

Polyphenol-enriched fraction of *Vaccinium uliginosum* L. protects selenite-induced cataract formation in the lens of Sprague-Dawley rat pups

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Purpose: As the aging population is increasing, the incidence of age-related cataract is expected to increase globally. The surgical intervention, a treatment for cataract, still has complications and is limited to developed countries. In this study, we investigated whether the polyphenol-enriched fraction of *Vaccinium uliginosum* L. (FH) prevents cataract formation in Sprague-Dawley (SD) rat pups.

Methods: Sixty rat pups were randomly divided into six groups: CTL, Se, FH40, FH80, FH120, and Cur80. The cataract was induced with subcutaneous injection of sodium selenite (18 μ mol/kg bodyweight) on postnatal (P) day 10. All groups, except CTL, were injected with sodium selenite, and the FH40, FH80, and FH120 groups were given gastric intubation with FH40 mg/kg, 80 mg/kg, and 120 mg/kg on P9, P10, and P11. The Cur80 group was also given gastric intubation with curcumin 80 mg/kg on P9, P10, and P11. All rat pups were euthanized on P30.

Results: Lens morphological analysis showed that FH dose-dependently inhibited cataract formation. In the Se group, soluble proteins were insolubilized, and the gene expression of the α -, β -, and γ -crystallins was downregulated. However, FH treatment statistically significantly inhibited insolubilization of soluble proteins and downregulation of the gene expression of the α -, β -, and γ -crystallins. In the Se group, the gene and protein levels of m-calpain were downregulated, which were attenuated with FH treatment. In addition, sodium selenite injection caused reduced antioxidant enzymes (superoxide dismutase (SOD) and glutathione peroxidase (GPx)), glutathione (GSH) depletion, and malondialdehyde (MDA) production in the lens. The administration of FH inhibited sodium selenite-induced oxidative stress in a dose-dependent manner. The mechanism of protection against oxidative stress by FH involves NF-E2-related factor (Nrf-2) and hemoxygenase-1 (HO-1). FH treatment inhibited decrease of Nrf-2 in the nucleus fraction and HO-1 in the cytosol fraction. Finally, the FH treatment protected poly (ADP)-ribose polymerase (PARP) from cleavage, determined with western blotting.

Conclusions: FH showed a preventive effect against cataract formation by inhibiting m-calpain-mediated proteolysis and oxidative stress in the lens. These results suggest that FH could be a potential anticataract agent in age-related cataract.

The eye is an indispensable sensory organ that enables us to interpret colors, shapes, and depth by receiving light. The eye has its own defensive systems against oxidative stress induced by light exposure (ultraviolet and visible light). However, the ability to protect against this stress diminishes as the eye ages; thus, diverse ocular diseases (like cataract, macular degeneration, and retinopathy) occur in the elderly [1]. Among eye diseases, cataract is the leading cause of vision loss worldwide [2]. Cataract is a clouding of the lens and mainly caused by aging. It is usually characterized by several symptoms such as decreased visual acuity,

glare, myopic shift, and monocular diplopia. Presently, age-related cataract can be easily treated through lens extraction and artificial intraocular lens implantation. However, the surgical treatment is invasive and still has complications, such as double vision, posterior capsule opacification, cystoid macular edema, and detached retina [3]. The treatment is also limited to developed countries, due to the cost of the surgery and the need for modern facilities and highly skilled personnel. As cataract is more prevalent in developing countries, preventive intervention for cataract must be investigated to reduce the burden of the surgery.

Oxidative stress is substantially involved in the onset and development of age-related cataract. The stress results from an imbalance between the production of reactive oxygen species (ROS) and the antioxidant system [4]. Oxidants accumulate with aging; however, antioxidant activities diminish

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progressively in the eye. In the prooxidant status, calcium ATPase in the plasma membrane is oxidized and loses its ion-transferring function; therefore, calcium begins to accumulate in the lens epithelial cells. The elevated calcium activates a calcium-dependent cysteine protease, called m-calpain. The activated m-calpain results in proteolysis of α - and β -crystallins, followed by insolubilization and coprecipitation of γ -crystallin [5]. The dysfunction and insolubilization of crystallins reduce transparency in the lens. In addition, accumulated ROS can damage intracellular macromolecules, especially mitochondrial DNA. It was reported that oxidative stress increases apoptosis in the rat lens determined by Bax/Bcl-2 balance and caspase 3 cleavage [6]. To prevent the onset of age-related cataract, maintaining the antioxidant activity is crucial. The nuclear factor (erythroid-derived 2)-like 2 (Nrf-2) and hemoxygenase-1 (HO-1) signaling pathway contribute to protection against oxidative stress in the lens and the retina [7,8]. Nrf-2 is generally activated when exposed to mild oxidative or electrophilic stress in the cells; then, it controls the upregulation of genes whose proteins are involved in the elimination and detoxification of ROS and electrophilic agents [9]. Little Nrf-2 activity could lead to the loss of cytoprotection, diminished antioxidant capacity, and reduced beta-oxidation [10]. HO-1 is induced by the Nrf-2-ARE pathway and degrades heme to CO, iron, and biliverdin. Due to the antioxidant property of biliverdin, HO-1 has long been considered a cytoprotective enzyme [11]. Therefore, natural products with antioxidant and antiapoptotic properties could be potential anticataract agents.

