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ARTICLE

Purple Rice Extract and Anthocyanidins of the Constituents Protect against Light-Induced Retinal Damage in Vitro and in Vivo

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This study evaluated the protective effects of purple rice (*Oryza sativa* L.) bran extract (PRE) and its major anthocyanidins (cyanidin and peonidin) against light-induced retinal damage. In an in vitro experiment, cultured murine photoreceptor cells (661W) were damaged by a 24 h exposure to light. Viability of 661W after light treatment, assessed by the tetrazolium salt (WST-8) assay and Hoechst 33342 nuclear staining, was improved by the addition of PRE, cyanidin, and peonidin. Intracellular radical activation in 661W, evaluated using the reactive oxygen species (ROS) sensitive probe 5-(and 6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA), was reduced by PRE and its anthocyanidins. Electron spin resonance (ESR) measurements showed that PRE, peonidin, and cyanidin all exhibited radical scavenging activities against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, superoxide anion radical ($^{\bullet}O_2^-$), and hydroxyl radical ($^{\bullet}$OH). In an in vivo mouse experiment, intravitreous injection of PRE significantly suppressed photoreceptor degeneration induced by exposure to light as revealed by histological analysis using hematoxylin−eosin staining. These findings suggest that PRE and its anthocyanidins possess protective effects with antioxidation mechanism in both in vitro and in vivo models of retinal diseases.

KEYWORDS: Anthocyanin; oxidative stress; photoreceptor; purple rice; retina

INTRODUCTION

Visible light is generated by the sun as well as by a wide variety of artificial illumination sources such as fluorescent lights, light-emitting diodes (LED), and the monitors of computers, mobile phones, and televisions. However, excessive exposure to light can be damaging to the eye, as evidenced by photoreceptor degeneration in rats and mice following prolonged light exposure (*1*, *2*). In addition, sunlight exposure has a role in the progression of age-related macular degeneration (AMD) (*3*) and can also contribute to retinitis pigmentosa (RP) as determined in a human population-based study (*4*). In the United States, AMD and RP are the most frequent causes of blindness in adults. Photoreceptor cell death is an irreversible injury and can cause night blindness and constriction of the visual field, leading to the loss of central vision. Light-induced photoreceptor cell death can be caused by a variety of cellular mechanisms that involve oxidative stress, reactive oxygen species (ROS), activation of caspase-1, and depletion of NF-κB (*5*).

The human retina, in particular, requires large amounts of oxygen; thus, it readily generates ROS, such as superoxide anion radical ($^{\bullet}O_2^-$) and hydrogen peroxide (H_2O_2). Although the oxidizing capability of these radicals is weak, they react with metals in living tissues and are immediately changed to hydroxyl radical ($^{\bullet}$OH) if exposed to ultraviolet (UV) light. This radical has great capacity to injure DNA and the cell membrane. The eye therefore depends on the presence of antioxidants such as ascorbic acid to protect the retina from light-induced free radical damage (*6*, *7*). However, oxidative stress conditions can overwhelm this internal antioxidant system, resulting in the progression of many diseases including retinal diseases.

Purple rice, as its name implies, is a colored variety of *Oryza sativa* L. (Gramineae) that is widely cultivated in South East Asia, China, and Japan. Unpolished purple rice contains a purple-black dye (anthocyanins) in the seed coat. Many studies on the chemical composition and biological activities of purple rice and its constituents have indicated this rice to have a number of beneficial properties including antioxidant (*8*−*10*), α-glucosidase inhibitory (*11*), antimutagenic (*12*, *13*), hepatoprotective (*14*), hyaluronidase inhibitory (*15*), antidiabetic (*16*), and anti-inflammatory (*17*) effects. In our previous study on chemical and biological properties of an extract derived from defatted purple rice bran, we isolated three anthocyanins: cyanidin 3-*O*-glucoside, peonidin 3-*O*-glucoside, and cyanidin 3-*O*-gentiobioside (*18*). These anthocyanins have been reported to protect A2E-laden retinal pigment epithelial cells against blue light-induced damage, suggesting that purple rice bran extract (PRE) may show protective activity against retinal damage (*19*).

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The purpose of the present study was to examine, using both in vitro and in vivo models, the potential protective effects of PRE and its constituents (cyanidin and peonidin) against photoreceptor cell death induced by light exposure. First, we carried out an examination of visible light injury in cultured murine photoreceptor cells (661W). We also determined the antioxidant activity of PRE and its constituent anthocyanidins using an electron spin resonance (ESR) method, to confirm the actual antioxidant capacity of PRE. Finally, we studied the in vivo protective effect of PRE on light-induced retinal damage in mice.

MATERIALS AND METHODS

Materials. Cyanidin 3-*O*-glucoside, peonidin 3-*O*-glucoside, cyanidin, and peonidin were purchased from Extrasynthese (Genay Cedex, France). A Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan). Hoechst 33342 and propidium iodide (PI) were purchased from Molecular Probes (Eugene, OR). 5-(and 6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA) was purchased from Invitrogen Co. (Eugene, OR). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich (St. Louis, MO). 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Hypoxanthine, xanthine oxidase, H_2O_2, and $FeSO_4$ were purchased from Wako Pure Chemical Co., Ltd. (Osaka, Japan). 661W cells were a kind gift from Dr. Muayyad R. Al-Ubaidi (University of Oklahoma Health Sciences Center, Oklahoma City, OK).

