·Basic Research·

Effect of Z,E-butylidedephthalide on experimental choroidal neovascularization in rat and ocular blood flow in rabbits

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Abstract

• AIM: To investigate the effect of Z,E-butylidedephthalide (Bdph)on laser-induced experimental choroidal neovascularization (CNV) in rat model and choroid blood flow in rabbits' eyes.

• METHODS: Male Brown Norway rats were treated with Nd:YAG laser to break Bruch's membrane. 30mg/kg and 15mg/kg Bdph were given daily through intraperitoneal injection for 4 weeks after laser treatment. Fluorescein angiography (FA) and choroidal flat mount were used to measure the development of CNV. Female New Zealand white rabbits' eyes were instilled with 10g/L Z,E-BdPh solution, and ocular blood flow was measured with colored microsphere technique.

• RESULTS: The intensity of fluorescein leakage, indicating the ocular lesion, decreased significantly in group Bdph 30mg/kg and 15mg/kg, as compared to the control at $P<0.01$. The area of neovascularization checked by FA in both groups of Bdph, at 30mg/kg and 15mg/kg decreased significantly compared to the control group at $P<0.05$. On the choroid flat mount, the areas of CNV were also smaller in both Bdph groups than that in control group. One percent drug solution instilled into rabbits' eyes could improve the choroid blood flow at 30 and 60 minutes after drug instillation ($P<0.05$).

• CONCLUSION: Z,E-butylidedephthalide can inhibit the development of CNV in the rat eyes and increase the choroid blood flow in the rabbit eyes. These results suggest that Z, E-butylidedephthalide may be a good agent for the treatment of age-related macular degeneration (AMD).

• KEYWORDS: Z,E-butylidedephthalide; choroid; neovascularization; blood flow; age-related macular degeneration

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INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of poor vision in the developed world among people aged over 65 [1,2]. Till now, the precise etiology of AMD is poorly understood and there are limited choices of treatment for this kind of disease. It is known that the development of choroidal neovascularization (CNV) indicates a severe late stage of AMD because it is responsible for almost 90% of severe visual loss [3]. So, various therapies were tried to reduce the development of CNV.They include transpupillary thermotherapy, photodynamic therapy[3], Pegaptanib[4] and Interleukin-1 Blockers[5]. Among the factors associated with the development of CNV, cardiovascular risk is considered as a very important one. Choroidal blood flow is closely related to the severity of AMD and associated with the risk for the development of CNV[6]. Besides, elevation of intravascular pressure is also a crucial hemodynamic factor in AMD , through which proteins and lipids could leak and depose within Bruch's membrane, leading to the formation of drusen and atrophy of RPE[7].

Butylidenephthalide (BdPh) was originally isolated and purified from neutral oil of Ligusticum wallichii Franch (newly name: Ligusticum chuaxiong Hort) [8]. The main function of this chemical is vasorelaxation without changing blood pressure[9-11]. So it could be used as a antianginal agent[12]. BdPh had selective affinities toward rat aortic smooth muscle cells just the same as verapamil, one of the calcium ion antagonists, which could potently inhibit the bFGF-stimulated vascular smooth muscle cells (VSMC) proliferation but had no effects on the normal VSMC growth[13]. Because it is able to cause vasorelaxation and to inhibit the abnormal proliferation of VSMC induced by bFGF, it may

be used to inhibit CNV development.

MATERIALS AND METHODS

Materials The chemical structure of butylidedephthalide is presented in Figure 1. The ratio between Z-Bdph and E-Bdph is about 85:15. Fluorescein sodium salt, fluorescein isothiocyanate-Dextran and dimethy sulfoxide (DMSO) were purchased from Sigma Chemical (St. Louis, MO).

Methods

Laser –induced CNV rat model The method was described previously [5]. Briefly, Brown Norway rats, weighing 150-220g, were anesthetized by intramuscular injection of ketamine (35mg/kg) and xylazine (5mg/kg). The pupils were dilated with 1g/L tropicamide and 2.5g/L phenylephrine. The fundus was visualized with a VOLK super pupil XL biomicroscopy lens (Keeler Instrument Inc., Broomall, PA). A double-frequency Nd:YAG laser (Laserex LP352; Lumenis Inc., Salt Lake City, UT) with a 532-nm wavelength was used. Laser parameters were 100-μm spot size, 0.15-second exposure, and 180-200mW power. A pattern of eight lesions was concentrically placed at approximately equal distances around the optic nerve of both eyes. Only laser spots with bubble formation were included in the study. Lesions with retinal hemorrhage interfered with the evaluation of the lesions were excluded.

