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Effects of Crocin Analogs on Ocular Blood Flow and Retinal Function

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ABSTRACT

Ischemic retinopathy and age-related macular degeneration are the leading ocular diseases that cause blindness. The etiology of these diseases is due in part to the reduction of blood flow in the retina and/or choroid. Crocin analogs isolated from *Crocus sativus L.* were found to significantly increase the blood flow in the retina and choroid and to facilitate retinal function recovery. Increased blood flow due to vasodilation presumably improves oxygenation and nutrient supply of retinal structures. These results indicated that crocin analogs could be used to treat ischemic retinopathy and/or age-related macular degeneration.

It was noted that disaccharide analogs of crocin, such as crocin-1 and crocin-2, were less potent than monosaccharide analogs of crocin, such as crocin-3 and crocin-4, constituting an interesting structure-activity relationship.

INTRODUCTION

Crocus sativus L. is a well-known Chinese herb used widely in Asia for the treatment of blood stasis, hematemesis, exogenous febrile diseases, amenorrhea, postpartum blood stasis and abdominal pain, and swelling pain due to traumatic injuries (1). Pharmacologically, *C. sativus* is reported to constrict the uterine muscle of mice, guinea-pigs, rabbits, dogs, and cats. The sensitivity is even higher in the pregnant uterus (1). The extract of *C. sativus* is known to cause vasodilation, hypotension, and even diastolic cardiac standstill (1). The heartbeat can be restored quickly without producing cardiac fibrillation. Most importantly, this herb has very little toxicity with LD_{50} of 20.7 g herb extract/kg p.o. (1).

Ischemic retinopathy and age-related macular degeneration (AMD) are leading ocular diseases to cause blindness, yet no effective drug is presently available for their treatment (2-5).

Since ischemic retinopathy and AMD are hard to treat pharmacologically, it is hoped that crocin and its analogs might increase the ocular blood flow, particularly in the retina and choroid, to improve retinal functions. The ocular blood flow was measured with colored microspheres in rabbits whereas the retinal functions were measured with the b-wave recovery of electroretinogram in rats.

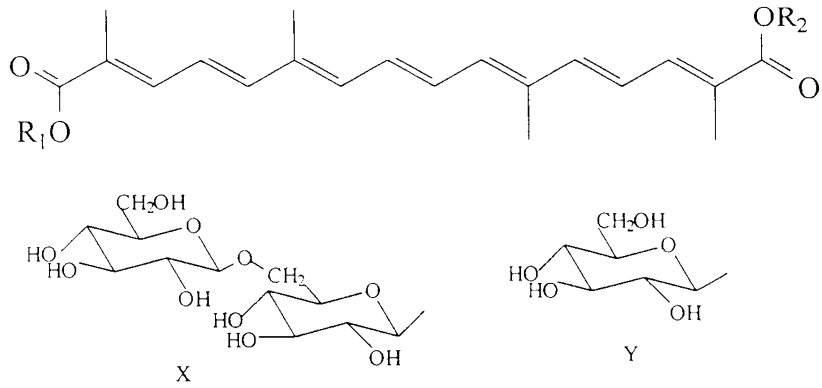
The chemical structures of crocin analogs are shown in Fig. 1. The sugar moieties are in the form of esters at the terminal ends. Basically, crocins are crocetin glycosides with 1 sugar as crocin-4, with 2 sugars as crocin-3, with 3 sugars as crocin-2 and with 4 sugars as crocin-1.

MATERIALS AND METHODS

Materials

Crocetin, crocin-1, crocin-2, crocin-3, and crocin-4 were isolated and purified from *C. sativus*, and were provided by Dr. Z.D. Min (Fig 1).

Colored microspheres and related reagents were purchased from E-Z Trac (Los Angeles, CA). The colored microsphere suspensions were prepared in saline containing trace amounts of a nonionic surfactant (Tween 80, 0.01% v/v) and a bacteriostatic (Thimerosal, 0.01% w/v) to prevent the microspheres from sticking together. The concentration of microspheres was 2 million/ml.



Crocin Analogs	R_1	R_2
Crocin-1	X	X
Crocin-2	X	Y
Crocin-3	X	H
Crocin-4	Y	H
Crocetin	H	H

FIGURE 1. Chemical Structures of Crocin Analogs.

