Original Article

Effect of Quercetin on Formation of Choroidal Neovascularization (CNV) in Age-related Macular Degeneration (AMD)

Pei Zhuang, Yi Shen, Baoqin Lin, Wanyu Zhang, George C.Y. Chiou
Institute of Ocular Pharmacology, College of Medicine, Texas A&M Health Science Center, College Station, TX 77843, USA

Abstract

Purpose: Age-related macular degeneration (AMD) as a disease entity is "dry" at early stage and made up of two main components at late stage: atrophic AMD and exudative AMD. Quercetin acts as an anti-oxidant to protect retinal pigment epithelial cells (RPE) from damaged by oxidative stress, but its effect on formation of choroidal neovascularization (CNV) in AMD is unclear. The aim of this study is to investigate the effect of quercetin on the formation of CNV in AMD.

Methods: The development of CNV induced by laser was detected by fluorescein angiography (FA). Colored microsphere technique was used to determine the choroidal blood flow in ocular hypertensive rabbit eyes.In in vitro studies, HUVECs were treated with $NaIO_3$, H_2O_2 and NaN_3 to induce oxidative cell damages. The effect of quercetin on various oxidations-induced injuries in HUVECs was measured by MTT assay. HUVECs migration was assessed using a wound healing assay.

Results: Quercetin significantly inhibited the formation of laser-induced CNV.The choroidal blood flow in rabbit eyes was significantly increased after quercetin instillation. In vitro results showed quercetin enhanced various oxidations-induced injuries in HUVECs and inhibited migration of HUVECs during wound healing.

Conclusion: Quercetin inhibited the formation of CNV both in vivo and in vitro and increased choroidal blood flow.It could become a promising candidate for the treatment of AMD.

Keywords: quercetin; age-related macular degeneration, choroidal neovascularization, ocular blood flow, human umbilical vein endothelial cell

Corresponding author: George C.Y.Chiou, Institute of Ocular Pharmacology, College of Medicine, Texas A&M Health Science Center, College Station, TX 77843, USA. Tel: +1 979 845 2817; Fax: +1 979 845 0699. E-mail address: chiou@medicine.tamhsc.edu

Introduction

Age-related macular degeneration (AMD) affects older adults and accounts for about half of all vision impairment or blind registrations in the developed world[1]. As the disease affects the central regions of the retina and choroid, central visual loss ensues. Approximately 30% of adults aged 75 or older have some signs of maculopathy, and 6% to 8% of these individuals are afflicted with the advanced stages of AMD[2,3]. Due to increased life expectancy and current demographics, the prevalence of AMD is expected to double by the year 2020[4].

The late stage of AMD as a disease entity is made up of two main components: atrophic AMD and exudative AMD[5].Exudative AMD (wet AMD) is marked by the growth of aberrant blood vessels under the retina along with epithelial proliferation and inflammation, which is referred to as choroidal neovascularization (CNV).CNV can leak fluid as well as hemorrhage in the subretinal space resulting in blurry vision, visual distortion and sudden loss of vision[6].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 widely distributed in the plant kingdom and are categorized as flavonol, flavanol, flavanone, flavone, anthocyanidin, and isoflavone. Quercetin is a typical flavonol-type flavonoid ubiquitously present in fruits and vegetables[7]. It has been proven to be an excellent antioxidant that also possesses anti-inflammatory, anti-proliferative and gene expression changing capacities in vitro[8]. Previous studies have shown that quercetin can protect retinal pigment epithelial cells (RPE) form oxidative damage in vitro[9–11]. The aim of our present study is to investigate

the effects of quercetin on the formation of CNV in wet AMD both in vivo and in vitro.

Materials and methods

Animals and materials

Eight-week-old male Brown-Norway rats and female New Zealand white rabbits weighing 2.5–3.0 kg, were purchased through LARR (Texas A&M University, USA). Animal care and treatment were followed by the institutional guidelines.

