· Original article ·

Effect of flavone on the ocular blood flow and formation of choroidal neovascularization

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Abstract

• AIM: To investigate the effect of flavone on ocular blood flow in rabbit eyes and the formation of choroidal neovascularization (CNV) in rat model of age - related macular degeneration (AMD).

• METHODS: In *in vivo* studies, colored microsphere technique was used to determine the ocular blood flow in ocular hypertensive rabbit eyes. The rat eyes were treated with 5g/L flavone eye drops 3 times a day for 1 week before and 4 weeks after laser - induced injury of Bruch's membrane. The development of CNV was determined by fluorescein angiography (FA) performed on weeks 2 and 4. In *in vitro* studies, the effect of flavone on the viability of HUVECs was measured by MTT assay.

• RESULTS: The ocular blood flow in rabbit eyes was significantly increased after flavone instillation. Flavone significantly inhibited the formation of laser induced CNV. *In vitro* results showed that flavone inhibited the proliferation of HUVECs.

• CONCLUSION: Flavone could increase ocular blood flow and inhibit the formation of CNV.

• KEYWORDS: flavone; age-related macular degeneration; ocular blood flow; choroidal neovascularization
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INTRODUCTION

Age-related macular degeneration (AMD) is the leading disease of visual impairment in western countries in patients over 50 years of age[1]. The prevalence of AMD increases with age so that up to one third of individuals aged 75 and older suffered from some form of AMD[2]. Given the enormous impact of AMD on the aging population, much public interest and research has been focused on this condition in the past decade.

AMD initially occurs in a "dry" form with pathological changes in the retinal pigment epithelium (RPE) and drusen formation, and can progress to geographic atrophy and/or "wet" form of AMD with choroidal neovascularization (CNV)[3]. The breakdown of Bruch's membrane under the detached RPE serves as an entrance for new and immature choroidal vessels to grow into the subretinal space that leads to the formation of CNV[4-6]. CNV can leak fluid as well as hemorrhage in the subretinal space resulting in blurry vision, visual distortion and sudden loss of vision[7]. If left untreated, these lesions progress to form an organized fibrous scar, termed a disciform scar, which typically results in irreversible central vision loss. Flavonoids are a class of more than 4000 phenylben- zopyrones that occur in many edible plants, like fruits and vegetables[8]. These polyphenolic compounds display a remarkable spectrum of biochemical activities including antioxidant activities[9]. This study is to observe the effects of flavone on ocular blood flow, laser induced CNV formation and human umbilical vein endothelial cells (HUVECs).

MATERIALS AND METHODS

Materials Eight-week-old male Brown- Norway rats and female New Zealand white rabbits weighing 2. 5-3. 0kg, were purchased through LARR (Texas A&M University, USA). Animal care and treatment were followed by the institutional guidelines. Flavone, thiazolyl blue tetrazolium bromide (MTT, purity ≥ 97. 5%), Dulbecco's phosphate buffered saline (DPBS) and sodium iodate ($NaIO_3$, purity≥99. 5%) were all purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Human umbilical vein endothelial cells (HUVECs), fetal bovine serum (FBS), vascular cell basal medium and endothelial cell growth kit were purchased from ATCC (Manassas, VA, USA).

Effect of Flavone on Ocular Blood Flow of Ocular Hypertensive Rabbits Rabbits were anesthetized with 35mg/kg ketamine and 5mg/kg xylazine by intramuscular injection. Half of the initial dose was given every one hour thereafter. The left ventricle was cannulated through the right carotid artery for injection of colored microspheres and the femoral artery was cannulated for collection of blood samples. The left eye was treated with one drop of proparacaine hydrochloride ophthalmic solution (Bausch & Lomb, Inc., Tampa, FL, USA). The needle was inserted directly into the anterior chamber of the left eye, which was connected to the 40mmHg saline manometer. The ocular hypertensive model reduced the ocular blood flow to approximately one third of the normal valued. 50μL of 10g/L flavone or vehicle (30% HP-β-CD solution) was instilled topically to the left eye 30 minutes after the ocular hypertensive model was built. The ocular blood flow was measured by

colored microspheres at 0, 30, 60 and 120 minutes after treatment with flavone or vehicle. At each timepoint, 2 million microspheres were injected as a reference, and blood samples were taken from the femoral artery for exactly one minute 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 iris, ciliary body, retina and choroid. All the tissues were weighed.

