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Hesperetin prevents selenite-induced cataract in rats

Yosuke Nakazawa,[1,2] Mikako Oka,[1,3] Masayasu Bando,[1] Makoto Takehana[1]

[1]Department of Molecular Function and Physiology, Faculty of Pharmacy, Keio University; [2]Division of Hygienic Chemistry, Faculty of Pharmacy, Keio University; [3]Center for Pharmaceutical Education, Yokohama University of Pharmacy

Purpose: This study investigated the ability of hesperetin, a natural flavonoid, to prevent selenite-induced cataracts in a rat model.

Methods: Animals were divided into four treatment groups: G1 (control group), G2 (hesperetin-treated group), G3 (selenite-induced cataract group), and G4 (hesperetin-treated selenite cataract group). Animals in the G1 and G3 groups were injected with vehicle alone, while those in the G2 and G4 groups received a subcutaneous injection of hesperetin (0.4 µg/g bodyweight on days 0, 1, and 2, corresponding to P13, P14, and P15). Sodium selenite (20 µmol/g bodyweight given 4 h after the hesperetin injection on day 0) was administered to rats in the G3 and G4 groups to induce cataract formation. Lenses were observed with slit-lamp microscopy, and filensin degradation and the decreased glutathione (GSH) and ascorbic acid levels in the lens were measured on day 6.

Results: Lenses in the G3 group showed mature central opacity, while some lenses in the G4 group lacked central opacity and had lower-grade cataracts. All lenses in the G1 and G2 groups were transparent. Expression of the 94 kDa and 50 kDa forms of filensin was significantly decreased in the lenses in the G3 group compared with those in the G1 and G2 groups. Interestingly, these forms of filensin rescued the rat lenses in the G4 group. In the G3 group lenses, the GSH and ascorbic acid levels were lower than in the control group but were normalized in the G4 group lenses.

Conclusions: The results suggest that hesperetin can prevent selenite-induced cataract formation.

Cataract is expressed by opacification of the lens, which leads to a progressive lack of vision. The lens of the eye contains high concentrations of proteins and antioxidants, which are necessary to maintain transparency. Mutation of lens-specific proteins such as crystallins, aquaporin 0, filensin, or phakinin is associated with the development of cataracts, which suggests that these proteins are vital in maintaining the transparency of the lens [1]. Filensin and phakinin copolymerize into structures known as beaded filaments [2]. Mutation or deletion of either filensin or phakinin leads to the formation of cataracts in humans and mice, and is associated with subtle phenotypic differences [3,4]. In particular, filensin knockout mice appear to develop cataracts at an earlier age than phakinin knockout mice [3]. This suggests that filensin may play an even more important role than phakinin in maintenance of lens transparency. In the normal lens, filensin is a 94 kDa protein that is processed into 50 kDa and 38 kDa proteins, while the 94 kDa and 50 kDa forms are decreased in the lenses of rats with hereditary cataracts [5]. Thus, the 94 kDa and 50 kDa forms of filensin may be vital for maintaining lens transparency.

Correspondence to: Makoto Takehana, Department of Molecular Function and Physiology, Faculty of Pharmacy, Keio University, 1-5-30 Shibakoen, Minato-ku, Tokyo 105-8512, Japan; Phone: +81-3-5400-2689; FAX: +81-3-5400-2689; email: takehana-mk@pha.keio.ac.jp

In addition, lenses exposed to ultraviolet (UV) radiation generate reactive oxygen species (ROS) [6]. The modification and denaturation of proteins in the lens by ROS is referred to as oxidative stress, which leads to the development of cataracts [7]. However, to protect the lens and prevent the formation of cataracts, high concentrations of antioxidants such as reduced glutathione (GSH) and ascorbic acid (AsA) are present, both of which limit oxidative damage. Cataract stage and formation correlate with the GSH and AsA concentrations in the lens in humans and in animal models [8,9]. Therefore, maintaining high concentrations of antioxidants in the lens is important to prevent cataract formation.

Many compounds, including curcumin, lutein, and zeaxanthin, may help maintain the GSH and AsA levels in the lens in animal models of cataract [10,11]. These naturally occurring compounds have free-radical scavenger activity and an antioxidant effect. Hesperetin, a natural flavonoid, is reported to aid in inhibiting reactive oxygen species (ROS) activities by activating catalase, GSH peroxidase, and glutathione reductase [12,13]. Hesperetin may also inhibit ROS activity in the lens and maintain GSH and AsA concentrations.

