Research Article

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The protective effects of berry-derived anthocyanins against visible light-induced damage in human retinal pigment epithelial cells

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Abstract

BACKGROUND: Studies have shown that anthocyanins (ACNs) in berries contribute to eye health. However, information on the relationship between the chemical structures and visual functions of ACNs is scarce. This study investigated the protection effects of ACNs with different structures against visible light-induced damage in human retinal pigment epithelial (RPE) cells.

RESULTS: Four ACNs with different aglycones, namely, pelargonidin-3-glucoside (Pg-3-glu), cyanidin-3-glucoside (Cy-3-glu), delphinidin-3-glucoside, and malvidin-3-glucoside (Mv-3-glu), were isolated from three berries (blueberry, blackberry and strawberry). Of these ACNs, Cy-3-glu exhibited the highest reactive oxygen species inhibitory capacity in RPE cells, with 40 μg mL^{-1} Cy-3-glu showing a ROS clearance of 57.5% ± 4.2%. The expression of vascular endothelial growth factor levels were significantly ($P < 0.05$) down-regulated by Cy-3-glu and Mv-3-glu in a visible light-induced damage RPE cell model. Cy-3-glu and Pg-3-glu treatments significantly ($P < 0.05$) inhibited the increase in β-galactosidase during the RPE cell ageing caused by visible light exposure.

CONCLUSION: Our findings suggest that the biological properties of different ACNs significantly vary. Cy-3-glu, which contains an *ortho* hydroxyl group in its B ring, possibly exerts multiple protective effects (antioxidant, anti-angiogenic and anti-ageing) in RPE cells. Therefore, Cy-3-glu may prove useful as a prophylactic health food for the prevention of retinal diseases.

Keywords: cyanidin-3-glucoside; oxidative stress; vascular endothelial growth factor; β-galactosidase

INTRODUCTION

The retinal pigment epithelium (RPE), which is a simple layer of cuboidal cells strategically situated behind the photoreceptor cells, is a vital factor in the visual function of the retina as well as in the phagocytosis of shed photoreceptor outer segments, absorption of scattered light, and maintenance of the structural integrity of the retina.[1,2] Recently, the wide use of computers, mobile phones, and televisions has increased the number of people suffering from eye symptoms such as red eyes, eyestrain, blurred vision, and occasional double vision.[3] The adult retina is protected from ultraviolet (UV) light by the lens, which absorbs most of the UV light, but can be damaged by visible light.[2]

The retina is particularly susceptible to light damage for its surroundings include high consumption of oxygen, large doses of visible light exposure, high proportion of polyunsaturated fatty acids, and numerous chromophores.[4] The RPE cells are vulnerable to the generation of reactive oxygen species (ROS), which are believed to be important causative factors underlying damage of the retina by light.[5] An imbalance between excess free radical production and the antioxidant defence results in cellular damage leading to lipid peroxidation in the retina. Evidence gathered from clinical studies showed that long-term stress on the RPE can result in age-related macular degeneration (AMD), which is a major cause of blindness in elderly people.[6]

The beneficial properties of berries, particularly their high anthocyanin (ACN) contents, have attracted the interest of consumers. The World Health Organization emphasises the importance of edible berries as potential sources of natural ACN antioxidants as well as their beneficial effects on vision.[7,8] In the United States, the daily intake of ACNs per person is 180–215 mg.[9] Berry ACNs are beneficial for vision in that they increase the circulation within the retinal capillaries, protect corneal limbal epithelial cells, improve night vision, as well as decrease macular degeneration and diabetic

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retinopathy.[9,10] Thus, ACNs may be used as a safe and inexpensive adjunct supplement to protect against visible light-induced damage in RPE cells.

Previous studies found that various ACN extracts and their major anthocyanidins protect against light-induced photoreceptor cells damage.[11–13] Visible light can cause retinal cellular damage to the RPE and photoreceptors.[14,15] Furthermore, although many papers have been published on the retina protection of anthocyanidins,[11,13,16] only a few systematic studies on the glycosylated forms of anthocyanidins have been conducted. It is of great importance to study the activities of the glycosylated compounds as they are the native forms occurring in plants and may be absorbed as intact glycosides into the circulation.[17,18] The most widespread anthocyanidin glycosides is 3-glucoside in plants,[19] so we study the 3-glucoside form of anthocyanidins. Our previous studies showed that blueberry ACN mixtures are effective in protecting RPE cells from light-induced damage.[20] The findings indicated that berry ACNs can prevent AMD and other retinal diseases related to RPE cells. However, information on the protection effects of ACN with different structures against visible light-induced damage in RPE cells remained scarce. Anthocyanidins vary in the different hydroxyl or methoxyl substitutions in their basic flavylium (2-phenylbenzopyrilium) structure. In this study, four types of ACNs with different aglycones, namely, pelargonidin-3-glucoside (Pg-3-glu), cyanidin-3-glucoside (Cy-3-glu), delphinidin-3-glucoside (Dp-3-glu), and malvidin-3-glucoside (Mv-3-glu), were investigated. The effects of these ACNs against visible light-induced RPE cell damage were then compared to determine the structure–activity relationship of ACNs during retinal protection.

MATERIALS AND METHODS

Plant materials

The four ACNs were extracted from blueberry, blackberry and strawberry. Fresh blueberries (*Vaccinium* spp.) were supplied by the Science and Technology Bureau of the Greater Hinggan Mountains District. Fresh blackberries (*Rubus* sp.) were supplied by the Merrycare Green Food Company. Fresh strawberries (*Fragaria ananassa*) were purchased from local farmers. The berries were stored at −20°C prior to use.