Vaccinium uliginosum L. (V.U), called bog bilberry, is a deciduous shrub found in Europe, North America, northern China, the Korean peninsula, and Japan. V.U is known to contain glycosides, anthocyanin, organic acids, and polyphenols. As flavonoids and anthocyanins in berry crops have been investigated for their ocular benefits, such as retinal cell protection, neuroprotection, night vision, and visual pigment function, berry crops (including bog bilberry) are consumed for vision and eye health [12]. In previous studies, we prepared various V.U fractions of water or ethanol or HP20 resin. Among the fractions, *Vaccinium uliginosum* L. using with Diaion HP-20 resin (FH) showed superior antioxidant and protective effects compared to the V.U extract and its other fractions (water and ethanol). In the preparation of FH, sugar/aid and polysaccharides were removed, which resulted in the enriched-polyphenol fraction of the V.U extract. We previously established the ultra performance liquid chromatography (UPLC) profile and identification of the V.U extract and isolated active compounds from the V.U extract fraction. FH contains several flavonoids (quercetin, hyperoside, guajaverin, myricetin, syringetin-3-O- β -d-galactopyranoside,

and myricetin-3-O- β -d-galactopyranoside), anthocyanin (cyanidin-3-O- β -d-glucopyranoside), phenyl propanoid (chlorogenic acid and methylchlorogenate), and iridoid (loganic acid and splendoside) [13]. Flavonoids, including quercetin, hyperoside, and rutin, have been reported to have antioxidant effects and inhibitory effects on advanced glycation end-product formation, sorbitol accumulation, and aldose reductase in healthy and diabetic lenses [14-18]. In addition, FH protects against RPE cell death via inhibiting oxidative stress in RPE cells and mice [13]. FH treatment inhibited oxidative stress-induced transcriptional activity of nuclear factor-kappa beta (NF- κ B) and AP-1, and apoptosis in ARPE-19 cells [19]. Moreover, oral administration of FH protected against retinal degeneration induced by blue light in mice, determined with histological analysis of photoreceptor cells in the retina [13]. As it has been demonstrated that FH has beneficial effects on eye health, we hypothesized that FH could inhibit cataract formation via maintenance of the antioxidant defense system and inhibition of apoptosis in the lens.

METHODS

Materials and chemicals: FH was obtained from a previous study [13]. Curcumin was purchased from Carl Roth GmbH Co. (Karlsruhe, Germany). Sodium selenite (Na_2SeO_3) was purchased from Sigma-Aldrich (St. Louis, MO). The primary antibodies of m-calpain (Cell Signaling Technology, Danvers, MA), Nrf-2 (Santa Cruz Biotechnology, Dallas, TX), HO-1 (Enzo Life Sciences, Plymouth Meeting, PA), poly (ADP)-ribose polymerase (PARP; Cell Signaling Technology), and β -actin (Santa Cruz Biotechnology) were purchased for western immunoblotting. All chemicals and reagents were of analytical grade.

Animals: Sixty Sprague-Dawley (SD) rat pups were purchased from DBL (Cheongju, Republic of Korea). The 6-day-old rat pups, weighting 16–19 g, were maintained along with their mothers in a controlled environment (25 ± 1 °C, 12 h:12 h light-dark cycle). The mothers were provided with a standard diet and water ad libitum. This study was performed in strict accordance with ARVO Statement for Use of Animals in Research, and the protocol was approved by the Institutional Animal Care and Use Committee guideline of Kyung Hee University (KHUASP(SE)-18–019).

Experimental design: The 60 SD rat pups were randomly divided into six groups as follows.

CTL group: rat pups treated with nothing.

Se group: rat pups treated with sodium selenite (18 $\mu\text{mol}/\text{kg}$ bodyweight)

FH40 group: rat pups treated with sodium selenite (18 $\mu\text{mol/kg}$ bodyweight) and FH (40 mg/kg bodyweight)

FH80 group: rat pups treated with sodium selenite (18 $\mu\text{mol/kg}$ bodyweight) and FH (80 mg/kg bodyweight)

FH120 group: rat pups treated with sodium selenite (18 $\mu\text{mol/kg}$ bodyweight) and FH (120 mg/kg bodyweight)

Cur80 group: rat pups treated with sodium selenite (18 $\mu\text{mol/kg}$ bodyweight) and curcumin (80 mg/kg bodyweight).

The 6-day-old rat pups were maintained for 3 days before treatment. Each group was orally administered each sample from P9 to P11. However, the CTL and Se groups were treated with vehicle (0.5% CMC). On P10, all groups, except CTL, were given a subcutaneous injection of sodium selenite to induce cataract formation. On P16, cataract staging was performed using an ophthalmoscope. Then, all rat pups were euthanized on P30 for the following studies. All rat pups were euthanized by cervical dislocation on P30 for the following studies. The dosage of curcumin, served as a reference control, was determined according to Manikandan et al. [20].

Classification of cataract: On P16, the degree of opacification in the lens was evaluated based on a scale ranging from 0 to 6 according to published methods [21]. Stage 0: clear transparent lens; stage 1: slight light scattering, only observed with a white light lamp; stage 2: light scattering with the naked eye and swollen fibers; stage 3: diffused form of nuclear opacity with the naked eye; stage 4: partial nuclear opacity; stage 5: dense nuclear opacity; and stage 6: mature dense opacity.

Soluble and insoluble protein assay: Soluble and insoluble proteins were estimated according to previous methods [6]. Briefly, the lenses were surgically obtained with the posterior approach on P30. One of the lenses was homogenized in ice-cold PBS (1X; 135 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 , 1.4 mM KH_2PO_4 , pH 7.4) and centrifuged (12,000 $\times g$, 20 min, 4 $^\circ\text{C}$). The supernatant was used for estimation of

the soluble protein and other enzymes. The remaining pellet was redispersed in sodium hydroxide and centrifuged. The supernatant was used for the insoluble protein. The protein assay was performed with the Pierce BCA protein assay kit (Thermo Fisher Scientific Co., Fair Lawn, NJ).