Ocular Blood Flow in Rabbits

New Zealand white rabbits, weighing 2.5~3.0 kg, were anesthetized with 35 mg/kg ketamine and 5 mg/kg xylazine intramuscularly. Half of the initial dose was given hourly to maintain anesthesia. An ocular hypertensive model was created by raising the introcular pressure of the left eye to 40 mmHg which reduced the ocular blood flow to approximately 1/3 of the normal value (6). 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 (50 µl) or vehicle (50 µl) 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 min thereafter. At each time point, 2 million microspheres in 0.2 ml were injected, and blood samples were taken as a reference from the femoral artery for exactly 1 min immediately after the injection of the microspheres. The blood samples were collected in heparinized tubes, and the volumes were recorded. The rabbits were euthanized with an injection of 100 mg/kg pentobarbital sodium after the last blood sampling. The left eyes were enucleated and dissected into the retina, choroid, iris, and ciliary body. 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, vortexed and centrifuged for 30 min at 6000 rpm. The supernatant was removed, and the Tissue/Blood Digest Reagent I and II were added. The tubes were capped and vortexed and again centrifuged for 30 min. The supernatant was removed, and the Counting Reagent was added, then vortexed and centrifuged for 15 min at the same revolution. The supernatant was removed, and the microspheres were resuspended in a precise volume of the Counting Reagent. The number of microspheres were 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°C for 15 min. The tubes were vortexed for 30 sec, then reheated and revortexed until all tissue samples were dissolved. The Tissue/Blood Digest Reagent II were added while the tissue samples were still hot, then the tubes were capped and vortexed for 30 min. 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 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 tissues, 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.

Electroretinogram (ERG) in Rats

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 inserted subcutaneously to the neck as a ground electrode. A photostimulator (Grass PS22 Flash) was used to produce flashes of light five 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 msec duration), white light stimuli was used to elicit ERG a- and b-waves. Peak b-wave amplitudes were measured from the trough of the a-wave to the peak of the b-wave.

Dark-adapted, female Long-Evans rats (200-250 g) were anesthetized with 35 mg/kg ketamine plus 5 mg/kg xylazine intramuscularly. Half of the initial dose was given thereafter at one hour intervals to maintain adequate anesthesia. The pupils were dilated with 1% tropicamide plus 10% phenylephrine (50 μl) 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 tightly drawn for 30 min to occlude the retinal vessels. The retinal ischemia was confirmed by the extinction of the ERG waves. After 30 min of retinal ischemia, the ligature was released and the retinal arteries allowed to reperfuse. ERGs were then measured at 0, 30, 60, 90, 120, 180 and 240 min thereafter.

All drugs and vehicles were administered intraperitoneally. These drugs were administered immediately prior to occlusion of the central retinal arteries.

Statistical Analysis

All data were presented as mean ± standard errors (SEM). Non-paired Student's t-test was performed to analyze the significance between two means at a certain time point. The differences were considered significant if $P<0.05$.

RESULTS

Ocular Blood Flow Results

When the intraocular pressure (IOP) was raised to 40 mmHg, the ocular blood flow in all tissues was reduced to 1/3 of the original value (6) and then gradually declined during the 2 hr experimental period (see controls in Tables 1-4). All crocin analogs except crocin-3 increased the retinal blood flow significantly at 120 min after drug instillation (Table 1). Although the retinal blood flow was not significantly increased by crocin-3 statistically at 120 min after drug instillation, it showed a tendency to increase. In case the SE were reduced, it would have shown the statistical difference between treated and control blood flows (Table 1).

The effects of crocin analogs on choroidal blood flow were quite impressive as can be seen from Table 2. Crocin-1 and crocin-2 were the least effective in increasing choroidal blood flow, if any, at 120 min after drug instillation. Crocin-3 increased choroidal blood flow significantly at 60 and 120 min after drug instillation whereas crocin-4 increased choroidal blood flow significantly at 30 and 60 min after drug instillation. The effects of crocetin were most impressive to increase the blood flow at all time points of 30, 60 and 120 min after drug administration (Table 2).

