Protection of Rat Retina Against Light-induced Damage by Intraperitoneal and Oral Administration of a Polyphenol Fraction From Seed Shells of Japanese Horse Chestnuts (*Aesculus turbinata* BLUME)

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Retinal light-induced damage model rats used in age-related macular degeneration (AMD) studies clarified that antioxidants decrease the damage. The seed shells of Japanese horse chestnuts have antioxidant activity with polyphenols. We studied that protective effect of the polyphenols from light-induced damage. Polyphenols fraction (PF) was extracted and separated from the seed shells. Antioxidant activities of PF were measured. Intraperitoneal administration (IP) and oral administration (PO) of PF was evaluated protective effects by flash electroretinograms (ERGs) and outer nuclear layer (ONL) thickness measurement. IP of PF (100 mg/kg body weight [BW] and phenyl N-tert-butyl nitrone (PBN) (50 mg/kg BW) suppressed decrease of ERG amplitude significantly. And then, decrease of ONL thickness was suppressed by PF (25, 50 and 100 mg/kg BW) and PBN (50 mg/kg BW). PO of PF (100 mg/kg BW) protects retinas from light-induced damage, as well as PBN (10 and 50 mg/kg BW). Our results indicate that PF alleviates light-induced retinal damage and potentially useful for the prevention of AMD.

Key words: retina protection, Japanese horse chestnuts, *Aesculus turbinata* BLUME, polyphenol, antioxidant

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INTRODUCTION

Age-related macular degeneration (AMD), a progressive blinding disease that is incurable currently [1], results from aging and long-term light exposure. About 11 million individuals have AMD in the United States alone [1]. In Japan, 700,000 patients have AMD, which is ranked fourth among the causes of visual disorders [2]. Therefore, AMD is the leading cause of worldwide blindness [1].

Anti-vascular endothelial growth factor drugs are one treatment option for AMD, but long-term treatment causes retinal atrophy [3]. There is no specific medical treatment for AMD. To delay disease progression and visual loss, experimental and clinical studies have suggested that high doses of antioxidant vitamins and zinc supplements are potential strategies [4, 5]. Therefore, preventive medicine is required, especially in countries with an aging population.

Retinal tissue has the highest oxidative consumption and consists of a unique fatty acid component [6]. Because oxidized retinal phospholipids increase with aging [7], oxidative stress might cause AMD. Oxidized phospholipids in the retinas of patients with AMD increase substantially more than in normal retinal tissue [7]. Strong visible light also increases lipid peroxidation and retinal damage [8]. A rat model of light damage used in AMD studies clarified that antioxidants, such as ascorbic acid [9], dimethylurea [8], N-acetylcysteine (NAC) [10], and phenyl N-tert-butyl nitrone (PBN) [11], decrease retinal light damage. In these studies, the drugs were administrated intraperitoneally.

The seeds of the Japanese horse chestnut (*Aesculus turbinata* BLUME) have been used as emergency

food since antiquity. The seeds are used now as an ingredient in rice cakes and rice balls. We reported previously that the seed shells of the Japanese horse chestnut, regarded as a waste byproduct, contain a large amount of polyphenols [12, 13], which have antioxidant activity [12, 13] and the ability to alleviate methotrexate (MTX)-induced intestinal injury in rats [14].

The current study measured the antioxidant activities of polyphenolic fraction (PF) from Japanese horse chestnut seed shells using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) and hydrophilic oxygen radical absorbance capacity (H-ORAC) methods. To compare the retinal protective effects of the PF with PBN, the PF was administered intraperitoneally in a rat model of retinal light damage. We found that oral administration of the PF prevents retinas from light-induced damage.

MATERIALS AND METHODS

MATERIALS

The seeds of the Japanese horse chestnut (*A. turbinata* BLUME) were purchased from Takaki Co. (Kurashiki, Japan). Filter paper No. 2 was purchased from Advantec (Tokyo, Japan). Diaion HP-20 and Chromatorex ODS 1024T for column chromatography were supplied by Mitsubishi Chemical (Tokyo, Japan) and Fuji Silysia (Kasugai, Japan), respectively. DPPH, 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), fluorescein sodium salt, and PBN were obtained from Sigma (St. Louis, MO, USA). Saline and 0.5% tropicamide and 0.5% phenylephrine hydrochloride eye drops were obtained from Otsuka Pharmacy (Tokyo, Japan) and Santen Pharmaceutical (Osaka, Japan). White fluorescent light bulbs (TL5 HE) were purchased from Philips Lighting (Tokyo, Japan). Reduced sugar syrup (PO30) was obtained from Towa Chemical (Tokyo, Japan). Eosin was obtained from Sakura Finetek (Tokyo, Japan). 2,2'-Azobis (2-amidinopropane) dihydrochloride, L-ascorbic acid and other chemicals of analytic grade were supplied by Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Extraction and separation of PF