Estimation of GSH, MDA, SOD, and GPx: Glutathione (GSH), malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GPx) were measured in the supernatant of the lens homogenates. GSH and MDA levels were measured with the enzyme-linked immunoassay (ELISA) kit from Elabscience (Bethesda, MD) according to the manufacturer's instruction. SOD activity was determined with the SOD assay kit (Cayman Chemical Co., Ann Arbor, MI), and GPx activity was estimated with the GSH-PX colorimetric assay kit from Elabscience. SOD activity was presented as units per milligrams of lens tissue, and GPx was presented as unit per minute per gram of lens tissue.

Real-time quantitative PCR: The lenses were homogenized in easy-RED reagent from iNtRON biotechnology (Seongnam, Republic of Korea) according to the manufacturer's protocol. After extraction of total RNA, cDNA was synthesized using a cDNA synthesis kit (TaKaRa, Tokyo, Japan). The gene expression was quantitatively measured using an ABI Step One PlusTM RT-PCR system (Applied Biosystems, Foster City, CA) with SYBR Green Premix Ex Taq (TaKaRa). The mixtures were incubated for an initial duration at 95 $^\circ\text{C}$ for 10 min, followed by 60 cycles of 95 $^\circ\text{C}$ for 5 s, and Tm temperature for 30 s and 72 $^\circ\text{C}$ for 30 s. Gene expression was normalized to glyceraldehyde 3-phosphated dehydrogenase (GAPDH, internal control). The mRNA levels were expressed as relative fold changes. The primer sequences are shown in Table 1.

Immunoblot analysis: The immunoblot analysis was performed according to previous methods [6]. Briefly, the lenses were homogenized in lysis buffer (20 mM Tris-HCL (pH 7.4), 0.5 M EDTA (pH 8.0), 1 mM Na_3VO_4 , 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.32 mM

TABLE 1. DETAILS OF PRIMERS USED FOR REAL TIME qPCR.

| No. | Gene | Sequence (Forward / Reverse) | |
|-----|--|------------------------------|-------------------------|
| 1 | $\alpha\text{A-crystalline}$ | TGGCAAACACAACGAGAGGCA | GCCAGCATCCAAGCCAGACT |
| 2 | bB1-crystalline | CACCAACACAGCATCAGAAACCAT | GTTTTCTGCTCAAAGACGACCA |
| 3 | rD-crystalline | CGACTCTGTCCGCTCCTGCC | TACTCCCCGGGCCTCAGCAG |
| 4 | m-calpain | ATCGCCTCCCTCACCTTGAAT | AGCTTTCAACCTCCTCGGCTCC |
| 5 | Nrf-2 | CACATCCAGACAGACACCAGT | CTACAAATGGGAATGTCTC TGC |
| 6 | HO-1 | CAGAAGGGTCAGGTGTCCAG | GAAGGCCATGTCCTGCTCTA |
| 7 | GAPDH | GCCAGCCTCGTCTCATAGACA | AGAGAAGGCAGCCCTGGTAAC |

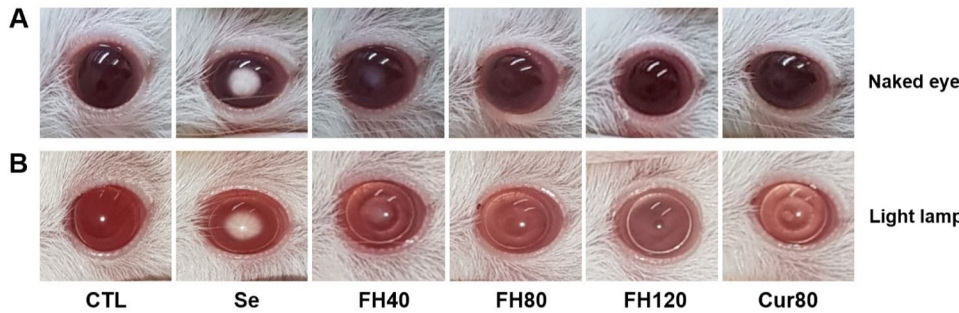


Figure 1. Effects of FH on cataract formation. Representative anterior images of all groups were taken (A) without or (B) with a white-light lamp on P16. The CTL group shows a clear lens (stage 0); however, the Se group shows severe cataract formation (stage 5). FH treatment dose-dependently inhibited cataract

formation. CTL: normal control, Se: selenite-treated, FH40/80/120: selenite/FH-treated (40, 80, and 120 mg/kg, respectively), Cur80: selenite/curcumin-treated (80 mg/kg).

sucrose, protease inhibitor cocktail). The lysates were sonicated for 30 min and centrifuged (16,000 ×g at 4 °C) for 15 min. The proteins were separated and transferred to the nitrocellulose membrane. Immunoblot analysis was performed using primary antibodies of m-calpain, Nrf-2, HO-1, PARP, and β-actin. The protein bands were visualized using a LAS3000 Luminescent image analyzer (Fuji Film, Tokyo, Japan). The protein expression levels were quantified with band density, with Image Lab statistical software (Bio-Rad, Richmond, CA).