TABLE 1
Effects of Crocin Analogs on the Blood Flow in the Retina

	Retinal Blood Flow (μl/min/mg)			
Drugs	0 min	30 min	60 min	120 min
Control	0.35 ± 0.09	0.22 ± 0.07	0.15 ± 0.05	0.03 ± 0.02
1% Crocin-1	0.41 ± 0.12	0.45 ± 0.08	0.31 ± 0.04	0.30 ± 0.06*
1% Crocin-2	0.20 ± 0.03	0.25 ± 0.05	0.19 ± 0.05	0.15 ± 0.04*
1% Crocin-3	0.15 ± 0.03	0.17 ± 0.05	0.20 ± 0.07	0.14 ± 0.06
1% Crocin-4	0.14 ± 0.03	0.16 ± 0.03	0.15 ± 0.03	0.11 ± 0.02*
1% Crocetin	0.18 ± 0.07	0.20 ± 0.04	0.16 ± 0.03	0.19 ± 0.04*

All data were mean ± SE of N = 6. Stars represent significant difference from corresponding controls at $P<0.05$.

TABLE 2
Effects of Crocin Analogs on the Blood Flow in the Choroid

	Choroidal Blood Flow (μl/min/mg)			
Drugs	0 min	30 min	60 min	120 min
Control	5.95 ± 0.75	3.89 ± 0.73	2.22 ± 0.57	1.71 ± 0.51
1% Crocin-1	5.08 ± 0.85	4.95 ± 0.87	2.52 ± 0.70	4.40 ± 1.06*
1% Crocin-2	6.66 ± 0.65	5.72 ± 0.99	4.03 ± 0.67	3.52 ± 0.86
1% Crocin-3	6.95 ± 1.05	5.66 ± 0.94	5.61 ± 0.75*	4.37 ± 0.70*
1% Crocin-4	5.84 ± 0.88	6.37 ± 0.60*	4.91 ± 1.04*	3.95 ± 0.95
1% Crocetin	6.97 ± 1.16	6.92 ± 0.96*	5.63 ± 1.17*	7.20 ± 1.63*

All data were mean ± SE of N = 6. Stars represent significant difference from corresponding controls at $P<0.05$.

Effects of crocin analogs on the blood flow in the ciliary body and iris were negligible as can be seen in Tables 3 and 4.

TABLE 3
Effects of Crocin Analogs on the Blood Flow in the Ciliary Body

Drugs	Ciliary Body Blood Flow (μl/min/mg)			
	0 min	30 min	60 min	120 min
Control	3.80 ± 0.53	2.59 ± 0.56	1.16 ± 0.26	0.83 ± 0.31
1% Crocin-1	2.49 ± 0.27	1.81 ± 0.39	1.00 ± 0.18	1.36 ± 0.36
1% Crocin-2	3.36 ± 0.43	2.38 ± 0.30	1.71 ± 0.36	1.29 ± 0.20
1% Crocin-3	4.11 ± 0.76	3.74 ± 0.62	3.63 ± 0.56*	2.35 ± 0.62
1% Crocin-4	2.83 ± 0.53	3.63 ± 0.22	1.74 ± 0.42	2.27 ± 0.79
1% Crocetin	3.67 ± 1.16	3.87 ± 1.16	3.32 ± 1.18	3.33 ± 1.23

All data were mean ± SE of N = 6. Stars represent significant difference from corresponding controls at $P<0.05$.

TABLE 4
Effects of Crocin Analogs on the Blood Flow in the Iris

Drugs	Iris Blood Flow (μl/min/mg)			
	0 min	30 min	60 min	120 min
Control	2.49 ± 0.71	1.28 ± 0.29	0.83 ± 0.31	0.47 ± 0.14
1% Crocin-1	2.38 ± 0.26	1.68 ± 0.33	0.96 ± 0.14	1.24 ± 0.22*
1% Crocin-2	1.92 ± 0.51	1.55 ± 0.48	1.11 ± 0.15	0.65 ± 0.11
1% Crocin-3	1.86 ± 0.49	1.26 ± 0.94	0.99 ± 0.18	0.85 ± 0.33
1% Crocin-4	1.47 ± 0.14	1.69 ± 0.26	0.93 ± 0.21	1.08 ± 0.34
1% Crocetin	1.99 ± 0.38	1.85 ± 0.39	1.85 ± 0.50	1.64 ± 0.58

All data were mean ± SE of N = 6. Stars represent significant difference from corresponding controls at $P<0.05$.