Preparation of PRE and Determination of Anthocyanins. Purple rice bran (100 g) was defatted with *n*-hexane (500 mL) to eliminate interference of lipid components in the subsequent aqueous ethanol extraction step. After drying at room temperature, the defatted bran was extracted with 50% ethanol (500 mL). The extract was loaded onto a Diaion HP-20 column (Mitsubishi Chemical Co., Tokyo, Japan), which was then washed with H_2O, followed by 70% ethanol. The 70% ethanol fraction was evaporated to obtain PRE (2.8 g). The anthocyanins in PRE were analyzed by HPLC (Shimadzu, Kyoto, Japan) equipped with a C18 column (Develosil ODS-UG-5, 4.6 mm i.d. × 250 mm, Nomura Chemical Co., Ltd., Aichi, Japan) (*20*). The mobile phase was composed of 10% formic acid solution v/v (A) and formic acid/CH_3CN/MeOH/H_2O (10:22.5:22.5:45 v/v) (B). The gradient was as follows: 0 min, 90% A, 10% B; 40 min, 65% A, 35% B. The flow rate was set at 1 mL/min. The wavelength for detection was 520 nm, and the column was kept at 25 °C. The contents of cyanidin-3-*O*-glucoside and peonidin-3-*O*-glucoside in the PRE preparation were 2.9 and 0.2%, respectively.

Cell Culture. 661W cells were maintained in DMEM containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin under a humidified atmosphere of 5% CO_2 at 37 °C. 661W cells were passaged by trypsinization every 2−3 days.

Cell Survival Following Visible Light Irradiation. 661W cells (5×10^3 cells/100 μL) were seeded into a 96-well white plate and cultured at 37 °C for 24 h. The cells reached 80−90% of confluence. The medium was replaced with 1% FBS−DMEM containing PRE at 3−30 μg/mL, anthocyanidins at 3−30 μM, or Trolox at 10−30 μM and cultured at 37 °C for 1 h. After preincubation, the cells were exposed to 2500 lx of white fluorescent light (Nikon, Tokyo, Japan) for 24 h at 37 °C. The viable cell numbers were measured using a water-soluble tetrazolium salt, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H* tetrazolium monosodium salt (WST-8). Briefly, 10 μL of CCK-8 was added to each well and incubated at 37 °C for 3 h, and the absorbance was measured at 492 nm (reference wavelength, 660 nm) using a spectrophotometer (Varioskan; Thermo Electron Corp., Vantaa, Finland) (*21*).

Hoechst 33342 Staining. At the end of the light exposure treatment, Hoechst 33342 (excitation, 360 nm; emission, 490 nm) and PI were added to the culture medium (final concentrations of 8.1 and 1.5 μM, respectively) and incubated for 15 min. Microscopic images through fluorescence filters for Hoechst 33342 (U-MWU; Olympus Co., Tokyo, Japan) and PI (U-MWIG; Olympus) were captured by a CCD camera (DP30BW; Olympus).

Measurement of ROS Production in 661W Cells. Intracellular radical activation within 661W cells was determined using CM-H_2DCFDA. CM-H_2DCFDA being taken into the cell is converted to dichlorodihydrofluorescin (DCFH) by an intracellular esterase. Then, the ROS oxidizes DCFH (nonfluorescent) to dichlorodihydrofluorescein (fluorescent). At the end of the light exposure period, CM-H_2DCFDA was added to the culture medium and incubated at 37 °C for 1 h at a final concentration of 10 μM. The 96-well plate was loaded into a plate holder in a fluorescence spectrophotometer. The reaction was carried out at 37 °C, and fluorescence was measured at 488 nm excitation and 525 nm emission. The number of cells was determined by Hoechst 33342 staining and used to calculate ROS production per cell (*20*).

Animals. All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and they were approved and monitored by the Institutional Animal Care and Use Committee of Gifu Pharmaceutical University. Male ddY mice (Japan SLC, Hamamatsu, Japan), aged 8−9 weeks (33−38 g), were used. They were kept under controlled lighting conditions (12 h/12 h light/dark).

Light-Induced Retinal Damage. Mice were adapted in the dark for 24 h, and PRE dissolved in saline was then intravitreously injected at a dose of 10 μg/eye 2 h before the light exposure. Control mice received intravitreous injection of an identical volume of saline. The pupils were dilated by eye drops with 1% cyclopentolate hydrochloride (Santen, Osaka, Japan) 30 min before exposure to light. Nonanesthetized mice were exposed to 8000 lx of white fluorescent light (Toshiba, Tokyo, Japan) for 3 h in cages with reflective interiors. The temperature during the exposure to light was maintained at 25.0 ± 1.5 °C. After the exposure to light, all mice were placed in dark adaptation for 24 h and then returned to the normal light/dark cycle.

Histological Analysis. Mice were sacrificed by cervical dislocation. Each eye was enucleated and kept immersed for > 24 h at 4 °C in a fixative solution containing 4% paraformaldehyde. Six paraffin-embedded sections (thickness = 4 μm) cut through the optic disk of each eye were prepared in the standard manner and stained with hematoxylin and eosin (HE). The damage induced by light exposure was then evaluated, with six sections from each eye used for the morphometric analysis described below. Light-microscope images were photographed, and the thickness of the outer nuclear layer (ONL) from the optic disk was measured at 240 μm intervals on the photographs in a masked fashion by a single observer (J.T.). Data from three sections (selected randomly from the six sections) were averaged for each eye.

ESR Measurement Conditions. The measurement conditions for ESR (JES-FA 200, JEOL, Tokyo, Japan) were as follows: center field, 330 mT; sweep width, 1.5 × 10 mT; sweep time, 4 min; field modulation width, 2 × 0.1 mT; amplitude, 5.0 × 100; time constant, 0.3 s; microwave power, 4.0 mW (DPPH radical) or 8.0 mW ($^{\bullet}O_2^-$ and $^{\bullet}$OH). DPPH radical, $^{\bullet}O_2^-$, and $^{\bullet}$OH scavenging activities were calculated from the integration values of the ESR spectra corrected with a Mn^{2+} marker. The conditions were determined by following the procedure of Izuta et al. (*22*).

