



Article

Panax ginseng Fraction F3 Extracted by Supercritical Carbon Dioxide Protects against Oxidative Stress in ARPE-19 Cells

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Abstract: In our previous work, the ethanolic extract of *Panax ginseng* C. A. Meyer was successively partitioned using supercritical carbon dioxide at pressures in series to yield residue (R), F1, F2, and F3 fractions. Among them, F3 contained the highest deglycosylated ginsenosides and exerted the strongest antioxidant and anti-inflammatory activities. The aim of this study was to investigate the protective effects of *P. ginseng* fractions against cellular oxidative stress induced by hydrogen peroxide (H₂O₂). Viability of adult retinal pigment epithelium-19 (ARPE-19) cells was examined after treatments of different concentrations of fractions followed by exposure to H₂O₂. Oxidative levels (malondialdehyde (MDA), 8-hydroxydeoxyguanosine (8-OHdG), and reactive oxygen species (ROS)) and levels of activity of antioxidant enzymes were assessed. Results showed that F3 could dose-dependently protected ARPE-19 cells against oxidative injury induced by H₂O₂. F3 at a level of 1 mg/mL could restore the cell death induced by H₂O₂ of up to 60% and could alleviate the increase in cellular oxidation (MDA, 8-OHdG, and ROS) induced by H₂O₂. Moreover, F3 could restore the activities of antioxidant enzymes suppressed by H₂O₂. In conclusion, F3 obtained using supercritical carbon dioxide fractionation could significantly increase the antioxidant capacity of *P. ginseng* extract. The antioxidant capacity was highly correlated with the concentration of F3.

Keywords: *Panax ginseng*; supercritical carbon dioxide fractionation; retinal pigment epithelium; hydrogen peroxide-induced oxidative damage

1. Introduction

The retinal pigment epithelium is the pigmented cell layer that constitutes the outer blood-retinal barrier. The combination of light and high oxygen consumption puts the retinal pigment epithelial cells under a strong oxidative stress. The oxidative damage of retinal pigment epithelia is known to be involved in the pathogenesis of age-related macular degeneration [1].

Panax ginseng is a widely used traditional Chinese medicine for the treatment of various diseases, such as cancer [2], diabetes [3], and cardiovascular diseases [4,5]. Because of its antioxidant activities [6–8], *P. ginseng* has been used for improving the immune system [9] and central nervous system functions, and relieving stress. Ginsenosides, also known as ginseng saponins, have been recognized as the principal components of *P. ginseng* and they are responsible for the therapeutic and pharmacological effects of ginseng [10]. Approximately 40 ginsenoside compounds have been identified and they can be categorized into three groups based on their structures: protopanaxadiol, protopanaxatriol, and deglycosylated ginsenosides [11]. Studies found that the functionalities of ginseng varied due to the diversities of ginsenoside structures. For instance, deglycosylated ginsenosides are generally easier to be absorbed and thus exhibit more bioactive functions [12]. In addition, studies demonstrated that deglycosylated ginsenosides could exert stronger anti-inflammation, antidepressant-like effects, and anti-cancer activity than protopanaxadiol and protopanaxatriol ginsenosides [13–15].

One important function of ginseng is its antioxidative property in protecting cells from reactive oxygen species (ROS)-induced damage. ROS are generated as byproducts during mitochondrial metabolism and excessive ROS can induce massive oxidation of proteins, lipid, and DNA, leading to cell death. Certain body tissues are especially prone to oxidative damage. For example, the ROS formation caused by the combination of focused light and oxygenated blood conditioned the retinal pigment epithelium of the eye to a constant oxidative stress [16]. The retinal pigment epithelium is the pigmented cell layer that constitutes the outer blood-retinal barrier and it is easily affected by oxidative stress [17], which is believed to be involved in the formation of age-related macular degeneration and can lead to blindness [18]. Despite the fact that a large number of studies have evaluated the protective effects of ginseng against free radical damage to various tissues, such as those of the cardiovascular, liver, and nervous system [19], the effects of different extractions of *P. ginseng* on retinal pigment epithelium is still unclear. Therefore, the aim of this study was to explore the protective effects of the different fractions of *P. ginseng* in retina cells against oxidative stress.