The details of sample processing and microsphere counting were provided by E-Z Trac (Los Angeles, CA, USA). In brief, the blood 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 then tissue/blood digest reagents I and II were added. The tubes were capped, vortexed, and centrifuged for 30 minutes. The supernatant was removed, and the counting reagent was added, vortexed, and centrifuged for 15 minutes. The supernatant was removed, and the microspheres were resuspended in a precise volume of the counting reagent. The number of microspheres was counted by the hemocytometer under the microscope. Tissue/blood digest reagent I was added to the microfuge tubes with the tissue samples, sealed, and heated at 95℃ for 15 minutes. Then the tubes were vortexed for 30 seconds, reheated, and revortexed until all tissue samples were dissolved. The tissue/blood digest reagent II was 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 sampled, and the microspheres were counted.

The blood flow of each tissue at a certain time point was calculated according to the following formula: $Q\text{m} = (C\text{m} \times Q\text{r})/C\text{r}$. Qm is the blood flow of a tissue in terms of μL/min/mg, Cm is the microsphere number/mg of tissue, Qr is the flow rate of blood sample in terms of μL/min, and Cr is the microsphere number in the referenced blood sample.

Effect of Flavone on Laser Induced CNV Formation in Rats The rats were randomly divided into 2 groups. Control group was instilled with vehicle (30% HP-β-CD solution). Flavone group was instilled with 5g/L flavone eye drops. Both eyes of all rats were instilled with 1 drop of ophthalmic solutions 3 times a day for 1 week before and 4 weeks after laser induced injury. The rats were anesthetized for all procedures with intramuscular injection of ketamine (35mg/kg) and xylazine (5mg/kg). The pupils were dilated with one drop each of 10g/L atropine, and 25g/L phenylephrine. The fundus was visualized by the VOLK super Pupil XL Biomicroscopy Lens (Keeler Instrument Inc., Broomall, PA, USA). A double-frequency Nd: YAG laser (Laserex LP3532; Lumenis Inc., Salt Lake City, UT, USA) was used at 532nm wavelength. Laser parameters were used by 100μm spot size, 0.15-second exposure and 150-200 mw powers. Five laser spots were made to the ocular fundus at approximately equal distances around the optic nerves. Acute vapor bubbles suggested rupture of Bruch membrane[10]. Only laser spots with bubble formation were included in the study. Lesions with subretinal hemorrhage were excluded.

Fluorescein angiography (FA) was performed after 2 and 4 weeks laser treatment with a digital fundus camera (TRC-50EX; TOPCON, Tokyo, Japan). 100g/L fluorescein sodium salt was injected (0.5mL/kg) through hypoglossal vein. Both early (under 2 minutes) and late (over 7 minutes) fluorescein phases were captured. 100g/L fluorescein isothiocyanate-dextran was injected (1.4mg/kg) through hypoglossal vein after 3 drops of fluorescein sodium salt injection. Fluorescein pictures were taken within 20 minutes. The clearest pictures were chosen for the areas of CNV formation measurement by Imagenet2000 digital imaging system (Topcon Medical Systems, Inc., Paramus, NJ, USA).

Cell Culture HUVECs were grown in vascular cell basal medium supplemented with endothelial cell growth kit. Cells were incubated in a humidified incubator at 37℃ under 50mL/L CO_2 and 950mL/L air.

Effect of Flavone on the Viability of HUVECs MTT assay was used to measure the viability of HUVECs. 1×10^5 cells were seeded in 96-well plates (100μL/well) and allowed to grow overnight. Negative control was prepared by adding 100μL medium without cells. The cells were then treated with fresh medium with flavone (flavone was dissolved in 30% HP-β-CD solution, the final concentration of HP-β-CD in cells is less than 0.3%). The vehicle control group was treated with 30% HP-β-CD solution with fresh medium (the final concentration of HP-β-CD in cells is less than 0.3%). 20μL MTT (5g/L) was added to wells, and incubated for another 4 hours. After incubation, the medium was discarded and 100μL DMSO was added to solubilize formazan produced from MTT by the viable cells. Absorbance was measured at 570nm using a microplate reader (Bio-Rad Laboratories, Inc., CA). Cells viability was calculated according to the following formula: Viability of cells (%) = (absorbance in tested sample-absorbance in negative control)/(absorbance in vehicle control-absorbance in negative control) × 100%.

Hypoxia Treatment Cells were allowed to attach overnight, and then exposed to flavone or vehicle under hypoxic condition for 72 hours. Hypoxic conditions (10mL/L O_2, 50mL/L CO_2 and 940mL/L N_2) were maintained by using a temperature and humidity controlled environmental C-chamber by O_2 and CO_2 controllers (Proox Model 110 and Pro CO_2 Model 120, Bio Spherix Ltd., Redfield, NY, USA) with N_2 and CO_2 gas sources.

