·Basic Research·

Effect of D-Timolol and L-Timolol on rat experimental choroidal neovascularization *in vivo* and endothelial cells *in vitro*

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Abstract

• AIM: Impairment of choroidal perfusion was found in age-related macular degeneration(AMD) patients. We postulated that vasoactive agents, which can reduce choroidal blood flow resistance, might prevent the development of choroidal neovascularization (CNV). D-Timolol and L-Timolol are hypotensive agents used in cardiovascular and glaucoma therapy. Their effects on laser-induced experimental CNV rat model and human umbilical vein endothelial cells (HUVEC) were thus evaluated.

• METHODS: Male Brown Norway rats were anesthetized to receive Nd:YAG laser to break the Bruch's membrane. D-Timolol and L-Timolol were given once daily through intraperitoneal injection after laser treatment for 4 weeks. Fluorescein angiography(FA) was performed on 2 weeks and 4 weeks. HUVEC were tested by proliferation assay and adhesion assay with D-Timolol and L-Timolol at different concentrations.

• RESULTS: D-Timolol reduced the fluorescein leakage to 83% of the control group in laser-induced rat's CNV model at a dosage of 15mg/ (kg·d). L-Timolol had no effect on CNV formation even at a higher dosage of 20mg/ (kg·d). D-Timolol inhibited the endothelial cells proliferation significantly by 300mg/L. L-Timolol also significantly inhibited the cell proliferation at 1 000mg/L. But at a lower dose such as 300mg/L, no significant inhibitory effect was found. Both drugs showed no effect on cell adhesion function in cell culture experiments.

• CONCLUSION: D-Timolol was found to prevent CNV development in laser-induced model *in vivo* and inhibit vascular endothelial cells proliferation *in vitro*. L-Timolol had no effect on cell proliferation at the same dose, and neither on rat CNV model. The results indicate these two isomers have different functions on rat's CNV prevention and on HUVEC cell proliferation.

• KEYWORDS: D-Timolol; L-Timolol; choroidal neovascularization;rat

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INTRODUCTION

D- and L-Timolols are hypotensive agents used in cardiovascular and glaucoma therapy. Application of L-Timolol to the clinical treatment of glaucoma has been a great success as it induces relatively few side effects compared with pilocarpine. D-Timolol was found to be as useful as L-Timolol in lowering the intraocular pressure and aqueous humor production. However, it was much less potent than L-Timolol in inhibiting cardiac contractility and heart rate stimulated by isoproterenol and was less active in blocking beta-adrenergic receptors in tracheal muscle [1]. Further investigation revealed the different action of these two isomers on ocular blood flow. D-Timolol increases blood flow in iris-ciliary body whereas L-Timolol does not change ocular blood flow using 85Sr-microsphere technique in rabbit eyes [2]. With laser Doppler method, L-Timolol (0.4mg/kg) was found to reduce retinal blood flow significantly(17%), whereas D-Timolol (4.0mg/kg) showed a slight (9%) increase [3]. Using colored microspheres technique, it was found that L-Timolol produced biphasic action on the blood flow of iris, ciliary body and choroid, with a significant reduction of blood flow initially at 30 minutes, then a marked increase later at 90 minutes in rabbit eyes. However, there was a tendency to increase the blood flow in all ocular tissues from the very beginning by D-Timolol. The increase became significant at 90 minutes and thereafter it was less potent than L-Timolol[4].

It is noteworthy that cho roidal blood flow is found to be

impaired in the aging eye of age-related macular degeneration (AMD)[5]. We postulated that vasoactive agents, which can reduce choroidal blood flow resistance and increase choroidal blood flow, might prevent the development of human umbilical vein endothelial cells(HUVEC).The effects of L-and D-Timolol on laser-induced experimental choroidal neovascularization (CNV) rat model and HUVEC were thus investigated.

MATERIALS AND METHODS

Laser-Induced CNV Rat Model The Brown Norway rats weighing 150-200g were anesthetized for all procedures with intramuscular injection of ketamine (35mg/kg) and xylazine (5mg/kg). The pupils were dilated with 10g/L tropicamide and 25g/L phenylephrine. The fundus was visualized with VOLK super pupil XL biomicroscopy lens. A double frequency Nd:YAG laser (Laserex LP352; Ellex Medical PTY. LTD., Australia) with a 532nm wavelength was used. Laser parameters were 100μm spot size, 0.15 second exposure, and 110-150mW power. A pattern of eight lesions was concentrically placed at approximately equal distances around the optic nerve of both eyes. Acute vapor bubbles suggested the rupture of Bruch's membrane. Only laser spots with bubble formation were included in the study. The animals with subretinal hemorrhage interfered with the evaluation of lesions were excluded.