Extraction and separation of PF from the seed shells of the Japanese horse chestnut were performed according to our previously reported procedures [12]. The seed shells (16.2 g, dry weight [DW]) were refluxed by boiling for 2 hours in 1 L of distilled water. The mixture was filtered through Advantec No. 2 filters to obtain the extracts of the PF. The solvent was removed by rotary evaporation in vacuo and resulted in a 4.2-g yield of dry material. To remove sugars, proteins, and lipids, the extracted material was subjected to column chromatography on the Diaion HP-20 (470 × 60-mm inner diameter) and eluted with 500 ml of methanol after washing with 1 L of distilled water. The resulting methanol extracts were evaporated to dryness and dissolved in 5% methanol. For further purification, the aliquots were subjected to column chromatography on the Chromatorex ODS 1024T (330 × 40-mm inner diameter). After the column was washed with 500 ml of 5% methanol, the polyphenols were eluted with 500 ml of 50% methanol. To obtain polyphenols, the 50% methanol fraction was evaporated to dryness, which resulted in 2.0 g of the separated PF obtained.

Measurement of antioxidant activities

Antioxidant activities were assayed using DPPH radical-scavenging activity and H-ORAC methods, according to the methods described previously [13]. DPPH radical scavenging activity and H-ORAC value were expressed as the Trolox equivalent (TE) (mmol TE/g DW).

Animals

All procedures were performed according to the ARVO Statement for the Use of Animal in Ophthalmic and Vision Research. The Institutional Animal Care and Use Committees of Shimane University reviewed and approved all protocols. Five-week-old male Sprague-Dawley (SD) rats were obtained from Charles River Laboratories Japan, Inc. (Kanagawa, Japan) and maintained in a colony room at 22°C for 1 week before the experiments. All rats were kept in a 12-hour (7 am-7 pm) light/dark cycle.

Evaluation of protective effect of intraperitoneally administrated PF against light-induced retinal damage in rats

The PF was dissolved in saline solution. After the rats fasted for 3 hours, the PF (25, 50, and 100 mg/kg of body weight [BW]) or PBN (10 and 50 mg/kg BW) was intraperitoneally administered 30 minutes before light exposure (5,000 lux, 6 hours) (Fig. 1A) as described previously [15, 16]. Thirty minutes after administering the fourth PF dose, the rats were exposed to intense light as reported previously with slight modification [17]. The pupils were dilated with 0.5% tropicamide and 0.5% phenyleph-rine hydrochloride eye drops 15 minutes before light exposure. The rats were exposed to 5,000 lux of diffuse, cool, and white fluorescent light (TL5 HE) while housed for 6 hours in clear plastic cages with wire tops. During exposure, the rats had free access to food and water. After exposure, the animals were returned to the dim cyclic light conditions.