DPPH Radical, $^{\bullet}O_2^-$, and $^{\bullet}$OH Scavenging Activity. DPPH (200 μM) and various concentrations of PRE, anthocyanidin, or Trolox were dissolved in ethanol. The DPPH solution (100 μL) and sample solution (100 μL) were mixed and transferred to an ESR spectrometry cell. The ESR spectrum of DPPH radical was measured 2 min after mixing (*22*).

The O_2^- was generated by reaction of the hypoxanthine−xanthine oxidase system (*23*). All solutions were dissolved in 0.1 M phosphate buffer (pH 7.4). Sample solution (50 μL), 0.5 M DMPO (50 μL), 5 mM hypoxanthine (50 μL), and 0.4 units/mL xanthine oxidase (50 μL) were mixed and transferred to an ESR spectrometry cell. Exactly 1 min after the addition of xanthine oxidase, the ESR spectrum of DMPO−OOH spin adducts was recorded.

The $^{\bullet}$OH was generated by the Fenton reaction (*24*). All solutions except $FeSO_4$ were dissolved in 0.1 M phosphate buffer (pH 7.4); $FeSO_4$ was dissolved in distilled water. Sample solution (50 μL), 1.8 mM DMPO (50 μL), 2 mM H_2O_2 (50 μL), and 0.2 mM $FeSO_4$ (50 μL) were mixed and transferred to an ESR spectrometry cell. Exactly 1 min after the addition of $FeSO_4$, the ESR spectrum of DMPO−OH spin adducts was recorded.

Statistical Analysis. Data are presented as the mean ± SEM. Statistical comparisons were made using a one-way analysis of variance (ANOVA) followed by a Student's *t* test or Dunnett's multiple-comparison test. A value of $p < 0.05$ was considered to indicate statistical significance.

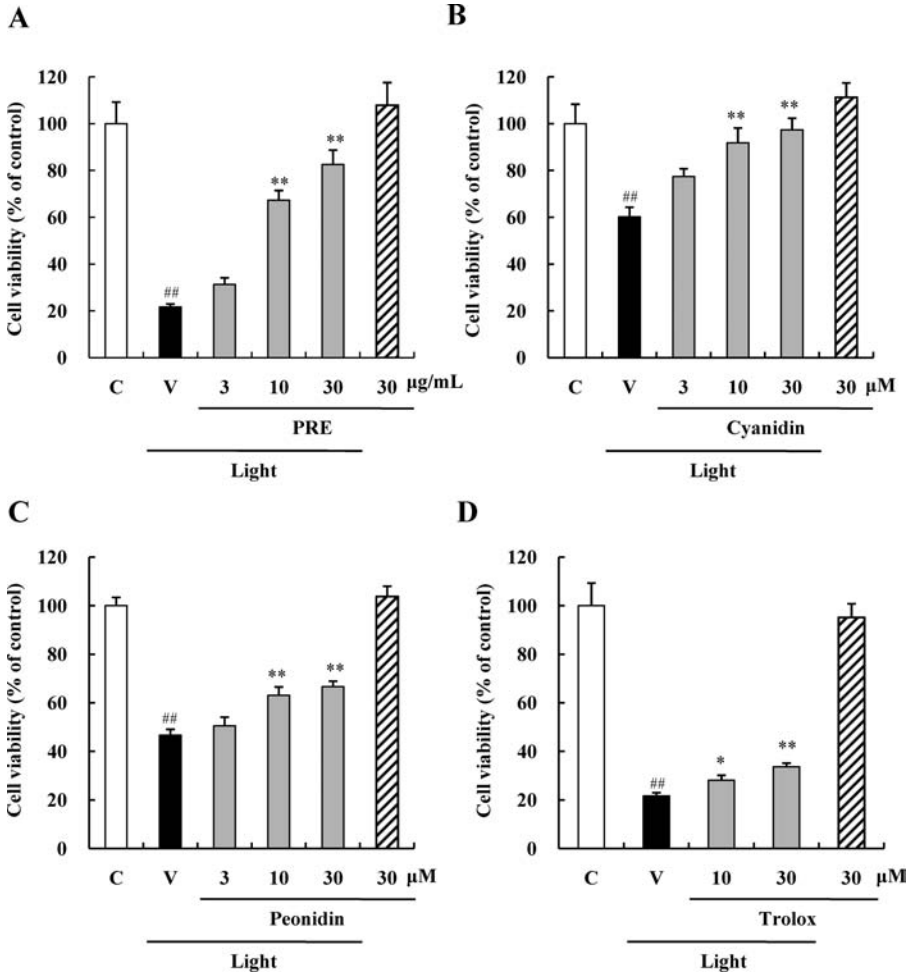

Figure 1. Effects of PRE and its anthocyanin constituents [(**A**) PRE, (**B**) cyanidin, (**C**) peonidin, and (**D**) Trolox] on visible light-induced 661W cell death, as determined by WST-8 assay. Cells were cultured in 96-well plates at a density of 5×10^3 cells/well and then incubated for a total of 48 h at 37 °C in 5% CO_2. Cell viability was assessed by immersing cells in CCK-8 for 3 h at 37 °C, with photometric data being recorded at 492/660 nm. The last bars in each figure show data obtained following the application of each sample alone but without light exposure. Data are shown as mean ± SEM ($n = 6$). C, control; V, vehicle. ##, $p < 0.01$ versus control; ∗, $p < 0.05$, and ∗∗, $p < 0.01$, versus vehicle.

