·Basic Research·

Antioxidant activity of naringenin on various oxidants induced damages in ARPE-19 cells and HUVEC

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Abstract

• AIM: To evaluate the antioxidant activity of naringenin in human retinal pigment epithelium (ARPE-19) cells and human umbilical vein endothelial cells (HUVEC).

• METHODS: MTT assay was used to measure the viability and proliferation of ARPE-19 cells and HUVEC.

• RESULTS: Three and 10mg/L naringenin significantly increased the proliferation of ARPE-19 cells by 10.8% and 11.4%, respectively. Ten mg/L naringenin increased hypoxia-, 0.3mmol/L NaN_3-, and 200μmol/L H_2O_2-induced damage of ARPE-19 cells by 55.2%, 69.2%, and 50.3%, respectively. One mg/L naringenin increased the viability of 50μmol/L t-BHP-, and 30mg/L $NaIO_3$-treated ARPE-19 cells by 20.2% and 30.4% , respectively. Thirty mg/L naringenin also increased the proliferation of 50μmol/L t-BHP-treated ARPE-19 cells by 32.2% , and 1mg/L naringenin increased the proliferation of 30, 100 and 300mg/L $NaIO_3$-treated ARPE-19 cells by 30.3% , 10.3% and 18.5% , respectively. The reduction of HUVEC was 23.9%, 70.4% and 77.9% in the 3, 10 and 30mg/L naringenin-treated groups, respectively. Furthermore, 1 and 3mg/L naringenin increased hypoxia-induced damage in HUVEC by 10.7% and 13.1% , and 300mg/L $NaIO_3$-induced damage in HUVEC by 41.2% and 37.7%. Three mg/L naringenin increased 200 and 400μmol/L H_2O_2-in-jured HUVEC by 20.1% and 21.5%, respectively.

• CONCLUSION: Naringenin increases the proliferation of ARPE-19 cells and inhibits the growth of HUVEC, and has potent antioxidant activity in ARPE-19 cells and HUVEC.

• KEYWORDS: naringenin; ARPE-19 cells; HUVEC; AMD

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INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of blindness in older persons in developed countries. It initially occurs in a "dry" form (pathological changes in the RPE and drusen formation), and can progress to geographic atrophy or "wet" form with choroidal neovascularization (CNV). RPE forms a monolayer between the neurosensory retina and choroid. Its main functions are to supply nutrients to the adjacent photoreceptors and to dispose of shed photoreceptor outer segments by phagocytosis [1]. One hypothesis places RPE dysfunction as the major etiology of AMD pathogenesis. When RPE is unable to remove the metabolic waste, it results in 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. The break down of Bruch's memebrane 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. Furthermore, loss of RPE may cause loss of choriocapillaries [2-4]. Therefore, RPE is a target for therapeutic approaches aimed at enhancement of photoreceptor survival in such ocular disease [5]. The mechanisms of the dysfunction or cell death of RPE may involve various factors, such as oxidative injury, degenerative changes in Bruch's membrane and damage to the choroidal vasculature [6-7]. Different types of oxidative stress results in different patterns of oxidative damage to proteins in RPE cells and different patterns of loss of viability [8].

CNV, the hallmark of wet AMD, is responsible for approximately 90% of cases of severe vision loss due to AMD. CNV is the result of angiogenesis, which include endothelial cell proliferation, migration and adhesion [9].

Naringenin is a flavonoid that is considered to have bioactivity *in vivo*[10-12] and *in vitro*[13] as an antioxidant.

The aim of this research was to study whether naringenin has the antioxidant activity on various oxidants induced injuries in RPE cells and HUVEC. If so, naringenin could become a candidate for the treatment of AMD.