Chemicals and reagents

Amberlite XAD-7 was purchased from Sigma (Sydney, Australia). Sephadex LH-20 was purchased from Amersham Biosciences AB (Uppsala, Sweden). Acetonitrile was of chromatographic grade and purchased from Merck (Darmstadt, Germany). Deionised water was produced using a Milli-Q water-purification system (Millipore, Billerica, MA, USA). Trifluoroacetic acid (TFA) was purchased from Sigma (St. Louis, MO, USA). Analytical-reagent grade solvents were used during extraction and chromatography. 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA), dimethyl sulfoxide (DMSO), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma–Aldrich, Inc. (St. Louis, MO, USA). Fetal bovine serum (FBS), penicillin, streptomycin, and Hank's balanced salt solution (HBSS) were obtained from Gibco Life Technologies (Grand Island, NY, USA). Dulbecco's modified Eagle's/Ham's F12 media were purchased from Invitrogen (Carlsbad, CA, USA). The vascular endothelial growth factor (VEGF) ELISA kit was purchased from Shanghai ExCell Biology, Inc. (Shanghai, China). The senescence-associated β-galactosidase staining kit was obtained from Beyotime Institute of Biotechnology (Jiangsu, China).

Extraction and purification of anthocyanins

ACNs were extracted from the three berries as previously described, with some modifications.[21] Fresh berries (50 g) and 100 mL of a 70:29:1 (v/v) ethanol/water/HCl mixture were mixed and then homogenised using a homogeniser (XHF-D; Ningbo Science & Biotechnology Co., Ningbo, Zhejiang, China) set at 5000 rpm for 1 min. The homogenised samples were further centrifuged for 10 min at 4000 × *g*. The supernate was filtered through a moderate-speed 102 qualitative filter paper (Hangzhou Special Paper Industry Company Ltd, Hangzhou, Zhejiang, China). The extraction procedure was repeated on the residue, and the filtrate was collected. The two filtrates of each sample were combined and subsequently concentrated by rotary evaporation at 40°C under vacuum. The resulting concentrated solution was the crude ACN berry extract. All samples were filtered through a 0.45 µm membrane filter. The crude ACN extract was applied into an Amberlite XAD-7 resin column. After 1 h, the XAD-7 column was washed with 600 mL of a 1% (v/v) aqueous formic acid solution to remove non-polyphenolic compounds. Polyphenolics were further eluted with approximately 600 mL of absolute methanol containing 1% (v/v) formic acid. The eluent was collected, concentrated at 40°C by a rotary evaporator, and lyophilised using a freeze dryer (Four-Ring Science Instrument Plant, Beijing Co., Ltd, Beijing, China). After 48 h, a dark-red powder was obtained. The lyophilised sample (polyphenol mixture) was resolubilised in a phosphate buffer (pH 2.0) and loaded onto a Sephadex LH-20 column. The ACNs were eluted with a 1% (v/v) aqueous formic acid solution, followed by a 19:80:1 (v/v) methanol/water/formic acid mixture, 49:50:1 (v/v) methanol/water/formic acid mixture, 70:29:1 (v/v) methanol/water/formic acid mixture, and finally by 1% (v/v) formic acid in methanol. The ACN-rich fractions were evaporated and dried in a vacuum at 40°C using a DZF-6021 vacuum-drying oven (Hangzhou Lihui Environmental Testing Equipment Co. Ltd., Hangzhou, China). The purified ACNs were kept at −20°C until use.

Liquid chromatography–electrospray ionisation tandem mass spectrometry analysis of purified anthocyanins

Liquid chromatography–electrospray ionisation tandem mass spectrometry (LC-ESI-MS/MS) analysis was performed using an Agilent 1260 series HPLC combined with an Agilent 6460 Series Triple Quad LC/MS equipped with a Jet Stream ESI source (Agilent Technologies, Santa Clara, CA, USA). The MS was operated in the positive-ion mode. Nitrogen was used as a collision gas. The analytical column was a 150 mm × 2.1 mm i.d. Agilent Zorbax SB-C18 column (Agilent, Palo Alto, CA, USA), which was maintained at 25°C. Prior to analysis, all samples were filtered through a 0.45 µm membrane filter. The injection volume was 20 µL. The elution solvents, namely (A) methyl cyanides with 2% formic acid and (B) H_2O with 2% formic acid, were applied as follows: isocratic 3% A for 3 min, from 3% to 15% A for 12 min, from 15% to 25% A for 13 min, from 25% to 30% A for 7 min, from 30% to 3% A for 3 min, and isocratic 3% A for 7 min. The flow rate was 0.4 mL min^{-1}, and detection was performed at 520 nm. The detailed MS conditions were as follows: collision energy 8 eV, gas temperature 300°C, gas flow 5 L min^{-1}, nebuliser pressure 45 psi, sheath gas temperature 250°C, sheath gas flow 11 L min^{-1}, capillary voltage 3.5 kV, and nozzle voltage 500 V.

Determination of the purity of the purified anthocyanins

To determine the purity of the purified ACNs, the samples were analysed by HPLC at 280 and 520 nm. The % area of the isolated ACNs in the 520 nm chromatogram was used to indicate purity.[22]

Cell culture

A human RPE cell line, ARPE-19 (CRL-2302; American Type Culture Collection, Manassas, VA, USA), was used. Cells were grown in Dulbecco's modified Eagle's/Ham's F12 media supplemented with 10% FBS and containing a 1% antibiotic mixture of penicillin (100 U mL^{-1}) and streptomycin (100 mg mL^{-1}). Incubation was performed at 37°C in a humidified 5% CO_2 atmosphere.

Anthocyanin cytotoxicity

Cell viability was measured by the MTT assay, as previously described.[20] The RPE cells were seeded in a 96-well plate at a concentration of 5×10^5 cells mL^{-1} and then allowed to attach after 24 h. The medium was replaced with a serum-free F12 medium containing 20, 40, 60 and 80 μg mL^{-1} of the four ACNs. After 24 h, 200 μL of the 0.5 mg mL^{-1} MTT serum-free F12 medium was added into each well. The plate was then incubated for 4 h. After removal of the MTT solution, 150 mL of DMSO was added. The absorbance was recorded at 570 nm using a microplate spectrophotometer system (Spectra Max M2^e; Molecular Devices, Sunnyvale, CA, USA). The experiment was conducted in triplicate. The results are expressed as the percentage of viable cells with respect to the untreated control cells.