RESULTS

Effects of PRE and Anthocyanidins on 661W Cell Death Induced by Light. We first investigated the effects of PRE and anthocyanidins on light-induced damage in 661W cell cultures. The WST-8 assay indicated that light induced the death of approximately 43−78% of the cells. PRE significantly inhibited this cell death at 10 and 30 μg/mL (**Figure 1A**). Cyanidin, peonidin, and Trolox at 10 and 30 μM also significantly inhibited the light-induced cell death (**Figure 1B−D**). At these concentrations, PRE, cyanidin, peonidin, and Trolox alone had no effects on the viability of the cells (**Figure 1**). Typical images of Hoechst 33342 staining are shown in **Figure 2A−C**. With this dye, condensed nuclei of damaged cells stained more intensely than did those of normal cells. Untreated control cells displayed normal nuclear morphology (**Figure 2A**), whereas the cells irradiated with light for 24 h revealed shrunken and condensed nuclei (**Figure 2B**). After light exposure, nuclei in the cells treated with PRE at 30 μg/mL were morphologically similar to those of the control cells (**Figure 2C**). As shown in **Figure 2D**, PRE at 10 and 30 μg/mL significantly reduced the number of apoptotic cells. Cyanidin at 10 and 30 μM, peonidin at 10 and 30 μM, and Trolox at 30 μM significantly inhibited cell apoptosis (**Figure 2E−G**). At these concentrations, PRE, cyanidin, peonidin, and Trolox alone had no effects on the viability of 661W cells (**Figure 2D−G**).

Effect of PRE on Light-Induced Photoreceptor Degeneration in Mice. Figure 3 shows the histological evaluation of representative retinal images between 240 and 360 μm from the optic nerve in the superior area at 5 days after light exposure. The ONL thickness was remarkably thinned in the saline-treated group (**Figure 3B**) versus the nonirradiated group (**Figure 3A**), but the group treated with 10 μg/eye of intravitreous PRE showed significantly suppressed photic damage (**Figure 3C**) compared to the saline-treated group (**Figure 3B**). PRE provided significant protection to the retinal superior area from 240 to 480 μm and to the inferior area from 240 to 960 μm (**Figure 3D**). The ONL thickness was reduced by 69% versus the nontreated retina, but PRE inhibited ONL degeneration to 23% versus saline in this area.

Effects of PRE and Anthocyanidins on Light-Induced ROS Production in 661W Culture. CM-H_2DCFDA, a cell-permeant indicator of ROS, is nonfluorescent until removal of its acetate groups by intracellular esterase. Within the cell, esterases cleave CM-H_2DCFDA to release CM-H_2DCFDH, which is converted to a fluorescent product (CM-H_2DCF) when exposed to ROS. PRE at 3−30 μg/mL, cyanidin at 3−30 μM, peonidin at 3−30 μM, and Trolox at 30 μM significantly inhibited the light-induced radical activity in 661W cells (**Figure 4**), decreasing intracellular radical formation by 25−82, 22−83, 32−53, and 49%, respectively (**Figure 4**).