Administration of drugs 30mg/kg and 15mg/kg Bdph were given once-daily through intraperitoneal injection after laser treatment for 4 weeks. DMSO was given ip as control.

Fluorescein angiography Fluorescein angiography was performed at week 2 and 4 post laser with a digital fundus camera (TRC-50EX; Topcon, Tokyo, Japan). 10mg of fluorescein sodium was injected intravenously (*i.v.*) through the hypoglossal vein. Both early (less 2 minutes) and late (over 7 minutes) phases of fluorescein were captured. Each photocoagulated lesion was classified as "leaky" or "not pronounced leaky," according to the intensity of fluorescein for determining the CNV formation [5]. Two days after injection of fluorescein sodium, 20mg fluorescein isothiocyanate-Dextran was injected *iv* also through the hypoglossal vein. Fluorescein pictures were captured every 5 minutes for each eye till 20 minutes after the injection. Choose the clearest pictures and use the Imagenet 2000 digital imaging systems (Topcon Medical Systems, Inc., Paramus, NJ) to measure the areas of CNV formation.

Measurement of choroidal flat mounts for CNV When the FA with fluorescein isothiocyanate-Dextran was done after 4-week treatment, rat were sacrificed and the eyes were removed and fixed for 2 hours in 100mL/L phosphate-buffered formalin. The cornea and lens were removed and the entire retina was carefully dissected from the eyecup.

Radial cuts (average 6) of the choroids were made from the edge to the equator and the eyecup was flat mounted with the sclera facing down. Flat mounts were examined by fluorescence microscopy on an Axioskop microscope (Zeiss, Thornwood, NY) and Image-Pro Plus software (Media cybernetics, Silver Spring, MD) was used to measure the each area of CNV.

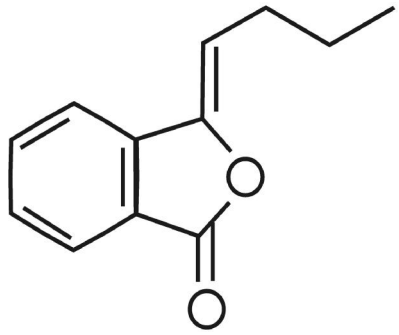

Figure 1 Chemical structure of butylidedephthalide

Ocular blood flow in rabbits Method was previously published [14]. New Zealand white rabbits, weighing 2.5-3.0kg, were anesthetized with 35mg/kg ketamine and 5mg/kg xylazine intramuscularly. Half of the initial dose was given hourly to maintain anesthesia. An ocular hypertensive model was created by raising the intraocular pressure of the left eye to 40mmHg which reduced the ocular blood flow to approximately 1/3 of the normal values. The left ventricle was cannulated through the right carotid artery for the injection of microspheres, and the femoral artery was cannulated for blood sampling. One percent drug solution 50μL or vehicle (50μL) was instilled topically to the left eye, and the ocular blood flow of the ocular hypertensive rabbits was measured with colored microspheres at 0, 30, 60, and 120 minutes thereafter. At each time point, 2 million microspheres in 0.2mL were injected and, as a reference, blood samples were taken from the femoral artery for exactly one minute immediately following injection of the microspheres. The blood sample was collected in a heparinized tube, and the volume was recorded. The rabbits were euthanized with an injection of 100mg/kg pentobarbital sodium after the last blood sampling. The left eyes were enucleated and dissected into the choroid and retina. The tissue samples were weighed.

The details of sample processing and microsphere counting were provided by E-Z Trac. The blood flow of each tissue at a certain time point was calculated from the following equation: $Qm=(Cm\times Qr)/Cr$, where Qm is the blood flow of a tissue in terms of μL/ (min•mg), Cm is the microsphere count per mg of tissue, Qr is the flow rate of blood sample in terms of μL/min, and Cr is the total microsphere count in the referenced blood sample.

Statistical Analysis Each group has 6 animals. Both eyes of each rat were used in the experiment. A Chi-square test was used for the analysis of FA with fluorescein sodium. A Student's *t* test was used for other analysis.

