·Basic Research·

Effects of ZX-5 and its optical isomers on ocular blood flow in rabbits and retinal function recovery in rats

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Abstract

• AIM: The effects of ZX-5, as nitric oxide (NO) donor, on ocular blood flow has been investigated using colored microsphere technique in previous study. The relationship between the production of NO by ZX-5 and ocular blood flow has been evaluated. ZX-5 has been shown to have strong positive effect on increasing choroidal blood flow. However, the effect of ZX-5 on retinal function recovery, the effects of its optical isomers, (R, R)-ZX-5 and (S, S)-ZX-5, on choroidal blood flow and retinal function recovery have not been studied and merit investigation.

• METHODS: Colored microsphere technique was used for *in vivo* experiments to determine choroidal blood flow of ocular hypertension (40mmHg) in rabbit eyes. Electroretinography was used to measure the b-wave recovery as an indication of retinal function recovery.

• RESULTS: (R, R)-ZX-5 increased choroidal blood flow at 10g/L, 50μL instillation into eyes at all time points ($P<0.05$). (S, S)-ZX-5 was not effective in increasing choroidal blood flow. ZX-5 and (R, R)-ZX-5 showed significant effects in retinal function recovery after ischemia of the retina at all time points ($P<0.05$); whereas (S, S)-ZX-5 did not show significant effect on recovery of b-wave after ischemia at most time points except at 120 and 240 minutes.

• CONCLUSION: ZX-5 and (R, R)-ZX-5 have high potency in increasing the choroidal blood flow and improving the retinal function recovery. It is hoped that they could be used for the prevention/treatment of ocular blood flow related eye diseases.

• KEYWORDS: ZX-5; (R,R)-ZX-5; (S,S)-ZX-5; choroid; retina

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INTRODUCTION

Certain eye diseases are closely related to the malfunction of ocular circulation/blood flows [1-10]. They are the most common causes of visual disorder and blindness. Therefore, searching for drugs that can improve ocular blood flow, particularly in retina and choroid, may be useful for the prevention/ treatment of these ocular blood flow related eye diseases.

ZX-5,1-phenyl-3-{3-methoxy-2-propoxy-5-[4-(3,4,5-trimethoxyphenyl)-1,3-dithiolane-2-yl]phenyl}thiourea, is a racemic form of (R, R)-ZX-5 and (S, S)-ZX-5, two optical isomers. ZX-5 is a nitric oxide (NO) releaser with both stereoisomerism and optic isomerism. ZX-5 and ZX-4 are two stereo-isomers which have been investigated in previous study. It has been reported that ZX-5, as a synthesized NO donor, could improve choroidal blood flow stereospecifically by topical administration in rabbit eyes. The corresponding ZX-4 was not effective on ocular blood flow nor released NO [11]. That finding shows that NO donor receptors in choroidal vasculatures are probably stereospecific. That explains why very few compounds can work effectively on choroidal vasculatures [12]. This finding makes us even more interested in further study on the optical

isomers of ZX-5. Because the retinal function is dependent on ocular blood flow, it is interesting to find out whether ZX-5 can improve retinal function recovery after ischemic insults. Meanwhile, it is important to find out whether its two optical isomers, (R, R)-ZX-5 and (S, S)-ZX-5, have the same effects on choroidal blood flow and retinal function recovery. It is hoped that this study will lead to the discovery of drugs that could be used for the prevention or treatment of eye diseases related to ocular blood flow.

MATERIALS AND METHODS

Materials ZX-5, (R, R)-ZX-5 and (S, S)-ZX-5 used in this study were synthesized and provided by Dr. Yihua Zhang. The chemical structure of them is presented as below (Figure 1). Dimethyl sulfoxide (DMSO) was purchased from Sigma Chemical (St. Louis, MO). Colored microspheres (15μm diameter) were purchased from E-Z Trac (Irvine, CA).

Methods

Measurement of ocular blood flow in ocular hypertensive rabbit eyes 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 artificially by inserting a butterfly infusion needle into the anterior chamber and raising the intraocular pressure (IOP) of the left eye to 40mmHg which reduced the ocular blood flow to approximately 1/3 of the normal values [1,2]. In this way there is room for blood flow increasing agents to show their efficacies. Since the blood flows of the choroid and the retina are very well autoregulated in normal condition, it is hard to show an increase of ocular blood flow by drugs. In the ocular hypertensive condition, the ocular blood flow is reduced as in the disease state and is easier to be increased by drug if the drug is capable of raising the ocular blood flow. The left ventricle was cannulated through the right carotid artery for the injection of colored 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 at time 0 minute with an IOP of 40mmHg, and the ocular blood flow of the ocular hypertensive rabbits was measured with colored microspheres at 0, 30, 60, and 120 minutes thereafter. All drugs were dissolved in DMSO and DMSO alone was used as the control. At each time point, 2 million microspheres in 0.2mL were injected and blood samples were taken from the femoral artery at exactly one minute immediately following injection of the microspheres as reference. Since the right carotid artery was cannulated, the ocular blood flow in the right eyes was consistently lower than that in the left eyes.