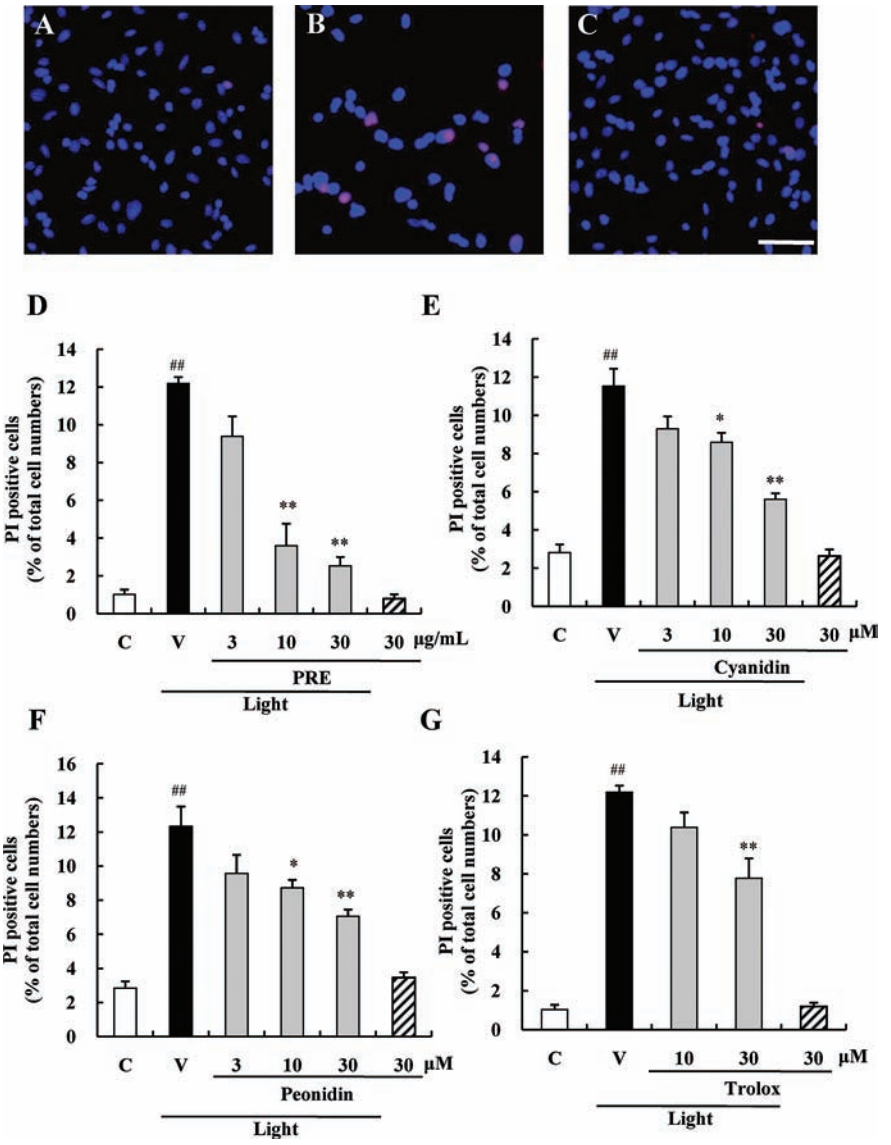

Figure 2. Effects of PRE and its constituents on photoreceptor cell damage induced by light in 661W cells. (**A**−**C**) Representative fluorescence microscopy of Hoechst 33342 staining at 24 h after light exposure: (**A**) nontreated cells showing normal nuclear morphology; (**B**) light-induced cell death, with cells showing nuclear condensation; (**C**) pretreatment with PRE at 30 μg/mL reduced nuclear condensation. (**D**−**G**) The number of cells exhibiting PI fluorescence was counted, and positive cells were expressed as the percentage of PI-positive to Hoechst 33342-positive cells. Effects of PRE, cyanidin, peonidin, and Trolox on light-induced 661W cell death. The last bars in each figure (**D**−**G**) show data obtained following application of each sample alone but without light exposure. Data are shown as mean ± SEM (n = 5 or 6). C, control; V, vehicle. ##, $p < 0.01$ versus control; ∗, $p < 0.05$, and ∗∗, $p < 0.01$, versus vehicle. The scale bar (**C**) represents 100 μm.

Effects of PRE and Anthocyanidins on DPPH Radical, $^{\bullet}O_2^-$, and $^{\bullet}$OH Scavenging Activities by ESR. To clarify the antioxidative mechanism of PRE, we measured three types of radical scavenging activities. First, PRE, cyanidin, peonidin, and Trolox exhibited potent scavenging activity against DPPH radical, with IC_{50} values of 14.9 μg/mL, 32.3 μM, 25.9 μM, and 31.2 μM, respectively (**Table 1**). The antioxidant activities of cyanidin and peonidin were almost equal to those of the typical antioxidant, Trolox. Typical ESR spectra of PRE scavenging activity against DPPH radical is shown in **Figure 5A**. Second, PRE, cyanidin, peonidin, and Trolox exhibited potent scavenging activity against $^{\bullet}O_2^-$, with IC_{50} values of 17.8 μg/mL, 40.5 μM, 39.6 μM, and 522.5 μM, respectively (**Table 1**). The IC_{50} values of cyanidin and peonidin on $^{\bullet}O_2^-$ scavenging activity were almost 10 times lower than that of Trolox. A typical ESR spectrum of PRE scavenging activity against $^{\bullet}O_2^-$ is shown in **Figure 5B**. Third, PRE and Trolox exhibited potent scavenging activity against $^{\bullet}$OH, and IC_{50} values were 84.3 μg/mL and 503.2 μM, respectively (**Table 1**). However, cyanidin and peonidin did not show any antioxidant activity (**Table 1**). A typical ESR spectrum of PRE scavenging activity against $^{\bullet}$OH is shown in **Figure 5C**.

DISCUSSION

In the present study, in vitro supply of PRE, its constituents (cyanidin and peonidin), and Trolox protected cultured 661W cells against visible light-induced cell death. Our previous study had indicated that cyanidin at 10 μM inhibited VEGF-induced angiogenesis (*25*) and SIN-1-induced retinal ganglion cell death (*20*). Trolox, a typical antioxidant, at 10 μM has been reported to protect the retinal ganglion cell from excessive visible light exposure-induced cell death, suggesting the involvement of ROS generation in the mechanism (*26*). On the basis of these findings, we used anthocyanidins at 3−30 μM and Trolox at 10−30 μM in the present in vitro studies.

