

# Effects of naringenin on ocular blood flow and choroidal neovascularization in experimental animals

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Received:2009-08-03 Accepted:2009-11-02

## Abstract

• **AIM:** To investigate the effects of naringenin on laser-induced experimental choroidal neovascularization (CNV) in rat models, ocular blood flow and retinal function recovery after ischemic insults in rat eyes.

• **METHODS:** Male Brown Norway rats were treated to break the Bruch's membrane. Naringenin 10g/L (20mg/kg) was given once per day through intraperitoneal injection for 4 weeks after laser treatment. The development of CNV was determined by fluorescein angiography (FA) performed on week 2 and 4. The colored microsphere technique and electroretinography method were used for the study of ocular blood flow and retinal function recovery, respectively.

• **RESULTS:** The choroidal blood flow in elevated intraocular pressure (IOP) rabbit eyes was significantly increased by 10g/L naringenin solution as compared to control group ( $P < 0.05$ ). The retinal function recovery after ischemic insults in rat eyes indicated significant increase of b-wave recovery in treated group, as compared to control group ( $P < 0.05$ ). The intensity of fluorescein leakage from the photocoagulated lesions significantly decreased in treated group, compared to the control group (75.8%-95.0%,  $P < 0.01$ ).

• **CONCLUSION:** Naringenin could prevent the development of CNV on laser-induced experimental rat models, increase the choroidal blood flow in elevated IOP rabbit eyes and be beneficial on retinal function recovery in ischemic rat eyes.

• **KEYWORDS:** naringenin; choroidal neovascularization; ocular blood flow; age-related macular degeneration

Ji J, Xu XR, Chiou GCY. Effects of naringenin on ocular blood flow and choroidal neovascularization in experimental animals. *Int J Ophthalmol* 2009;2(4):320-323

## INTRODUCTION

The development of choroidal neovascularization (CNV) in patients with age-related macular degeneration (AMD) often leads to a significant decrease in visual function. Several mechanisms have been proposed to explain the pathogenesis of CNV, and they include, among others, the presence of senescence changes in the RPE-Bruch's membrane-photoreceptor complex, the accumulation of oxidative damage, and the development of ischemia of the outer retina. Various therapies were tried to reduce the development of CNV, including transpupillary thermotherapy<sup>[1]</sup>, photodynamic therapy, anti-VEGF and anti-inflammation drugs<sup>[2,3]</sup>. Alterations of blood flow in the subfoveal choroidal circulation and metabolic changes of the retinal pigment epithelium have been suggested important in the development of neovascular AMD<sup>[4]</sup>. Choroidal blood flow changes affecting the metabolism of the adjacent external retinal layers lead either to the development of atrophic lesions or to subretinal neovascular lesions<sup>[5]</sup>. Furthermore, vascular changes in the choroid have potentially deleterious effects on the RPE and, in addition to metabolic changes of the RPE caused by senescence, may induce the early clinical findings in AMD.

Our previous studies have suggested that naringenin can increase ocular blood flow<sup>[6]</sup>, it was of interest to determine the effect of naringenin on CNV experimental models *in vivo*

## MATERIALS AND METHODS

**Materials** Naringenin were purchased from Pfaltz and Bauer (Waterburg, CT). Sodium fluorescein and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical (St. Louis, MO).

## Methods

**Measurement of ocular blood flow in ocular**

**hypertensive rabbit eyes** New Zealand white rabbits of either sex, 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 (IOP) 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 (50 $\mu$ L) or vehicle (50 $\mu$ L) 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 of 10 $\mu$ m in diameter in 0.2mL 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 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 (Los Angeles, CA). The blood flow of each tissue at a certain time point was calculated from the following equation:  $Q_m = (C_m \times Q_r) / C_r$ . where  $Q_m$  is the blood flow of a tissue in terms of mL/min/g,  $C_m$  is the microsphere counted per mg of tissue,  $Q_r$  is the flow rate of blood sample in terms of mL/min, and  $C_r$  is the total microsphere counted in the referenced blood sample.