Flash electroretinograms (ERGs) were recorded 7 days after light exposure using an ERG recording system (LS-W, Mayo Corporation, Aichi, Japan). All animals were dark-adapted overnight before the measurement. Anesthesia was induced by intramuscular injection of a mixture of ketamine (100 mg/kg BW) and xylazine (20 mg/kg BW). The pupils were dilated with 0.5% tropicamide and 0.5% phenylephrine hydrochloride eye drops. Light-emitting diodes (LED) electrodes were placed on both eyes. An identical reference electrode was placed in the mouth; a ground electrode was placed on the tail. A single flashlight (10,000 cd/mm^2, 5 ms) from the LED was used as the light stimulus. The a-wave amplitude was measured as the difference in voltage between the baseline value before the flash and the peak of the a-wave; the b-wave was measured as the difference in voltage between the peaks of the a- and b-waves. The a- and b-wave amplitudes obtained from the right and left eyes were averaged for each animal.
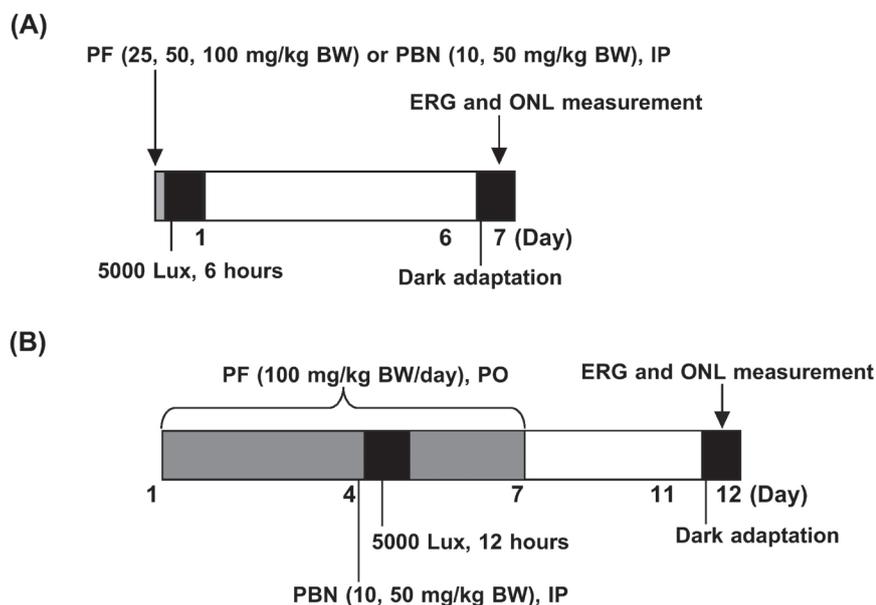

Fig. 1. Experimental design. (A) Experiment by intraperitoneal administration (IP). The rats received PF (25, 50, and 100 mg/kg body weight [BW]) or phenyl N-tert-butyl nitrone (PBN) (10 and 50 mg/kg BW) by IP. Thirty minutes after the injection, the rats were exposed to intense light (5,000 lux) for 6 hours, after which they were returned to the dim cyclic light conditions. Flash electroretinograms (ERGs) were recorded using an ERG recording system 7 days after light exposure. After ERG measurement, the outer nuclear layer (ONL) thickness was measured in the retinal sections. (B) Experiment by oral administration (PO; per os). The rats received oral PF (100 mg/kg BW/day) once daily for 7 days (4 days before and 3 days after light exposure). IP of PBN (10 and 50 mg/kg BW) were administered 30 minutes before light exposure. On the fourth day, the rats were exposed to intense light (5,000 lux) for 12 hours. After light exposure, the animals were returned to the dim cyclic light conditions. ERGs were recorded using an ERG recording system 7 days after light exposure. After ERG measurement, the outer nuclear layer (ONL) thickness was measured in the retinal sections.

After ERG measurement, the outer nuclear layer (ONL) thickness was measured in the retinal sections as described previously with slight modification [17]. After euthanasia by an overdose of anesthesia and cervical dislocation, both eyes were enucleated and fixed in 4% paraformaldehyde containing 20% isopropanol, 2% trichloroacetic acid, and 2% zinc chloride for 24 hours at room temperature. After alcohol dehydration, the eyes were embedded in paraffin, and 4-μm-thick sagittal sections containing the whole retina including the optic nerve head (ONH) were cut. The sections were stained with hematoxylin and eosin. For each section, digitized images of the entire retina were captured with a digital imaging system (Eclipse E800, Nikon, Tokyo, Japan) at × 100 magnification. To cover the entire retina, 10 images were obtained from each section. The ONL thicknesses were measured at 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mm superior and inferior to the ONH and at the periphery 100 μm from the superior and inferior edges of the retina (periphery) using ImageJ 1.32 software (National Eye Institute, Bethesda, MD, USA). The thickness values obtained from the right and left eyes were averaged for each animal.