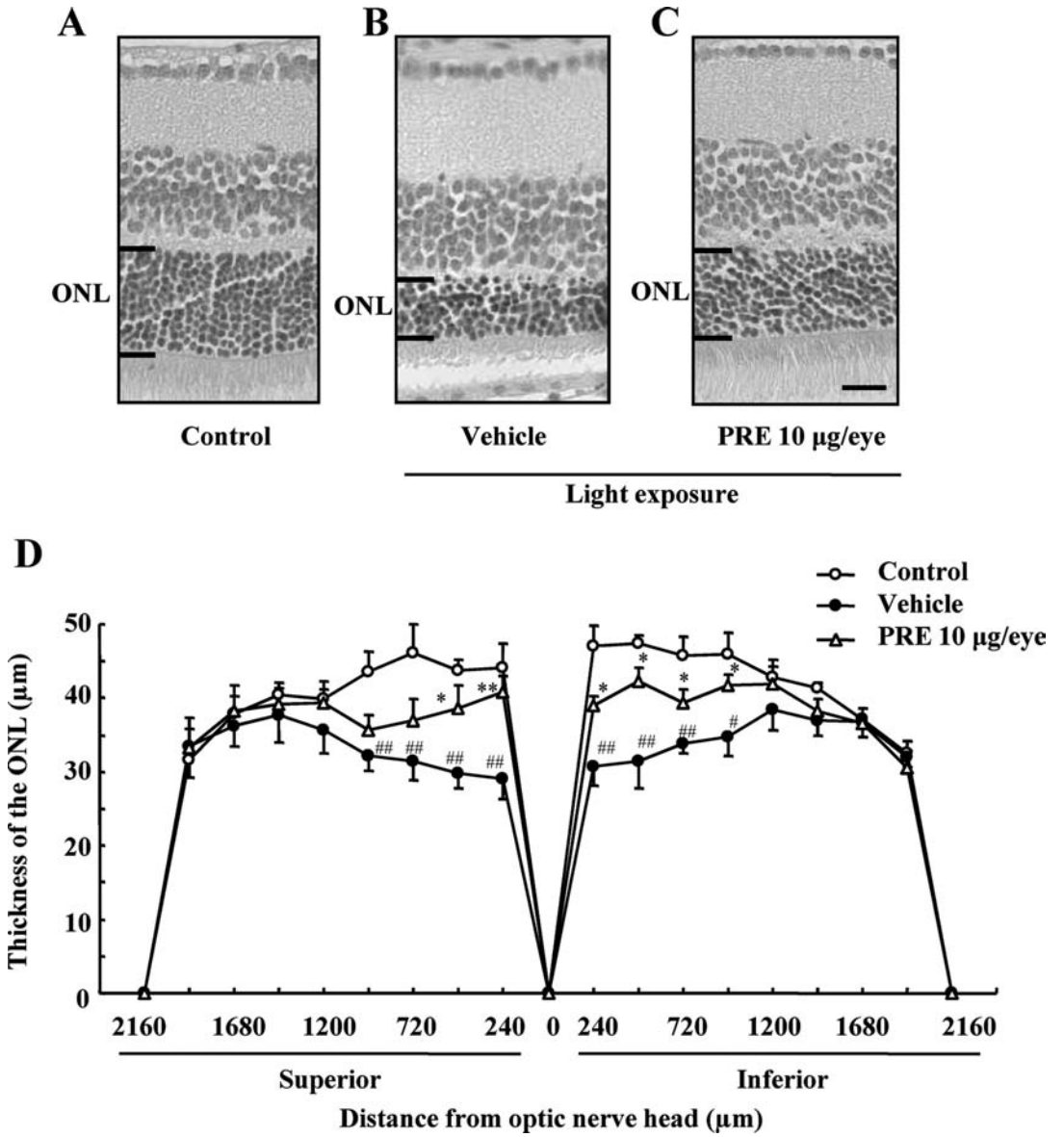

Figure 3. Effects of PRE on retinal damage induced by exposure to light in mice: (**A**) nontreated; (**B**) light exposure (8000 lx) plus vehicle-treated; (**C**) light exposure plus PRE-treated (10 μg/eye) retinal cross sections at 5 days after light exposure in mice. (**D**) Measurement of the thickness in the outer nuclear layer (ONL) at 5 days after light exposure. Data are shown as mean ± SEM (n = 5 or 6). #, $p < 0.05$, and ##, $p < 0.01$, versus control; ∗, $p < 0.05$, and ∗∗, $p < 0.01$, versus vehicle. The scale bar (**C**) represents 25 μm.

The 661W cells served as a useful in vitro model for photoreceptor cell responses to visible light, as the cells in culture also showed light-induced cell death pathways that are similar to those observed in photoreceptor cells in vivo (*5*). Irradiation with white fluorescent light (2500 lx for 24 h) in the present study was sufficient to induce cell damage, such as necrosis and apoptosis, as shown in **Figures 1** and **2**. PRE, cyanidin, peonidin, and Trolox treatments were able to inhibit the light-induced 661W cell death. Cyanidin and cyanidin glycoside (glucoside, galactoside, and arabinoside) have been previously reported to protect against A2E oxidation induced by blue light, whereas cyanidin galactoside protected A2E-laden retinal pigment epithelial cells against blue light-induced damage (*19*). Consequently, these data indicate that cyanidin and peonidin are active anthocyanin components of PRE.

As excessive light exposure is known to induce retinal dysfunction (*1*, *2*), we investigated the effect of PRE on light-induced photoreceptor degeneration in mice by HE staining. After 5 days of light exposure, the saline-treated (vehicle group) mice showed significant atrophy of the ONL thickness. Intravitreous injection of PRE suppressed visible light-induced retinal damage, and PRE-treated mice showed significantly less light-induced photoreceptor degeneration. Previous studies demonstrated that light exposure triggered apoptosis in the ONL (*27*) and that apoptosis was prevented by antioxidants such as ascorbic acid (*6*, *7*), dimethylthiourea (*28*), and thioredoxin (*29*). The main effects of light-induced cell death have been reported to depend on the apoptotic pathway and also on ROS production (*5*). ROS such as H_2O_2, $^{\bullet}O_2^-$, and $^{\bullet}OH$, have been implicated in the regulation of many important cellular events, including transcription-factor activation (*30*), gene expression (*31*), and cell proliferation (*32*). However, excessive production of ROS gives rise to events that lead to the death of several types of cells (*33*). The ROS level of 661W cells increased at 24 h after light exposure in the vehicle group, whereas PRE, cyanidin, peonidin, and Trolox treatment prevented this increase (**Figure 4**). Cyanidin has been reported to enhance cellular antioxidant capacity, including activation of glutathione-related enzymes and inhibition of caspase-3 induced