MATERIALS AND METHODS

Materials Naringenin was purchased from Pfaltz & Bauer,

Inc. (Waterbury, CT, USA). Sodium iodate ($NaIO_3$, purity ≥ 99.5%), thiazolyl blue tetrazolium bromide (MTT, purity ≥ 97.5%), hydrogen peroxide (H_2O_2, 500g/L solution in water), tert-buryl hydroperoxide (t-BHP, 700g/L in water), dimethyl sulfoxide (DMSO, purity ≥99.9%), sodium azide (NaN_3, purity ≥99.5%), and Dulbecco's modified Eagle's medium/ Ham's F12 (DMEM/F12, 1 :1) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from GIBCO (Grand Island, NY, USA). HUVEC and EGM-2 Bulletkit that contains EBM-2 basal medium and EGM-2 singlequot Kit were bought from Lonza Walkersville, Inc. (Walkersville, MD, USA). ARPE-19 cells were purchased from ATCC (Manassas, VA, USA).

Cell Culture ARPE-19 cells were grown in DMEM/F12 medium supplemented with 100mL/L FBS, 1×10^5/L penicillin G, and 100mg/L streptomycin sulfate. HUVEC were grown in EBM-2 basal medium supplemented with EGM-2 singlequot Kit. Cells were incubated in a humidified incubator at 37℃ under 50mL/L CO_2 and 950mL/L air.

Naringenin on RPE Cells and HUVEC MTT assay was used to measure the viability and proliferation of ARPE-19 cells and HUVEC. 8×10^4 cells were seeded in 96-well plates (100L/well) and allowed to grow overnight. Blanks were prepared by adding 100L medium (A_{blank}). The cells were then treated with fresh medium with naringenin and/or oxidizing agents at the same time for 12, 24, or 72 hours (200L/well, $A_{compound}$). The vehicle control group ($A_{control}$) was treated with 7.6mL/L alcohol and/or PBS. 20L MTT (5g/L) was added to wells, and incubated for another 4 hours. After incubation, the medium was discarded and 150L DMSO was added to solubilize formazan produced from MTT by the viable cells. Absorbance was measured at 570nm using a microplate reader (Packard BioScience Co. Meriden, CT, USA). Cell viability was calculated according to the following formula: Viability of cells (%) = $(A_{compound\text{-}Ablank})/(A_{control\text{-}Ablank})\times100\%$.

Hypoxia Treatment Cells were allowed to attach overnight, and then exposed to naringenin and solvent 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) with N_2 and CO_2 gas sources.

Statistical Analysis Results were presented as means ± SEM. Multiple comparisons between groups were made with Student's *t*-tests (two-tail). Different values at $P<0.05$ were considered significant.

RESULTS

Cytotoxicity of Naringenin Naringenin significantly improved the formation of formazan on ARPE-19 cells in a concentration-dependent manner (Figure 1A). If ARPE-19 cells were rinsed with PBS before adding MTT, 3 and 10mg/L naringenin still significantly improved the proliferation of ARPE-19 cells by 10.8% and 11.4%, respectively (Figure 1B). However, naringenin significantly inhibited the proliferation of HUVEC. The reduction of the endothelial cells was 23.9%, 70.4% and 77.9% in the 3, 10 and 30mg/L naringenin-treated groups, respectively (Figure 1C).

Effect of Naringenin on Hypoxia –induced Damage in Cells Naringenin significantly increased the hypoxia-induced damage in RPE cells in a concentration-dependent manner. Ten mg/L naringenin increased the viabilily of ARPE-19 cell by 55.2% in hypoxic condition (Figure 2A). However, if the cells were rinsed with PBS before adding MTT solution, the result showed that naringenin had no effect on the proliferation of ARPE-19 cells in hypoxic condition (data not shown). Naringenin increased hypoxia-induced damage by 10.7% and 13.1% at the concentrations of 1 and 3mg/L naringenin-treated groups as compared with vehicle control group in HUVEC (Figure 2B).

Effect of Naringenin on NaN_3–induced Injury in Cells Naringenin significantly increased 0.3, 1 and 3mmol/L NaN_3-induced injury in ARPE-19 cells concentration-dependently. Ten mg/L naringenin increased the viability of 0.3mmol/L NaN_3-injured ARPE-19 cells by 69.2%(Figure 3). However, if the cells were rinsed with PBS before adding MTT solution, the result showed that naringenin had no effect on the proliferation of NaN_3-injured ARPE-19 cells (data not shown). Naringenin had no effect on NaN_3-induced injury in HUVEC either (data not presented).

