· Original article ·

Effects of flavone on the oxidation-induced injury of retinal pigment epithelium cells

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Abstract

• AIM: To investigate the effect of flavone on oxidation-induced injury in retinal pigment epithelium (RPE) cells.

• METHODS: In *in vivo* studies, $NaIO_3$-induced RPE degeneration in rat eyes were treated with 5g/L flavone eye drops 3 times a day for 1 week before and 4 weeks after $NaIO_3$ injection. At the end of 2 and 4 weeks, all rats were measured c-wave by electroretinogram (ERG). In *in vitro* studies, ARPE-19 cells were treated with hypoxia, H_2O_2, NaN_3 and t-BHP to induce cell damages. MTT assay was used to measure the viable cells.

• RESULTS: The ERG c-wave results showed that flavone reversed $NaIO_3$-induced injury at the end of 4 weeks. *In vitro* results showed flavone reversed the various oxidants-induced injuries in RPE cells.

• CONCLUSION: Flavone could prevent the RPE from oxidation-induced injury both *in vivo* and *in vitro*.

• KEYWORDS: flavone; age-related macular degeneration; retinal pigment epithelium; oxidation

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INTRODUCTION

Age-related macular degeneration (AMD) is one of the most common irreversible causes of severe loss of vision, in the elderly population[1-3]. In 2002, the World Health Organization estimated that 14 million persons worldwide are blind or severely visually impaired because of AMD. As the population in the United States and Europe is growing older, and higher expectations of better quality of life, including the ability of reading and driving, are being demanded by patients, the morbidity resulting from AMD is becoming increasingly significant[4]. Given the enormous impact of AMD on an 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[5]. RPE plays a key role in maintaining retinal function by assuming a strategic position as the metabolic gatekeeper between photoreceptors (PRs) and the choriocapillaries[6]. The aging RPE monolayer incurs annual cell losses at a rate of approximately 0.3%[7] and may as a result impose an increased metabolic demand for each cell[8]. When RPE disabled to remove the metabolic wastes, it will result in the accumulation of drusen. RPE dysfunction causes the breakdown of the blood-retinal barrier and the leakage of plasma and proteins that leads to exudative retinal detachment.

Flavonoids are a class of more than 4000 phenylbenzopyrones that occur in many edible plants, like fruits and vegetables[9]. These polyphenolic compounds display a remarkable spectrum of biochemical activities including antioxidant activities[10]. This study is to observe the effects of flavone on $NaIO_3$ induced RPE degeneration and various oxidants induced injury in human retinal pigment epithelium (ARPE-19) cells.

MATERIALS AND METHODS

Materials Eight-week-old male Brown-Norway rats 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), hydrogen peroxide (H_2O_2, 50wt% solution in water), tert-butyl hydroperoxide (t-BHP, 70wt% in water), sodium iodate ($NaIO_3$, purity ≥ 99.5%), sodium azide (NaN_3, purity ≥ 99.5%) and Dulbecco's modified Eagle's medium/Ham's F12 (DMEM/F12, 1∶1) were all purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Human retinal pigment epithelium (ARPE-19) cells and fetal bovine serum (FBS) were purchased from ATCC (Manassas, VA, USA).

Effect of Flavone on $NaIO_3$-induced RPE Degeneration in Rat Eyes The rats were randomly divided into 3 groups. Control group was instilled with vehicle (30% HP-β-CD); $NaIO_3$ group was instilled with vehicle plus 35mg/kg $NaIO_3$ injection; flavone + $NaIO_3$ group was instilled with 5g/L flavone eye drops plus 35mg/kg $NaIO_3$ injection. All eye drops were instilled 3 times a day for 1 week before and 4 weeks after $NaIO_3$ injection. At the end of 2 and 4 weeks, all rats were measured c-wave of ERG.

The rats were dark adapted for 2 hours, and then anesthetized with ketamine 35mg/kg plus xylazine 5mg/kg intramuscular