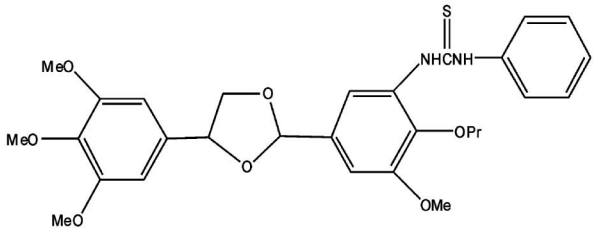

Figure 1 Chemical structures of ZX−5, (R, R)−ZX−5 and (S, S)−ZX−5

As a result, only the drug actions on the left eyes were studied in this research. 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 iris, ciliary body, retina, and choroid. The tissue samples were weighed.

The details of sample processing and microsphere counting were provided by E-Z Trac. In brief, Hemolysis Reagent was added to the microfuge tubes with the blood sample, then vortexed and centrifuged for 30 minutes at 6 000rpm. The supernatant was removed, and Tissue/Blood Digest Reagents I and II were added. The tubes were capped, vortexed and centrifuged for 15 minutes at the same revolutions as above. The supernatant was removed, and the microspheres were re-suspended in a precise volume of the Counting Reagent. The number of microspheres was counted with a hemocytometer.

The Tissue/Blood Digest Reagent I was added to the microfuge tubes with the tissue samples, sealed, and heated at 95℃ for 15 minutes. The tubes were vortexed for 30 seconds, then reheated and re-vortexed until all tissue samples were dissolved. Reagent II was then added while the tissue samples were still hot. Then the tubes were capped, vortexed, and centrifuged for 30 minutes. The protocol thereafter, was the same as that used to process the blood samples, and the microspheres were counted.

The blood flow of each tissue at a certain time point was calculated according to the following equation: $Q\text{m} = (C\text{m} \times Q\text{r})/C\text{r}$ 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.

Measurement of retinal function recovery after ischemic insult in rat eyes Electroretinograms (ERGs) were determined to provide the assessment of the retinal function prior to and following the ischemic insult. ERGs were recorded by means of Ag/AgCl electrodes placed in contact with the cornea. One stainless-steel needle was

inserted subcutaneously between the two eyes as a reference electrode, and another needle was inserted subcutaneously to the neck as a ground electrode. A photostimulator (Grass PS22 Flash) was used to produce flashes of light 5 inches from the eye, and the ERG potentials were recorded with a polygraph system. The ERG machine was purchased from LKC Technologies, Inc.(Gaithersburg, MD). A single flash (10-millisecond duration), white light stimuli were used to elicit ERG a-and b-waves. Peak b-waves amplitudes were measured from the trough of the a-wave to the peak of the b-wave.

Dark-adapted (at least 2 hours), female Long-Evans rats (200-250g) were anesthetized with 35mg/kg ketamine plus 5 mg/kg xylazine intramuscularly. Half of the initial dose was given thereafter at 1-hour intervals to maintain adequate anesthesia. The pupils were dilated with 10g/L tropicamide (50μL) for ERG experiments. Retinal ischemia was produced by occlusion of the central retina and posterior ciliary arteries by means of a ligature placed around the optic nerve and the posterior ciliary artery. The ligature was then drawn tightly through a micropipette tip placed at the base of the eyeball in the socket to occlude the retinal vessels for 30 minutes. The retinal ischemia was confirmed by the extinction of the ERG waves. After 30 minutes of retinal ischemia, the ligautre was released and the retinal arteries were allowed to reperfuse. ERGs were then measured at 0, 30, 60, 90, 120, 180, and 240 minutes thereafter. At the end of the experiments, the animals were euthanized with 100mg/kg of pentobarbital sodium.

All drugs and vehicles at 10mg/kg were injected intraperitoneally (ip.) right before the occlusion of central retinal arteries. All drugs were dissolved in DMSO and DMSO alone was used as the control.

Statistical Analysis All data were presented as mean ± standard deviation of the mean (SD). Analysis of variance (ANOVA) was used for multiple means comparisons at a certain time point. The differences were considered significant at $P<0.05$.

RESULTS

When the IOP was raised from normal values around 18-20mmHg to 40mmHg, the ocular blood flow was reduced to 1/3 of the original values. The blood flow continued to drop gradually over the time period of 2 hours during the experiments (Table1).