Effect of Naringenin on t–BHP–induced Injury in Cells Naringenin significantly improved the viability of 50 and 100mol/L t-BHP-treated ARPE-19 cells concentration-dependently (Figure 4A). At the concentration of 1mg/L, naringenin increased the 50mol/L t-BHP-induced damage by 20.2%. If the cells were rinsed with PBS before adding MTT solution, the result showed that naringenin still increased the proliferation of t-BHP-treated ARPE-19 cells. At higher concen-tration of 30mg/L, naringenin increased the proliferation of ARPE-19 cells by 32.2% (Figure 4B). Naringenin had no effect on t-BHP-induced injury in HUVEC(data not shown).

Effect of Naringenin on H_2O_2–induced Injury in Cells Naringenin significantly increased the H_2O_2-induced damage

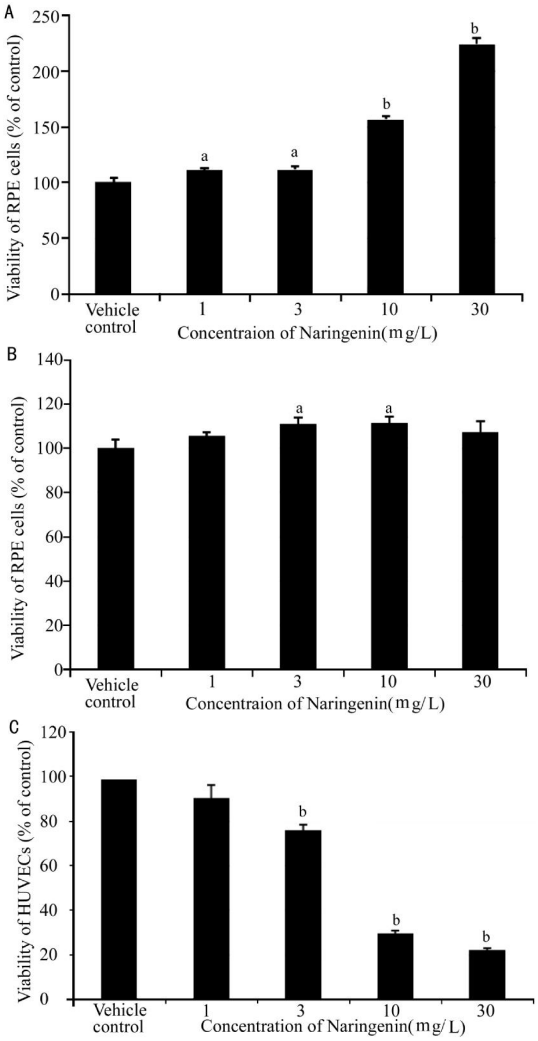


Figure 1 Effect of naringenin on proliferation of cells incubated for 72h ($^{a}P<0.05$, $^{b}P<0.01$ *vs* vehicle control, $\bar{x}\pm s$, $n=6$)

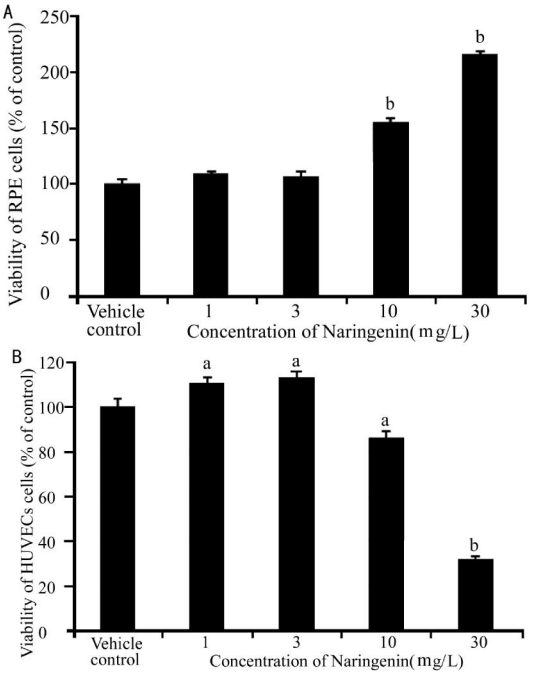


Figure 2 Effect of naringenin on hypoxia-induced injury in cells for 72h ($^{a}P<0.05$, $^{b}P<0.01$ *vs* vehicle control, $\bar{x}\pm s$, $n=6$)