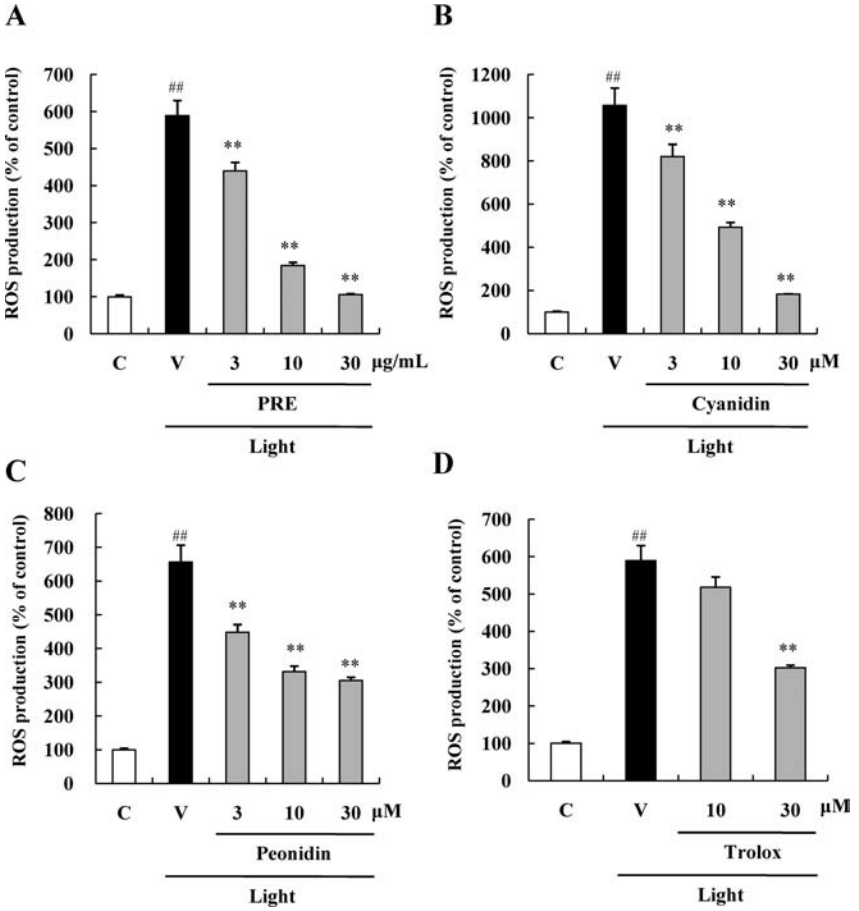

Figure 4. Effects of PRE and its constituents [(**A**) PRE; (**B**) cyanidin; (**C**) peonidin; (**D**) Trolox] on light-induced production of ROS. Intracellular ROS levels were determined by measuring the fluorescence of CM-H_2DCFDA (at excitation 488 nm/emission 525 nm) after light exposure at 24 h in 661W. Data are shown as mean ± SEM (n = 6−8). C, control; V, vehicle. ##, $p < 0.01$ versus control; **, $p < 0.01$ versus vehicle.

Table 1. DPPH Radical, $^{\bullet}O_2^-$, and $^{\bullet}$OH Scavenging Activity of PRE and Its Constituent Anthocyanins[a]

		radical scavenging activity, IC_{50} (95% confidence limits)		
treatment	unit	DPPH	$^{\bullet}O_2^-$	$^{\bullet}$OH
PRE	μg/mL	14.9 (13.2−16.9)	17.8 (14.8−22.3)	84.3 (67.9−111.5)
cyanidin	μM	32.3 (26.0−40.3)	40.5 (33.4−50.3)	>1000
peonidin	μM	25.9 (21.3−31.9)	39.6 (32.4−49.4)	>1000
Trolox	μM	31.2 (25.8−39.2)	522.5 (453.0−615.4)	503.2 (394.4−684.9)

[a] DPPH, 1,1-diphenyl-2-picrylhydrazyl; PRE, purple rice extract.

by H_2O_2 in rat liver Clone 9 cells (*34*). Hence, cyanidin and peonidin may be two of the active components in PRE that cause inhibition of ROS production in 661W cells following irradiation by visible light. Overall, the findings of the present study indicate that PRE is effective at attenuating the dysfunction of photoreceptor and inner retinal cells induced by light exposure and, thus, PRE can ameliorate visual function.

Mitogen-activated protein kinases (MAPKs) are stress-related kinases, and members of the MAPKs subfamily (JNK, p38, and ERK1/2) have been implicated in neuronal injury and diseases. Activation of JNK and p38 MAPK, which are stimulated by various stresses, including ischemia UV exposure, and oxidative stress, are involved in cell differentiation and apoptosis. It has been reported that light-induced photoreceptor apoptosis was related to MAPKs activation in vitro and in vivo (*35*, *36*). In addition, activator protein-1 (AP-1) is an important factor for light-induced retinal degeneration (*37*). AP-1 is a complex that consists either of heterodimers of members of the Fos and the Jun family of proteins or of homodimers of members of the Jun family of proteins. Light exposure leads to activation of AP-1 in mouse retina, and their complexes are mainly composed of c-Fos and c-Jun proteins. Furthermore, AP-1 is activated through phosphorylations of c-Jun and c-Fos, induced by JNK and p38 in retinal pigment epithelium cells (*38*). These reports suggest that AP-1 activation through p38 and/or JNK is essential for light-induced photoreceptor apoptosis in mouse retina. Cyanidin-3-*O*-glucoside has been shown to have inhibitory effects against activation of ERK, JNK, p38, and AP-1 induced by UV in JB6 cells (*39*). Therefore, there is a possibility that PRE containing cyanidin-3-*O*-glucoside may inhibit AP-1 activation by inhibiting p38 and/or JNK activation. However, further studies will be needed to clarify the precise mechanisms in light-induced retinal degeneration in vivo.

Finally, we investigated the radical-scavenging activity of PRE and anthocyanidins using ESR to examine further the involvement of their protective antioxidant functions. The DPPH radical is a reactive hydrogen acceptor and has been found to be useful for antioxidant measurements. Excessive light exposure induces large amounts of ROS, including free radicals, and their production can be overcome by a retinal defensive mechanism, such as superoxide dismutase (SOD) (*40*). In the present study, PRE exhibited potent scavenging activities against DPPH radical, $^{\bullet}O_2^-$, and $^{\bullet}$OH that were even stronger than those of Trolox. PRE has previously been reported to have 10−25 times stronger $^{\bullet}O_2^-$ scavenging activity than the same concentration of Trolox (*8*). PRE treatment also inhibited $^{\bullet}O_2^-$ and ROS in HepG2