It has been reported that ZX-5 could increase choroidal blood flow stereo-specifically by topical administration in rabbit eyes. The effects of optical isomers, (R, R)-ZX-5 and (S, S)-ZX-5, on choroidal blood flow were investigated in this study. When 10g/L compounds were instilled to the eyes, the choroidal blood flow was significantly increased by (R, R)-ZX-5 at all time points (30, 60, 120 minutes after drug instillation) as compared with the corresponding controls. On the other hand, (S, S)-ZX-5 did not show any effect on choroidal blood flow at any time point.

Table 1 Effects of two optical isomers of ZX-5 on choroidal blood flow

Compounds (10g/L,50 μL)	Choroidal Blood Flow (μL/min/mg)			
	0min	30min	60min	120min
Control(DMSO)	12.57±2.20	7.16±1.86	4.33±1.07	1.97±0.78
(R, R)-ZX-5	23.26±4.48	16.41±2.52[a]	14.27±2.87[a]	10.53±1.81[a]
(S, S)-ZX-5	17.31±1.05	9.91±1.51	5.11±1.09	3.07±1.25

[a] Statistically higher than corresponding controls at $P<0.05$; All values are mean ± SD with all $n=5$ except controls $n=7$

Although samples of iris, ciliary body and retina were processed in the same way as that of choroid, no significant changes in their blood flows were noted.

When the blood flow to retina was blocked, the b-wave of ERG diminished rapidly. When the blood flow was resumed 30 minutes later, the b-wave recovered slowly and partially up to approximately 30% of the original value in 3-4 hours (Figure 2).

When the animal was pretreated with 10 mg/kg ip. of ZX-5, the b-wave recovery was markedly enhanced and reached approximately 60% of the original value in 3-4 hours. Its effect on retinal function recovery was significantly higher than the corresponding controls at all time points (Figure 2a). When (R, R)-ZX-5 was given alone, the b-wave recovery was also markedly enhanced and reached approximately 70% of the original value in 3-4 hours. Its effect on retinal function recovery was also significantly higher than the corresponding controls at all time points (Figure 2b). However, when (S, S)-ZX-5 was given to rats alone, the b-wave recovered approximately 40% of the original amplitude in 3-4 hours. Its effect on retinal function recovery was much less significant as compared with the corresponding controls at most time points except at 120 minutes and 240 minutes (Figure 2c).

(R, R)-ZX-5 and (S, S)-ZX-5, as two optical isomers of ZX-5, were also compared. The b-wave recovery with (R, R) -ZX-5 was significantly higher than that with (S, S)-ZX-5 at all time points (Figure 2d).

When compared with ZX-5, the retinal function recovery with (R, R)-ZX-5 was significantly higher at 120, 180 and 240 minutes. There was no significant difference found before 120 minutes (Figure 2e). However, the b-wave recovery with (S, S)-ZX-5 was significantly lower than that with ZX-5 at all time points (Figure 2f). Among all compounds tested, (R, R)-ZX-5 showed the strongest effect on retinal function recovery after ischemic insult (Figure 2).