Subcutaneous injection of sodium selenite (Na_2SeO_3) in suckling rats (10−18 days old) rapidly induces bilateral nuclear cataracts and is used as a model for evaluating anti-cataract agents [14]. Cataract induced by sodium selenite has characteristics similar to those seen clinically in humans,

for example, cytoskeletal loss, suppression of mitosis and decreased rate of epithelial cell differentiation, and calpain-induced proteolysis [14,15]. In addition, GSH and AsA concentrations are decreased in the lens of selenite-induced cataracts in rats [16]. We used this model to evaluate the anticataract activity of hesperetin.

METHODS

Animals: Sprague Dawley (SD) rats were obtained from Sankyo Labo Service Corporation (Tokyo, Japan) and housed in temperature-controlled cages (25 °C ± 5 °C) with a 12 h:12 h light-dark cycle. Rats were fed a balanced commercial rat chow (CE-2, Clea Japan, Inc., Tokyo, Japan) and allowed water ad libitum. The Keio University Animal Research Committee approved all animal procedures performed in this study. Rats were euthanized with an overdose of 5% isoflurane inhalation (Wako Pure Chemical Industries Ltd., Osaka, Japan). All of the animals in this work were treated according to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

Selenite-induced cataracts and hesperetin treatment: 108 female rats of 13 days-old were randomized into four groups: the control group (G1), the treatment with hesperetin group (G2), the treatment with sodium selenite group (G3), and the treatment with hesperetin and sodium selenite group (G4). Hesperetin (Wako Pure Chemical Industries Ltd.) was dissolved in 7% ethanol (Wako Pure Chemical Industries Ltd.) and 93% olive oil (Wako Pure Chemical Industries Ltd.). The hesperetin solution was administered to the G2 and G4 groups (0.4 µg/g bodyweight per day), while solvent alone was given to the G1 and G3 groups. Sodium selenite (Na_2SeO_3; 20 µmol/g bodyweight; Wako Pure Chemical Industries Ltd) was dissolved in PBS (1X; 130 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) and administered to rats in the G2 and G4 groups, while rats in the G1 and G3 groups received PBS alone. Sodium selenite was injected subcutaneously into 13-day-old rats (day 0) 4 h after hesperetin or solvent was administered. A subcutaneous injection of hesperetin was given on days 0, 1, and 2. On day 6 (19 days old), the rat lenses were observed with slit-lamp microscopy (Topcon Corp., Tokyo, Japan), and the rats were euthanized. Enucleated eyes were analyzed for degradation of filensin, and the GSH and AsA levels were determined.

Slit-lamp observation and cataract classification: Opacity of rat lenses was classified using slit-lamp microscopy (Topcon Corp.) after eye drops containing tropicamide (Midorin P, Santen Pharmaceutical Company, Osaka, Japan) were administered. Classification of cataracts was based on a scale of 1–6 as reported by Hiraoka [17].

Western blot analysis: Rat lenses were homogenized in 0.1 M Tris buffer (pH 8.0) and centrifuged at 20,000 ×*g* for 20 min at 4 °C. The precipitate was then washed twice with the same buffer. The pellet was extracted with 8 M urea, incubated for 15 min at room temperature, and then centrifuged at 20,000 ×*g* for 20 min at room temperature. The supernatant protein concentrations were measured with a Bradford protein assay kit (Bio-Rad, Hercules, CA) using bovine serum albumin (BSA) as the standard. Proteins (1 µg) were separated with 12.5% polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane (Bio-Rad). Western blot analysis was performed using an anti-filensin rod domain antibody and anti-beta-actin antibody (C-11 Santa Cruz, Santa Cruz, CA), and proteins were visualized with the horseradish peroxidase/3,3'-diaminobenzidine (HRP/DAB) system (Wako Pure Chemical Industries Ltd.) [5,18]. The band intensity was quantified using the National Institutes of Health's ImageJ 64 software.