Administration of Drugs D-Timolol was given once daily through intraperitoneal injection after laser treat-ment at 5mg/(kg·d) and 15mg/(kg·d) for 4 weeks. L-Timolol was given intraperitoneally at 20mg/(kg·d) for 4 weeks as well. DMSO alone was used as control.

Fluorescein Angiography Fluorescein angiography (FA) was performed on the 2nd and 4th week post laser treatment with a digital fundus camera (TRC-50EX; TOPCON, Japan) and standard fluorescein filter. Ten milligrams of sodium fluorescein (Sigma-Aldrich Inc.; St. Louis, MO, USA) was injected intravenously via hypoglossal vein. Both the early (<2 minutes) and late (>7 minutes) fluorescein phases were captured. CNV formation was determined with fluorescein angiogram. Each laser lesion was classified as leaky or non-minimally leaky by consensus of two observers (XXR, ZYH).

Human Umbilical Vein Endothelial Cell Culture HUVECs purchased from Cambrex Bioscience Walkersville, Inc. (Walkersville, MD) were cultured in a 37℃ humidified 50mL/L CO_2/950mL/L air incubator.Culture media, EGM, purchased from Cambrex Bioscience Walkersville, Inc. (Walkersville, MD), were subsequently changed every other day. Confluent cultures were released by weak digestion with trypsin-EDTA (0.5+0.2)g/L.

Cell Proliferation Assay Cultured cell viability was determined by MTT cell proliferation assay. MTT was purchased from Sigma (St. Louis, MO, USA) and dissolved in PBS at 5mg/mL and sterile filtered. HUVECs from passage 3-5 were seeded in 24-well culture plates. After incubation of 24 hours, the medium was changed, and the cells were further incubated with the appropriate medium containing 100, 300, 1 000mg/L D-Timolol or L-Timolol. Control cultures were incubated with DMSO alone. After removing the medium at 24, 48 and 72 hours, cells were washed with DPBS and incubated with 10μL MTT in 100μL phenol-red free medium for exactly 4 hours. Supernatant media were then removed by aspiration. The resulting formation product was solubilized by addition of 200μL DMSO to each well and dishes were shaken for 2 minutes to mix. Absorbency in each well was read at 570nm using Spectra Count plate reader. Blanks were formed by identical incubation of 100μL media with 10μL MTT in cell-free wells, followed by removal of media and addition of 200μL DMSO. A standard curve of MTT absorbency at 570nm *vs* cell number was constructed by comparing MTT absorbency and cells counting with Trypan blue dye (Gibco, Grand Island, NY, USA).

Cells Adhesion Assay HUVEC were seeded in 24-well tissue culture plates precoated with fibronectin. D-and L-Timolols were added into medium as 300, 1 000mg/L. Medium without any drug was used as control. After incubation at 37℃ for 1 hour, attached cells were counted and percentage of attached cells was calculated.

Statistical Analysis There are 5 rats in the control group and 3 rats in each treated group. Both eyes of the animal were used in the experiment. Cell culture experiments were carried out in three fold and performed at least four times. Chi-square test was used for analysis of FA. Student's *t*-test was used for other experiments.

RESULTS

Incidence of Angiographically Defined CNV The incidence of angiographyically defined CNV was significantly reduced in D-Timolol treated group. The prominent leaking lesions were reduced to 83% of the control group at a dosage of 15mg/(kg·d) (P<0.05). (Figure 1 and 2, Table 1). L-Timolol had no effect on CNV even at a higher dosage 20mg/(kg·d) (Figure 3).

Effect on HUVEC Proliferation D-Timolol inhibited the endothelial cells proliferation significantly (Figure 4, Table 2). Cells population was reduced to 69% of the control group after incubation with 300mg/L D-Timolol for 72 hours. Higher concentration of D-Timolol at 1 000mg/L significantly reduced the cell population after 24 hours incubation and cells remained only 4% of the control group after 72 hours.

L-Timolol also significantly inhibited the cell proliferation at 1 000mg/L. But at 300mg/L, no significant inhibitory effect was found (Figure 5, Table 3).