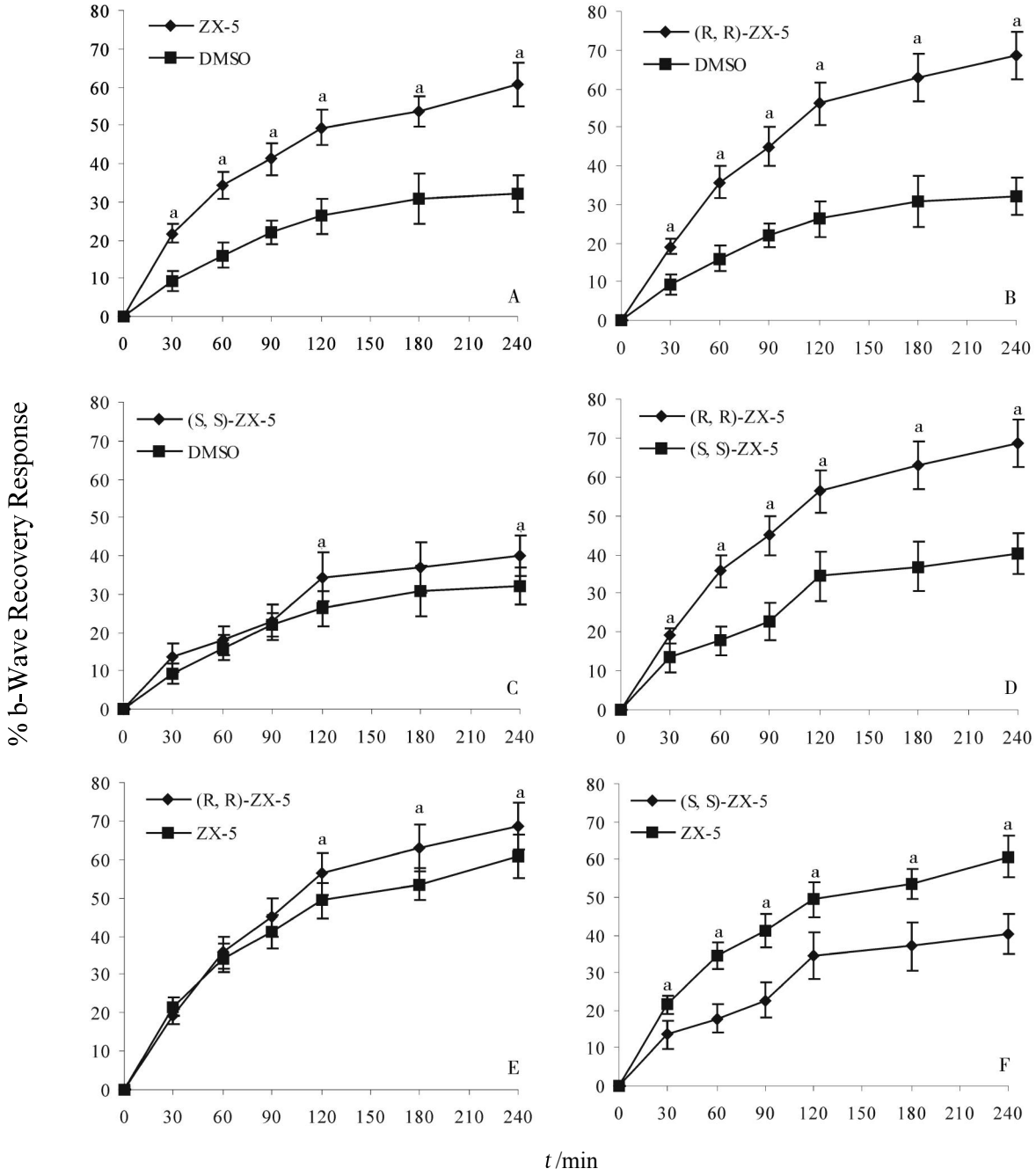

Figure 2 Effects of ZX−5 and its optical isomers on retinal function recovery after ischemic insult in rat eyes [a] Significant difference of b-wave recovery from corresponding controls at $P<0.05$

DISCUSSION

The blood to human retina is supplied by two sources: (a) the choriocapillaries, nurturing the retinal pigment epithelium and the photocell layer of the retina, and (b) the branches of the central retinal artery, nurturing the neuronal layer of the retina. The choroidal circulation has a blood flow volume approximately 40 times greater than that of retinal blood flow and is important to the normal functions of the photocell layer, particularly in the metabolic exchange of the avascular fovea of the retina [13].

The relationship between the production of NO by two stereo-isomers, ZX-5 as trans-form and ZX-4 as cis-form, and their ocular blood flow has been evaluated in the previous study. It has been reported that ZX-5 could increase choroidal blood flow stereo-specifically by topical instillation in rabbit eyes. The corresponding ZX-4 was not effective on ocular blood flow nor released NO. The increase of choroidal circulation caused by ZX-5 could be due to the release of NO in the choroid [11]. NO raises C- GMP via the stimulation of guanylate cyclase, which leads to vasodilation and an increase in blood flow [11,14-19]. In this study, the relative effect of the racemic form of ZX-5 was explored further along with its

optical isomers on improvement of retinal function recovery after ischemic insult.

It was found that (R, R)-ZX-5 and racemic-ZX-5 could improve choroidal blood flow by topical administration in rabbit eyes, whereas (S, S)-ZX-5 showed no effect on choroidal blood flow. Similar effects were observed for both in improving the retinal function recovery after ischemic insult. (R, R)-ZX-5 greatly facilitated the retinal function recovery, whereas (S, S)-ZX-5 could not.

Among all compounds studied, (R, R)-ZX-5 showed higher potency in increasing the choroidal blood flow and facilitating the retinal function recovery than racemic-ZX-5, whereas (S, S)-ZX-5 showed no effect on choroidal blood flow and retinal function recovery. These results further indicate that there are stereo as well as optic isomerism in choroidal vasculatures. (R, R)-ZX-5 and racemic-ZX-5 could improve retinal function through increasing the ocular blood flow, particularly in the retina and choroid. They may be useful for the prevention/treatment of certain eye diseases.

There are a large number of compounds, natural [20-26] as well as synthetic [11,14,18,27,28], which have been found to facilitate ocular blood flow and retinal function recovery after ischemic insult. It is hoped that some of these compounds could be developed into useful drugs for the prevention/ treatment of ocular blood flow related eye diseases.

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