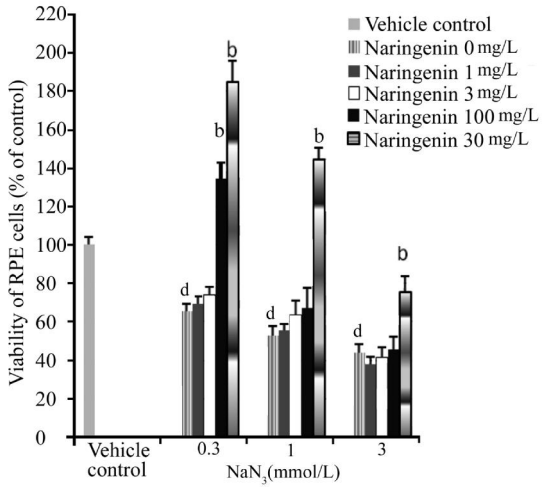


Figure 3 Effect of naringenin on NaN_3-induced injury in ARPE-19 cells for 72h ($^{a}P<0.05$, $^{b}P<0.01$ *vs* model control, $^{c}P<0.05$, $^{d}P<0.01$ *vs* vehicle control, $\bar{x}\pm s$, $n=6$)

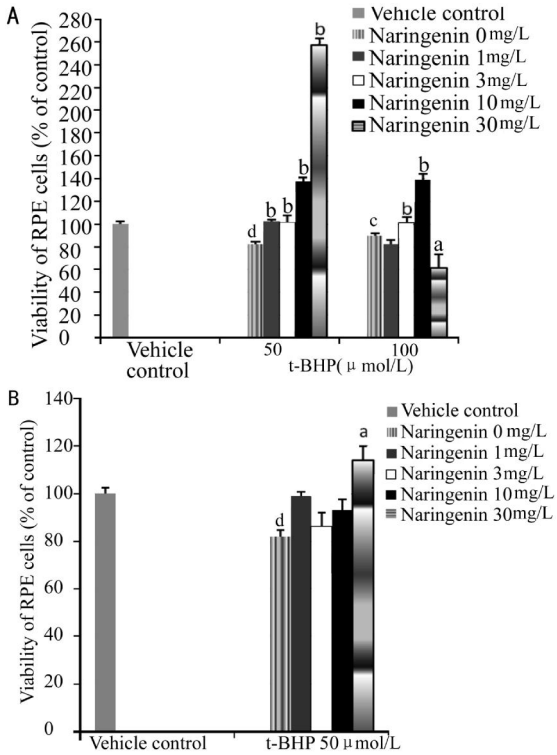


Figure 4 Effect of naringenin on t-BHP-induced injury in ARPE-19 cells for 12h A: not rinsed with PBS before adding MTT; B: washed with PBS before adding MTT ($^{a}P<0.05$, $^{b}P<0.01$ *vs* model control, $^{c}P<0.05$, $^{d}P<0.01$ *vs* vehicle control, $\bar{x}\pm s$, $n=6$)

in ARPE-19 cells in a concentration-dependent manner. Ten mg/L naringenin increased the viability of H_2O_2-injured ARPE-19 cells by 50.3% (Figure 5A). However, if the cells were rinsed with PBS before adding MTT solution, the result showed that naringenin had no effect on the proliferation of H_2O_2-treated ARPE-19 cells (data not shown). Naringenin also increased H_2O_2-induced injury in HUVEC. At the concentration of 3mg/L, naringenin increased the viability