**Electroretinogram** Electroretinogram (ERG) were determined to provide assessment of the retinal function prior to and following 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 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 (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 a-wave to the peak of the b-wave. Dark-adapted, female Long-Evans rats (200-250g) were anesthetized with 35mg/kg ketamine plus 5mg/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 100g/L phenylephrine (50 $\mu$ L) 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 minutes to occlude the retinal vessels. The retinal ischemia was released and the retinal arteries were allowed to re-perfuse. ERGs were then measured at 0, 30, 60, 90, 120, 180 and 240 minutes thereafter. Naringenin (20mg/kg) and vehicle were administered intraperitoneally. Drug was administered immediately prior to occlusion of the central retinal arteries.

**Laser -induced CNV rat model** Brown Norway rats, weighing 150-200g, were anesthetized for all procedures with an intraperitoneal (*i.p.*) injection of chloral hydrate (10mg/kg). The pupils were dilated with mixture of 0.5% tropicamide and 5g/L phenylephrine. The fundus was visualized with a VOLK super pupil XL biomicroscopy lens (Keeler Instrument Inc., Broomall, PA). A krypton laser (Lumenis Inc., Santa Clara, CA) with a 532-nm wavelength was used. Laser parameters were 200 $\mu$ m spot size, 0.2-s exposure, and 150-200mW 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. If lesions with subretinal hemorrhage interfered with the evaluation of the lesions, they were excluded.

**Fluorescein angiography** FA was performed on weeks 2 and 4 post laser with a digital fundus camera (Heidelberg Engineering GmbH, Dossenheim, Germany). Ten milligrams of sodium fluorescein was injected intravenously (*i.v.*) through the hypoglossal vein. Both early (under 2 minutes) and late (over 7 minutes) fluorescein phases were captured. CNV formation was determined with fluorescein angiogram. Each photocoagulated lesion was classified as "leaky" or "not pronounced leaky", according to the intensity of fluorescein leakage by observers (XXR and JJ).

**Table 1** Effects of naringenin on blood flow in rabbit eyes (n=6, mean±SD, mL/min/g)

| Group   |            | 0min       | 30min                   | 60min                  | 120min    |
|---------|------------|------------|-------------------------|------------------------|-----------|
| Choroid | Control    | 3.18±0.16  | 1.20±0.07               | 1.11±0.09              | 0.70±0.17 |
|         | Naringenin | 19.38±1.67 | 17.09±1.67 <sup>a</sup> | 8.70±2.09 <sup>a</sup> | 6.41±1.33 |
| Retina  | Control    | 3.18±0.16  | 1.20±0.07               | 1.11±0.09              | 0.70±0.17 |
|         | Naringenin | 10.15±0.73 | 5.76±1.44               | 4.81±1.36              | 3.29±0.86 |

<sup>a</sup>P<0.05 vs Retina group

**Administration of drugs** Naringenin was given once-daily through an intraperitoneal (ip) injection after laser treatment at 20mg/kg/d for 4 weeks as a positive control. DMSO alone was used as a negative control.

**Statistical Analysis** Each group has 15 rats. Both eyes of each animal were used in the experiment. A chi-square test was used for the analysis of FA. A Student's *t* test was used for other experiments.

**RESULTS**

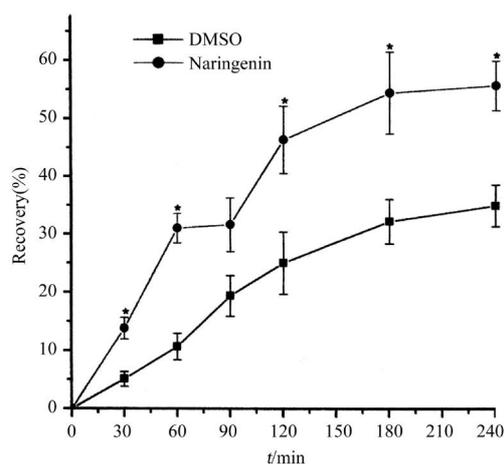
**Choroidal Blood Flow in the Rabbit Eyes** The choroid blood flow was significantly increased by 10g/L naringenin at 30 and 60 minutes after drug instillation as compared with corresponding controls (Table 1). On the other hand, naringenin did not show any effect on the retina blood flow at any time point after drug instillation (Table 1). Since the retina of albino rabbits is vascular, the drug is hard to show any effects on it.