injection. Half of the initial dose was given every one hour thereafter. The rat pupils were dilated with one drop of 10g/L atropine and 25g/L phenylephrine respectively. One drop of 5g/L tetracaine was given for surface anesthesia before recording. All rats were kept warm during ERG measurement. DC-ERG recording methods by Peachey *et al* [11] were followed. Briefly, the 1mm diameter glass capillary tube with filament (Sutter Instruments, Novato, CA, USA) which was filled with Hanks balanced salt solution (Invitrogen, Carlsbad, CA, USA) was used to connect with the Ag/AgCl wire electrode with the attached connector. The capillary tube was contacted with rat's corneal surface completely. Another similar electrode placed on the surface of the other eye served as a reference lead. Responses were amplified (dc-100 Hz; gain = 1000X; DP-31, Warner Instruments, Hamden, CT, USA) and digitized at 10Hz or 1000Hz. Data were analyzed by iWORX LabScribe Data Recording Software (iWorx0CB Sciences, Dover, NH). Light stimuli were derived from an optical channel using the fiber-lite high intensity illuminator (Dolan-Jenner Industries, Inc., MA, USA) with neutral density filters (Oriel, Stratford, CT, USA) placed in the light path to adjust stimulus luminance. The stimulus luminance used in this experiment was 3.22 log cd/m^2 and stimulated for 4 minutes. Luminance calibration was made by the Minolta (Ramsey, NJ, USA) LS-110 photometer focused on the output side of the fiber optic bundle where the rat eye was located.

Cell Culture ARPE-19 cells were grown in DMEM/F12 medium supplemented with 10% FBS, 100 units/mL penicillin G, and 100μg/mL streptomycin sulfate. 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 ARPE-19 Cells MTT assay was used to measure the viability of ARPE-19 cells. 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, the final concentration of HP-β-CD in cells is less than 0.3%) and/or oxidizing agents (H_2O_2, NaN_3 and t-BHP) for 12, 24, or 72 hours (200μL/well). 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.

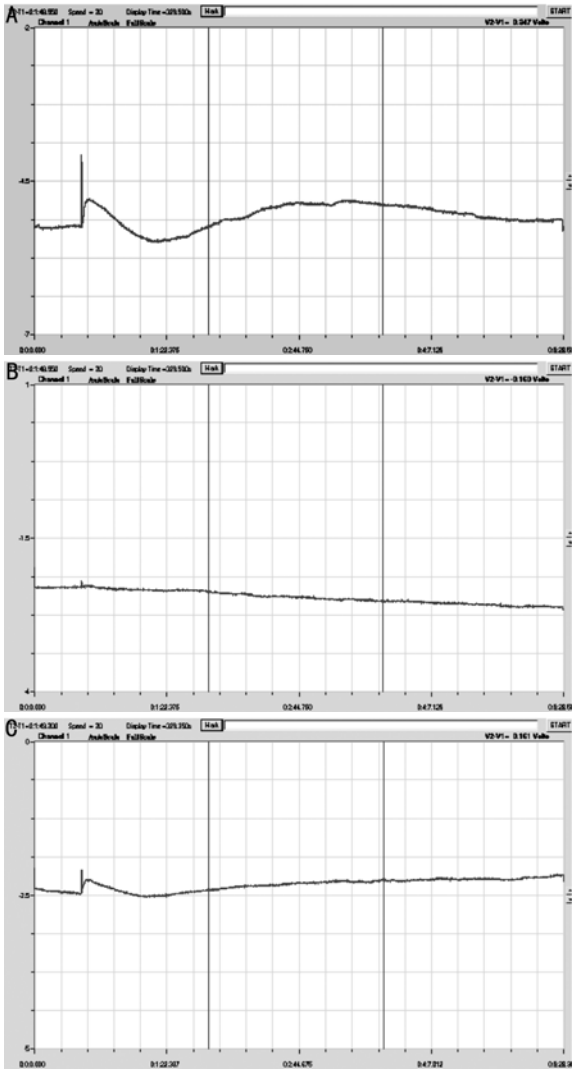

Figure 1 Representative ERG wave form at 4 weeks after $NaIO_3$ injection A: vehicle control group; B: $NaIO_3$ group; C: flavone + $NaIO_3$ group.

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 $NaIO_3$-induced RPE Degeneration in Rats Eyes Four weeks after $NaIO_3$ injection, the amplitude of ERG c-wave was (0.330 ± 0.036) Volts in the control group, (0.029 ± 0.005) Volts in the $NaIO_3$ group, and (0.070 ± 0.006) Volts in the flavone + $NaIO_3$ group. There was a significant reversal of the ERG c-wave by flavone as compared with $NaIO_3$ group ($P<0.05$, Figure 1).

Cytotoxicity of Flavone in ARPE-19 Cells The results showed that flavone did not affect cell growth of ARPE-19 up to the concentration of 10μg/mL. However, the proliferation of ARPE-19 cells was significantly inhibited at the concentrations of 30 and 100μg/mL ($P<0.01$, Figure 2).