Evaluation of the protective effect of oral PF against light-induced retinal damage in rats

PF was dissolved in 50% (w/w) PO30 (Towa Chemical, Tokyo, Japan) aqueous solution. After a 3-hour fast, the PF solution was administered via a stomach tube once daily orally into the stomachs of the rats for 7 days (4 days before and 3 days after light exposure for 12 hours at 5,000 lux) (Fig. 1B). The dose of the PF was 100 mg/kg BW. PBN was the positive control used to compare the protective effect of the PF. PBN dissolved in saline was injected intraperitoneally into the rats 30 minutes before light exposure as described previously [15, 16] at doses of 10 and 50 mg/kg BW. Thirty minutes after the PF dose, the rats were exposed to intense light as reported previously with slight modification [10]. All exposure to light began at 6 pm. The pupils were dilated with 0.5% tropicamide and 0.5% phenylephrine hydrochloride eye drops 15 minutes before light exposure. The rats were exposed to 5,000 lux of diffuse, cool, and white fluorescent light (TL5 HE) while housed for 12 hours in clear plastic cages with wire tops. During exposure, the rats had free access to food and water. After exposure, the animals were returned to the dim cyclic light conditions. The ERG and ONL measurement methods were the same as described previously.

Statistical analysis

The data are expressed as the mean ± standard error of the mean in each group (n = 6). The statistical significance was evaluated by one-way analysis of variance (ANOVA) with Mini StatMate software (ATMS Co., Tokyo, Japan). Comparisons of multiple data were performed by ANOVA followed by the post-hoc Dunnett's test. $P < 0.05$ and $P < 0.01$ were considered statistically significant.

RESULTS

Antioxidant activity of PF

The antioxidant activity of PF assayed using the H-ORAC and DPPH methods showed that the H-ORAC value and DPPH radical-scavenging activity were 6.90 ± 0.46 mmol TE/g DW and 4.94 ± 0.22 mmol TE/g DW, respectively.

Evaluation of the protective effect of intraperitoneal PF against light-induced retinal damage in rats

After PF was administered intraperitoneally and the animals were exposed to light, a significant decrease of the ERG a- and b-wave amplitudes occurred in the group that did not receive PF or PBN (negative control [NL]) compared with the normal control (Fig. 2A). The PF significantly ($P < 0.05$) suppressed retinal damage in a dose-dependent manner, especially in the b-wave of the rats treated with the PF 100 mg/kg. The dose of 50 mg/kg of intraperitoneal PBN (positive control) suppressed the retinal damage compared with the negative control.