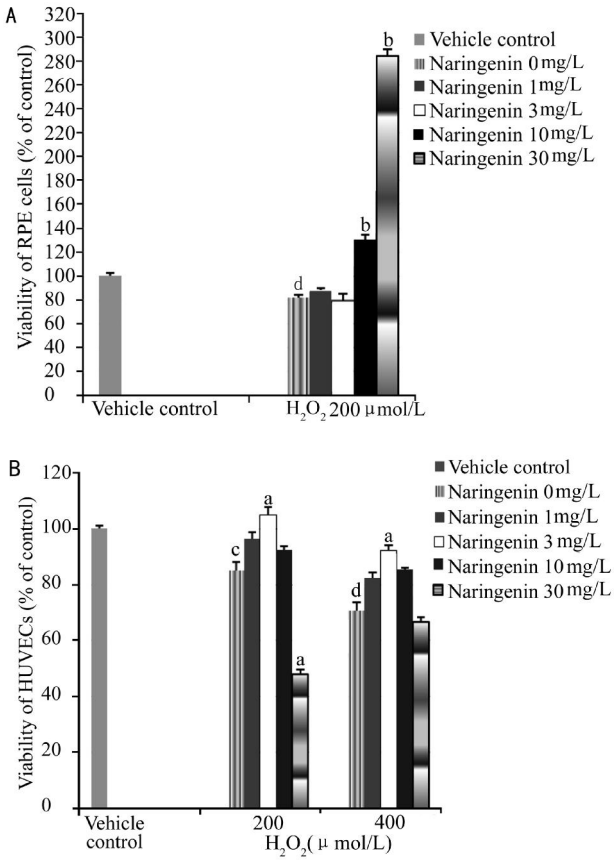
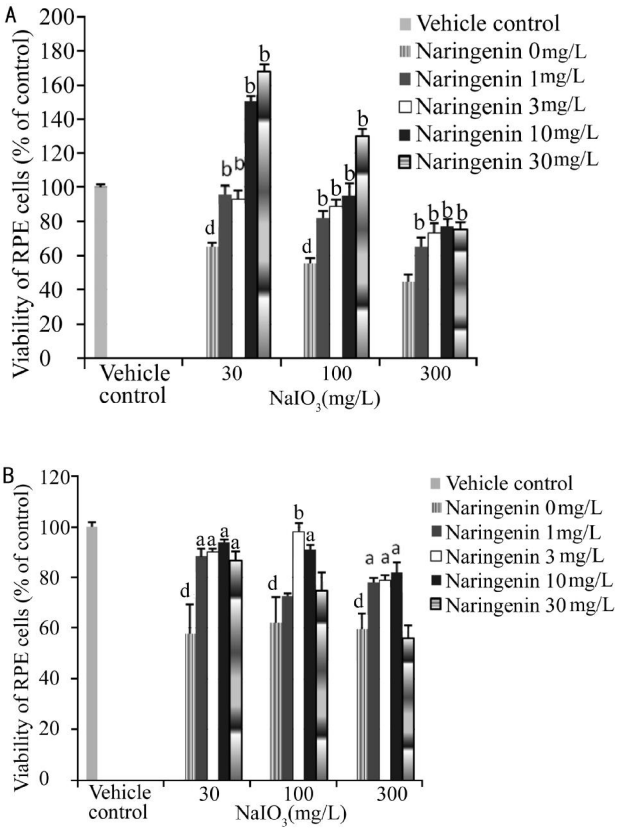


Figure 5 Effect of naringenin on H_2O_2–induced injury in cells for 24h ($^aP<0.05$, $^bP<0.01$ *vs* model control, $^cP<0.05$, $^dP<0.01$ *vs* vehicle control, $\bar{x}\pm s$, n=6)