Quercetin (purity ≥99%) was purchased from Pfaltz & Bauer Inc. (Waterbury, CT, USA). Thiazolyl blue tetrazolium bromide (MTT, purity ≥97.5%), Dulbecco's phosphate buffered saline (DPBS), sodium iodate ($NaIO_3$, purity ≥99.5%), hydrogen peroxide (H_2O_2, 50 wt.% solution in water) and sodium azide (NaN_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). Growth-reduced Matrigel was bought from Becton Dickinson (Bedford, MA, USA).

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). Quercetin group was instilled with 1% quercetin 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 method of laser-induced CNV formation was published previously[12]. Briefly, the fundus was visualized with 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 532 nm wavelength. Laser parameters were used by 100 μm 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's membrane[13]. 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 wks laser treatment with a digital fundus camera (TRC-50EX; TOPCON, Tokyo, Japan). The clearest pictures were chosen for the areas of CNV formation. And the size of CNV lesion was measured by Imagenet2000 digital imaging system (Topcon Medical Systems, Inc., Paramus, NJ, USA).

Choroidal blood flow in rabbits

Rabbit was anesthetized with 35 mg/kg ketamine and 5 mg/kg xylazine i.m.. And half of the initial dose was given each 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 40 mmHg saline manometer. The ocular hypertensive model reduced the ocular blood flow to approximately one third of the normal valued. 50 μl of 1% quercetin or vehicle (30% HP-β-CD solution) was instilled typically into the left eye 30 min after the ocular hypertensive model was built. The ocular blood flow was measured by colored microspheres at 0, 30, 60 and 120 min after the treatment with quercetin or vehicle. At each time point, 2 million different color microspheres were injected as a reference, and blood samples were taken from the femoral artery for exactly 1 minute following injection of the microspheres. The blood sample was collected in a heparinized tube and the volume was recorded. The rabbits were euthanized after the last blood sampling. The left eyes were enucleated and the choroid was dissected. The tissue was weighted. The details of sample processing and microsphere counting were provided by E-Z Trac (Los Angeles, CA, USA).

The blood flow of the tissue at a certain time point was calculated according to the following formula: $Qm = (Cm \times Qr)/Cr$. Qm is the blood flow of a tissue in terms of $\mu l \cdot min^{-1}/mg$; 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.

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 5% CO_2 and 95% air.

Various oxidant-induced injuries in HUVECs

MTT assay was used to measure the viability of HUVECs. 1×10^5 cells/ml were seeded in 96-well plates (100 μl/well) and allowed to grow overnight. Negative control was prepared by adding 100 μl medium without cells. The cells were then exposed to various concentrations of quercetin (1 and 10 μg/ml) and/or oxidants ($NaIO_3$, H_2O_2, t-BHP and NaN_3) for 12, 24 or 72 hours (200 ml/well). The vehicle control group was treated with vehicle (HP-β-CD solution, the final concentration of HP-β-CD in cells is less than 0.3%). Twenty μl MTT (5 mg/ml) was added to wells, and incubated for another 4 h. After incubation, the medium was discarded and 100 μl DMSO was added to solubilize formazan produced from MTT by the viable cells. Absorbance was measured at 570 nm using a microplate reader (Bio-Rad Laboratories, Inc., CA). Cell 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%.

Migration of HUVECs during wound healing

Wound healing method by Matsunaga[14] was followed with a minor modification. Briefly, when HUVECs in the 24-well plate reached 90% confluence, the monolayer of HUVECs was scratch wounded to approximately in a straight line by pipet tips (1–200 μl, VWR, West Chester, PA, USA), and then rinsed with DPBS twice. The cells were then treated with vehicle or various concentrations of quercetin (1 and 10 μg/ml), and incubated for 24 hours. Images were taken at the time of the wounding and 24 hours thereafter, using a phase-contrast microscope (Olympus, Tokyo, Japan). Migrated cells were estimated by counting the cell numbers in the wounded region.

Statistical analysis

All data were expressed as means ± S.E.M. Data were analyzed by one-way ANOVA followed by Tukey test. A value of $P<0.05$ was considered to be statistically significant.