Statistical analyses: The Shapiro-Wilk test was performed for the normality of the data: Normally distributed data were soluble and insoluble protein levels, GSH, MDA, SOD, and GPx. These results were analyzed with one-way ANOVA followed by the Tukey multiple comparison test. Not normally distributed data were analyzed with the Mann-Whitney test. Statistically significant differences were assessed using SPSS (Chicago, IL).

RESULTS

Effects of FH on cataract formation: We first examined morphological changes in the lenses of rat pups on P16. The degree of opacification was determined and classified as six stages according to a previous study [21]. Anterior views of the lenses were taken with the naked eye or a white-light lamp. In the Se group, mature and dense opacities were observed in most lenses; however, little light scattering or diffuse opacities were observed in the lenses of the FH-treated rat pups (Figure 1). Light scattering, not observed with the naked eye, was easily detected in the FH80 and FH120 groups with the light lamp. Most of the Se group belonged to stage 5, and FH treatment inhibited opacity formation in a dose-dependent manner according to the median and mean values (Table 2). Curcumin, which served as the reference control, also prevented cataract formation, but it showed higher median and mean values compared to the same dose of FH.

Effects of FH on soluble and insoluble proteins and α-, β-, and γ-crystallins in the lens: The loss of transparency in the lens usually results from insolubilization, coprecipitation,

TABLE 2. CLASSIFICATION OF CATARACT FORMATION IN RAT PUPS.

| Groups (n=10) | Number of lenses with different degree of opacification | | | | | | | Mean | SD |
|---------------|---|---------|---------|---------|---------|---------|---------|-----------|------|
| | Stage 0 | Stage 1 | Stage 2 | Stage 3 | Stage 4 | Stage 5 | Stage 6 | | |
| CTL | 10 | - | - | - | - | - | - | 0 | 0 |
| Se | - | - | - | - | 2 | 6 | 2 | 5### | 0.67 |
| FH40 | - | - | 1 | 5 | 3 | 1 | - | 3.4*** | 0.84 |
| FH80 | - | - | 4 | 5 | 1 | - | - | 2.7*** | 0.67 |
| FH120 | - | 3 | 6 | 1 | - | - | - | 1.8***YYY | 0.63 |
| Cur80 | - | - | 2 | 5 | 2 | 1 | - | 3.2*** | 0.92 |

###p<0.001 statistically significant different from CTL. ***p<0.001 statistically significant different from Se. YYYp<0.001 statistically significant different from CTL. ***p<0.001 statistically significant different from Cur80. CTL: normal control, Se: selenite-treated, FH80: selenite/FH-treated (80 mg/kg), Cur80: selenite/curcumin-treated (80 mg/kg).

and aggregation of lens-soluble proteins, including crystallins. Among the crystallins, α -crystallin is predominant in lens-soluble proteins, constituting up to 50%. α -crystallin has chaperone activity under mild oxidative and osmotic stress; however, excessive stress can impair the chaperone function [22]. Its activity and mRNA expression are also impaired in galactosemic and selenite-induced cataract [23]. The β - and γ -crystallins also have crucial roles in maintaining lens transparency, and the gene expression of the crystallins was downregulated in the selenite cataract model [24]. In this study, we estimated water-soluble and insoluble protein levels in the lenses of rat pups. The water-soluble protein level was statistically significantly decreased; however, the water-insoluble protein level showed a substantial increase in the lenses of the Se group (Figure 2A,B). Treatment of FH dose-dependently inhibited insolubilization of water-soluble proteins in the lenses. In the RNA analysis of α A-, β B1-, and γ D-crystallins, all mRNA levels in three crystallins were downregulated in the Se group (Figure 2C). FH and curcumin treatment inhibited downregulation of the crystallins. These results suggest that FH protects selenite-induced protein insolubilization and abnormal changes in lens crystallins.

Effects of FH on m-calpain in the lens: In the pathogenesis of nuclear cataract, calpain-induced proteolysis is highly associated with the onset of opacification [25]. Calpains are classified as μ -calpain, m-calpain, and lenticular preferred calpains (Lp82 and Lp85). Among the calpains, m-calpain is the predominant proteolytic enzyme in the human lens

and rodent lens [26]. In this study, we estimated the gene and protein expression levels of m-calpain in rodent lenses (Figure 3A,B). In the Se group, the gene and protein expression levels were statistically significantly reduced compared to the normal control. However, the decrease in both expression levels was compromised with the treatment of FH. This result demonstrated that FH showed inhibitory effects on calpain-induced proteolysis.

Effects of FH on GSH, MDA, SOD, and GPx in the lens:

The lens is susceptible to oxidative stress caused by UV irradiation, metabolic changes, and osmotic stress [27]. The lens is continually damaged by ROS production with age, leading to a decrease in the antioxidant system. Selenite cataract, a model of age-related cataract, is characterized by impaired antioxidant systems and the accumulation of oxidants. A healthy lens contains GSH, SOD, and GPx. GSH usually protects cellular components from ROS. SOD is an antioxidant enzyme catalyzing the dismutation of the superoxide radical into oxygen and hydrogen peroxide. GPx has peroxidase activity reducing lipid hydroperoxides. We measured GSH, MDA, SOD, and GPx activity in the lenses (Figure 4A-D). The GSH, SOD, and GPx levels were statistically significantly diminished, while the level of MDA, a marker of lipid peroxidation, was substantially increased in the Se group. The depletion of GSH and the reduced activities of SOD and GPx were inhibited by administration of FH. Therefore, the results suggest that FH maintains protective status against oxidative stress in the lens.

