating EP4 receptors *via* PI3K/Akt and ERK signaling pathways

and vascular endothelial growth factor 3<sup>[24]</sup>. Extrapolation of these results may be helpful in understanding why topical administration of latanoprost eye drops can induce development of new lymphatic capillaries in the conjunctiva. However, the detailed information on the signaling pathways of endothelial cells in the conjunctival initial lymphatics remains unclear and further studies are warranted to investigate.

In conclusion, administration of latanoprost eye drops can induce conjunctival lymphangiogenesis that may have clinical implications.

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