Table 1 FA evaluation in laser induced CNV rat model

Group	G1	G2	%	*P*
control	2	60	100	
D-Timolol 15mg/(kg · d)	9	39	83	<0.05
D-Timolol 5mg/(kg · d)	5	43	92	>0.05
L-Timolol 20mg/(kg · d)	3	46	97	>0.05

G1:laser lesion classified as 'non-minimally leaky'; G2: laser lesion classified as 'leaky';%: percent of response with control as 100%

Table 2 Effect of D-Timolol on HUVEC proliferation assay

Group	Cell number		
	24h	48h	72h
DMSO	23390 ± 8164	26999 ± 7040	33707 ± 11673
D-Timolol 1000mg/L	19848 ± 7508[a]	9082 ± 5577[a]	1276 ± 1551[b]
D-Timolol 300mg/L	24040 ± 6446	23847 ± 3812	23246 ± 9391[a]
D-Timolol 100mg/L	22862 ± 7308	20986 ± 8150	23799 ± 7486

[a] $P<0.05$; [b] $P<0.01$

Table 3 Effect of L-Timolol on HUVEC proliferation assay

Group	Cell number		
	24h	48h	72h
DMSO	25786 ± 10610	26825 ± 10443	30509 ± 15586
L-Timolol 1000mg/L	27498 ± 12305	14743 ± 8547[b]	2069 ± 2339[a]
L-Timolol 300mg/L	27671 ± 11855	25016 ± 7228	21780 ± 7777
L-Timolol 100mg/L	26421 ± 11182	28306 ± 8364	27094 ± 10571

[a] $P<0.05$; [b] $P<0.01$

Table 4 Cell adhesion on HUVEC

	Cell adhesion %
DMSO	75.7 ± 2.2
L-Timolol 1000mg/L	68.5 ± 10.3
L-Timolol 300mg/L	84.5 ± 14.1
D-Timolol 1000mg/L	79.6 ± 3.4
D-Timolol 300mg/L	86.0 ± 7.3

%: percent of response with control as 100%

Effect on HUVEC Adhesion Assay Both drugs showed no effect on cell adhesion function in cell culture experiments (Table 4).

DISCUSSION

AMD is the leading cause of legal blindness in individuals aged over 65 in the United States and other industrialized nations [6,7]. Till now, the precise etiology is not completely understood despite intensive research. Thus, limited choices of treatment are available for this kind of eye disease.

It is exciting to find that D-Timolol can reduce the prevalence of CNV in laser-induced rat model while L-Timolol has no effect. CNV is the result of angiogenesis, which includes endothelial cells proliferation, migration and adhesion. These processes are complicated and a lot of factors may influence the CNV development[8].

The mechanism of D-Timolol to prevent CNV may not be

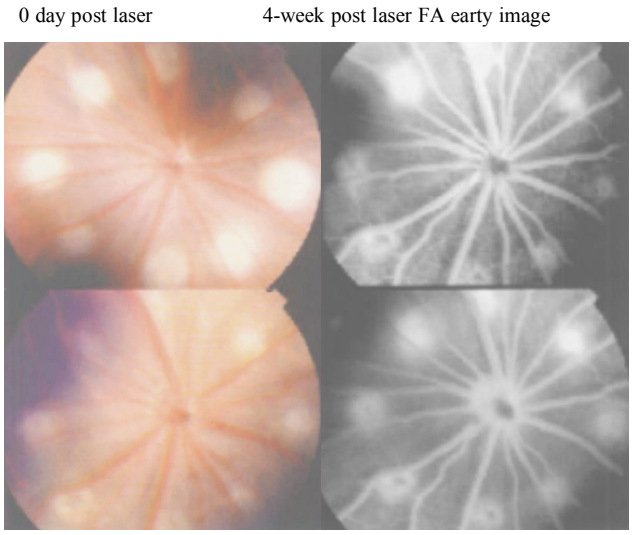

Figure 1 Control group, treated with DMSO

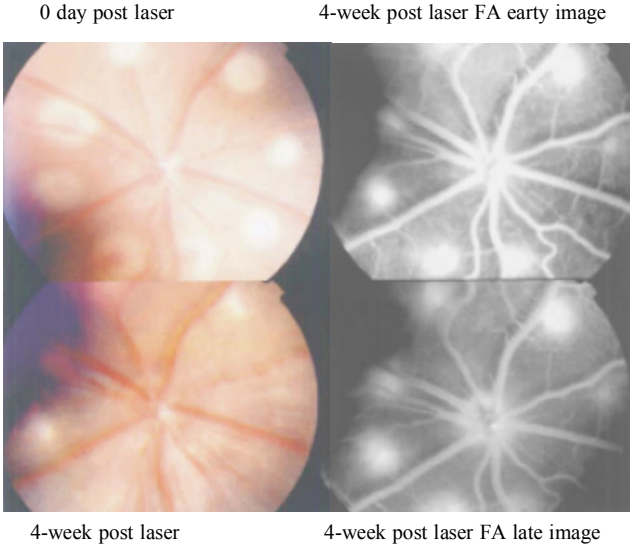

Figure 2 D-Timolol treated group 15mg/(kg·d)