2. Results

2.1. Cytotoxicity of H₂O₂ on ARPE-19 Cells

In the present study, we examined whether extractive fractions of *P. ginseng* C. A. Meyer could inhibit oxidative stress-induced cytotoxicity through a reduction of intracellular ROS. To test this hypothesis, we first analyzed the cytotoxicity of ARPE-19 cells under different treatments. As expected, the treatment of H₂O₂ significantly induced cytotoxicity in ARPE-19 cells in comparison with normal control cells in both dose and time-dependent manners (Figure 1A,B). The results showed that 100 μM of H₂O₂ could reduce the cell viability over 90% after exposure to H₂O₂ for 48 h.

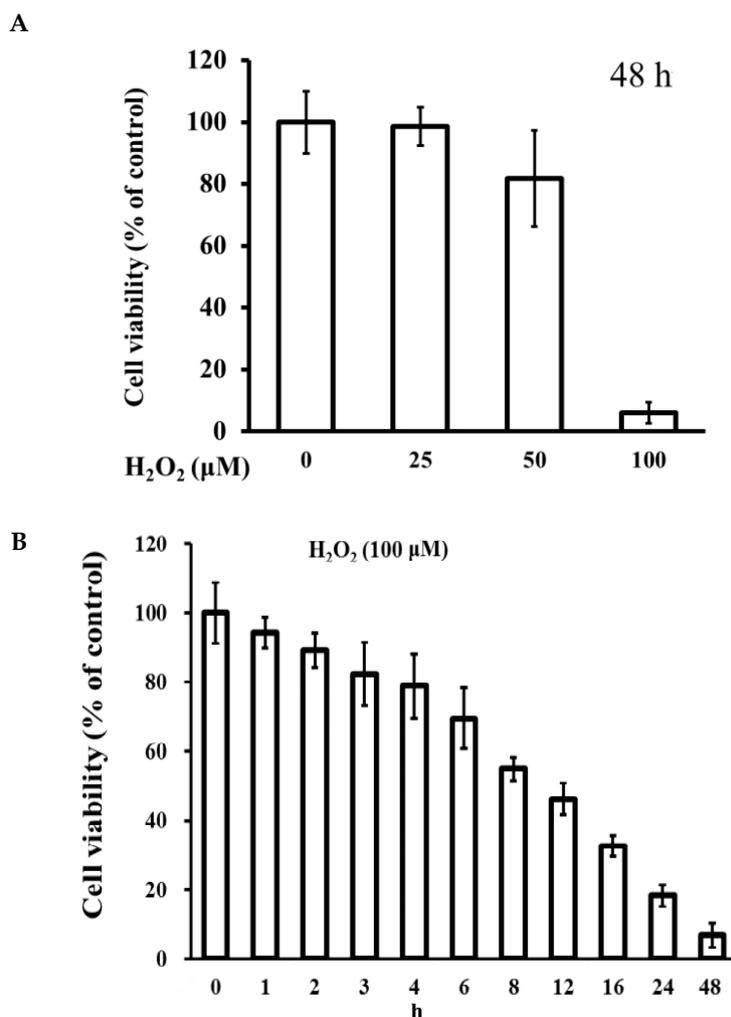


Figure 1. Cytotoxic effect of H₂O₂ on adult retinal pigment epithelium-19 (ARPE-19) cells. Cell viability of ARPE-19 cells, following different concentrations of H₂O₂ exposure, measured by trypan blue exclusion test. Dose (A); and (B) time effect of H₂O₂ (100 μM) on ARPE-19 cells. Results are expressed as percentages of control, and each value represents the mean ± SD of three independent experiments.