Retinal Function Recovery

When the blood flow of the central retinal artery was concluded for 30 min, the b-wave amplitude disappeared completely and returned to approximately 39% of initial values in 4 hr (Fig. 2). When crocin-1 (10 mg/kg) was injected intraperitoneally, the b-wave of ERG returned to approximately 75% of the initial values or twice as high as the corresponding control values (Fig. 2). Similar results were obtained by the injection of 10 mg/kg of crocin-2 and crocin-3 except that the recovery of b-wave amplitude was more pronounced to reach approximately 84% of the initial values (Figs. 3 and 4). Crocin-4 was the most potent. Even a reduced dose of 3 mg/kg still caused significant recovery over control values to 62% of initial values (Fig. 5). The potency of crocetin was about the same as those of crocin-2 and crocin-3 to reach 84% of the initial value (Fig. 6).

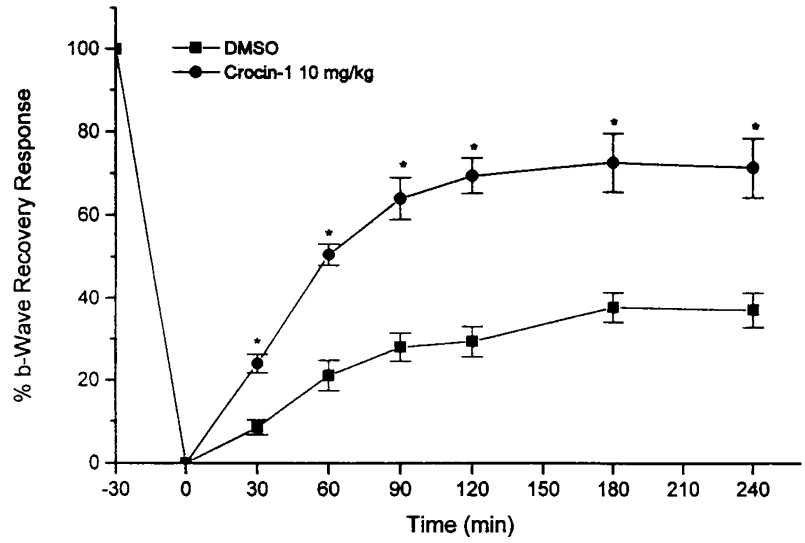


FIGURE 2. Effects of Crocin-1 (10 mg/kg ip) on Recovery of ERG b-wave after 30 min Occlusion of Central Retinal Artery in Rat Eyes. Each point is a mean of 6 values from 6 animals and bars represent SE. Stars indicate significant difference from corresponding control (DMSO) at $P<0.05$.

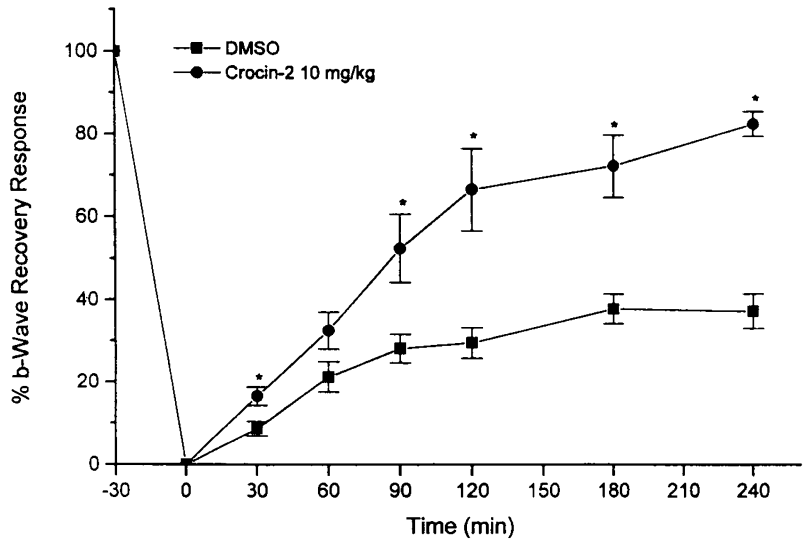


FIGURE 3. Effects of Crocin-2 (10 mg/kg ip) on Recovery of ERG b-wave after 30 min Occlusion of Central Retinal Artery in Rat Eyes. Each point is a mean of 6 values from 6 animals and bars represent SE. Stars indicate significant difference from corresponding control (DMSO) at $P<0.05$.