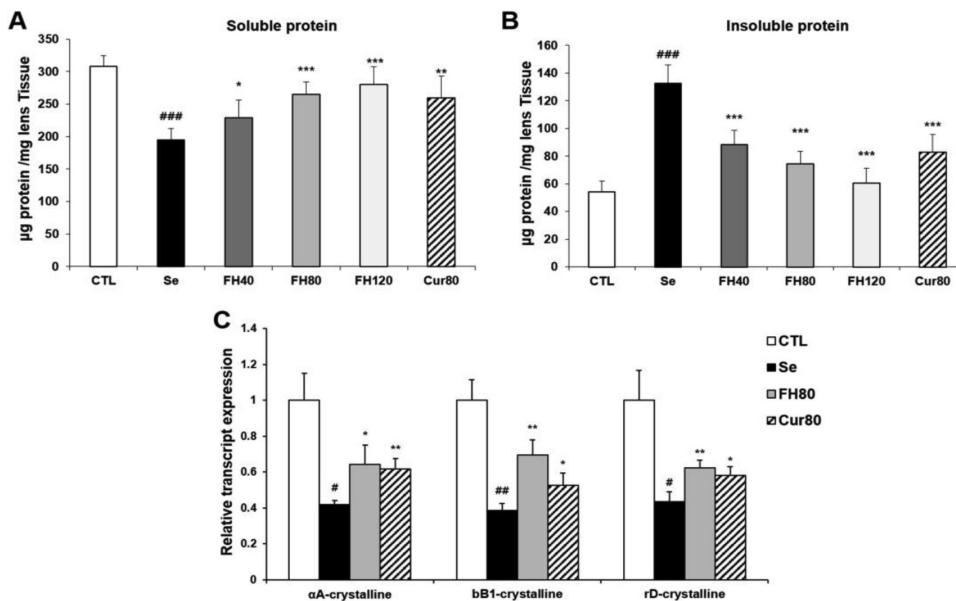


Figure 2. Effects of FH on soluble and insoluble proteins and α -, β -, and γ -crystallins in the lens. (A) Soluble and (B) insoluble proteins of lenses were analyzed in all groups. The results were represented as the mean \pm standard deviation (SD) of three independent experiments (n=6). C: The gene expression of the α -, β -, and γ -crystallins were analyzed using real-time polymerase chain reaction, and then normalized to glyceraldehyde 3-phosphated dehydrogenase (GAPDH). The transcript levels were expressed as the mean \pm standard deviation (SD) of three independent experiments (n=4). #p<0.05, ##p<0.01, ###p<0.001

statistically significant different from CTL. *p<0.05, **p<0.01, ***p<0.001 statistically significant different from Se. CTL: normal control, Se: selenite-treated, FH40/80/120: selenite/FH-treated (40, 80, 120 mg/kg, respectively), Cur80: selenite/curcumin-treated (80 mg/kg).

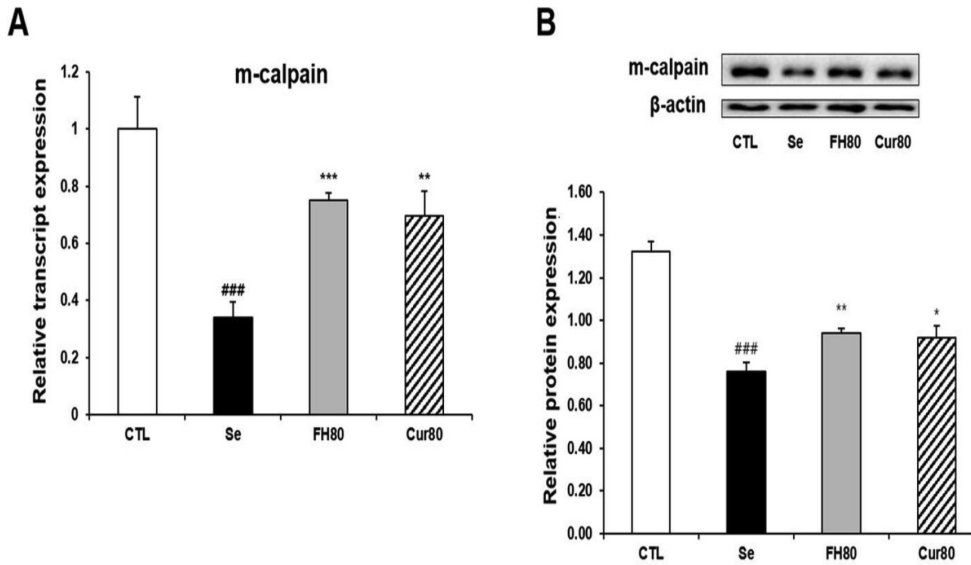


Figure 3. Effects of FH on m-calpain in the lens. **A:** Gene expression of m-calpain was analyzed using real-time polymerase chain reaction, and then normalized to glyceraldehyde 3-phosphated dehydrogenase (GAPDH). The transcript levels were expressed as mean \pm standard deviation (SD) of three independent experiments (n=4). **B:** Protein expression of m-calpain was analyzed using western blotting. The protein levels were quantified with band density. The protein levels were presented as mean \pm standard deviation (SD) of three independent experiments (n=4). ###p<0.001 statistically significant different from CTL. *p<0.05, **p<0.01, ***p<0.001 statistically significant different from Se. CTL: normal control, Se: selenite-treated, FH80: selenite/FH-treated (80 mg/kg), Cur80: selenite/curcumin-treated (80 mg/kg).

p<0.01, *p<0.001 statistically significant different from Se. CTL: normal control, Se: selenite-treated, FH80: selenite/FH-treated (80 mg/kg), Cur80: selenite/curcumin-treated (80 mg/kg).