Light-induced damage model

Light exposure

The RPE cells were exposed to visible-light radiation at 420 nm to 800 nm using an integrated light-emitting diode lamp system designed by the authors. The MTT assay was used to assess the effect of light time and light intensity on the loss of RPE cell viability. The experimental instrumentation was reported in a previous study.[20] RPE cells in an actively growing state were planted into plates with serum-containing F12 medium at a concentration of 5×10^5 cells mL^{-1}. The media for the vehicle control (no visible light exposure, no ACN treatment) and the model group (visible light exposure, no ACN treatment) were replaced with serum-free F12 media when 85% of the cells adhered to the wall. For the ACN treatment groups (visible light exposure, ACN treatment), serum-free F12 media containing 10, 20, or 40 μg mL^{-1} of the respective ACNs were added. The illumination intensity and time were confirmed through cell viability based on the MTT assay. The RPE cells were then exposed to 2500 lx visible light for 12 h. A black adhesive tape was placed on the plate covers of the vehicle control group to prevent light exposure.

Measurement of cellular reactive oxygen species

The ROS level in the RPE cells was monitored using the fluorescent dye DCFH-DA. RPE cells were cultured in 200 μL of the growth medium in a transparent black 96-well plate at a density of 5×10^5 cells mL^{-1}. After the cells had been incubated for 24 h, the media in the treatment groups were replaced with serum-free F12 media containing 10, 20 or 40 μg mL^{-1} of the respective ACNs. For the vehicle control and model groups, no ACNs were present in the medium. The RPE cells in the treatment groups and model group were then exposed to 2500 lx visible light for 12 h. Afterwards, the medium was replaced with serum-free F12 medium containing 25 mmol L^{-1} DCFH-DA. The cells were incubated sequentially for another 1 h at 37°C. After the supernate had been removed, the cells were carefully washed twice with HBSS. The fluorescence of the cells from each well was recorded using the microplate spectrophotometer system (Spectra Max M2^e; Molecular Devices) at 530 nm emission and 485 nm excitation.

Detection of vascular endothelial growth factor

After visible light irradiation, the supernate of the RPE culture was collected and then centrifuged at 1000 × g for 5 min. The VEGF level in the medium was determined using a VEGF ELISA kit. Absorbance at 450 nm was measured using the microplate spectrophotometer system (Spectra Max M2^e; Molecular Devices).

Senescence-associated β-galactosidase activity

Senescence was assayed using the senescence-associated β-galactosidase staining kit. The fourth passage of the RPE cells were washed twice with PBS and then fixed with 2% formaldehyde and 0.2% glutaraldehyde in PBS at room temperature for 4 min. Afterwards, the cells were washed twice with PBS and incubated in the dark for 8 h at 37°C with fresh β-galactosidase staining solution (1 mg mL^{-1} 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside, 40 mmol L^{-1} citric acid/sodium phosphate, 5 mmol L^{-1} potassium ferrocyanide, 5 mmol L^{-1} potassium ferricyanide, 150 mmol L^{-1} NaCl, and 2 mmol L^{-1} $MgCl_2$ in PBS; pH 6.0). Blue-stained cells were considered as senescent cells and photographed by a light microscope. The active β-galactosidase cell percentage was calculated as the number of blue-stained cells divided by the number of total cells. Five visual fields containing at least 150 cells of each passage group were chosen for analysis. The results were recorded as the means and standard deviations of the five counts.

Statistical analysis

Data were analysed by one-way ANOVA using Origin 8.0 (OriginLab, Northampton, MA, USA). Results are expressed as the means and standard deviations of each measurement. The significance of the difference ($^*P < 0.05$) between two groups was assessed using one-way ANOVA followed by Tukey's test.

RESULTS AND DISCUSSION

Extraction and purification of anthocyanins

The Amberlite XAD-7 resin successfully separated non-ACN compounds (salts, carbohydrates, and other soluble compounds) from the ACNs.[23] The ACNs were further purified by a Sephadex LH-20 column based on their different molecular sizes. Two major ACNs (Mv-3-glu and Dp-3-glu) were isolated from blueberry. Two major ACNs (Cy-3-glu and Pg-3-glu) were extracted from blackberry and strawberry, respectively.

Determination of anthocyanins

HPLC-ESI-MS in the positive detection mode was used to identify individual ACNs after extraction and purification from the berries. MS/MS resulted in the cleavage of glycosidic bonds between the flavilium ring and sugar of ACN.[24] The MS data were compared with the published data to identify the individual ACNs. The identified ACNs are presented in Table 1. All these results are consistent with those of previous studies.[25]

Determination of anthocyanin purity

The purity of the isolated ACNs obtained from the Sephadex LH-20 column was determined by monitoring the HPLC chromatogram at 280 and 520 nm. The % areas of the isolated ACNs were used as purity indicators.[22] The results indicate that high-purity ACN were successfully isolated. The peak areas of the four isolated ACNs accounted for more than 90% of the total peak area at 520 nm. The peak areas are as follows: 95.3% for Mv-3-glu (blueberry), 90.1% for Dp-3-glu (blueberry), 96.7% for Cy-3-glu (blackberry), and 91.7% for Pg-3-glu (strawberry) (Table 1). Therefore, we successfully isolated, purified and identified the four common ACNs from blueberry, blackberry and strawberry. The individual protective effects of these ACNs against visible light-induced damage in RPE cells were then evaluated.

Cytotoxicity of the four anthocyanins to retinal pigment epithelial cells

Different concentrations (20, 40, 60 and 80 μg mL^{-1}) of the four ACNs were incubated with the RPE cells to determine the cytotoxicity of these compounds (Fig. 1). The four ACNs exhibited dose–response cytotoxicities in the cells. The results show that 20 μg mL^{-1} and 40 μg mL^{-1} of all four ACNs did not significantly reduce ($P > 0.05$) the cell viability over a 24 h period. However, 60 and 80 μg mL^{-1} Dp-3-glu inhibited cell growth, whereas 80 μg mL^{-1} Pg-3-glu exhibited cytotoxicity. Therefore, concentrations of these ACNs below 40 μg mL^{-1} were used in the subsequent experiments.