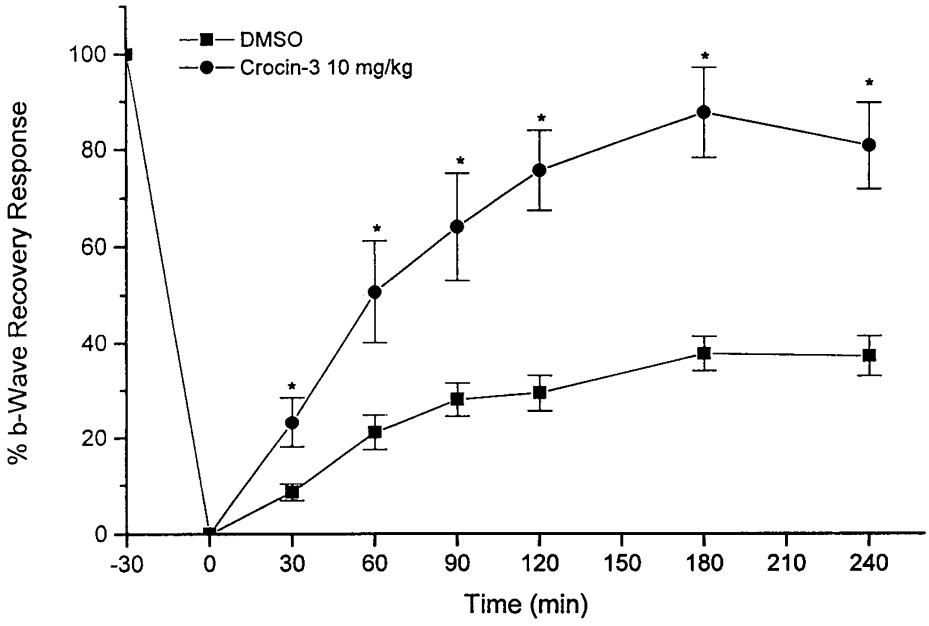


FIGURE 4. Effects of Crocin-3 (10 mg/kg ip) on Recovery of ERG b-wave after 30 min Occlusion of Central Retinal Artery in Rat Eyes. Each point is a mean of 6 values from 6 animals and bars represent SE. Stars indicate significant difference from corresponding control (DMSO) at $P<0.05$.

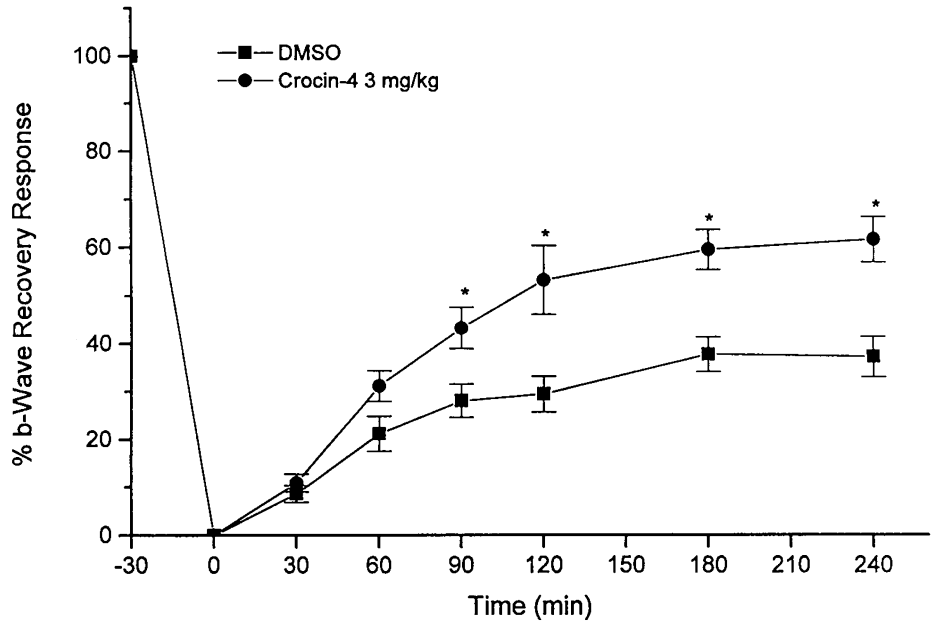


FIGURE 5. Effects of Crocin-4 (3 mg/kg ip) on Recovery of ERG b-wave after 30 min Occlusion of Central Retinal Artery in Rat Eyes. Each point is a mean of 6 values from 6 animals and bars represent SE. Stars indicate significant difference from corresponding control (DMSO) at $P<0.05$.