Statistical Analysis All data were expressed as mean SEM. Statistical analysis was performed using the Student's t-test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Effect of Flavone on Ocular Blood Flow of Ocular Hypertensive Rabbits The ocular blood flow (including iris, ciliary body and choroid) declined gradually due to the ocular hypertension in the vehicle control group. Flavone significantly increased the iris blood flow at 120 minutes after

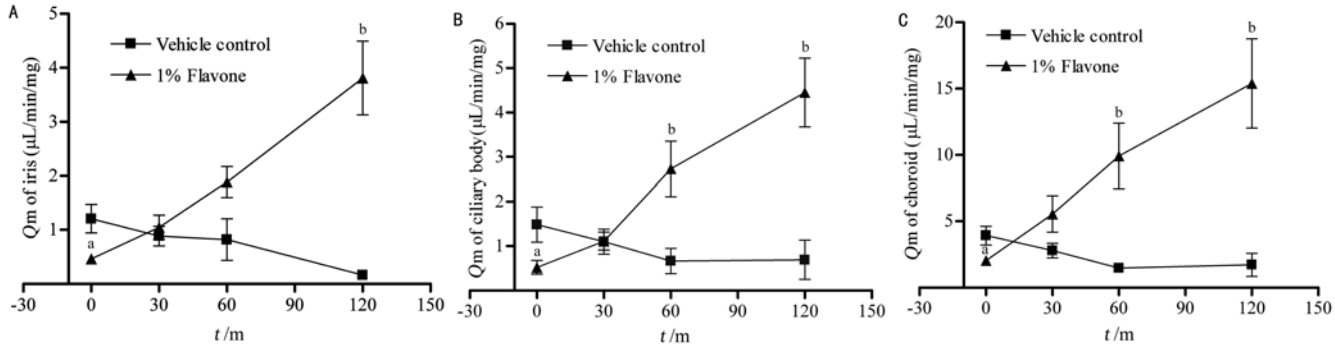

Figure 1 Effect of flavone on ocular blood flow of experimental ocular hypertensive rabbits n =6 in each group; $^{a}P<0.05$ and $^{b}P<0.01$ *vs*the vehicle control group.

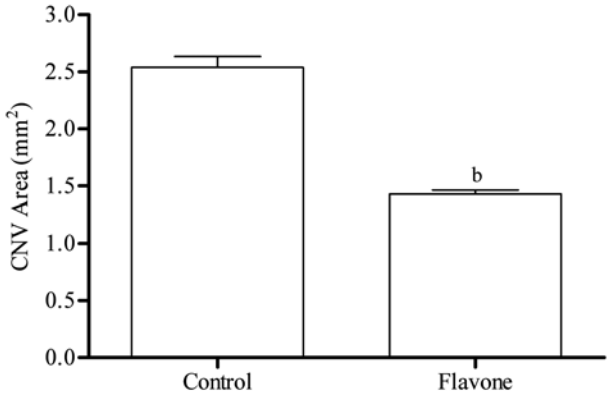

Figure 2 Effect of flavone on laser induced CNV rat model $^{b}P<0.01$ *vs*vehicle control group.

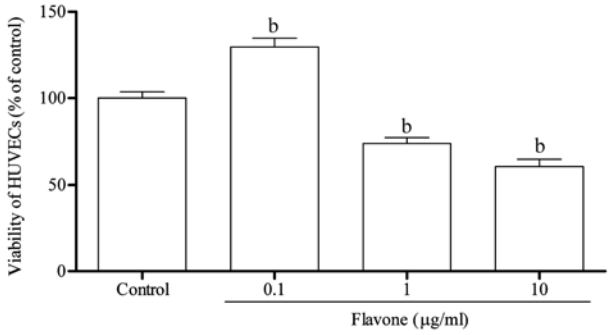

Figure 3 Effect of flavone on proliferation of HUVECs HUVECs were incubated with flavone for 72 hours n = 6 in each group; $^{b}P<0.01$ *vs*vehicle control group.