Effect of visible light on the viability of retinal pigment epithelial cells

Light exposure results in RPE cell damage that is related to light intensity and illumination time. Thus, we used the MTT assay to assess the effect of visible light on the loss of RPE cell viability (Fig. 2). The results show that visible light reduced the RPE cell viability in a dose-dependent manner. At low light intensity (500 lx for 12 h), the cell viability exceeded 90%. The cell viability decreased to 87.4% ± 1.4% after exposure to 2500 lx for 12 h. The RPE cells were severely damaged after exposure to high light intensity (4000 lx for 12 h), and the cell viability decreased to below 80%. Thus, a light intensity of approximately 2500 lx for 12 h was used to irradiate the RPE cells *in vitro* in order to establish the visible light-induced damaged model, which was used to evaluate the protective effects of individual ACNs on RPE cells.

Protective effects of anthocyanins against light-induced damage in reintal pigment epithelial cells

Effects of anthocyanins on intracellular generation of reactive oxygen species

The effects of ACNs on ROS generation in the RPE cells under visible-light irradiation were investigated. After exposing the RPE cells to light at 2500 lx for 12h, the ROS level in the model group significantly increased ($P < 0.05$). At 10, 20 and 40 μg mL^{-1} concentrations, all four ACNs reduced the visible light-induced intracellular ROS levels. Cy-3-glu caused the highest reduction in intracellular ROS level among the four ACNs. The DCFH-DA fluorescence intensity ratios for Cy-3-glu were 73.4% ± 3.8%, 44.4% ± 1.9%, and 42.5% ± 4.2% at concentrations of 10, 20 and 40 μg mL^{-1}, respectively (Fig. 3A). Dp-3-glu inhibited intracellular ROS in a dose-dependent manner, and its DCFH-DA fluorescence intensity ratios were 82.4% ± 3.0%, 73.4% ± 0.2%, and 61.6% ± 4.8% at concentrations of 10, 20 and 40 μg mL^{-1}, respectively (Fig. 3B). Mv-3-glu, and Pg-3-glu also exerted dose-dependent effects on ROS inhibition (Fig. 3C and D). The ROS levels in the presence of 10 μg mL^{-1} Pg-3-glu and Mv-3-glu were higher than those measured in the presence of 10 μg mL^{-1} Cy-3-glu and Dp-3-glu.

The pathogenesis of retinal degeneration is closely related to increased oxidative stress as a result of a reduction in protective mechanisms or increases in the ROS levels.[26] Under intense illumination from focal light and high oxygen tension in the macular area, ROS generated from phagocytosis is particularly significant in the RPE and leads to lipid peroxidation, protein oxidation, and DNA damage.[27] Moreover, *in vitro* studies showed that oxidative stress contributes to RPE cell apoptosis during early-phase AMD.[28] Cai *et al.* proposed a possible mechanism, in which oxidative stress triggers mitochondrial permeability transition to induce

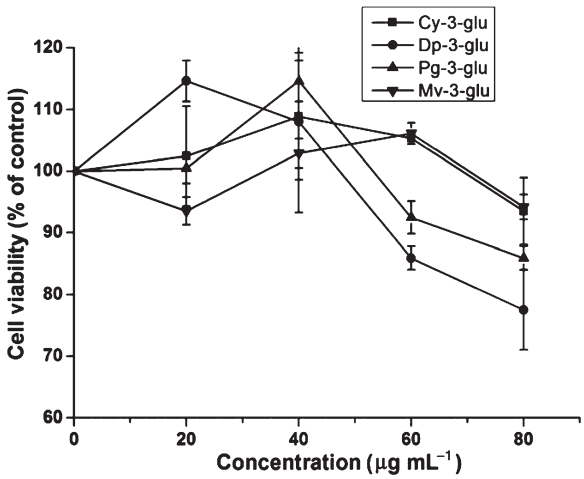

Figure 1. Cytotoxic effect of four anthocyanins (ACNs) on retinal pigment epithelium (RPE) cells. After RPE cells (5×10^5 cells mL^{-1}) were incubated with Dulbecco's modified Eagle's/Ham's F12 medium containing 10% serum for 24 h, the cells were exposed to serum-free F12 medium containing 20, 40, 60 and 80 μg mL^{-1} of the four ACNs for 24 h. Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and is expressed as a percentage of control cells with ACNs-free F12 medium. Values are means, with standard deviations represented by vertical bars ($n = 3$).

Table 1. High-performance liquid chromatography–electrospray ionisation tandem mass spectrometry results for the anthocyanins from different berries

Berry	Retention time (min)	$[M]^+$ (*m/z*)	MS/MS (m/z)	Anthocyanin	Purity (%)
Blueberry	30.0	493	331	Malvidin-3-glucoside (Mv-3-glu)	95.3
Blueberry	24.6	465	303	Delphinidin-3-glucoside (Dp-3-glu)	90.1
Blackberry	27.0	449	287	Cyanidin-3-glucoside (Cy-3-glu)	96.7
Strawberry	29.2	433	271	Pelargonidin-3-glucoside (Pg-3-glu)	91.7

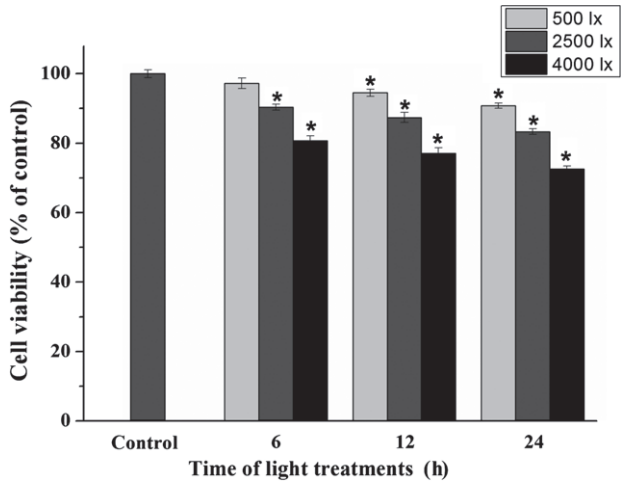

Figure 2. The cytotoxic effects of various light time and light intensity on the retinal pigment epithelial (RPE) cells. The RPE cells not treated with light irradiation were used as control. Mean values were significantly different when $*P < 0.05$ (one-way ANOVA).