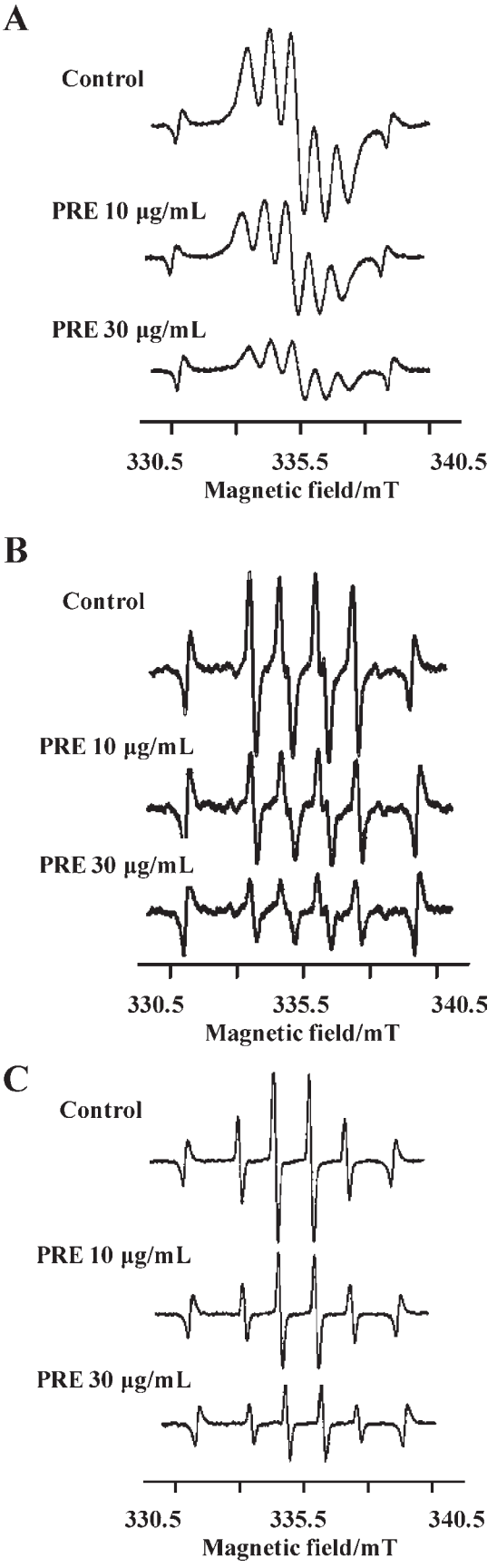

Figure 5. Representative ESR spectra of the DPPH radical, $^{\bullet}O_2^-$, and $^{\bullet}OH$: (**A**) antioxidant effect of PRE at 10 and 30 μg/mL against ESR spectra of DPPH radical; (**B**) antioxidant effect of PRE at 10 and 30 μg/mL against ESR spectra of $^{\bullet}O_2^-$; (**C**) antioxidant effect of PRE at 10 and 30 μg/mL against ESR spectra of $^{\bullet}OH$.

cells and resulted in increased SOD and catalase activities (*9*). Cyanidin and peonidin also showed strong scavenging activities against DPPH radical and $^{\bullet}O_2^-$ compared to Trolox, but their activity against $^{\bullet}OH$ was weaker than that of Trolox. These anthocyanidins therefore may not be involved in the $^{\bullet}OH$ scavenging activity of PRE; instead, there may be other polyphenolic compounds that are responsible for this effect. Han et al. (*41*) isolated oryzafuran, quercetin, and protocatechuic acid from PRE, and these compounds showed strong DPPH radical scavenging activity when compared to ascorbic acid. Quercetin and protocatechuic acid have also been reported to show potent $^{\bullet}O_2^-$ and $^{\bullet}OH$ scavenging activities (*42*, *43*). Therefore, it is considered that cyanidin and peonidin were active components in part for PRE, but not all for it. Further experiments will be needed to clarify synergistic or additional effects of anthocyanins and other polyphenols in PRE. Taken together, these findings indicate that PRE has strong antioxidant activity and can scavenge light-induced free radicals, including $^{\bullet}O_2^-$ and $^{\bullet}OH$, resulting in a protective effect against photoreceptor cell death.

Anthocyanins have been reported not to be detectable in the spleen, thymus, heart, muscle, brain, white fat, or eyes in mice by oral administration (*44*). On the other hand, Matsumoto et al. have reported that anthocyanins were identified in the plasma and whole eye after oral and intraperitoneal administration in rats and after intravenous administration in rabbits (*45*). In rats given intraperitoneal administration, the concentrations of total anthocyanins in the whole eye and some ocular tissues were higher than that measured in plasma. This study demonstrated that anthocyanins were absorbed and distributed in ocular tissues as intact forms. In addition, Kalt et al. have identifed anthocyanins at the eyes, liver, and brain in pig by oral administration (*46*). Therefore, these findings indicate that purple rice anthocyanins also are detectable in the eyes and pass thorough the blood−retinal barrier in rat, rabbit, and pig by oral administration.

In conclusion, we demonstrated that PRE, in part through the activity of its constituent anthocyanidins (cyanidin and peonidin), was able to protect retinal cells against light-induced photoreceptor degeneration in both in vitro and in vivo models. As the light-induced retinal damage model is one of the better models of dry-atrophic AMD (*47*), PRE may prove useful as a prophylactic health food for the prevention of retinal diseases.

ABBREVIATIONS USED

CM-H_2DCFDA, 5-(and 6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate acetyl ester; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; DPPH, 1,1-diphenyl-2-picrylhydrazyl; ESR, electron spin resonance; ONL, outer nuclear layer; PI, propidium iodide; PRE, purple rice bran extract; ROS, reactive oxygen species; WST-8, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H* tetrazolium monosodium salt.

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