GSH measurement: GSH concentrations in the lens were determined according to the method described by Sedlak and Lindsay, with minor modifications [19]. Briefly, lenses were homogenized in 500 µl of 0.1 M sodium phosphate buffer (pH 8.0) and centrifuged at 20,000 ×*g* for 20 min at 4 °C. The supernatant was mixed with an equal volume of 20% trichloroacetic acid, incubated for 15 min at 4 °C, and then centrifuged at 20,000 ×*g* for 30 min at 4 °C. Supernatant was diluted with 50 µl sodium phosphate buffer per 1 mg wet lens weight, and then 500 µl was added to 50 µg of dithionitrobenzene (DTNB; Wako Pure Chemical Industries Ltd.) and incubated for 30 min at room temperature. Absorbance at 412 nm was then measured in a microplate reader infinite M200 (Tecan Ltd., Männedorf, Switzerland). The GSH concentration in the lens was determined with a standard curve prepared using GSH (Wako Pure Chemical Industries Ltd.).

AsA measurement: The AsA concentration was determined using 2,6-dichlorophenol-indophenol (DCPIP; Merck, Darmstadt, Germany) as described previously [9]. Briefly, lenses were homogenized in four volumes of PBS (w/v). Samples were mixed with an equal volume of 5% metaphosphoric acid for deproteinization and titrated with DCPIP. Absorbance at 540 nm was measured in a microplate reader infinite M200 (Tecan Ltd.). The AsA concentration in the lens was determined using a standard curve prepared with AsA (Wako Pure Chemical Industries Ltd.).

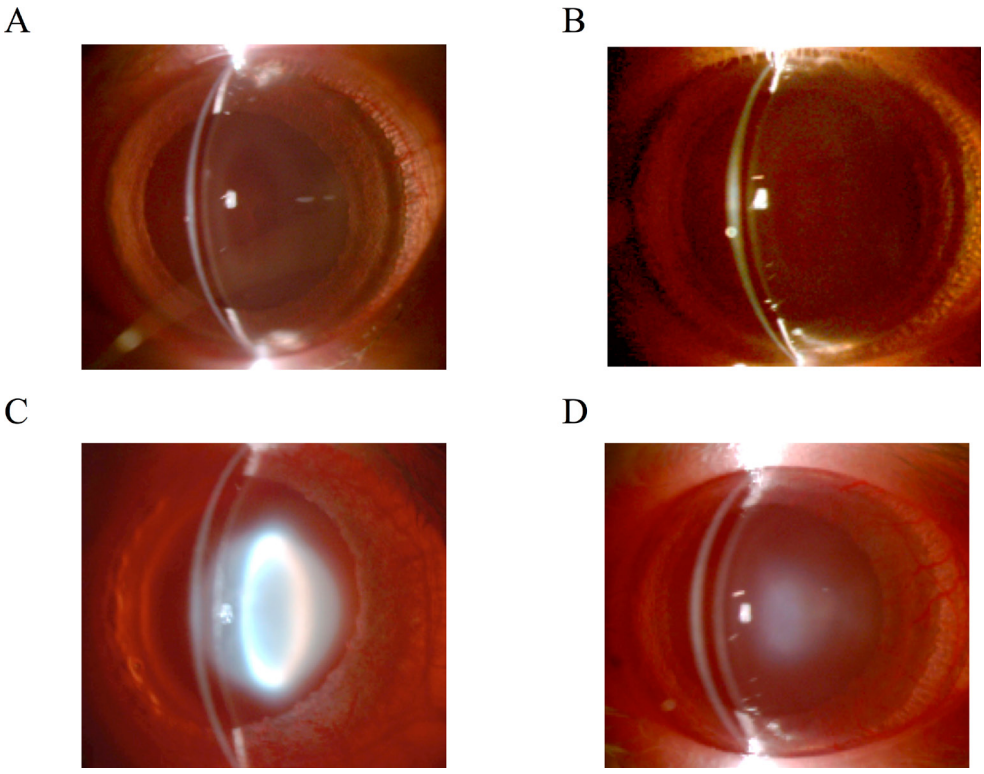

Figure 1. Lenses from rats with selenite-induced cataracts observed with a slit-lamp. **A**: Lens from a control rat (G1). **B**: Lens from a rat treated with hesperetin (G2). **C**: Lens from a rat treated with selenite (G3). **D**: Lens from a rat treated with selenite and hesperetin (G4).