The ONL thickness decreased in the light-exposed retina, especially in the superior retina 1.0 to 2.0 mm from the optic nerve (Fig. 3A). The decreases of the ONL thickness in all groups that received PF (25, 50, and 100 mg/kg BW) and PBN (50 mg/kg BW) were suppressed (Fig. 3A, B). The ONL areas under the curve (AUC) of the rats that received the PF (50 and 100 mg/kg) were larger than that of the rats in the 10 mg/kg PBN group but not in
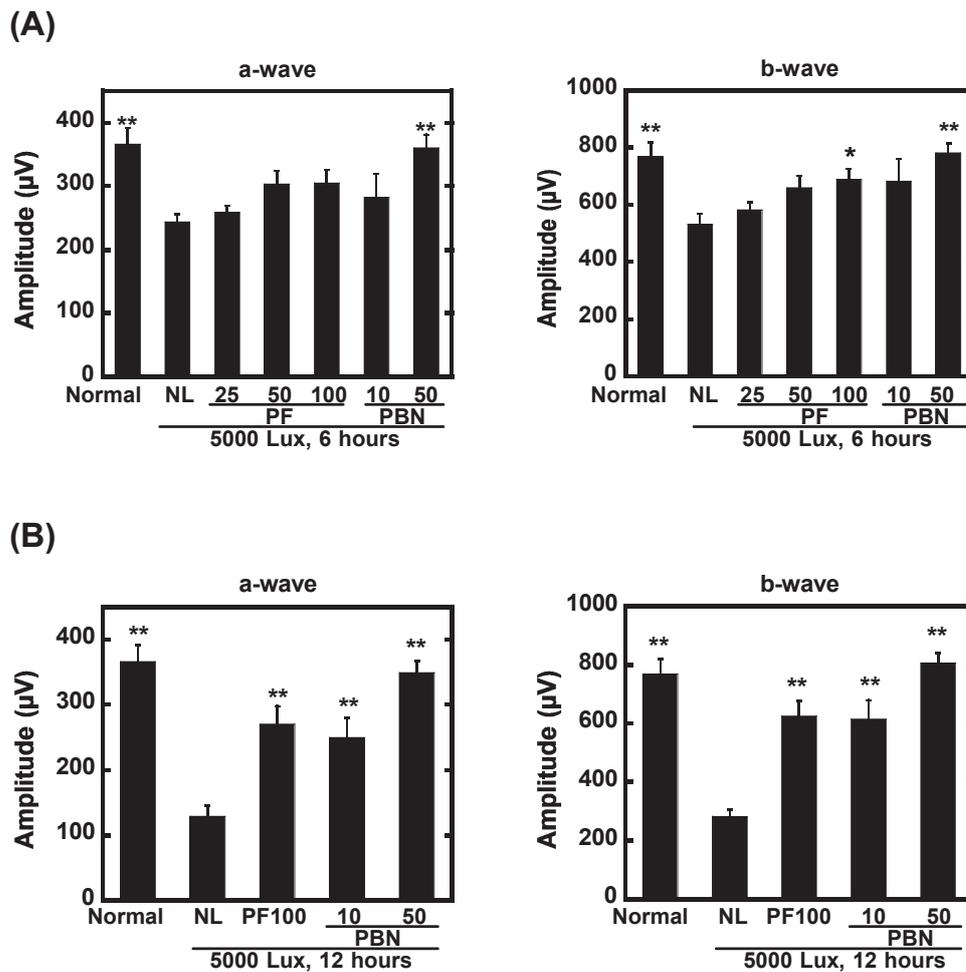
Fig. 2. Protective effect of the polyphenol fraction（PF）against light-induced retinal functional damage.（A）Flash electroretinograms（ERGs）of intraperitoneally administrated（IP）rats. The left and right graphs, respectively, shows the a- and b-waves. Normal = normal control; no light exposure = untreated; NL the negative control = light exposure; IP of solvent; PF 25, light exposure, IP of PF 25 mg/kg body weight（BW）; PF 50, light exposure, IP of PF 50 mg/kg BW; PF 100, light exposure, IP of PF 100 mg/kg BW; PBN 10, light exposure, IP of phenyl N-tert-butyl nitrone（PBN）10 mg/kg BW; and PBN 50, light exposure, IP of PBN 50 mg/kg BW.（B）ERGs of orally administrated（per os; PO）rats. The left and right graphs indicate the a- and b-waves. Normal indicates the normal control, no light exposure, untreated; NL the negative control, light exposure, PO of solvent; PF, light exposure, PO of 100 mg/kg BW; PBN 10, light exposure, IP of 10 mg/kg BW; PBN 50, light exposure, IP of 50 mg/kg BW. The data represent the mean ± standard error of the mean（SEM）（n = 6）.

These data show the results of analysis by the Dunnett's method（vs. negative control *$P < 0.05$, **$P < 0.01$）.

the 50 mg/kg PBN group.

Evaluation of the protective effect of oral PF administration against light-induced retinal damage in rats

To evaluate the protective effect of oral PF against light-induced retinal damage, the effects of 100 mg/kg BW of the PF were compared with the NL. The PF significantly（$P < 0.01$）suppressed the decreases in the ERGs（Fig. 2B）and the ONL thickness 1.0 to 2.0 mm from the optic nerve in the superior retinas（Fig. 3C）. The ONL AUC showed that the PF also significantly（$P < 0.01$）suppressed the damage compared with the NL（Fig. 3D）.