2.2. Effects of *P. ginseng* Supercritical CO₂ Fractions on Cell Viability of H₂O₂-Induced Cytotoxicity in ARPE-19 Cells

In the subsequent experiment, APRE-19 cells were left untreated or pretreated with various dosages of ginseng supercritical CO₂ fractions F1, F2, and F3, followed by treatment with H₂O₂. The results showed that H₂O₂ could reduce the viability of APRE-19 cells below 10% with chromosome condensation (Figure 2). The pretreatment of either F1 or F2 at a level of 2 mg/mL could rescue the cell death induced by H₂O₂ only up to 30%–40% accompanied by a reduction of chromosome condensation (Figure 2A,B,D,E). However, the pretreatment of F3 at a level of only 1 mg/mL showed stronger rescued effects of up to 60% on APRE-19 cell death induced by H₂O₂, with a significant reduction of chromosome condensation in the presence of H₂O₂ (Figure 2C,F). These results indicated that the F3 fraction could be an important component of *P. ginseng* in preventing cell death induced by oxidative stress.

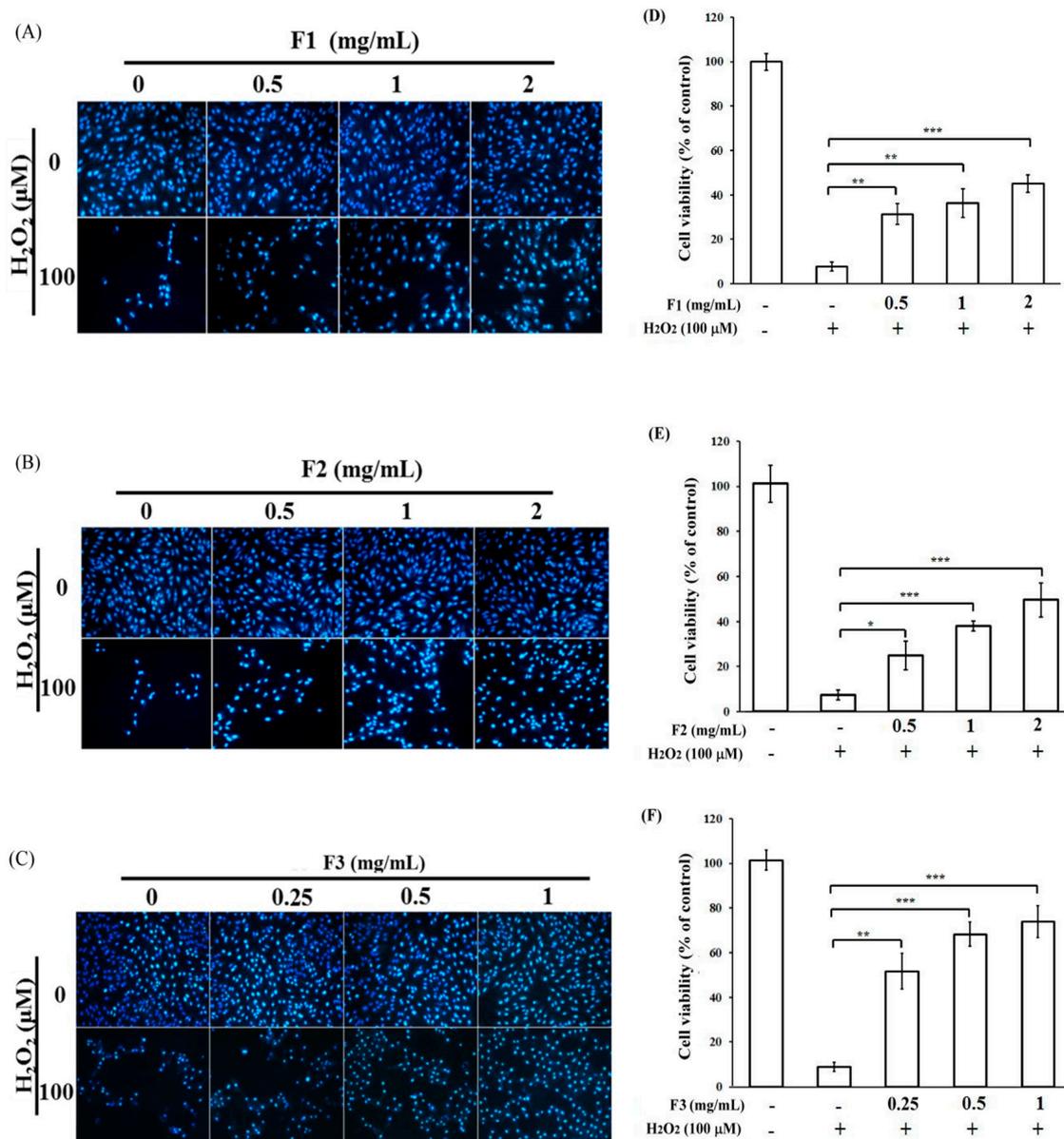


Figure 2. Effects of *P. ginseng* supercritical CO₂ extracted fractions on cell viability of H₂O₂-induced cytotoxicity in ARPE-19 cells. ARPE-19 cells were treated with or without a series of concentrations of *P. ginseng* supercritical CO₂ fractions for 2 h, and then treated with H₂O₂ for another 24 h. (A–C) Hoechst 33342 staining; (D–F) Quantitative analysis of panel (A–C). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus H₂O₂ group. Results are expressed as percentages of control, and each value represents the mean \pm SD of three independent experiments.

2.3. Effects of *P. ginseng* F3 Fraction on Cellular Oxidation Induced by H₂O₂ in ARPE-19 Cells

To confirm how F3 rescued ARPE-19 cells from H₂O₂-induced cell death, ARPE-19 cells were either untreated or pretreated with F3 fraction for 2 h followed by the administration of H₂O₂ for another 24 h, the cells were then collected and the contents of ROS were determined. The results showed the F3 fraction could effectively reduce the generation or accumulation of H₂O₂ in a dose-dependent manner (Figure 3A). Furthermore, both the lipid oxidation (MDA assay) (Figure 3B) and the DNA damage (8-OHdG assay) (Figure 3C) induced by H₂O₂ were also significantly attenuated in the presence of F3 in a dose-dependent manner. These results suggested that F3 might be able to prevent ARPE-19 cell death induced by H₂O₂ via attenuating the generation or accumulation of ROS and the subsequent lipid and DNA damages.