RESULTS

Characteristics of selenite-induced cataracts: All lenses from rats in the G1 and G2 groups were transparent, and all had grade 1 cataract on day 6 (Figure 1A,B), determined with slit-lamp microscopy. A mature nuclear cataract was observed in all rats in the G3 group (cataract grade 6; Figure 1C). In contrast, some lenses in the G4 group lacked central opacity and/or had lower-grade cataracts. In this group, 20%, 30%, 25%, 5%, and 20% of rats had cataract grades 6, 5, 4, 2, and 1, respectively (Table 1). Figure 1D shows a grade 4 nuclear cataract in a rat from the G4 group.

Degradation of filensin: The degradation of filensin in selenite-induced cataract lenses was investigated with western blot analysis. The lenses from rats in the G1 and G2 groups contained the same proportions of the 94 kDa, 50 kDa, and 38 kDa forms of filensin (Figure 2, lanes 1 and 2). The 94 kDa and 50 kDa forms of filensin were significantly less abundant in lenses from the G3 group than in lenses from the G1 and G2 groups (Figure 2, lane 3). However, the intensities of the 94 kDa and 50 kDa form of filensin in lenses were rescued with the hesperetin treatment with selenite administered to rats (Figure 2, lane 4). The protein expression of β-actin was detected as an internal control of all groups (Figure 2B). The 94, 50, and 38 kDa forms of filensin normalized for β-actin were assessed using densitometry, and data are depicted graphically in Figure 3C. The 94 kDa and 50 kDa forms of filensin were significantly decreased in the

Table 1. Stage of selenite-induced cataracts in the different treatment groups.

Groups	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5	Grade 6
Group 1	100	0	0	0	0	0
Group 2	100	0	0	0	0	0
Group 3	0	0	0	0	0	100
Group 4	20	5	0	25	30	20

Cataract grade was classified and shown the incidence (%) of the cataract grade in each group according to the Hiraoka system as described in the Materials and methods [17]. Group 1; control animals. Group 2; hesperetin-treated animals. Group 3; selenite-treated animals. Group 4; animals treated with both selenite and hesperetin. (n = 15 in G1 and G2 groups, and n = 20 in G3 and G4 groups).

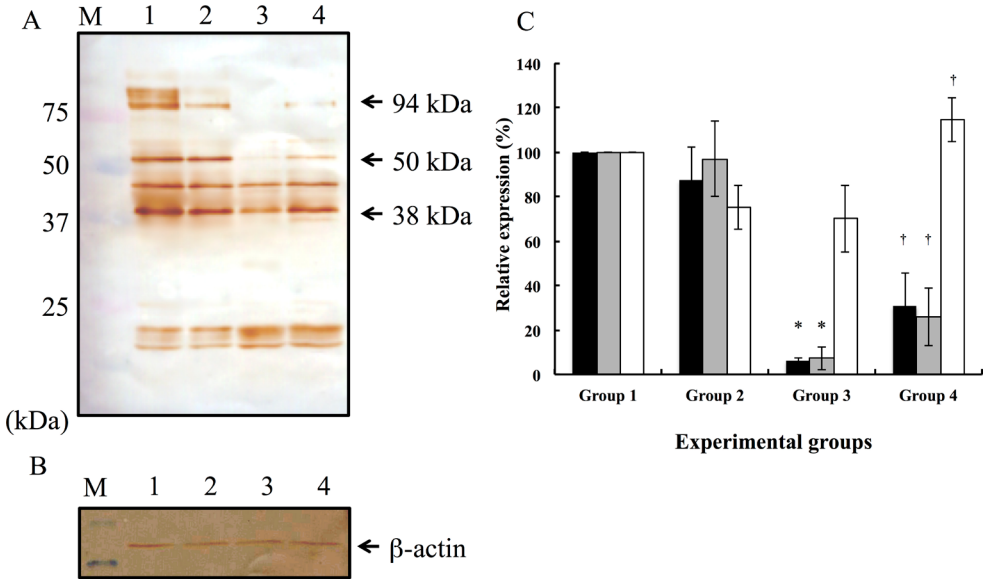

Figure 2. Degradation of filensin. Western blot analysis with an anti-filensin antibody. **A**: The 94 kDa form of filensin is processed into the 50 kDa and 38 kDa forms in vivo (arrow). **B**: Western blot analysis with anti-β-actin antibody as an internal control. Lane 1: control group (G1). Lane 2: hesperetin-treated group (G2). Lane 3: selenite-treated group (G3). Lane 4: selenite and hesperetin-treated group (G4). M shows the molecular weight standards. **C**: The respective bar diagrams show the band intensity (densitometric value) of the 94 kDa (black bar), 50 kDa (gray bar), and 38 kDa (white bar) forms of filensin levels relative to the levels in the control G1 group. All bar diagrams are expressed as the mean ± SD, and statistical analysis was determined using the unpaired Student t-test. *p<0.05 versus control (G1 group) and †p<0.05 versus rats with selenite-induced cataracts without hesperetin treatment (G3 group; n = 9 in each group).