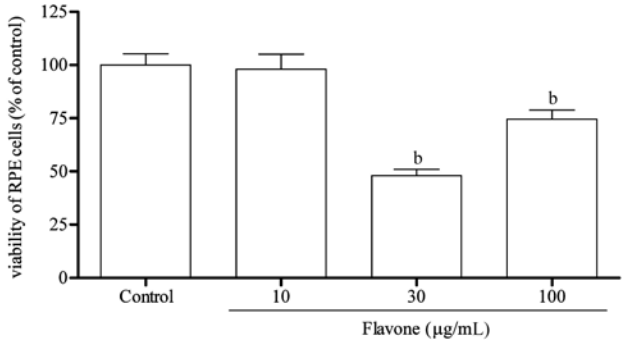

Figure 2 Effect of flavone on proliferation of ARPE-19 cells ARPE-19 cells were incubated with flavone for 72 hours. $n = 6$ in each group; $^{b}P < 0.01$ *vs*vehicle control group.

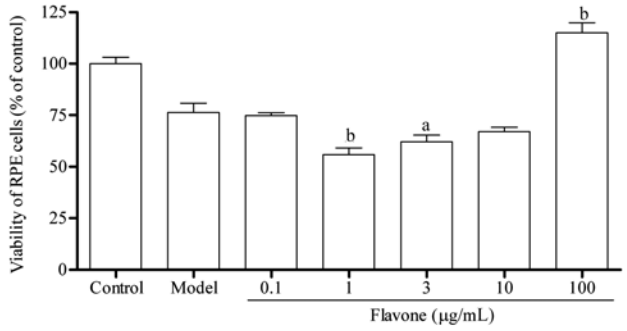

Figure 3 Effect of flavone on hypoxia-induced injury in ARPE-19 cells ARPE-19 cells were incubated with flavone for 72 hours. Control group treated with vehicle (30% HP-β-CD solution) under normal condition (50mL/L CO_2 and 950mL/L air) for 72 hours; model group treated with vehicle under hypoxic condition (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.

Effect of Flavone on Hypoxia-induced Injury in ARPE-19 Cells Flavone significantly decreased the viability of ARPE-19 cells in hypoxic condition at concentration of 1 and 3μg/mL (Figure 3). However, at the concentration of 100μg/mL, flavone significantly increased the viability of ARPE-19 cells by 38% ($P < 0.01$) in hypoxic condition.

Effect of Flavone on H_2O_2-induced Injury in ARPE-19 Cells At 0.3, 30 and 100μg/mL, flavone reversed 200μmol/L H_2O_2-induced injury by 35%, 15% and 56% respectively in ARPE-19 cells. And 30μg/mL flavone reversed 400μmol/L H_2O_2-induced injury by 25% in ARPE-19 cells (Figure 4).

Effect of Flavone on NaN_3-induced Injury in ARPE-19 Cells Flavone significantly reversed 0.3, 1 and 3mmol/L NaN_3-induced injury in ARPE-19 cells by 34%, 86% and 72% ($P < 0.01$), respectively, at the concentration of 100μg/mL (Figure 5).

Effect of Flavone on t-BHP-induced Injury in ARPE-19 Cells At the concentrations of 30 and 100μg/mL, flavone significantly increased the viability of 50 and 100μmol/L t-BHP treated ARPE-19 cells (Figure 6).

DISCUSSION

AMD is the leading cause of blindness in the elderly worldwide, affecting 30-50 million individuals. It is particularly prevalent in the United States and European countries.

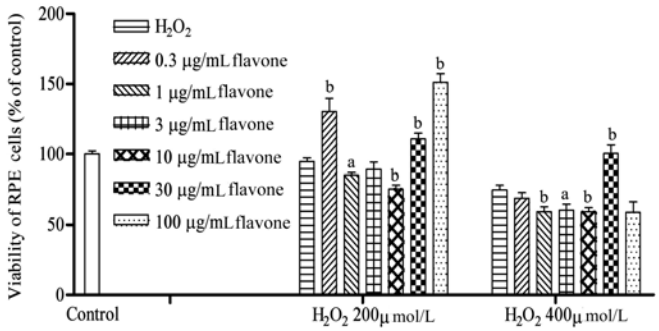

Figure 4 Effect of flavone on H_2O_2-induced injury in ARPE-19 cells ARPE-19 cells were incubated with flavone and H_2O_2 for 24 hours. $n = 6$ in each group; $^{a}P < 0.05$ and $^{b}P < 0.01$ *vs*model group.