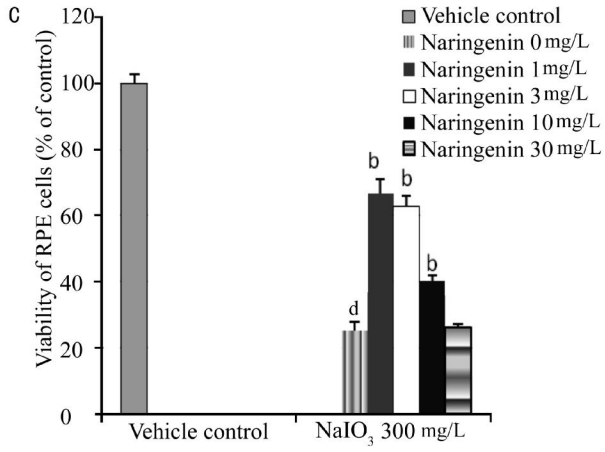


Figure 6 Effect of naringenin on $NaIO_3$–induced injury in cells for 72h ($^aP<0.05$, $^bP<0.01$ *vs* model control, $^cP<0.05$, $^dP<0.01$ *vs* vehicle control, $\bar{x}\pm s$, n=6)

of 400mol/L H_2O_2-injured HUVEC by 20.1% and 21.5% as compared with 200 and 400mol/L H_2O_2-treated groups, respectively (Figure 5B).

Effect of Naringenin on $NaIO_3$–induced Injury in Cells

As shown in Figure 6A, naringenin increased the viability of 30, 100 and 300mg/L $NaIO_3$-induced injuries in ARPE-19 cells concentration-dependently. One mg/L naringenin increased the viability of 30, 100 and 300mg/L $NaIO_3$-treated ARPE-19 cells by 30.4%, 26.6%, and 20.7%, respectively. Naringenin also increased $NaIO_3$-induced reduction of ARPE-19 cells in a concentration-dependent manner. One mg/L naringenin increased the proliferation of 30, 100 and 300mg/L $NaIO_3$-treated ARPE-19 cells by 30.3% , 10.3% and 18.5% , respectively (Figure 6B). Naringenin only increased 300mg/L $NaIO_3$-induced injuries in HUVEC. One mg/L naringenin increased the viablility of HUVEC that injured by 300mg/L $NaIO_3$ by 41.2% (Figure 6C).

DISCUSSION

The present data demonstrated that naringenin differed in its cytotoxicity and antioxidative capacity toward different oxidants and cell lines. Naringenin increased the viability of ARPE-19 cells alone and hypoxia-, NaN_3-, t-BHP-, H_2O_2-, and $NaIO_3$-treated ARPE-19 cells in a concentration-dependent manner. The results indicate that naringenin could prevent the oxidative damage of RPE in AMD patients. The mechanism of significant increase of formazan in ARPE-19 cells requires further study.Naringenin improved the proliferation of ARPE-19 cells, and t-BHP- and $NaIO_3$-treated ARPE-19 cells. These results indicate that naringenin could reverse the reduction of RPE in AMD patients.

Endothelial cells played an important role in the process of CNV development[14]. 3, 10, and 30mg/L naringenin inhibited

the proliferation of HUVEC, which suggested that naringenin might inhibit the development of CNV *in vivo*. These results indicate that naringenin might be useful to prevent wet AMD. It is interesting to find out that 3mg/L naringenin increased hypoxia-, H_2O_2-, and $NaIO_3$-induced damage in HUVEC. These results indicate that naringenin alone could inhibit the formation of CNV, and could also protect the endothelial cells of blood vessel from oxidative stress if the endothelial cells were attacked by some oxidants. The result of antioxidant effect of naringenin on H_2O_2-induced damage in HUVEC was consistent with previous publication [15] which reported that naringenin was effective as an inhibitor of H_2O_2-induced oxidative stress in HUVEC and could protect cell viability with ≥85% viable cells compared with the control without apparent nuclear condensation or DNA fragmentation.

In conclusion, the present study showed that naringenin could reverse hypoxia-, NaN_3-, t-BHP-, H_2O_2-, and $NaIO_3$-induced injury in ARPE-19 cells, and improve the proliferation of ARPE-19 cells. Furthermore, naringenin could inhibit the proliferation of HUVEC and increased hypoxia-, H_2O_2-, and $NaIO_3$-induced damage at the same time. These results indicate that naringenin is not only useful to treat dry AMD but also the wet AMD. Thus, naringenin might become a promising candidate for treating AMD in the future.

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