**Retinal Function Recovery in Rat Eyes** The retinal function recovery after ischemic insults in rat eyes indicated significant increase of b-wave recovery from corresponding controls at 30, 60, 120, 180 and 240 minutes (Figure 1).

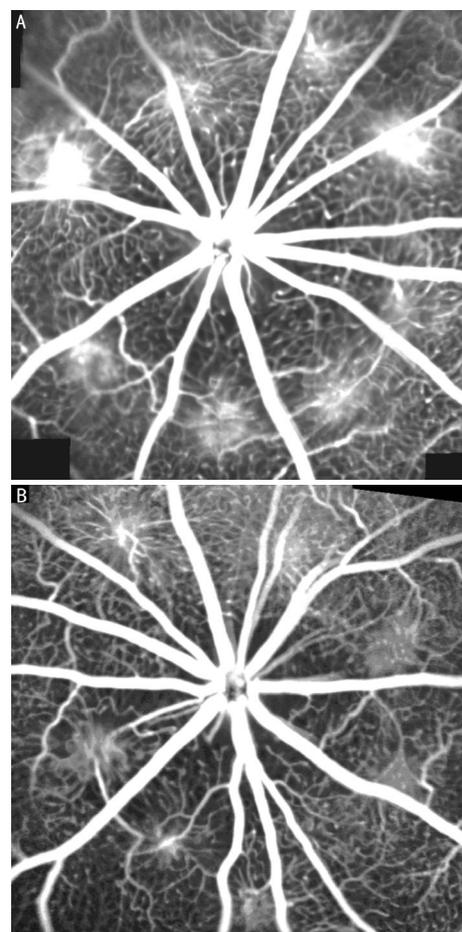
**Incidence of Angiographically Defined CNV** A fluorescein angiogram showed early hyperfluorescence of the laser lesions, which increased in size and intensity in the late phase (Figure 2A). The incidence of angiographically defined CNV was reduced significantly in naringenin-treated group (75.8%-95.0%, P<0.01) (Figure 2B), compared to the DMSO-treated control group, after 4 weeks treatment.

**DISCUSSION**

Several reports have suggested a role for decreased choroid blood flow in the formation of CNV. Preliminary work of Ross *et al*<sup>[7]</sup> has shown an association between the location of macular choroidal watershed vascular filling zones detected by FA and choroidal neovascular membranes. During angiography, watershed-filling zones correspond to the last areas of the choroid that fill with the dye. These areas are most probably the boundaries between adjacent choroidal lobules. The presence of CNV in close proximity



**Figure 1** Effects of naringenin on retinal function recovery after ischemic insults in rat eyes



**Figure 2** Fluorescein angiography of rats treated for 4 weeks A: Control group; B: Naringenin

to these areas, which are the most prone to ischemia and hypoxia in conditions of decreased choroidal blood flow, suggests that ischemia may have a role in the development of AMD-related CNV. Similarly, Guagnini *et al* [8] in a recent case report study have shown an association between areas of early choroidal hypofluorescence on angiography, which presumably correspond to areas of acute choroidal ischemia, and later development of large neovascular membranes.

The most purpose of this work was to examine the effects of naringenin on development of CNV, and the results of our study indicate that naringenin could significantly prevent the development of CNV induced by laser on rat models, which could be seen from fluorescein leakage. The mechanism of naringenin to prevent CNV may be related to its effect on choroidal blood flow. As shown in this research, naringenin can improve the choroidal blood flow on acute elevated IOP rabbit eyes, when IOP was raised from normal values around 18-20mmHg to 40mmHg, the ocular blood flow reduced to one third of the original values. Furthermore, this compound could significantly increase retinal function recovery after ischemic insults in rat eyes. These findings strongly support the role of hypoperfusion and possibly ischemia of choroid as an inciting factor for CNV formation. In summary, our study suggests that naringenin could prevent the development of CNV, and improve the choroidal

blood flow. Thus, naringenin might be a good candidate for the prevention and treatment of ocular neovascularization especially in the AMD. Since exact mechanism of naringenin on the development of CNV is not clear, further investigation is warranted.

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