Results

Effect of quercetin on laser-induced CNV formation in rats

The angiograms of FD70-FA showed the diminished lesion size in quercetin group as compared with control group. Four weeks after laser treatment, the size of the CNV lesion was 2.42 ± 0.12 mm^2 in the control group whereas it was only 1.19 ± 0.03 mm^2 in the quercetin group ($P<0.01$, Fig. 1).

Effect of quercetin on choroidal blood flow in rabbits

The choroidal blood flow declined gradually in the vehicle control group. Quercetin significantly increased the choroidal blood flow at 30, 60 and 120 min after drug instillation compared to the corresponding vehicle control group (Fig.2).

Cytotoxicity of quercetin in HUVECs

At the concentrations of 1 and 10 μg/ml quercetin had no significant effect on the proliferation of HUVECs (Fig.3).

Effect of quercetin on $NaIO_3$-induced injury in HUVECs

HUVECs were incubated with 30, 100 and 300 μg/ml $NaIO_3$ for 72 hours. As the concentration of $NaIO_3$ increased, the viability of HUVECs decreased. Although 1 μg/ml quercetin did not affect cell viability in 30 and 300 μg/ml $NaIO_3$ group, it enhanced 100 μg/ml $NaIO_3$-induced injury of HUVECs. At the concentration of 10 μg/ml, quercetin significantly enhanced $NaIO_3$-induced injury of HUVECs ($P<0.01$, Fig.4).

Effect of quercetin on H_2O_2-induced injury in HUVECs

The viability of HUVECs treated with different concentrations of H_2O_2 for 24 hours showed a gradu-

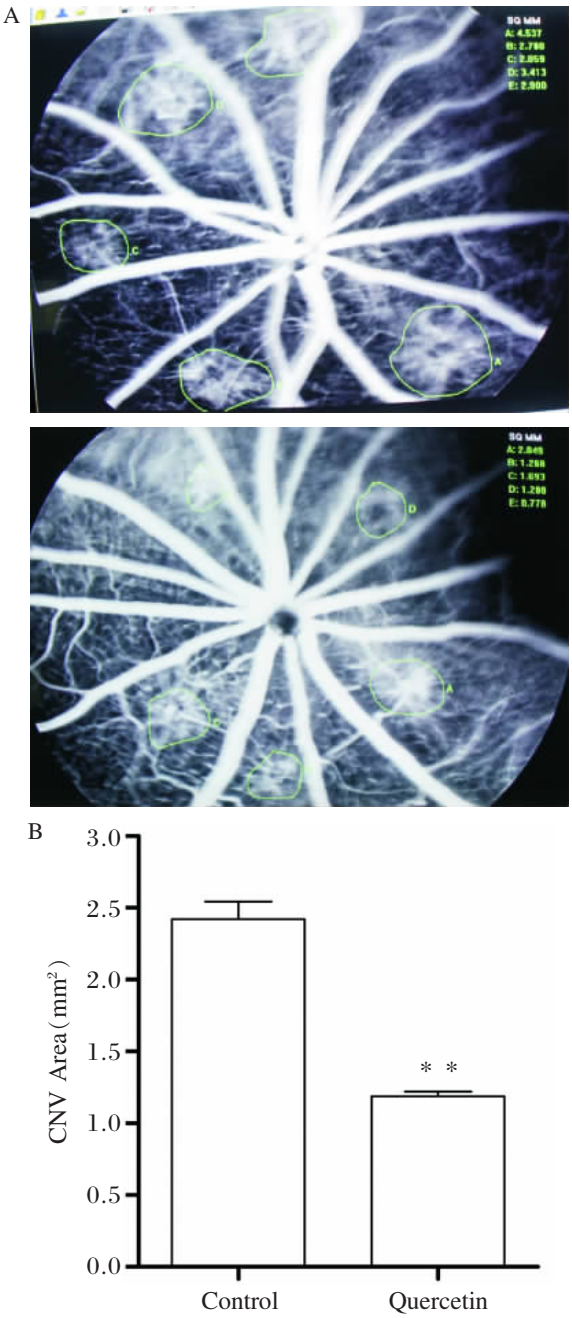

Figure 1 Effect of quercetin on laser-induced CNV rat model.The pictures showed the areas of CNV formation (A). The size of the CNV lesion was measured by Imagenet2000 digital imaging system (B). Data were expressed as means± SEM. n=10 in each group; $^{**}P < 0.01$ quercetin group vs. vehicle control group.