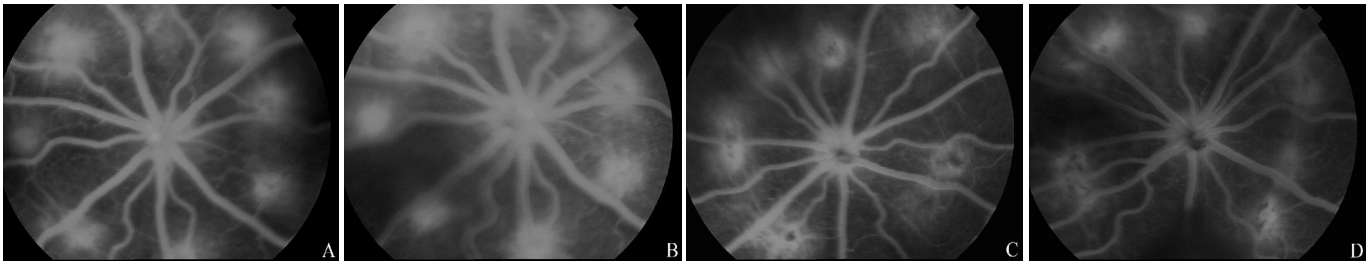

Figure 2 Fluorescein angiography photos with the injection of fluorescein sodium A: fluorescein angiogram in DMSO group after 2-week treatment, the size and intensity of the laser lesions were much more obvious; B: fluorescein angiogram of the same eye after 4-week treatment; C: Bdph 15mg/kg group after 4-week treatment; D: Bdph 30mg/kg group after 4-week treatment

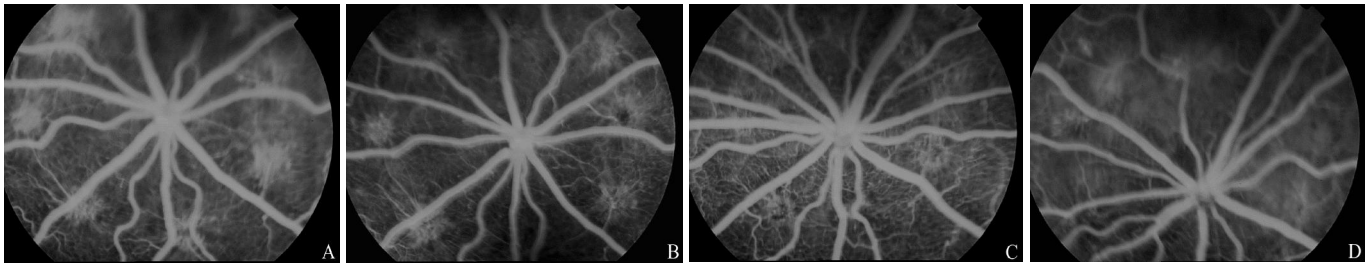

Figure 3 Fluorescein angiography photos with the injection of fluorescein isothiocyanate-dextran A: CNV area could be seen and measure from the fluorescein angiography photo, this is from DMSO group after 2-week treatment; B: the same eye after 4-week treatment; C: Bdph 15mg/kg group after 4-week treatment; D: Bdph 30mg/kg group after 4-week treatment

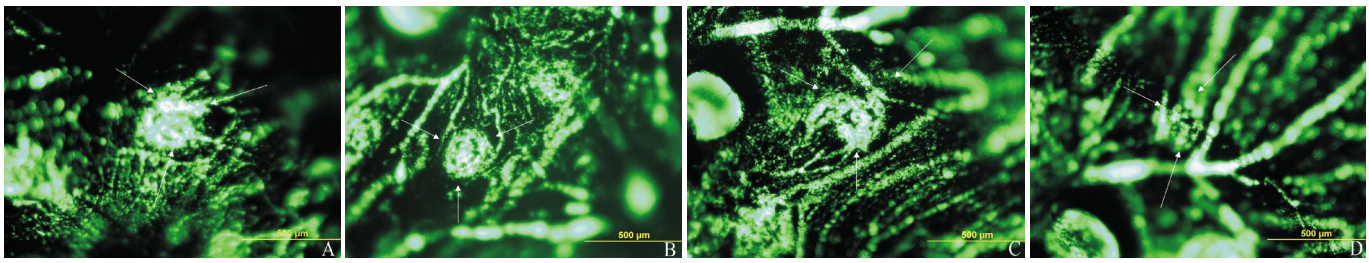

Figure 4 Photos of choroidal neovascularization in choroidal fat mounts examined by fluorescein microscopy A: CNV in eyes treated with DMSO after 4 weeks; B: considerably smaller areas of CNV at 4-week treatment of 15mg/kg Bdph; C and D: Bdph 30mg/kg group after 4-week treatment

RESULTS

Incidence of Angiographically Defined CNV A fluorescein angiogram showed early hyperfluorescence of the laser lesions, which increased in size and intensity in the late phase on the DMSO group (Figure 2). The incidence of the angiographyically defined CNV was reduced significantly in both Bdph groups (P <0.01), compared to the DMSO-treated control group, after 2-week and 4-week treatment (Figure 2, Table 1).