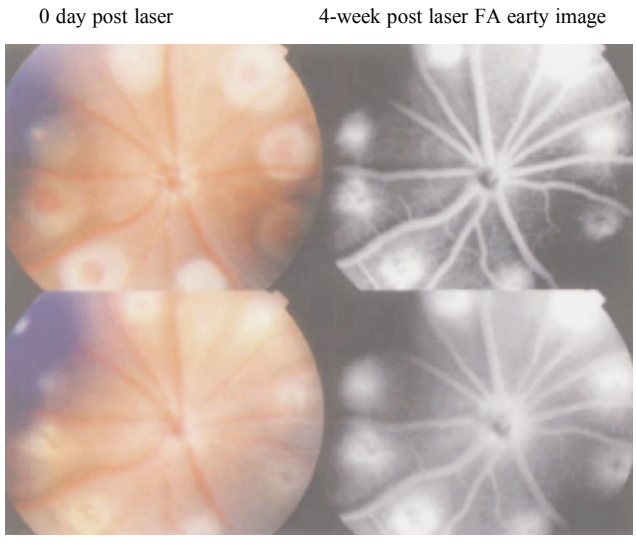

Figure 3 L-Timolol treated group 20mg/(kg·d)

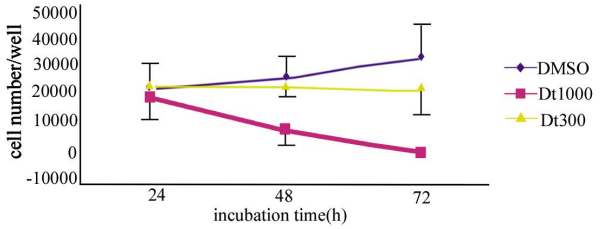

Figure 4 Effect of D-Timolol on HUVEC proliferation Dt1 000, treated with D-Timolol 1 000mg/L; Dt300, treated with D-Timolol 300mg/L

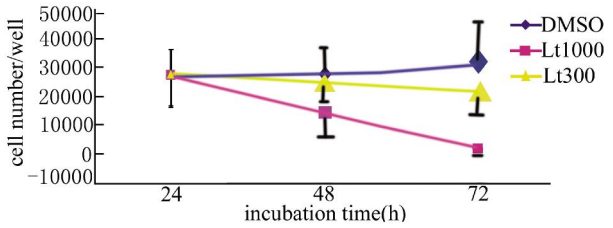

Figure 5 Effect of L-Timolol on HUVEC proliferation Lt1 000, treated with L-Timolol 1 000mg/L; Lt300, treated with L-Timolol 300mg/L

related to its effect on endothelial cells directly. Both D-Timolol and L-Timolol have no effect on the adhesion function of HUVEC in cell culture experiments. D-and L-Timolols reduced the number of cells at very high concentration such as 1 000mg/L, which was probably due to the cytotoxicity of the drug. Although D-Timolol can inhibit the growth of HUVEC at a lower dosage than L-Timolol, it is questionable whether the drug concentration in the choroid tissue can reach that high.

Their different activity on β-anrenergic antagonism may be one of the mechanisms. In an experiment comparing their ability to inhibit L-isoproterenol-stimulated cyclic AMP synthesis in the rabbit iris-ciliary body preparation *in vitro*, it was found that D-Timolol had little of the β-adrenergic antagonistic activity of L-Timolol and the I_{50}s for L-and D-Timolols differ by about 1.5 log units[9]. It was also found that cAMP might modulate vascular endothelial cell migration in an inhibitory fashion using bovine aortic endothelial cells [10]. Even though this is true in the animal model, it can only explain the difference between L-and D-Timolols, while the mechanism of D-Timolol remains unclear.

Their different activity on ocular blood flow, especially on choroidal blood flow, may contribute to the mechanism. Changes of blood flow supply may lead to changes in the environment condition of laser lesions, and changes in balance of positive and negative regulators of angiogenesis. Thus, vasoactive drugs might be a new class of drugs that should be taken into consideration for AMD treatment although further investigation is needed.

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