RPE apoptosis.[29] Thus, the protective effects of ACNs against visible light-induced damage in RPE cells may be associated with oxidative stress suppression. A general statement regarding antioxidant capacity is difficult to make because of the existence of several antioxidant assays, some of which require the donation of a hydrogen atom (e.g. chain reactions of lipid peroxidation), whereas others require electron donation (e.g. scavenging of free radicals). In the oxygen radical absorbance capacity (ORAC) assay, Cy-3-glu exhibited the highest antioxidant activity among the four ACNs.[30] This finding is consistent with the strong inhibitory effect of Cy-3-glu on ROS generation in the RPE cells. These results suggest that Cy-3-glu has potential protective effects against visible light-induced RPE cell damage. The ACNs tested exhibited antioxidant activity of variable efficiency. The hydroxyl groups on the B (benzene) ring of ACNs are fundamental to the antioxidant activity of these compounds because these hydroxyl moieties can donate hydrogens to scavenge radicals.[31] Generally, increasing the number of hydroxyl groups on the ACNs increases the ACN antioxidant capacity. Among the aglycones with the same hydroxylation patterns in the A and C rings, Cy-3-glu with two OH groups at ortho positions on the B ring, showed higher antioxidant activities compared with compounds with only one OH substitution (Pg-3-glu and Mv-3-glu). Wang *et al.* obtained a similar conclusion in their study on the antioxidant activities of ACNs.[30] The donation of a hydrogen by ACNs serves to scavenge oxygen radicals and break the chain reactions involved in lipid peroxidation.[32]

Effects of anthocyanins on vascular endothelial growth factor expression

VEGF is a cytokine secreted by the RPE cells and serves a vital function in regulating vascular permeability and angiogenesis.[33] Numerous studies have proposed that RPE cells are a major VEGF source.[2] However, VEGF over-expression in RPE cells was shown to induce vascular leakage, new choroidal blood vessel growth, neovascular AMD development, and neural retinal degeneration.[34] The regulatory effects of the four ACNs on VEGF levels in cell supernatants are shown in Fig. 4. The VEGF levels in the model group and in the sample groups of Pg-3-glu and Dp-3-glu (10, 20 and 40 μg mL^{-1}) showed no significant difference. Compared with the model group, 40 μg mL^{-1} Cy-3-glu significantly ($P < 0.05$) reduced the VEGF level from 145.5 ± 2.8 pg mL^{-1} to 125.1 ± 1.6 pg mL^{-1}. Meanwhile, 20 and 40 μg mL^{-1} Mv-3-glu significantly reduced the VEGF levels ($P < 0.05$).

Excessive visible light exposure and oxidative stress can result in VEGF over-expression in RPE cells.[20,35] Because ROS are a class of intracellular angiogenic mediators, ACNs as antioxidants can potentially be used in anti-angiogenic therapies.[36] Cy-3-glu and Mv-3-glu can inhibit VEGF over-expression, which is probably due to the antioxidant activity. Edible berries, such as black raspberry, blueberry and strawberry, exhibit anti-angiogenic properties and ACNs may be the main active constituents.[37–39] In addition, the anthocyanidins (cyanidin, malvidin and delphinidin) in bilberry inhibit VEGF-induced angiogenesis mainly because of their radical scavenging activities.[40] However, a previous study showed that a grape seed proanthocyanidin extract possessing high antioxidant capacity failed to inhibit inducible VEGF expression suggesting that the antioxidant activity alone may not account for the anti-angiogenic property.[38] These findings indicate that the structural characteristics of ACNs may affect the signal pathways in the cell and be responsible for the inhibitory effects of these compounds on VEGF expression and release.

Monocyte chemotactic protein-1 (MCP-1), which has been shown to be responsible for recruiting macrophages to infection or inflammation sites, induces VEGF gene expression and the subsequent VEGF-induced angiogenesis.[37,41] Atalaya *et al.*[42] found that various combinations of berry anthocyanin-rich extracts significantly inhibit basal MCP-1 and inducible NF-κB transcriptions to exhibit the potent inhibitory effect on inducible VEGF expression. In addition, it was reported that bilberry anthocyanosides inhibited angiogenesis both *in vitro* and *in vivo*, promptly inhibit the VEGF induced signalling, which were presumably by inhibiting the phosphorylations of extracellular signal-regulated kinase 1/2 (ERK 1/2) and serine/threonine protein kinase family protein kinase B (Akt).[43] Therefore, ACN as a VEGF inhibitor may act as a chemotherapeutic agent that can effectively improve vision in neovascular AMD.[44] Cy-3-glu exhibits superior protective activities by reducing oxidative stress and inhibiting VEGF over-expression. The antioxidant and anti-angiogenic properties of Cy-3-glu possibly act synergistically to protect the RPE cells.

Effects of anthocyanins on the β-galactosidase activity of retinal pigment endothelial cells

β-Galactosidase activity is an effective marker for cellular senescence, and its expression in the RPE cells was demonstrated to be age dependent.[45] Thus, β-galactosidase activity can be used to determine RPE cell senescence *in vitro*. The results show that RPE cells exhibited premature senescence after exposure to 2500 lx for 12 h (Fig. 5A). Compared with the vehicle control ($8.3\% \pm 1.5\%$ of senescent cells), the percentage of senescent cells in the model group increased to $23.0\% \pm 1.7\%$ (Fig. 5B). When the RPE cells were treated with 40 μg mL^{-1} Cy-3-glu, and Pg-3-glu the percentage of senescent cells significantly ($P < 0.05$) decreased to $15.0\% \pm 2.0\%$ and $17.3\% \pm 2.1\%$, respectively. Meanwhile, 40 μg mL^{-1} Dp-3-glu and Mv-3-glu did not sufficiently suppress light-induced cell ageing. These results indicate that Cy-3-glu and Pg-3-glu showed protective effects against light-induced ageing in RPE cells.