FH inhibits the selenite-induced decrease in Nrf-2 and HO-1 in the lens: The Nrf-2/HO-1 signaling pathway has been considered a cellular defense system against oxidative stress. In healthy or unstressed lens, Nrf-2 is kept inactivated by binding with Kelch-like ECH associating protein 1 (Keap1) in the cytoplasm. Nrf-2 breaks free from Keap1 under oxidative stress; then Nrf-2 is translocated into nucleus, resulting

in binding to the antioxidant response element (ARE) in the DNA. The activation of Nrf-2 regulates many antioxidant genes (including HO-1, SOD, and glutathione reductase) and increases synthesis of GSH [7,28]. HO-1 plays an important role in cellular protection with antioxidant and anti-inflammatory effects [29]. In this study, real-time polymerase chain reaction (PCR) and western blotting were performed

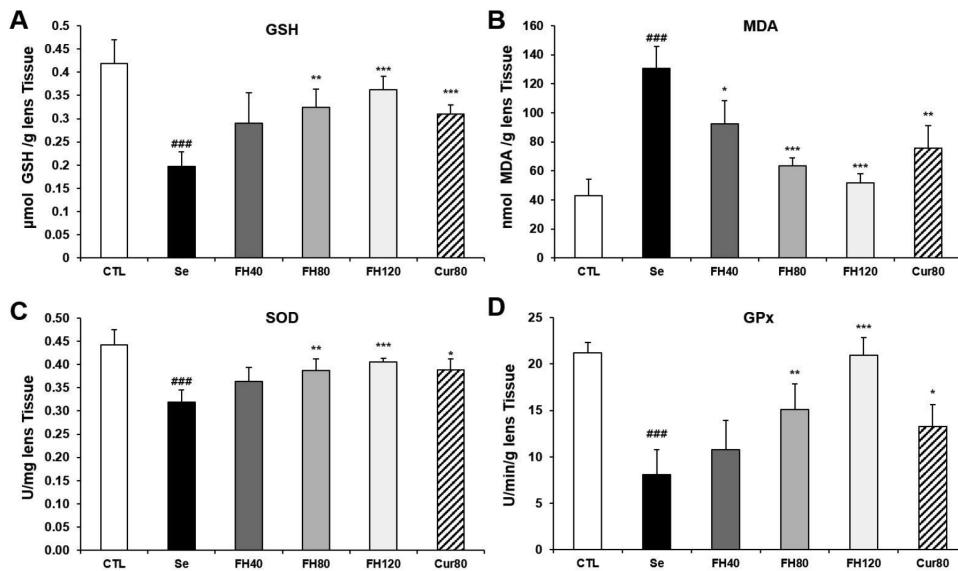


Figure 4. Effects of FH on GSH, MDA, SOD, and GPx in the lens. We measured the (A) glutathione (GSH), (B) malondialdehyde (MDA), (C) superoxide dismutase (SOD), and (D) glutathione peroxidase (GPx) levels to find antioxidant effects of FH in the lens. Each value represents the mean \pm standard deviation (SD) of three independent experiments (n=6). ###p<0.001 statistically significant different from CTL. *p<0.05, **p<0.01, ***p<0.001 statistically significant different from Se. CTL:

normal control, Se: selenite-treated, FH40/80/120: selenite/FH-treated (40, 80, 120 mg/kg, respectively), Cur80: selenite/curcumin-treated (80 mg/kg).

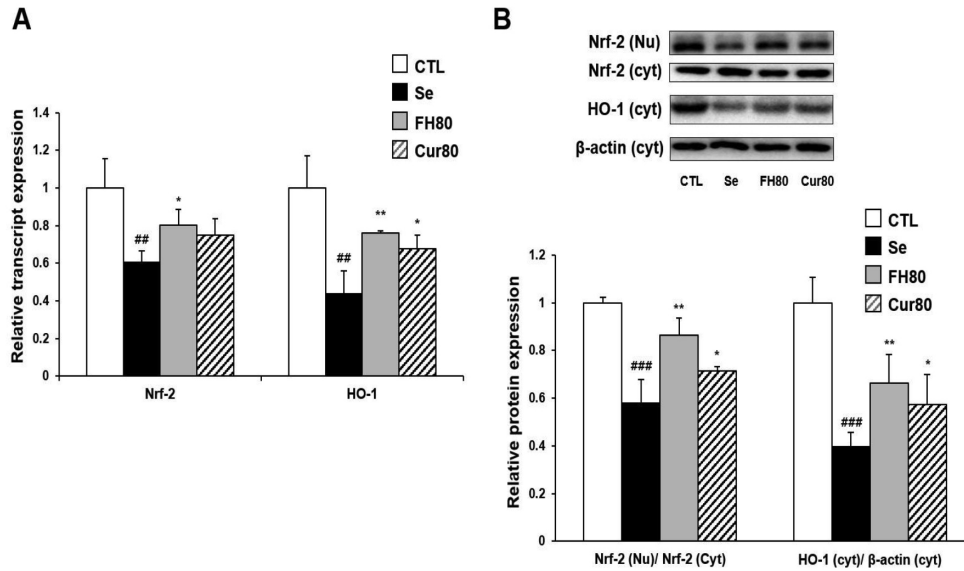


Figure 5. FH inhibits the selenite-induced decrease in Nrf-2 and HO-1 in the lens. **A:** Gene expression levels of Nrf-2 and HO-1 were analyzed with real-time polymerase chain reaction and normalized to glyceraldehyde 3-phosphated dehydrogenase (GAPDH) in the lens. The transcript level represents the mean \pm standard deviation (SD) of three independent experiments (n=4). **B:** The protein level of Nrf-2 was measured in the nuclear fraction of the lens, and the protein level of HO-1 was measured in the cytosol fraction of the lens using western blotting. The protein level represents the mean \pm standard deviation (SD) of three independent experiments (n=4). ##p<0.01, ###p<0.001 statistically significant different from CTL. *p<0.05, **p<0.01 statistically significant different from Se. CTL: normal control, Se: selenite-treated, FH80: selenite/FH-treated (80 mg/kg), Cur80: selenite/curcumin-treated (80 mg/kg).