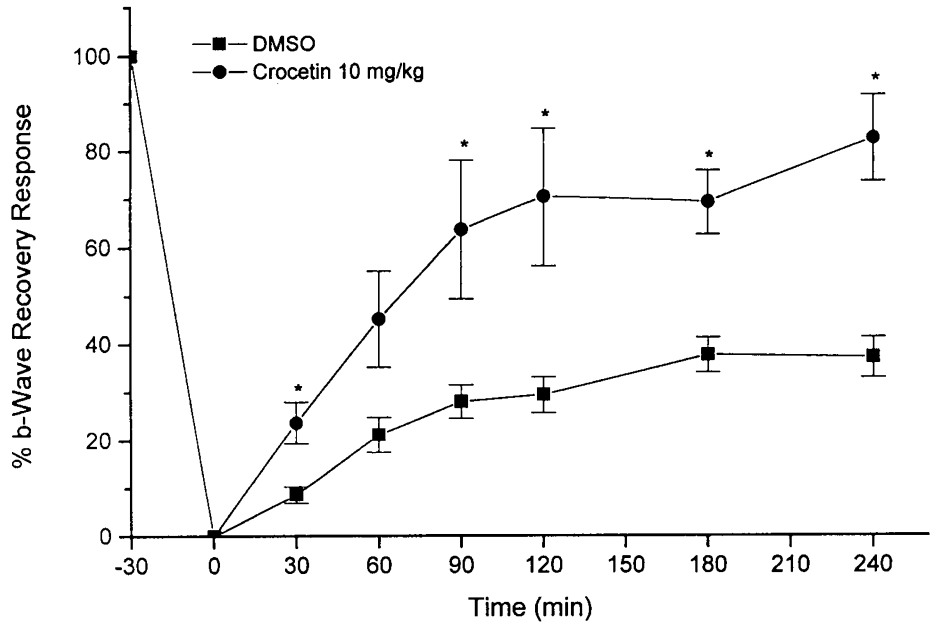


FIGURE 6. Effects of Crocetin (10 mg/kg ip) on Recovery of ERG b-wave after 30 min Occlusion of Central Retinal Artery in Rat Eyes. Each point is a mean of 6 values from 6 animals and bars represent SE. Stars indicate significant difference from corresponding control (DMSO) at $P<0.05$.

DISCUSSION

Ischemic retinopathy and age-related macular degeneration (AMD) are leading diseases that cause reduced vision and blindness. Tremendous efforts have been made to treat these diseases with little success so far (2-5). Although numerous agents have been found to increase the ocular blood flow (6-10), none of them showed any specificity to increase blood flow at the problem areas, the retina and choroid.

It is interesting to note that crocin analogs have specific actions to increase the blood flow in the retina and choroid. Since fovea is avascular and the pathogen of AMD has been related to the abnormalities of choroidal vasculatures and decreased blood flow (2-5), it would be reasonable to predict that agents which can improve choroidal blood flow may likely produce beneficial actions to AMD.

The recovery of retinal functions after ischemic insult with crocin analogs is quite exciting. These results clearly demonstrated that crocin analogs could be used for the treatment of ischemic retinopathy and/or AMD.

In both ocular blood flow increase and retinal function recovery, crocin analogs showed consistent structure activity relationship (SAR), i.e. those compounds with larger molecules (with 3 and 4 sugars) such as crocin-2 and crocin-1 showed lower potency than those smaller molecules (with only 1 or 2 molecules of sugars) such as crocin-4 and crocin-3. Crocetin has no molecule of sugar attached to it and thus produced its potency similar to crocin-3 and crocin-4. The reasons for this SAR could be due to the fact that larger molecules have more difficulty reaching the receptor site for reaction or the equal weight larger molecules have less molar equivalence to smaller molecules. Further, crocetin, the aglycone of crocin-1, crocin-2, crocin-3 and crocin-4, exhibited the activity similar to the corresponding glycoside forms, presumably the carboxylic ester forms are hydrolyzed and the rate of hydrolysis may determine the activity level.