RPE cellular senescence can lead to an abnormality in the physiological function of this layer. This abnormality is closely related to the development of AMD and other eye diseases.[46] Previous research has shown that oxidative stress and telomere shortening

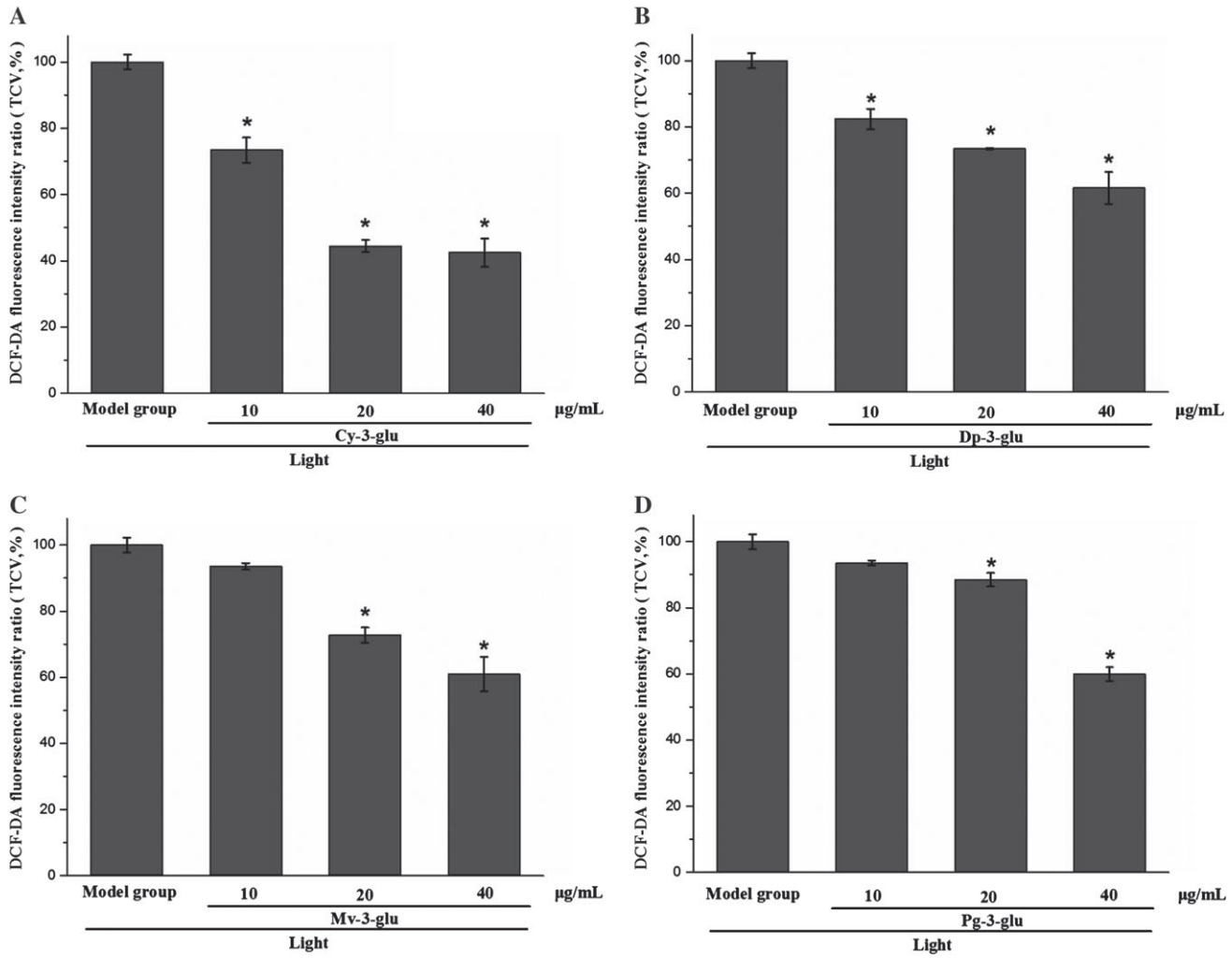

Figure 3. Effects of four anthocyanins (ACNs) on visible light-induced (420–800 nm) increases in intracellular reactive oxygen species (ROS). Confluent cultures were exposed to 2500 lx light for 12 h. The intracellular ROS levels were monitored using 2′,7′-dichlorodihydro-fluorescein diacetate (DCFH-DA). Model group: light exposure, no ACN treatment. DCFH-DA fluorescence intensity ratios [toward the control value (TCV), %] are expressed as means, and standard deviations are represented by vertical bars (n = 3). Mean values were significantly different when $^*P < 0.05$ (one-way ANOVA). (A) cyanidin-3-glucoside (Cy-3-glu), (B) delphinidin-3-glucoside (Dp-3-glu), (C) malvidin-3-glucoside (Mv-3-glu), and (D) pelargonidin-3-glucoside (Pg-3-glu).

contribute to premature cellular senescence.[47] The anti-ageing effects of ACNs on RPE cells may be due to the antioxidant activities of these compounds. In previous studies, various ACN extracts are able to modulate the inflammatory mediators and transcription factor of ageing *in vitro* and *in vivo*.[48–50] Furthermore, it has reported that cyanidin may possess anti-ageing effects by attenuating oxidative stress under the stress-induced premature senescence cellular model.[51]

A number of studies suggest that long-term exposure to light is significantly correlated with AMD pathogenesis; moreover, pathological changes in RPE causes primary lesions in AMD and can strongly affect the quality of life of ageing people.[28,52] In this study, visible-light exposure induced the increase in intracellular ROS levels, promoted VEGF over-expression, and caused RPE cell senescence. In addition, ACNs in berries were shown to exhibit alleviation effects. Increasing evidence indicates that ACNs exhibit preventive or curative effects on eye diseases because of the potent antioxidant and free-radical scavenging properties of these compounds as well as their ability to modulate numerous cellular enzyme functions. Blackcurrant ACNs can promote rhodopsin regeneration to improve dark adaptation and can accelerate recovery from transient refractive changes induced by video display terminal work.[53,54] In our previous studies, blueberry ACNs were shown to protect against light-induced damage *in vitro* and ameliorate light-induced retinal damage *in vivo*.[20,55]