to analyze the gene and protein expression levels of Nrf-2 and HO-1. To find Nrf-2 translocation into the nucleus, we separated the nuclear and cytosol fractions of the lenses. Figure 5A shows the gene expression levels of Nrf-2 and HO-1 in the lenses. Selenite injection downregulated both gene expression levels compared to the CTL group. FH treatment statistically significantly inhibited the downregulation of both genes to some extent. In the protein levels (Figure 5B), the selenite injection reduced Nrf-2 level in the nucleus, which resulted in HO-1 reduction in the cytosol fraction of the lenses. However, the downregulation of the protein levels was statistically significantly attenuated with FH treatment. These data demonstrated that FH protected against inhibition of the Nrf-2/HO-1 signaling pathway caused by selenite.

FH inhibits selenite-induced apoptosis in the lens: When the production of ROS overwhelms the cell's antioxidant system, oxidative stress occurs and impairs the homeostasis in the cells. Oxidative stress then induces apoptosis in the lens epithelial cells. Caspase 3, 8, and 9 (apoptotic cascade proteins) were cleaved in selenite-treated lenses [30]. However, PARP has not been investigated in cataractous lenses. PARP has essential roles in DNA repair, modification, and programmed death. PARP is a specific apoptotic marker in some organs (including the retina) because it is generally cleaved by activation of caspase 3 [31]. In this study, PARP was statistically significantly cleaved in the Se group (Figure 6). However, the cleavage of PARP was inhibited by FH treatment. This result demonstrated that FH

inhibits selenite-induced apoptosis in the lens, as determined by PARP cleavage.

DISCUSSION

The aim of this study was to evaluate the preventive effect of FH on cataract formation in selenite-induced cataract. It has been reported that V.U extract and its fraction have antioxidant, antiradical, and anti-inflammatory activities, which contribute to biological benefits especially in the eye [13,19,32]. It was demonstrated that FH protects blue light-induced retinal cell damage in vitro and in vivo [13,19]. As FH has never been investigated in age-related cataract, FH could have the possibility to show therapeutic potential. An anticataract effect was demonstrated using the selenite-induced cataract model. The selenite cataract model has been used to investigate nuclear cataract in many studies since 1978 [33]. Selenite administration induces cataract formation and biochemical changes, such as changes in calcium ATPase, calcium accumulation, calpain-induced proteolysis, insolubilization of the crystallin, decreased GSH and soluble proteins, and lens epithelial cell death [34]. These pathogenic changes are similar to human cataract; however, there are also dissimilarities. In the model, young rats are rapidly induced by excessive oxidative stress; cataract in humans is caused by continual oxidative stress with aging [35]. Despite the limitation, the selenite cataract model is still useful for investigating nuclear cataract and anticataract agents. This study suggests that FH treatment could possibly prevent cataract

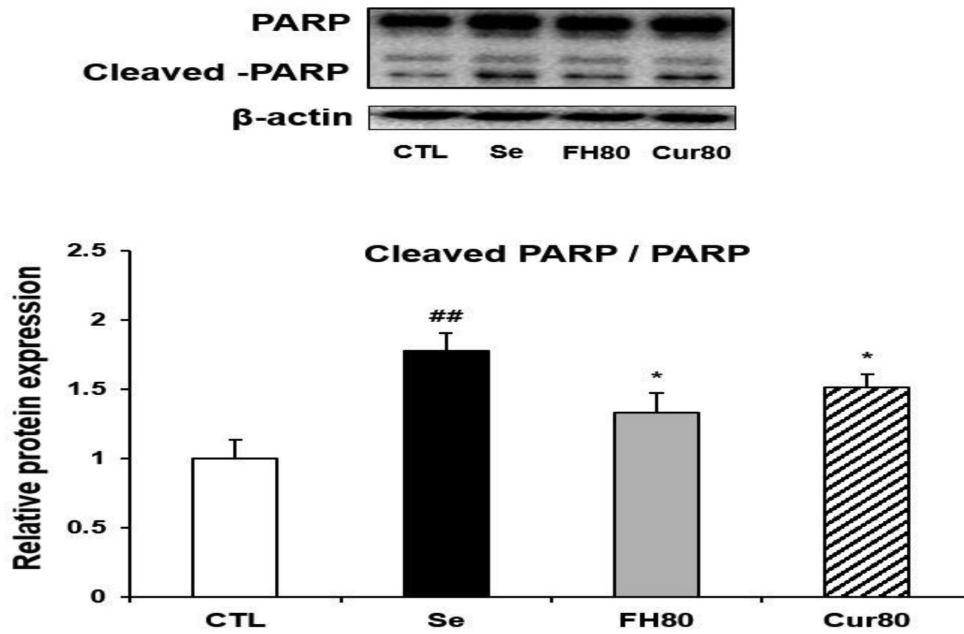


Figure 6. FH inhibits selenite-induced apoptosis in the lens. Poly (ADP)-ribose polymerase (PARP) and a cleaved form of PARP were analyzed using western blotting in the lens. The protein level represents the mean \pm standard deviation (SD) of three independent experiments (n=4). ##p<0.01 statistically significant different from CTL. *p<0.05 statistically significant different from Se. CTL: normal control, Se: selenite-treated, FH80: selenite/FH-treated (80 mg/kg), Cur80: selenite/curcumin-treated (80 mg/kg).