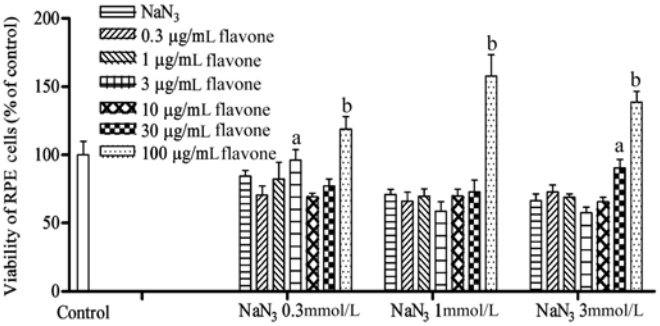

Figure 5 Effect of flavone on NaN_3-induced injury in ARPE-19 cells ARPE-19 cells were incubated with flavone and NaN_3 for 72 hours. $n = 6$ in each group; $^{a}P < 0.05$ and $^{b}P < 0.01$ *vs*model group.

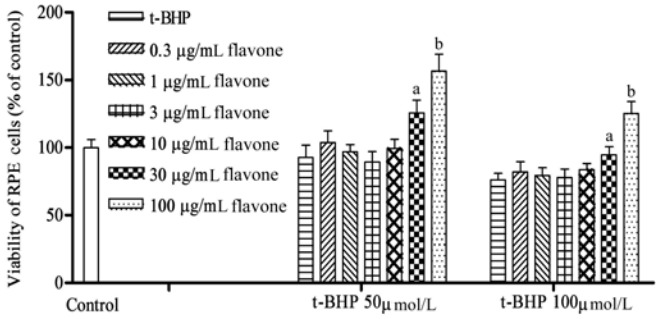

Figure 6 Effect of flavone on t-BHP-induced injury in ARPE-19 cells ARPE-19 cells were incubated with flavone and t-BHP for 12 hours. $n = 6$ in each group; $^{a}P < 0.05$ and $^{b}P < 0.01$ *vs*model group.

It is generally accepted that the impairment of RPE cell function is an early and crucial event in the molecular pathways leading to clinically relevant AMD[12,13]. RPE serves a variety of metabolic and supportive functions that are of vital importance for retinal photoreceptors, including maintenance of the blood-retinal barrier, participation in the visual cycle, and phagocytic uptake and degradation of constantly shed apical photoreceptor outer segments[14]. The direct toxic effect of $NaIO_3$ on RPE cells with secondary effects on photoreceptors and the choriocapillaries *in vivo* is well known[15]. The results of this research showed that flavone reversed $NaIO_3$-induced injury in RPE by 141% at the end of 4 weeks. This result might indicate that flavone had protective effect against $NaIO_3$ induced RPE degeneration in rat eyes and

might slow down the development of AMD.

RPE cells are particularly susceptible to oxidative stress because of high concentration of polyunsaturated fatty acids in the outer segments[16] and exposure to visible light[17-20]. Different types of oxidative stress results in different patterns of oxidative damage to proteins in RPE cells and different patterns of loss of viability[21]. In addition to this indirect evidence in support of oxidative stress as a pathogenic factor in AMD, clinical data provide direct validation of the antioxidant approach to AMD treatment. It is a general consensus that oxidative damage plays an important role in pathogenesis of dry AMD[22]. Clearly, anti-oxidants are needed for dry AMD treatment. In this study, H_2O_2, NaN_3 and t-BHP was used as oxidants to induce injuries in RPE cells. The results showed flavone reversed the various oxidants induced injuries in RPE cells. In other words, flavone could prevent the oxidative injury of RPE in AMD.

In conclusion, flavone acts as an anti-oxidant to protect the RPE from damage by oxidative stress. Thus, flavone might be a promising candidate for the treatment of AMD.

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黄酮对视网膜色素上皮细胞氧化损伤的保护作用

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摘要

目的:观察黄酮对氧化剂所诱导的视网膜色素上皮细胞(RPE)的保护作用。

方法:在体研究中,预先给予 5g/L 黄酮滴眼液(3 次/d),1wk 后舌下静脉注射 $NaIO_3$ 诱导大鼠 RPE 变性,在 2 和 4wk 末,采用视网膜电图(ERG)测量 C 波。离体研究中,采用缺氧、H_2O_2、NaN_3 和 t-BHP 诱导 RPE 细胞损伤,并用 MTT 法检测细胞的存活率。

结果:ERG 的 C 波结果表明,第 4wk 末,黄酮抑制了由 $NaIO_3$ 诱导的大鼠 RPE 变性。离体研究结果表明,黄酮对多种氧化剂所诱导的 RPE 细胞损伤具有保护作用。

结论:黄酮对氧化诱导的在体和离体视网膜色素上皮细胞均具有保护作用。

关键词:黄酮;老年黄斑变性;视网膜色素上皮;氧化作用