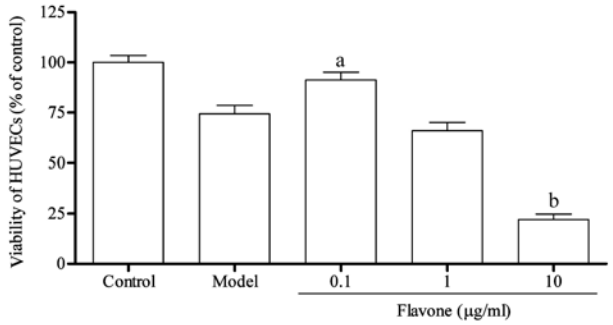

Figure 4 Effect of flavone on hypoxia-induced injury in HUVECs HUVECs were incubated with flavone for 72 hours. Control group treated with vehicle (30% HP-β-CD solution) under normal condition (50mL/L CO_2 and 950mL/L air) for 72 hours; model group treated with vehicle under hypoxic condition (10mL/L O_2, 50mL/L CO_2 and 940mL/L N_2) for 72 hours. n = 6 in each group; $^{a}P<0.05$ and $^{b}P<0.01$ *vs*model group.

drug instillation compared with the corresponding vehicle control group ($P<0.01$, Figure 1A). Furthermore, flavone also significantly increased the ciliary body and choroid blood flow at 60 and 120 minutes ($P<0.01$, Figure 1B and 1C).

Effect of Flavone on Laser Induced CNV Model in Rats The angiograms of FD70-FA showed diminished lesion size in flavone group as compared with control group. Four weeks after laser treatment, the size of the CNV lesion was (2.54 ± 0.10) mm^2 in the control group, whereas it was only (1.43 ± 0.03) mm^2 in the flavone group ($P<0.01$, Figure 2).

Cytotoxicity of Flavone in HUVECs Flavone increased the proliferation of HUVECs at the concentration of 0.1μg/mL, yet inhibited the proliferation of HUVECs at the concentrations of 1 and 10μg/mL ($P<0.01$, Figure 3).

Effect of Flavone on Hypoxia-induced Injury in HUVECs Flavone significantly increased the viability of HUVECs by 16% in hypoxic condition at concentration of 0.1μg/mL ($P<0.05$, Figure 4). At 10μg/mL, flavone significantly decreased the viability of HUVECs by 52% ($P<0.01$).

DISCUSSION

AMD is the leading cause of blindness in the elderly worldwide, affecting 30-50 million individuals. Given the enormous impact of AMD on an aging population, much public interest and research has been focused on it in the past decade.

CNV in AMD means a severe late stage of the disease and often damages central vision[11]. The reason CNV is formed in AMD patients is mainly because of the malfunction of choroid circulation during the early stage of AMD which leads to break down of Bruch's membrane. Improvement and facilitation of ocular blood flow are necessary to prevent CNV occurring[12]. It is exciting to find flavone can increase ocular (iris, ciliary body and choroid) blood flow in rabbit eyes. The increase of ocular blood flow may facilitate removal of metabolic wastes and replenish nutrients to RPE and photocells. Thus, it might change the microenvironment, and change the balance between pro- and anti-angiogenesis factors.

Eyes with the CNV in the macular region can have widely varying degrees of distortion and scotomata. New vessels or hemorrhages can obscure the visual axis in normally avascular

transparent ocular tissues such as the cornea lens and vitreous. Later, the final fibrovascular scar formation causes degeneration of the retina and other ocular tissues. Due to weak, curled, and leaky vessels, the CNV is a major cause of visual loss in AMD[13,14]. Under normal conditions, endothelial cells lining blood vessels are resistant to neovascular stimuli, and negligible endothelial cell proliferation takes place in the retinal vessels. Hypoxia or ischemia may play a role in the development of CNV[15]. Flavone significantly inhibited the formation of laser induced CNV. This study also showed that flavone inhibited vascular endothelial cells proliferation in vitro. These results suggest that flavone might be useful to delay wet AMD progression.

In conclusion, flavone increased ocular blood flow and inhibited the formation of CNV. Thus flavone might be a promising candidate for the treatment of AMD.

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黄酮对眼部血流量和脉络膜新生血管的作用

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摘要

目的:观察黄酮对兔眼部血流量和大鼠脉络膜新生血管(CNV)的作用。

方法:在体研究中,我们采用彩色微球技术测定黄酮对兔眼部血流量的影响。预先给予 5g/L 黄酮滴眼液(3 次/d),1wk 后用激光诱导大鼠 CNV 的生成,在 2 和 4wk 末,采用荧光血管造影(FA)测量 CNV 面积。离体研究中,采用 MTT 法检测黄酮对人脐静脉内皮细胞(HUVECs)存活率的影响。

结果:黄酮显著的增加了兔眼部血流量,抑制了由激光诱导的 CNV 的生成。离体研究结果表明,黄酮抑制了 HUVEC 的增生。

结论:黄酮能够增加眼部血流量,并且抑制 CNV 的生成。

关键词:黄酮;老年黄斑变性;眼血流量;脉络膜新生血管