Measurement of CNV Area by Fluorescein Angiography When fluorescein isothiocyanate-Dextran was injected into the vascular, the CNV in the ocular foundus could easily be seen and measured. CNV's in the DMSO group were much bigger than those in the Bdph groups (Figure 3, Table 2). A rat died in Bdph 15mg/kg group because of over dose of anesthesia when being measured after 4-week treatment.

Areas of CNV on Choroidal Flat Mounts The neovascular agglomerates could be clearly seen on the choroidal flat mounts, the mean areas of CNV of DMSO group was bigger than the Bdph 30mg/kg and Bdph 15mg/kg group after 4-week treatment (Figure 4, Table 3).

Choroid Blood Flow The choroid blood flow was significantly increased by 10g/L Z,E-Bdph at 30 and 60 minutes after drug instillation as compared with corresponding controls (Table 4). On the other hand, Z,E-Bdph did not show any effect on the retina blood flow at any time point after drug instillation (Table 5). Since the retina of albino rabbits is avascular, the drug is hard to show any effects on it.

DISCUSSION

Age-related macular degeneration (AMD) is a common eye disease among the elder people around the world. Many researchers and clinicians are making great efforts to find effective treatments. It has been found that a natural compound, butylidenephthalide (BdPh), can relax the blood vessels and inhibit bFGF-stimulated VSMC proliferation. Since the formation of CNV is related to cardiovascular risk and over production of endothelial cells, BdPh may produce

Table 1 FA evaluation in laser-induced CNV rat model (n=6)

Group	2wk treatment		P^a	4wk treatment		P^a
	G2	G1		G2	G1	
Control	54	9	<0.01	52	11	<0.01
Bdph30mg/kg/d	34	32	<0.01	37	29	<0.01
Bdph15mg/kg/d	20	38		23	36	

FA: fluoroscein angiography; CNV: choroidal neovascularization; G1, not pronounced leaky points; G2, leaky points; [a]Chi-square test, compared with the control group

Table 2 FA measurement of CNV on laser-induced rat model

(Mean±SEM)

Group	2wk treatment	P^a	4wk treatment	P^a
Control	2.665±0.438		2.928±0.600	
Bdph30mg/(kg • d)	1.875±0.501	<0.05	1.587±0.487	<0.01
Bdph15mg/(kg • d)	1.979±0.344	<0.05	2.071±0.483	<0.05

FA: fluoroscein angiography; CNV: choroidal neovascularization; [a]Student's t-test, compared with control group. For 2wk, n=6 in each group; for 4wk, n = 5 in Bdph 15mg/kg because of a death of one rat, n =6 in all other groups

Table 3 Areas of CNV on choroidal flat mounts (Mean±SEM)

Group	4wk treatment (μm^2)	P^a
Control	167.436±10.738	
Bdph30mg/(kg • d)	127.737±13.778	<0.01
Bdph15mg/(kg • d)	139.377±24.897	<0.05

CNV, choroidal neovascularization; [a]Student's t test, compared with the control group. n=6 in control and 30mg/ kg Bdph groups; n=5 in 15mg/kg Bdph group

Table 4 Effects of Z,E-butylidenephthalide(10g/L,50 μ L)on choroid blood flow in rabbit eyes (n=6, Mean±SEM)

Group	Blood flow [(μ L /(min • mg)]			
	0min	30min	60min	120min
Control(DMSO)	5.212 ± 3.287	3.721 ± 3.007	2.045 ±1.066	1.601 ± 1.450
BNP	6.405 ±2.970	7.056 ± 2.720[a]	3.740±1.260[a]	2.689 ±2.250

[a]P<0.05 *vs* control

Table 5 Effects of Z,E-butylidenephthalide(10g/L,50 μ L) on retina blood flow in rabbit eyes (n=6, Mean±SEM)

group	Blood flow[(μ L /min • mg)]			
	0min	30min	60min	120min
Control	0.107 ± 0.170	0.143 ± 0.194	012 ±0.168	0.064 ± 0.076
BNP	0.129 ±0.207	0.025 ± 0.061	0.046±0.054	0.186±0.176

inhibitory effect on CNV formation of AMD.

As shown in this research, Z,E-butylidedephthalide could effectively prevent the development of CNV induced by laser on rat model, which could be seen from both fluorescein leakage and the area of CNV formation. Further, this compound can also improve the choroid blood flow on the rabbit eyes. Thus, Z,E-butylidedephthalide might be a good candidate for the prevention and treatment of ocular neovascularization especially in the age-related macular degeneration. Since the mechanism of this compound is not very clear, further investigation is warnted.

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