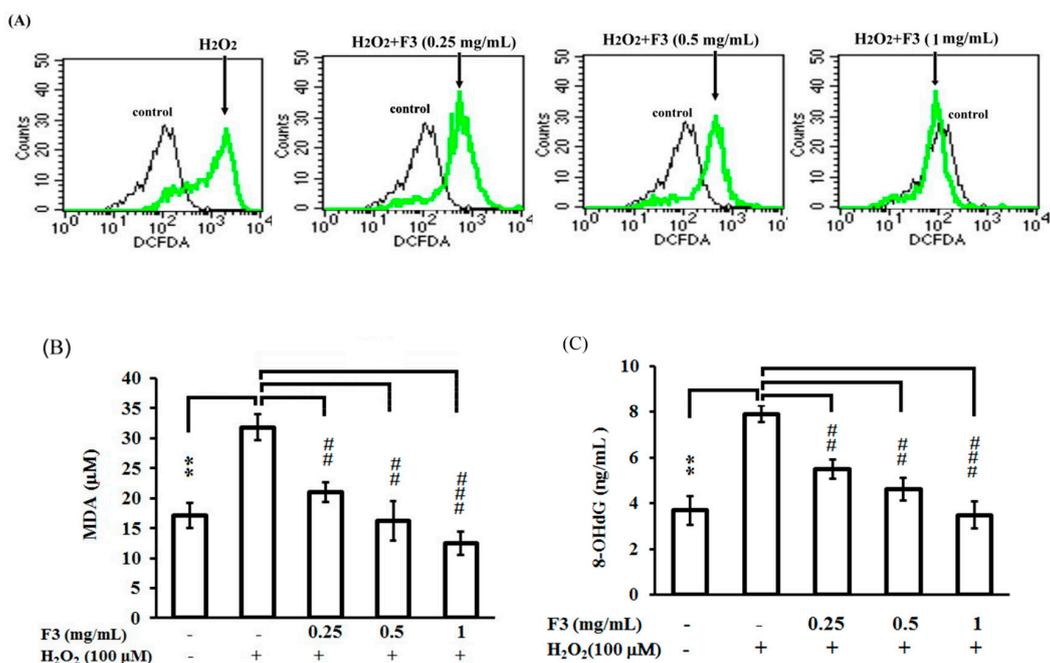


Figure 3. Effects of *P. ginseng* supercritical CO₂ extracted fraction F3 on H₂O₂-induced cellular oxidation in ARPE-19 cells. ARPE-19 cells were pretreated with or without a series of concentrations of F3 for 2 h, and then treated with 100 μM H₂O₂ for another 24 h. **(A)** Intracellular reactive oxygen species levels were measured by flow cytometry analysis; **(B)** malondialdehyde (MDA); and **(C)** 8-hydroxydeoxyguanosine (8-OHdG) contents were measured on a microplate reader (VersaMax). MDA and 8-OHdG levels in the H₂O₂ group increased significantly compared with the untreated group. F3 attenuated the H₂O₂-induced changes in MDA and 8-OHdG. ** $p < 0.01$ versus untreated group. ## $p < 0.01$, ### $p < 0.001$ versus H₂O₂ group. Each value represents the mean \pm SD of three independent experiments.

2.4. Effects of *P. ginseng* F3 Fraction on Antioxidant Enzymes Activities Induced by H₂O₂ in ARPE-19 Cells

In the next experiment, to confirm the relationships between ROS reduction and F3, we continued to analyze the effects of F3 in regulating the activity of antioxidant enzymes in the presence of H₂O₂. The results showed that the addition of H₂O₂ significantly decreased the activities of superoxide dismutase, catalase, and glutathione peroxidase in ARPE-19 cells (Figure 4). The pretreatment with F3 effectively restored the activities of all three tested antioxidant enzymes back to the normal levels even in the presence of H₂O₂. These results suggested that F3 might increase the activity of antioxidant enzymes to avoid the oxidative stress and subsequent cellular damage induced by H₂O₂.

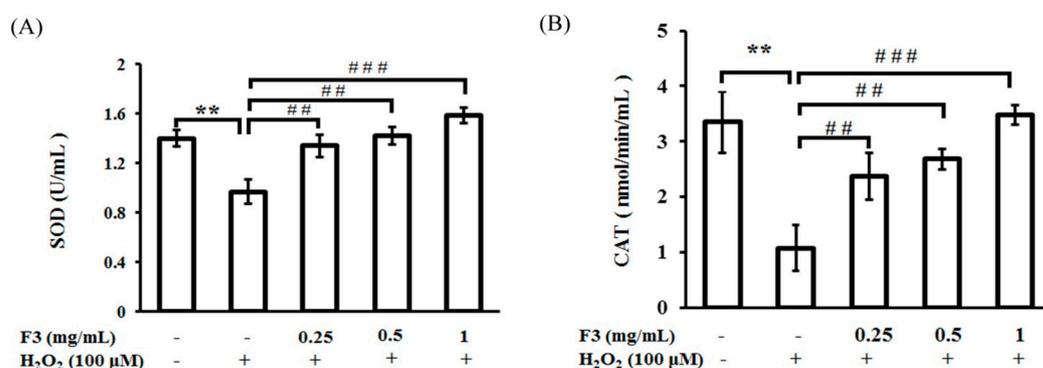


Figure 4. Cont.