DISCUSSION

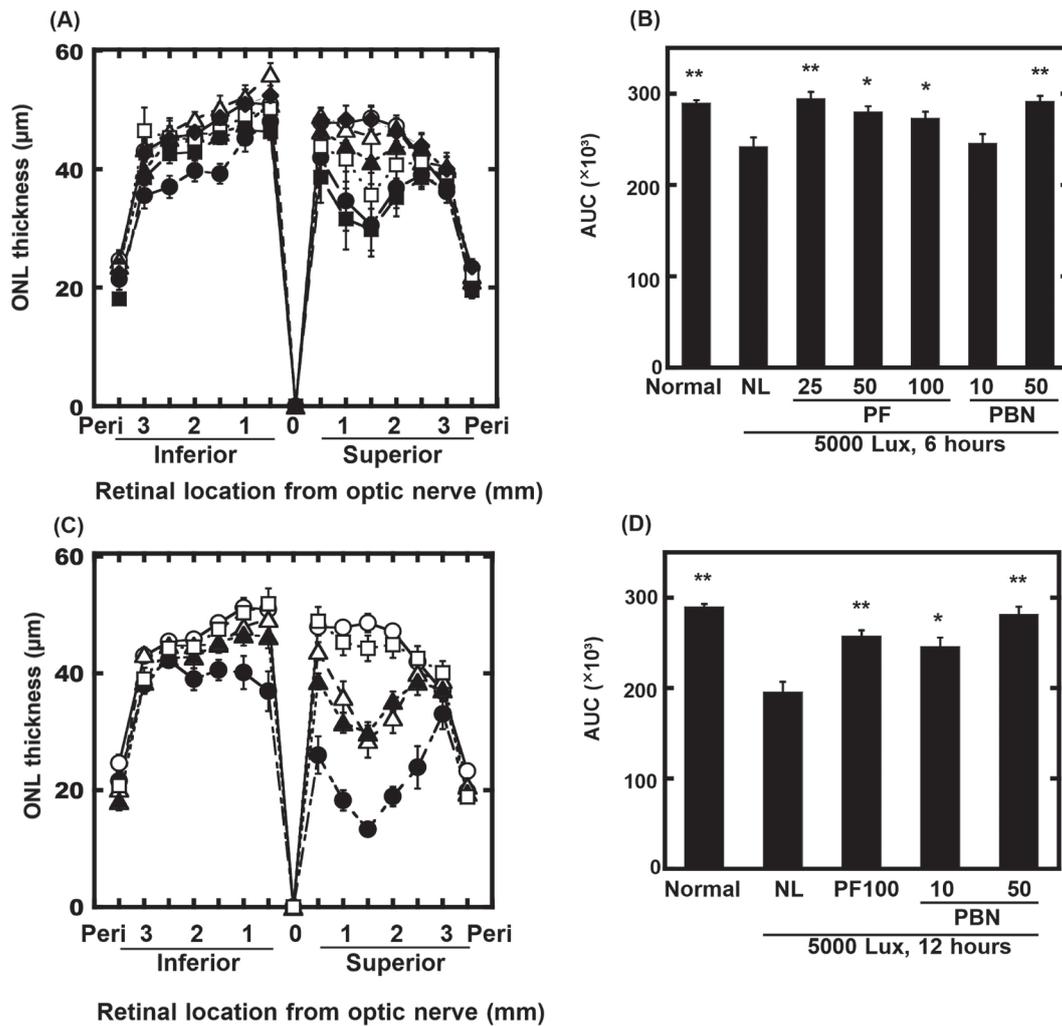We found that the PF separated from the seed

Fig. 3. Protective effect of the polyphenol fraction (PF) against light-induced retinal histologic damage. After electroretinography measurement, the outer nuclear layer (ONL) thickness was measured in the retinal sections. (A) The ONL thickness of intraperitoneally administrated (IP) group is shown. The data are expressed as the mean ± standard error of the mean (n = 6). Normal (○) indicates normal control, no light exposure, untreated; NL (●) negative control, light exposure, IP of solvent; PF 25 (△), light exposure, IP of PF 25 mg/kg of body weight (BW); PF 50 (▲), light exposure, IP of PF 50 mg/kg BW; PF 100 (□), light exposure, IP of PF 100 mg/kg BW; phenyl N-tert-butyl nitrone (PBN) 10 (■), light exposure, IP of PBN 10 mg/kg BW; PBN 50 (◆), light exposure, IP of PBN 50 mg/kg BW; and peri, periphery 100 μm from the superior and inferior edges of the retina. (B) The area under the curve (AUC) of Fig. 3A. (C) The ONL thickness of orally administrated (per os; PO) groups is shown. The data are expressed as the mean ± standard error of the mean (n = 6). Normal (○) indicates normal control, no light exposure, untreated; NL (●) negative control, light exposure, PO of solvent; PF 100 (△), light exposure, PO of PF 100 mg/kg BW; PBN 10 (▲), light exposure, IP of PBN 10 mg/kg BW; PBN 50 (■), light exposure, IP of PBN 50 mg/kg BW; and peri. (D) AUC in Fig. 3C. The data represent the mean ± standard error of the mean (SEM) (n = 6). *$P < 0.05$, **$P < 0.01$ (vs. negative control followed by Dunnett's test).

shells of Japanese horse chestnuts have high antioxidant capacity and protect eyes from light damage. Retinal degeneration caused by genetic or external factors is produced by oxidative damage caused by lipids, protein, and nucleic acids, resulting in apoptotic cell death. These phenomena have been observed after intense light exposure [18-21].