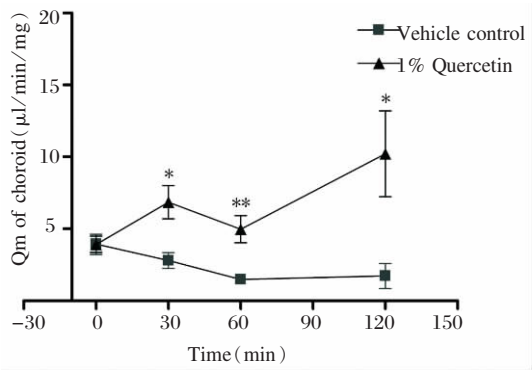

Figure 2 Effect of quercetin on choroidal blood flow of experimental ocular hypertensive rabbits.Data were expressed as means ± SEM.n=6 in each group; $^{*}P<0.05$ and $^{**}P<0.01$ quercetin group vs. the vehicle control group.

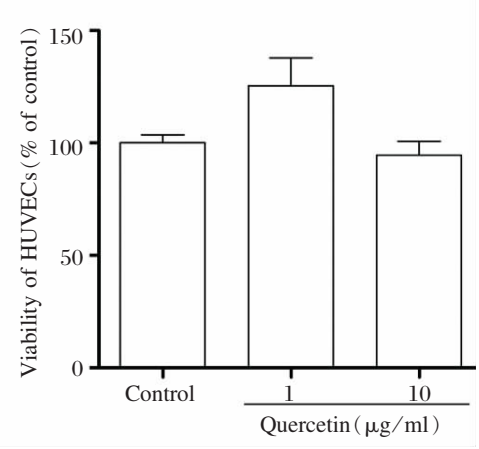

Figure 3 Effect of quercetin on proliferation of HUVECs. HUVECs were incubated with quercetin for 72 h.Data were expressed as means±SEM. n=6 in each group.

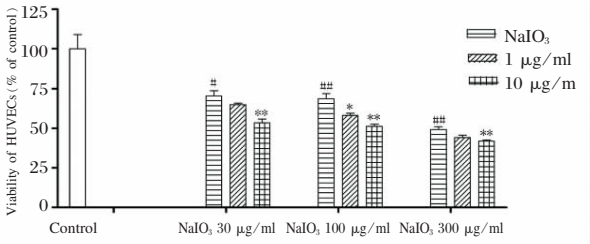

Figure 4 Effect of quercetin on $NaIO_3$-induced injury in HUVECs.HUVECs was incubated with quercetin and $NaIO_3$ for 72 h.Data were expressed as means ±SEM.n=6 in each group; $^{*}P<0.05$ and $^{**}P<0.01$ quercetin vs.$NaIO_3$ group, $^{\#}P<0.05$ and $^{\#\#}P<0.01$ vehicle control group vs.$NaIO_3$ group.

al decrease with increasing concentrations. Cell viability at 400 and 600 μM H_2O_2 decreased to 78% and 71% compared with vehicle control, respectively. One and 10 μg/ml quercetin enhanced H_2O_2-induced injury further in HUVECs (Fig.5).