G3 group compared to the G1 and G2 groups, and hesperetin treatment for rat cataract (the G4 group) inhibited the protein loss observed in the selenite-induced rat cataracts (the G3 group). Administration of hesperetin and/or selenite did not affect the 38 kDa form of filensin.

Decreased GSH and AsA concentrations: The GSH and AsA concentrations in lenses were measured to evaluate the effects of hesperetin administration on antioxidant levels. The GSH concentration in the G1 group was 1.40 µmol/g wet lens weight, similar to that seen in the G2 group (1.52 µmol/g). Although the GSH concentration in the G3 group was significantly lower than that in the G1 group (0.77 µmol/g), which was 55.0% concentration compared to the G1 group, that in the G4 group was increased to that in the G3 group (1.33 µmol/g), which was 95.0% concentration compared to the G1 group (Figure 3). These data indicated that hesperetin administration inhibits the decrease in GSH concentration in selenite-induced cataracts.

The AsA concentration was 30.0 µg/g wet lens weight in the G1 group, similar to that in the G2 group (28.9 µg/g). The AsA concentration was significantly lower in the G3 group than in the control group (17.2 µg/g), which was 57.3% concentration compared to the G1 group. The cotreatment with hesperetin and selenite (the G4 group) rescued the decreasing AsA concentration in the selenite-induced rat cataracts (25.0 µg/g), which was 83.3% concentration compared to the G1 group (Figure 4).

DISCUSSION

This report shows that hesperetin has anticataract activity as shown with slit-lamp observation and evaluation of filensin degradation and antioxidant levels. Coadministration of hesperetin and selenite (but not selenite alone) to rats

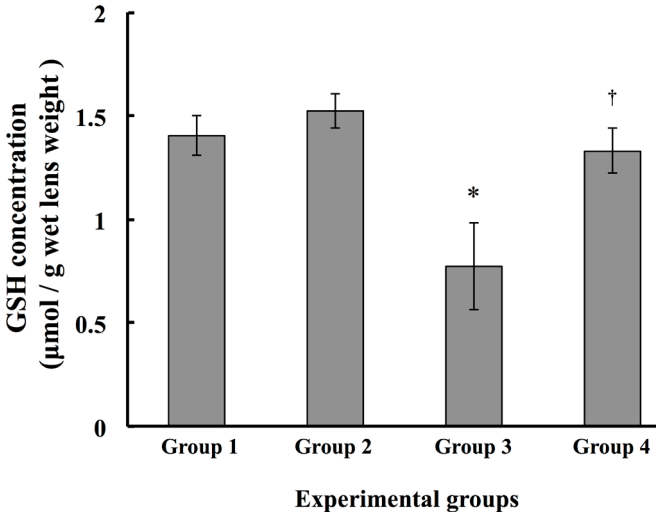

Figure 3. GSH levels in lenses with selenite-induced cataracts. Group 1: control group. Group 2: hesperetin-treated rats. Group 3: selenite-treated rats. Group 4: rats cotreated with selenite and hesperetin. All results are expressed as the mean ± SEM, and statistical analysis was determined using the unpaired Student t-test. *p<0.05 versus control (Group 1) and †p<0.05 versus rats with selenite-induced cataracts without hesperetin treatment (Group 3; n = 9 in each group).