ACNs are not substantially metabolised after ingestion, and native ACNs have been identified in ocular tissues.[56,57] The ACN concentration was 6.89 μg g^{-1} in the retinal tissue after intraperitoneal administration in rats.[57] ACNs can cross the blood–retinal barrier after normal feeding and after acute administration by intraperitoneal and intravenous means.[58] The stability of the different ACNs will probably be important to their ameliorative effects. At neutral pH, ACNs have consistently been shown to degrade to their constituent phenolic acids, such that cyanidin, malvidin, and pelargonidin degrade to form protocatechuic, syringic, and 4-hydroxybenzoic acids, respectively.[59] Thus, the protective effects may be partially attributed to ACN degradation products. For example, protocatechuic acid significantly suppressed VEGF production in vitro and was a potential anti-ageing agent.[60,61] Recent reports have shown that bilberry ACNs had been identified in

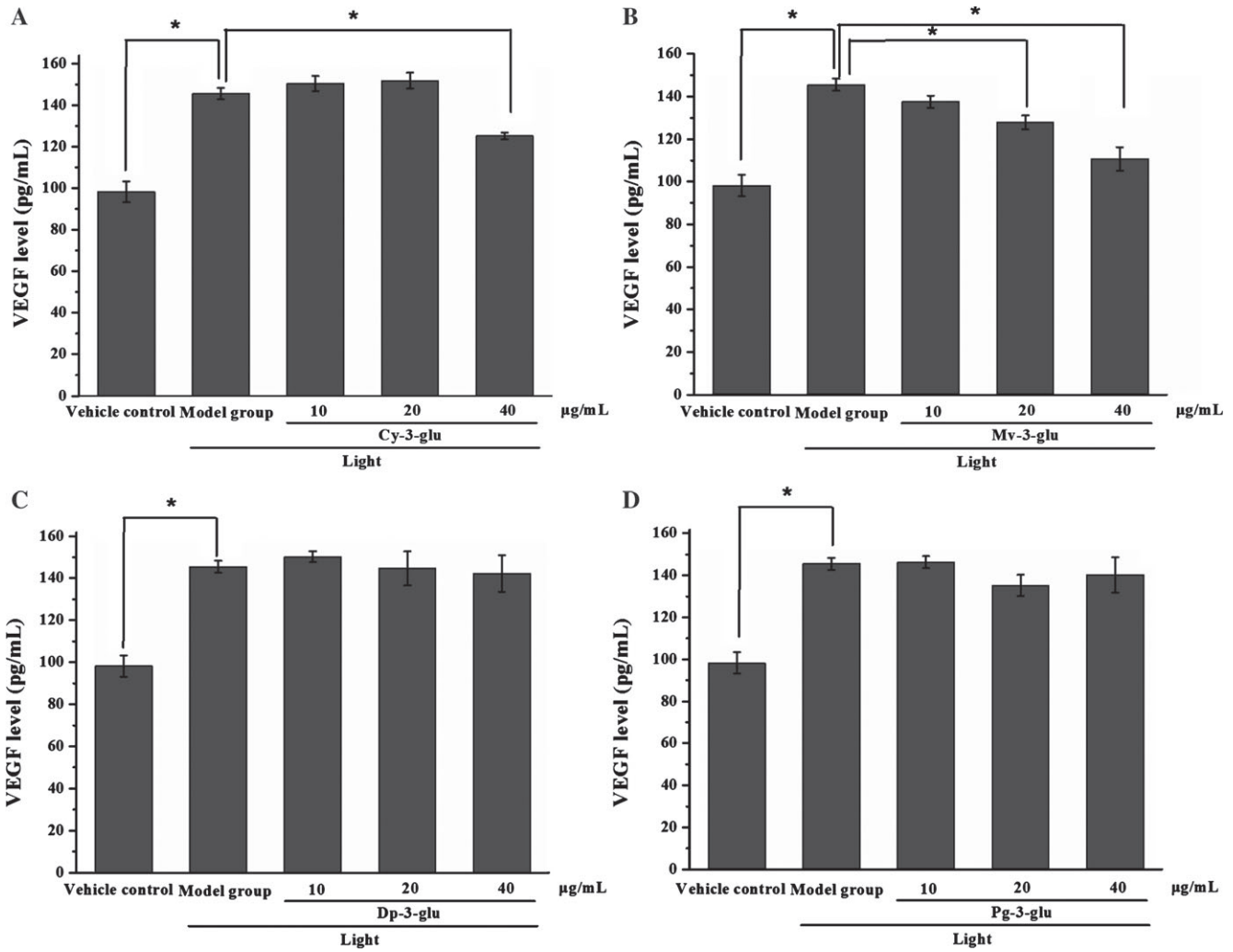

Figure 4. Cytoprotective effect of four anthocyanins (ACNs) on vascular endothelial growth factor (VEGF) secretion in the retinal pigment epithelium (RPE) cells after exposure to light (420–800 nm) at 2500 lx for 12 h. Vehicle control: no light exposure, no ACN treatment; model group: light exposure, no ACN treatment. Values are expressed as means, and standard deviations are represented by vertical bars ($n = 3$). Mean values were significantly different when $^*P < 0.05$ (one-way ANOVA). (A) cyanidin-3-glucoside (Cy-3-glu), (B) malvidin-3-glucoside (Mv-3-glu), (C) delphinidin-3-glucoside (Dp-3-glu), and (D) pelargonidin-3-glucoside (Pg-3-glu).

the eyes in pig by oral administration,[56] suggesting the possible protective effect of oral berry for retinal diseases. Although the retina concentration of ACNs after oral administration may be lower than the concentrations of ACNs showing *in vitro* effects in this study, ACNs may be able to reach ocular tissues and may possess potential efficacy for eye health. Thus, further *in vivo* experiments would be necessary to confirm the effect of ACNs against visible light-induced damage in RPE cells. In addition, the other phenolic compounds, particularly the flavonols (quercetin or keampferol derivatives) in berry extracts, may serve an important function as antioxidants that protect RPE cells.