Numerous agents including antiglaucoma drugs (6), dopamine antagonists (7, 8), L-arginine derivatives (9, 10) and phytogenic compounds (4-15) have been studied to improve the retinal and choroidal blood flow and to facilitate retinal function recovery after central retinal artery occlusion in animals. However, most of these drugs increase ocular blood flow in general without any specific actions in the retina or choroid. Consequently, naturally occurring pharmacologically active compounds (16) can provide valuable leads to the discovery of newer drugs for eye disorders.

REFERENCES

1. Jiangsu New Medical College, Encyclopedia of Chinese Herbs, Shanghai People's Publ. Co., Shanghai, China, 1975.

2. Ciulla, T.A., Danis, R.P. and Harris, A. Age-related macular degeneration: A review of experimental treatments. *Curr. Res.* 43:1-13, 1998.

3. Ciulla, T.A., Harris, A. and Danis, R.P. Choroidal perfusion defects in age-related macular degeneration. *Amer. J. Ophthalmol.*, in press.

4. Harris, A., Chung, H.S., Ciulla, T.A. and Martin, B. Regulation of retinal and optic nerve blood flow. *Arch. Ophthalmol.*, in press.

5. Ciulla, T.A., Danis, R.P. and Chakravarthy, U. Radiation therapy for exudate age-related macular degeneration. *Retina, J. Retinal Vitreous Diseases* 18:19-20, 1998.

6. Chiou, G.C.Y. and Chen, Y.J. Effects of antiglaucoma drugs in ocular blood flow in ocular hypertensive rabbits. *J. Ocular Pharmacol.* 9:13-24, 1993.

7. Chiou, G.C.Y. and Chen, Y.J. Effects of dopamine agonist, bromocriptine, and some dopamine antagonists on ocular blood flow. *J. Ocular Pharmacol.* 8:285-294, 1992.

8. Chiou, G.C.Y. and Li, B.H.P. Effects of dopamine antagonists on retinal b-wave recovery after retinal ischemia. *J. Ocular Pharmacol.* 9:179-185, 1993.

9. Chiou, G.C.Y., Liu, S.X.L., Li, B.H.P., Varma, R.S. and Chiang, C.H. Ocular hypotensive effects of arginine compounds and their actions on ocular blood flow. *J. Ocular Pharmacol. Ther.* 11:1-10, 1995.

10. Liu, S.X.L., Chiou, G.C.Y. and Varma, R.S. Improvement of retinal function after ischemia with L-arginine and its derivatives. *J. Ocular Pharmacol. Ther.* 11:263-268, 1995.

11. Chiou, G.C.Y., Li, B.H.P. and Wang, M.S. Facilitation of retinal function recovery by natural products after temporary ischemic occlusion of central retinal artery. *J. Ocular Pharmacol.* 10:493-498, 1994.

12. Chiou, G.C.Y. Treatment of open angle glaucoma and ischemic retinopathy with dopamine antagonists. *J. Ocular Pharmacol.* 10:371-377, 1994. (Review)

13. Liu, S.X.L., Chiou, G.C.Y., Chiang, C.H. and Yao, Q.S. Increase of ocular blood flow by some phytogenic compounds. *J. Ocular Pharmocol. Ther.* 12:95-101, 1996.

14. Liu, S.X.L. and Chiou, G.C.Y. Effects of Chinese herb products on mammalian retinal function. *J. Ocular Pharmacol. Ther.* 12:377-386, 1996.

15. Liu, S.X.L., Wang, M.S., Kapingu, M.C. and Chiou, G.C.Y. Facilitation of retinal functions recovery by coumarin derivatives. *J. Ocular Pharmacol. Ther.* 13:69-79, 1997.

16. Varma, R.S. and Chiou, G.C.Y. Chemistry and vascular dilating actions of pharmacologically active natural products from medicinal plants. *Int. J. Oriental Med.* 14:63-91, 1989.

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