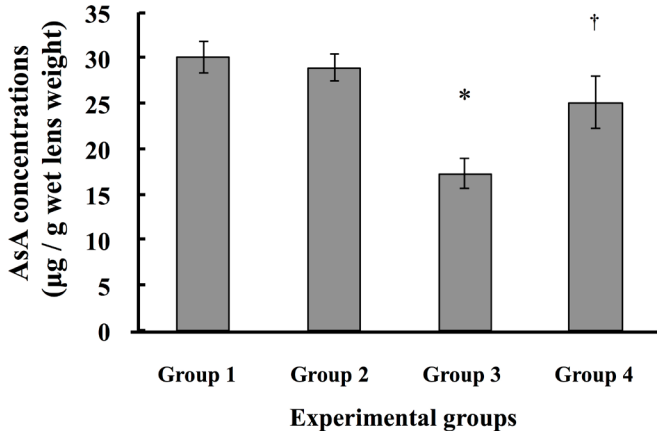

Figure 4. AsA concentrations in lenses with selenite-induced cataracts. Group 1: control group. Group 2: hesperetin-treated rats. Group 3: selenite-treated rats. Group 4: rats cotreated with selenite and hesperetin. All results are expressed as the mean ± SEM, and statistical analysis was determined using the unpaired Student t-test. *p<0.05 versus control (Group 1) and †p<0.05 versus rats with selenite-induced cataracts without hesperetin treatment (Group 3; m = 9 in each group).

prevented the cataracts from progressing (Figure 1, Table 1). Epidemiological studies have suggested that dietary intake of antioxidants, such as AsA, GSH, curcumin, lutein, and zeaxanthin, is associated with a reduced risk of cataract formation in humans and animal models [9-11,20]. Hesperetin exhibits antioxidant activity by regulating the expression of antioxidant enzymes such as catalase, glutathione peroxidase, and glutathione reductase [21,22]. Hesperetin also has several health benefits, including antitumor activity [23]. Our data suggest that hesperetin may also have anticataract activity.

A previous study reported that the GSH and AsA concentrations in the lens are lower in human and animal cataract models than in the normal lens. Thus, high levels of antioxidants in the lens appear to reduce the risk of cataract formation [20]. GSH and AsA have antioxidant activity by acting as ROS inhibitors. The thiol group in the cysteine residue within GSH acts as a strong reducing agent. GSH forms oxidized glutathione (GSSG) upon oxidative stress, which is then reduced to GSH by the glutathione reductase redox regulating system in the lens [24]. AsA also has antioxidant activity, which protects the lens from the early stages of damage caused by oxidative stress [25]. AsA is oxidized to the ascorbate free radical (AFR) and dehydroascorbic acid (DHA). The AFR and DHA are immediately reduced by the AFR reductase and DHA reductase systems by thioltransferase, respectively [26,27]. Thus, GSH and AsA act cooperatively in the antioxidant system and help to maintain a reduced state in the lens. In this regard, the GSH and AsA levels are reduced in selenite-induced cataracts [16]. Although hesperetin was not detected with high-performance liquid chromatography (HPLC) in the serum and the lens within 4 h after the hesperetin injection [data not shown], we found that the GSH and AsA levels in the selenite-induced cataract model were partially normalized by treatment with hesperetin (Figure 3 and Figure 4). Hesperetin did not reach the lens. It was supposed that the decrease in GSH and AsA was inhibited by hesperetin administration indirectly to maintain a reduced state in the lens and prevent cataract formation. We tested whether hesperetin could inhibit the cataract development for the hereditary Shumiya cataract rat (SCR), but hesperetin did not affect this cataract model. SCR is a hereditary cataract model unrelated to oxidative stress and thus was not affected by the hesperetin treatment.

It was reported that the 94 kDa and 50 kDa forms of filensin decrease in preclinical cataract formation in SCR [5]. We found that these forms of filensin were decreased in the lenses of SD rats following the administration of selenite (Figure 2, lane 3). Beaded filaments are lens-specific intermediate filaments composed of filensin and phakinin. Although filensin is essential for lens transparency, the relationship between filensin and selenite-induced cataracts has not been previously investigated. The results of the present study suggest that intermediate filaments in lenses are degraded following the administration of selenite and that cataract formation might be induced by disruption of the cytoskeleton. This may indicate cataract development, with concomitant degradation of the 94 kDa and 50 kDa forms of filensin, in addition to the decreased GSH and AsA concentrations.

In conclusion, this study provides insight into the positive effects of hesperetin on selenite-induced cataracts, filensin degradation, and GSH and AsA levels in rat lenses. Cataract is a major cause of blindness worldwide, and cataract surgery is the most common treatment. To decrease the burden of surgery in older adults, it would be of interest to develop other treatments that can delay or prevent the development of cataracts. Hesperetin is an effective cytoprotective agent against oxidative stress. Further studies of the molecular mechanisms involved in the protective effects of hesperetin against the formation of cataracts are currently in progress.

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