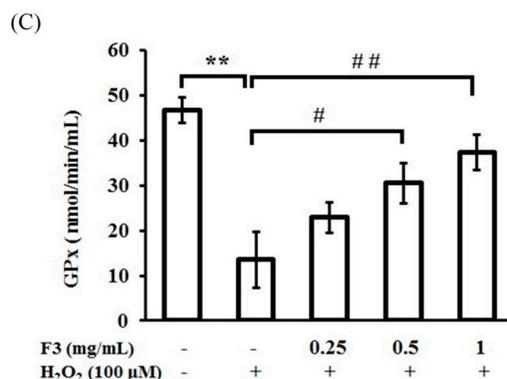


Figure 4. Effect of *P. ginseng* supercritical CO₂ extracted fraction F3 on cellular antioxidant enzymes: (A) superoxide dismutase (SOD); (B) catalase (CAT); and (C) glutathione peroxidase (GPx) activities in ARPE-19 cells subjected to H₂O₂-induced oxidative stress. ARPE-19 cells were pretreated with or without a series of concentrations of F3 for 2 h, and then treated with 100 μM H₂O₂ for another 24 h. Antioxidant enzymes activities were determined using commercially available kits following the manufactures' instructions. ** $p < 0.01$ versus untreated group, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ versus H₂O₂ group. Each value represents the mean \pm SD of three independent experiments.

3. Discussion

In this study, we investigated the antioxidation ability of supercritical CO₂ extracted fractions of the root powder of *P. ginseng* C. A. Meyer and explored the reasons for the high antioxidative activity exhibited by F3. Results from our previous study [20] showed that the E, R, F1, and F2 fractions were composed mainly of protopanaxadiol and protopanaxatriol and F3 contained a higher level of deglycosylated ginsenosides comparing with other fractions of *P. ginseng*. Since deglycosylated ginsenosides are involved in the oxidative stress-resistant process, it is reasonable to infer that the highest antioxidation ability of F3 could contribute to the presence of deglycosylated ginsenosides. Both protopanaxadiol and protopanaxatriol exhibited minor effects in protecting ARPE-19 cells from H₂O₂-induced oxidative stress. In addition, deglycosylated ginsenosides showed the strongest protective effects in ARPE-19 cells against cytotoxicity induced by H₂O₂. Furthermore, deglycosylated ginsenosides recovered the activity of antioxidant enzymes, including the H₂O₂-induced oxidative damage, catalase, and glutathione peroxidase, suggesting that a possible pathway of deglycosylated ginsenosides of *P. ginseng* in reducing oxidative stress.

Under normal physiological conditions, cells need to maintain an optimal level of ROS to manipulate a proper signal transduction and to avoid damage by free radicals. The free radical theory of aging suggests that aging and its related disorders are caused by the continuous accumulation of ROS, which can react with multiple molecules, such as DNA, protein, and lipids. In the retinal system, the oxidative damage from ROS induced by the combination of focused light and oxygen in retina epithelial cells is involved in the pathogenesis of age-related macular degeneration [1]. Woo et al. showed that curcumin could protect retinal pigment epithelial cells against oxidative stress by inducing the levels of phase II enzymes heme oxygenase-1 [21]. Wankun et al. also demonstrated a protective effect of paeoniflorin against oxidative stress via the mitogen-activated protein kinase pathways in retinal pigment epithelial cells [22]. Furthermore, Li et al. showed that astaxanthin could protect retinal pigment epithelial cells from oxidative stress via upregulating phase II enzymes in accompanied with the activation of PI3K/Akt [23]. These results showed that the upregulation of metabolic enzymes is a major pathway for protection against oxidative stress. However, these reports did not mention the effects of these compounds in the activities of these metabolic enzymes. Conversely, our results clearly showed that an activity induction of metabolic enzymes, including superoxide dismutase, catalase, and glutathione peroxidase by supercritical CO₂ extracted fraction F3 of *P. ginseng* in the condition of

oxidative stresses primed by H₂O₂ (Figure 4). Therefore, the role of F3 in the pathogenesis of oxidative stress-related ocular diseases warrants further investigation.