formation in three ways: 1) FH acts as an antioxidant and maintains a balance in the Nrf-2/HO-1 system, 2) FH inhibits m-calpain-induced proteolysis, and 3) FH inhibits selenite-induced apoptosis.

Oxidative stress is thought to be the major cause in age-related cataract. The aged lens loses its antioxidant activity and fails to scavenge ROS, which leads to lipid peroxidation, DNA damage, cross-linking, and aggregation of proteins. Recent studies reported that some plant extracts or compound protected lens opacification and have antioxidant effect, determined by enzyme estimation [6,36,37]. However, protective substances should not only maintain the antioxidant condition but also inhibit proteolysis of crystallin to prevent cataractogenesis in the lens. In this study, FH prevented cataract formation via maintenance of the redox balance and inhibition of m-calpain-induced proteolysis in the lens. FH treatment inhibited the GSH depletion, MDA production, and reduced antioxidant enzymes (SOD and GPx) caused by the selenite injection. These results suggest that FH treatment reduced selenite-induced oxidative stress. Therefore, the inhibition of calcium ATPase by oxidative stress was attenuated to some extent. Then, m-calpain-induced proteolysis was also reduced in the cytosol, followed by less opacity in the lens.

Among the calpains, m-calpain (calpain 2) exists in human and rat lenses, and it is associated with age-related cataract [30]. In the pathogenesis of cataract, calcium accumulates in the cytoplasm, followed by m-calpain activation. Activated m-calpain leads to proteolysis of cytoskeleton

proteins and crystallins, resulting in insolubilization and aggregation of proteins. In this study, selenite injection provoked the decline of soluble proteins in the lens. To further underline this investigation, we performed gene and protein analysis of m-calpain in the lens. The gene and protein levels of m-calpain were lower in the Se group than in the CTL, FH, and Cur groups. This finding is in accordance with previous studies investigating calpains in the selenite cataract [6,25,30]. The mRNA levels of m-calpain were increased before nuclear cataract formation (early stage of cataract formation); however, the mRNA level was statistically significantly decreased compared to that in normal rats after nuclear cataract formation. This reduction was caused by autolysis of m-calpain after cataract formation [34]. Consequently, normal levels of m-calpain indicate that calpain-mediated proteolysis did not occur in the lens.

Previous studies reported that Nrf-2 is highly associated with oxidative stress in the lens. A study also indicated that Nrf-2 plays pivotal role in the pathogenesis of cataract (age-related cataract and diabetic cataract). In the aging lens with diabetes, the hypoxia condition induces the unfolded protein response (UPR) and ROS production in the lens epithelial cells (LECs). In this mild oxidative or hypoxia condition, Nrf-2 is generally activated and translocated into the nucleus, followed by the Nrf-2-dependent antioxidant defense. However, when the oxidative stress overwhelms the intrinsic capacity in the lens or cortical lens fiber cells have less Nrf-2 dependent antioxidant protection in diabetic condition, Nrf-2 level is suppressed, and the changes result in crystalline

aggregation and opacities in the lens [38]. A previous in vitro study using human lens epithelial cells reported that homocysteine-induced ROS production suppressed the Nrf-2 level, which resulted from demethylation of Keap1 promoter DNA and expression of Keap1 protein [39]. In another study using human LECs, selenite-induced endoplasmic reticulum (ER) stress and UPR in the cells, which led to downregulation of the Nrf-2, catalase, and glutathione reductase levels. In selenite-injected suckling rats, production of intracellular ROS was observed in the LECs and the newly differentiated lens fiber cells with nuclear cataract formation [40]. In this in vivo model, we demonstrated that selenite induces the suppression of Nrf-2 and HO-1 in the rat lens. Due to the suppression, the cells lose their antioxidant capacity and eventually result in apoptosis. However, FH treatment statistically significantly maintained a higher Nrf-2 level than the Se group; thus, it maintained lens transparency.

All the cells in the lens are derived by differentiation of LECs. The lens epithelial cells play a pivotal role in the transparency, growth, and development of the entire lens [41]. The cells have antioxidant systems to protect the lens epithelium; however, the epithelium loses its metabolic function in the age-related cataract. It has been widely suggested that lens epithelial cell apoptosis is associated with development of age-related cataract. In the selenite cataract model, the imbalance of bcl-2/bax and cleavage of caspase-3 were observed and considered apoptotic markers in the lens, which was also demonstrated by our previous study [6]. In this study, we aimed to find whether PARP was cleaved in the lens when injected with selenite. Protein analysis of PARP indicated that selenite-induced apoptosis results in PARP cleavage in the lens.

In summary, this study showed that FH prevents cataract formation by maintaining Nrf-2/HO-1 expression, antioxidant enzymes, and soluble proteins, including crystallins. FH also inhibits m-calpain-mediated proteolysis and PARP cleavage. Thus, FH could exert a therapeutic effect on age-related cataract.

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