Although antioxidants, such as ascorbic acid [9], dimethyl urea [8], and NAC [10], prevent light damage in retinas, those experiments were not performed to assess the antioxidant activity of the test samples. In our study, the H-ORAC value of ascorbic acid was 2.14 mmol TE/g DW. The H-ORAC value of the PF was 3.2-fold higher than that of ascorbic acid. On the other hand, the DPPH radical-scavenging activity of ascorbic acid was 8.15 ±

0.36 mmol TE/g DW. The DPPH radical-scavenging activity of the PF was 0.6-fold of the activity of ascorbic acid. These varying degrees of the difference between two methods were attributed to each reaction mechanisms. The action of antioxidants can be mainly classified into two reaction mechanisms of hydrogen atom transfer and electron transfer. The H-ORAC method is based on a hydrogen atom transfer mechanism by which the peroxy radical is reduced by the transfer of hydrogen atom from antioxidants. For this reason, ORAC method was reported similar to in vivo reaction [22].

On the other hand, the DPPH method is an electron transfer-based assay to evaluate the capacity of antioxidants to donate one electron to DPPH radical. A previous study has shown that antioxidant activities of different compounds do not necessarily exhibit consistent relationship between two methods with different reaction mechanisms [23].

We evaluated the protective effect of intraperitoneal and oral PF against light-induced retinal damage in rats. The ERGs were recorded to estimate the ocular function, and the ONL thickness was measured to evaluate the retinal tissue. PBN has been used extensively as a positive control in the retinal light damage model, but PBN is not approved for human use because it cannot be administered orally. Therefore, to compare PF and PBN, like PBN, we tested the PF by intraperitoneal administration (Figs. 1A, 2A, 3A, B). The results showed decreased suppression of the ERG b-wave amplitude, and only PF in a dose of 100 mg/kg BW differed significantly from the negative control in ONL thinning. We then examined the protective effect of oral administration of PF against light-induced retinal damage using a dose of 100 mg/kg BW (Figs. 1B, 2B, 3C, D). The ERG results showed that retinal damage was suppressed significantly ($P < 0.01$) (Fig. 2B). ONL thinning that progressed as the result of light damage was inhibited ($P < 0.01$) by administration of the PF (Fig. 3C, D). Therefore, the results suggested that oral PF protects eyes from light exposure. Recently, the retinal protective effects of oral bilberry extract (containing about 39% anthocyanin) [24] and oral epigallocatechin gallate [25] have been reported. However, in those experiments, bilberry extract and epigallocatechin gallate were administered

at doses of 750 and 400 mg/kg BW, respectively. However, PF was effective using a lower dose than those experiments.

We reported previously that the PF mainly contained highly polymeric A-type proanthocyanidin [12, 13]. Absorption of proanthocyanidin depends on the degree of polymerization. Dimers and trimers were clearly absorbed through an intestinal epithelium cell monolayer [26, 27]. While the absorption of these polymers was difficult, they were degraded by the colonic microflora into low-molecular-weight compounds and absorbed [26].

The results of the ORAC assay indicate that these metabolites have antioxidant capacity [28]. Therefore, the PF containing highly polymeric proanthocyanidins might be decomposed by the colonic microflora and absorbed as low-molecular-weight compounds. These metabolites might have high antioxidant properties.

Antioxidant activity, ERG, and ONL results showed that the PF, which has antioxidant activity, protects the retina from light damage. A previous study showed decreased glutathione (GSH) after light exposure, but the change was suppressed by NAC treatment in mice [10]. MTX caused intestinal morphologic injury and increased malondialdehyde (MDA) levels, decreased GSH levels, and glutathione peroxidase (GSH-Px) activities in the small intestine of rats. However, oral PF ameliorated MTX-induced intestinal injury and inhibited the increase in MDA and decreases in GSH and GSH-Px activity in the small intestine. Those results indicate that oral PF alleviates MTX-induced intestinal injury through its antioxidant properties [14]. For this reason, the PF would reinforce antioxidant activity in vivo. Based on these findings, the PF might suppress the decrease in the GSH level after light exposure.

In summary, the PF of the seed shells in Japanese horse chestnuts protected the retina from light exposure damage by inhibiting oxidative stress. Consequently, intake of supplementary the polyphenols from Japanese horse chestnut seed shells appear to have a protective effect on the retina against AMD caused by oxidative stress.

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