Although a number of studies have demonstrated the positive effects of ACNs on visual function, most of these studies used berry extracts as ACN test samples. In the current study, four ACN components were isolated, and their protective effects against visible light-induced damage in RPE cells were compared. Cy-3-glu, the principal ACN in most fruits, may prove useful as a prophylactic health food for the prevention of retinal diseases. Cy-3-glu can reduce the intracellular ROS level, inhibit VEGF expression, and reduce senescence in RPE cells. In the previous study, cyanidin-3-glycosides promoted rhodopsin regeneration; however, delphinidin-3-glycosides had no effect on the process, possibly because the cyanidin form is hydrophobic compared with the delphinidin form, and 11-*cis*-retinal is a non-polar substance.[54] In addition, cyanidin and cyanidin glycoside (glucoside, galactoside and arabinoside) protect against blue light-induced A2E (a major component of lipofuscin) oxidation.[62] However, further research is needed to elucidate the mechanisms of ACNs against visible light-induced damage in RPE cells.

CONCLUSION

We successfully isolated and purified four ACNs (Cy-3-glu, Mv-3-glu, Dp-3-glu, and Pg-3-glu) from blackberry, blueberry and strawberry. Cy-3-glu, which contains an *ortho* hydroxyl group in its B ring, exhibited antioxidant, anti-angiogenic, and anti-ageing properties in the visible light-induced RPE damage model *in vitro*. Therefore, ACNs, specifically Cy-3-glu, are promising candidates as nutritional supplements for the prevention of early pathological changes in the retina. Furthermore, the structural characteristics

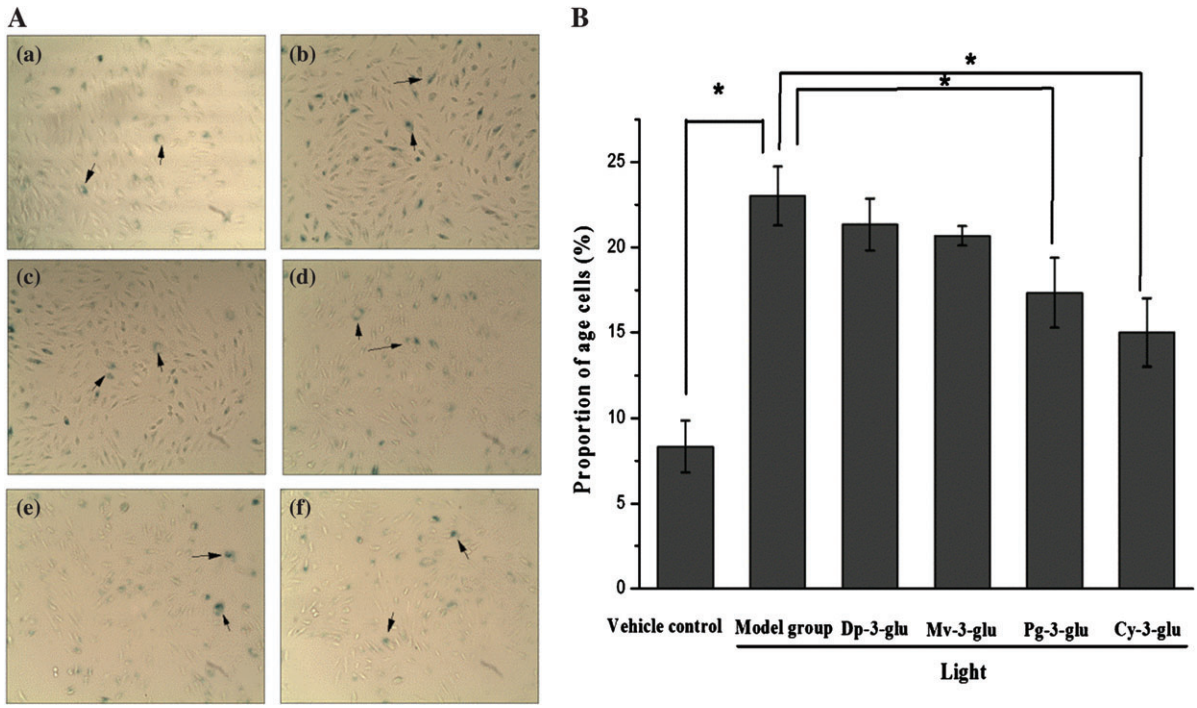

Figure 5. Protective effects of four anthocyanins (ACNs) on visible-light-induced (420–800 nm) senescence of retinal pigment epithelium (RPE) cells. Confluent cultures were exposed to light (2500 lx) or maintained in the dark for 12 h. (A) Morphology of RPE cells observed under a light microscope (magnification, ×100) after β-galactosidase staining. Senescent cells are indicated by black arrows. (B) Senescence percentage of RPE cells in the vehicle control, model group, and treatment groups. Vehicle control: no light exposure, no ACN treatment; model group: light exposure, no ACN treatment; and treatment group: light exposure, ACN (40 μg mL^{-1}) treatment. Values are expressed as means, and standard deviations are represented by vertical bars (n = 3). Mean values were significantly different when $^*P < 0.05$ (one-way ANOVA). (a) Vehicle control, (b) model group, (c) cyanidin-3-glucoside (Cy-3-glu), (d) malvidin-3-glucoside (Mv-3-glu), (e) delphinidin-3-glucoside (Dp-3-glu), and (f) pelargonidin-3-glucoside (Pg-3-glu).

of ACNs may affect the signal pathways in the RPE cell for the VEGF expression and β-galactosidase activity. Further studies are needed to investigate these hypotheses.

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