Ginseng is a popular medicinal herb that contains plenty of its active constituent ginsenosides. Some components of ginsenosides, for example, Rb1, Rg1, and Re, might activate certain antioxidant enzymes and thereby offer protection against cell death. An in vitro study of oxidative stress and cell death in brain cells showed that the protective effects of *P. notoginseng* in astrocytes were associated with attenuation of ROS accumulation. Such effect was involved the activation of Nrf2 (nuclear translocation) and upregulation of downstream antioxidant systems, which included heme oxygenase-1 and glutathione S-transferase pi 1 [24]. Betts et al. described that Rb1 ginsenoside could potentially induce ARPE-19 proliferation and thereby reduced the release of vascular endothelial growth factor [25]. When vascular endothelial growth factor is overexpressed, it can contribute to vascular disease in the retina of the eye. Furthermore, Li et al. demonstrated that Rg1 ginsenoside could protect retinal pigment epithelial cells against hypoxia and cobalt chloride assaults [26], indicating a protective role of *P. ginseng* in retinal pigment epithelial cells. In the present study, we showed that H₂O₂ could strongly induce intracellular ROS generation and mortality of ARPE-19 cells, and the effect could significantly be alleviated by the treatment of F3 (Figure 3).

4. Materials and Methods

4.1. Materials

P. ginseng C. A. Meyer was obtained from Wah Hong Ginseng Company, Kaohsiung City, Taiwan. 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA), fetal bovine serum, H₂O₂, bisBenzimide H33342 trihydrochloride, and trypan blue solution were purchased from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle's medium was purchased from Invitrogen. Lipid Peroxidation (malondialdehyde, MDA) Colorimetric/Fluorometric Assay Kit, Superoxide Dismutase (SOD) Activity Assay Kit, Catalase Activity Colorimetric/Fluorometric Assay Kit and Glutathione Colorimetric Assay Kit were purchased from Biovision (Milpitas, CA, USA). DNA damage ELISA kit (8-hydroxydeoxyguanosine, 8-OHdG) was purchased from Enzo Life Sciences, Inc. (Farmingdale, NY, USA).

4.2. Extraction and Fractionation

Ethanolic extracts from *P. ginseng* C. A. Meyer was fractionated into R, F1, F2, and F3 according to the method described in our previous work [20]. The root powder of *P. ginseng* was extracted twice using 85% ethanol at a ratio of 1:2 (*w/v*) for 24 h per extraction. The operating conditions of the fractionation were set at a temperature of 45 °C, a system pressure of 30 MPa, a flow rate of CO₂ at 4 mL/min, and a sample inflow rate of 1 mL/min. A series of separation vessels was operated at pressures of 30, 25, 20 and 15 MPa to obtain three fractions (F1, F2, and F3) and a residue (R). The obtained samples were freeze-dried and stored at −20 °C until use.

4.3. Cell Culture and Viability Assay

ARPE-19 cells (ATCC[®] CRL-2302), originated from human retinal pigment epithelial cells, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (*v/v*) heat-inactivated fetal bovine serum and 100 U/mL penicillin and streptomycin. The cells were incubated at 37 °C in a humidified 5% CO₂ atmosphere. The cells were plated in 12-well culture dishes at a density of 2 × 10⁵ cells/mL and incubated overnight. Cells were pretreated with each ginseng fraction (0.25–2 mg/mL) for 2 h and then stimulated with H₂O₂ (100 μM) for indicated time points. The number of viable cells was determined by the trypan blue dye exclusion method. Cell viability was expressed as a percentage of the value in the control.