Effect of quercetin on NaN_3-induced injury in HUVECs

Viability of HUVECs was reduced by 33% when exposed to 3 mM NaN_3 for 72 hours. Quercetin sig-

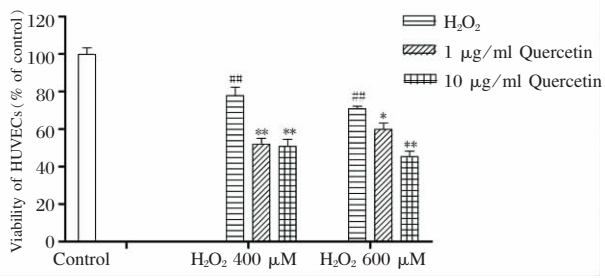

Figure 5 Effect of quercetin on H_2O_2-induced injury in HUVECs.HUVECs was incubated with quercetin and H_2O_2 for 24 h.Data were expressed as means±SEM.n=6 in each group; $^{*}P$<0.05 and $^{**}P$<0.01 quercetin vs.H_2O_2 group, $^{\#\#}P$< 0.01 vehicle control group vs. H_2O_2 group.

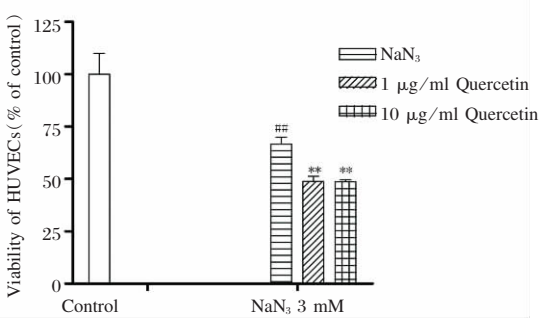

Figure 6 Effect of quercetin on NaN_3-induced injury in HUVECs.HUVECs was incubated with quercetin and NaN_3 for 72 h.Data were expressed as means±SEM.n=6 in each group; $^{**}P$<0.01 quercetin group vs.NaN_3 group, $^{\#}P$<0.01 vehicle control group vs. NaN_3 group.

nificantly enhanced NaN_3-induced injury in HUVECs at the concentration of 1 and 10 μg/ml (P<0.01, Fig.6).

Effect of quer cetin on migration of HUVECs during wound healing

Quercetin significantly inhibited the HUVECs migration. One and 10 μg/ml quercetin inhibited the migration of HUVEC by 43% and 75%, respectively (Fig.7).

Discussion

AMD affects approximately 5% of people aged 75 or older, is among the most debilitating of chronic human diseases. The prevalence and severity of the condition are predicted to increase as human life expectancy increases. It is particularly prevalent in the United States and European countries. Given the

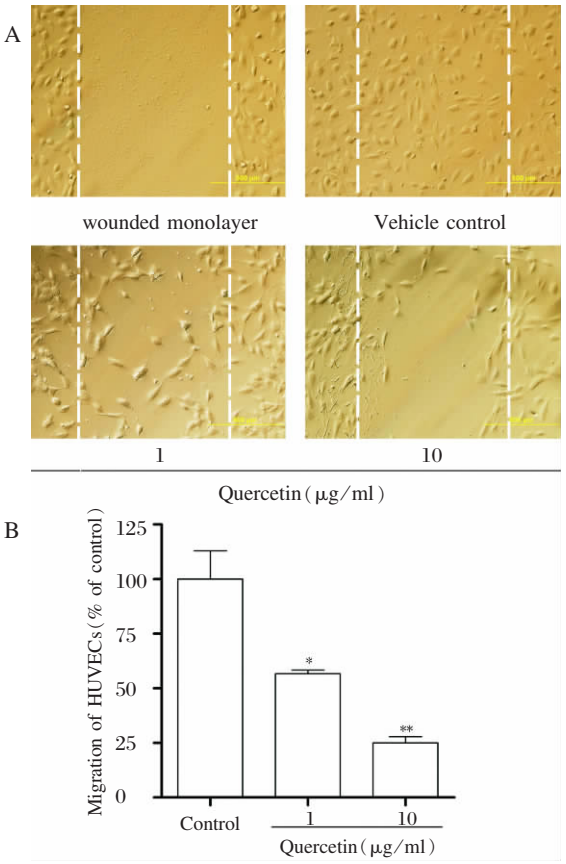

Figure 7 Effect of Quercetin on Migration of HUVECs during Wound healing. The wounded region is indicated by broken lines. Scale bar represents 500 μm (A). Migration was estimated by measurement of cell numbers within the wounded region (B). Migration of HUVECs (%)=cell numbers in quercetin group/cell numbers in vehicle control group × 100%. Data were shown as mean±SEM, n=3. $^{*}P$<0.05 and $^{**}P$<0.01 quercetin group vs. vehicle control group.

enormous impact of AMD on an aging population, much public interest and research has been focused on it.