4.4. Hoechst 33342 Staining

Hoechst 33342 staining was used to observe changes of nuclei morphology of ARPE-19 cells after H₂O₂ treatment. After treatment with the indicated concentrations of H₂O₂ and fractions, cells were stained with 10 μM Hoechst 33342 for 15 min at room temperature. The stained cells were rinsed three times with phosphate-buffered saline and then observed using a fluorescence microscope with standard excitation filters.

4.5. Measurement of Intracellular Reactive Oxygen Species

Intracellular ROS levels were measured using 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA), which is converted into a non-fluorescent derivative by intracellular esterases. ARPE-19 cells were plated at a density of 5 × 10⁵ cells per 6-well plates and incubated overnight. Cells were pretreated with each F3 fraction (0.25–1 mg/mL) for 2 h and then exposed to 100 μM H₂O₂ for 24 h. The cells were incubated with H₂DCFDA at 5 μM for 30 min at 37 °C. At the end of incubation, the cells were washed three times with phosphate-buffered saline and harvested. Fluorescence was measured with a FACScan flow cytometer. ROS levels were measured using excitation and emission wavelengths of 488 nm and 525 nm, respectively. Enzyme-linked immunosorbent assays (ELISA) were used to measure 8-OH-dG levels, according to the manufacturer's instructions. MDA was measured at a wavelength of 532 nm by reacting with thiobarbituric acid (TBA) and the results were expressed as thiobarbituric acid reactive substances (TBARS).

4.6. Determination of Superoxide Dismutase, Catalase, and Glutathione Peroxidase Activities

ARPE-19 cells were plated at a density of 5 × 10⁵ cells per 6-well plates and incubated overnight. Cells were pretreated with each F3 fraction (0.25–1 mg/mL) for 2 h and then exposed to 100 μM H₂O₂ for 24 h. After cultured, the cells were washed with phosphate-buffered saline. The cells were then scraped from the plates into ice-cold phosphate-buffered saline and homogenized. Intracellular superoxide dismutase, catalase, and glutathione peroxidase activities were determined using commercially available kits and measured spectrophotometrically at 450, 520 and 415 nm, respectively.

4.7. Statistical Analysis

All data are expressed as mean ± standard deviation of at least three independent experiments. One-way analysis of variance with Duncan's multiple range tests were used for comparisons. Two-tailed $p < 0.05$ was considered statistically significant.

5. Conclusions

In conclusion, the present study showed that F3, but not F1 and F2, from supercritical CO₂ extracted fractions of root powder of *P. ginseng* C. A. Meyer, could effectively protect ARPE-19 cells against H₂O₂-induced cell death via a reduction in oxidative stress. Further results demonstrated that F3 could potentially increase the activity of phase II enzymes, including superoxide dismutase, catalase, and glutathione peroxidase in the presence of H₂O₂. Therefore, the confirmative component of F3 responsible for the strong protective effect of retinal pigment epithelial cells should be elucidated further.

Author Contributions: Chao-Chin Yang, Be-Jen Wang, and Zer-Ran Yu conceived and designed the experiments; Chao-Chin Yang and Chiu-Yuan Chen performed the experiments; Chiu-Yuan Chen and Chun-Chi Wu analyzed the data; Be-Jen Wang and Chun-Chi Wu contributed reagents/materials/analysis tools; Chao-Chin Yang and Malcolm Koo wrote the paper. All authors read and approved the final manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

8-OHdG	8-hydroxydeoxyguanosine
ARPE-19	adult retinal pigment epithelium-19
CAT	catalase
CO ₂	carbon dioxide
H ₂ DCFDA	2',7'-dichlorodihydrofluorescein diacetate
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
GPx	glutathione peroxidase
H ₂ O ₂	hydrogen peroxide
MDA	malondialdehyde
ROS	reactive oxygen species
SD	standard deviation
SOD	superoxide dismutase
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substances

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