CNV in AMD means a severe late stage of the disease and often damages central vision[13]. During the past four decades there have been some highly encouraging and much needed improvements in the management of CNV secondary to AMD. CNV secondary to AMD is now the leading cause of blindness and severe vision loss among people over the age of 50 in the Western world[15]. Its prevalence is certain to increase substantially as the population ages. In in vivo study, laser was used to induce the formation of CNV in rat. The fluorescein angiography (FA) of CNV consists of a discrete, well-demar-

cated focal area of hyperfluorescence that can be discerned in the early phases of the angiogram, sometimes before dye has completely filled the retinal vessels during choroidal filling[16-18]. The angiograms result showed that the size of CNV lesion diminished after treatment with quercetin. This indicates that quercetin significantly inhibited the formation of laser-induced CNV formation in rats.

As the human eye ages, it develops choroidal vascular atrophy, oxidative stress and hypoxia, which result in local inflammation in the sub-retinal layers. This inflammation causes over production of vascular endothelial growth factor (VEGF), a protein which among other pro-angiogenic factors stimulates the formation of new blood vessels[15]. In response to angiogenic growth factors, endothelial cells degrade the basement membrane of the parent vessel, migrate into the surrounding intercellular matrix towards angiogenic stimulus, proliferate to prolong new blood vessels, and differentiate into contiguous tubular sprouts which subsequently anastomose to form functional capillary loops[19,20]. VEGF also upregulates the production and release of matrix metalloproteinases. This degrades the extracellular matrix and leads to endothelial leakage, as well as vascular endothelial cell migration and proliferation.CNV then penetrates Bruch's membrane and the RPE, leading to vision loss[21]. As endothelial cell played an important role in the process of CNV development, HUVEC was used to investigate the effect of quercetin on CNV in vitro in this study[22,23]. Although quercetin didn't show cytotoxic effect on HUVECs, it enhanced various oxidants-induced injuries and inhibited the cells migration during wound healing on HUVECs at the concentrations of 1 and 10 μg/ml. These results suggested that quercetin could inhibit the formation of CNV in in vitro.Previous study revealed that quercetin protected RPE cells from oxidative stress at concentrations ranging between 3 and 15 μg/ml in vitro[10]. It is interesting to find out that 10 μg/ml quercetin enhanced oxidants induced injuries in HUVECs, however, it reversed oxidants induced injuries in RPE cell.This result may have profound clinical implication. Therefore, quercetin plays a double role in AMD: to act as an anti-oxidant to protect the RPE cells from damage by oxidative stress and to inhibit the formation of CNV.

It is believed that the accumulation of visual pathway metabolites and retinal fluorophores often precede damage and death of RPE cells and photoreceptors. These metabolites have been shown to be toxic to the retina, resulting in increased oxidative damage, destabilization of cell membranes and activation of inflammation including the complement and cytokine systems.Visual cycle inhibition has been hypothesized to decrease the rate of accumulation of toxic metabolites[24]. In addition, eyes with more AMD fundus features associated with risk for the development of CNV tend to show more pronounced decreases in choroidal blood flow.Large drusen, RPE hyperpigmentary changes and the presence of CNV have been shown to be associated with increased risk[25]. Based on this, it is important to find quercetin could increase choroidal blood flow in rabbit eyes.The increase of choroidal blood flow may facilitate removal of metabolic wastes and replenish nutrient to RPE and photocells, thus it may change the microenvironment, change the balance between pro-and anti-angiogenesis factors, and then change the process of angiogenesis.

In conclusion, quercetin could inhibit the formation of CNV both in vivo and in vitro and might be useful to delay the progression of AMD.Thus, quercetin could become a promising candidate for the treatment of AMD.

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