

# Oral intake of $\alpha$ -glucosyl-hesperidin ameliorates selenite-induced cataract formation

YOSUKE NAKAZAWA<sup>1</sup>, MIKI AOKI<sup>1</sup>, SHO ISHIWA<sup>1</sup>, NAOKI MORISHITA<sup>2</sup>, SHIN ENDO<sup>2</sup>,  
NORIAKI NAGAI<sup>3</sup>, NAOKI YAMAMOTO<sup>4</sup>, MEGUMI FUNAKOSHI-TAGO<sup>1</sup> and HIROOMI TAMURA<sup>1</sup>

<sup>1</sup>Department of Hygienic Chemistry, Faculty of Pharmacy, Keio University, Tokyo 105-8512;

<sup>2</sup>R&D Division, Hayashibara Co., Ltd, Okayama 702-8006; <sup>3</sup>Department of Advanced Design for Pharmaceuticals, Faculty of Pharmacy, Kindai University, Osaka 577-8502; <sup>4</sup>Regenerative Medicine Support Promotion Facility, Center for Research Promotion and Support, Fujita Health University, Toyoake, Aichi 470-1192, Japan

Received August 28, 2019; Accepted November 27, 2019

DOI: 10.3892/mmr.2020.10941

**Abstract.** Hesperetin is a natural flavonoid with robust antioxidant properties. Our previous study reported that hesperetin can prevent cataract formation. However, an important consideration regarding hesperetin consumption is the limited bioavailability due to its poor solubility. The present study investigated the anti-cataract effects of  $\alpha$ -glucosyl hesperidin *in vivo* and *in vitro* using a selenite-induced cataract model. SD rats (age, 13 days) were orally administered PBS (0.2 ml) or  $\alpha$ -glucosyl hesperidin (200 mg/kg) on days 0, 1 and 2. Sodium selenite was subcutaneously administered to the rats 4 h after the first oral administration on day 0. Antioxidant levels in the lens and blood were measured on day 6. *In vitro*, human lens epithelial cells were treated with sodium selenite (10  $\mu$ M) and/or hesperetin (50 or 100 mM) for 24 h and analyzed for apoptosis markers using sub-G<sub>1</sub> population and Annexin V-FITC/propidium iodide staining and DNA ladder formation.  $\alpha$ -glucosyl hesperidin treatment significantly reduced the severity of selenite-induced cataract. The level of antioxidants was significantly reduced in the selenite-treated rats compared with in the controls; however, they were normalized with  $\alpha$ -glucosyl hesperidin treatment. *In vitro*, hesperetin could significantly reduce the number of cells undergoing apoptosis induced by sodium selenite in human lens epithelial cell lines. Overall, oral consumption of  $\alpha$ -glucosyl hesperidin could delay the onset of selenite-induced cataract, at least in

part by modulating the selenite-induced cell death in lens epithelial cells.

## Introduction

The lens of the eye contains high concentrations of antioxidant compounds, such as reduced glutathione (GSH) and ascorbic acid (AsA), which prevent oxidative stress caused by ultraviolet light or reactive oxygen species (ROS) (1). In addition to GSH and AsA, catalase (CAT) is well known to protect the lens from damage induced by hydrogen peroxide, by decomposing it to water and oxygen (2). Approximately 80% of the information we get daily is through the eye, and the lens is necessary to maintain transparency. Opacification of the lens causes lack of vision, which is called cataract. Given that lens opacity is a direct result of oxidative stress, GSH and AsA levels and CAT activities are frequently used as markers of cataract formation for both human and in animal models (3,4).

It has been reported that the levels of oxidative stress markers in the blood are increased in patients with cataract (5). Oxidative stress in the blood causes cataract and other life-style related diseases due to an imbalance in the ratio of ROS and antioxidants (6). Superoxide dismutase (SOD) is a reaction enzyme that catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide and maintains the body redox state in coordination with CAT (7). In the current study, we measured the plasma SOD and CAT activities to assess the redox state of the body and lens by antioxidant compound consumption.

We previously reported that a subcutaneous injection of hesperetin (Hst; Fig. 1A) can prevent or delay the onset of cataracts, as assessed using selenite-induced animal cataract models (8,9). Hst, which is an abundant and inexpensive plant flavanone largely derived from citrus species, has a flavanone backbone structure and strong antioxidant activity. It is the aglycone of hesperidin (Hsd; Fig. 1B). Hst and Hsd are called bioflavonoids and were previously called Vitamin P because of their various biological activities, including anti-inflammatory, anti-oxidative, anti-diabetic, anti-hypertensive, improvement of very low-density lipoprotein (VLDL) metabolic abnormality, and their ability to decrease the capillary permeability (10-12).

**Correspondence to:** Dr Yosuke Nakazawa, Department of Hygienic Chemistry, Faculty of Pharmacy, Keio University, 1-5-30 Shibako-en, Minato-ku, Tokyo 105-8512, Japan  
E-mail: nakazawa-ys@pha.keio.ac.jp

**Abbreviations:** Hst, hesperetin; Hsd, hesperidin; G-Hsd,  $\alpha$ -glucosyl hesperidin; GSH, reduced glutathione; AsA, ascorbic acid; ROS, reactive oxygen species; CAT, catalase; SOD, superoxide dismutase

**Key words:**  $\alpha$ -glucosyl hesperidin, hesperetin, anti-cataract effect, hesperidin, anti-oxidants

However, the bioavailability of Hsd and Hsd after oral consumption is reported to be slow and irregular because of their poor solubility. To address these weaknesses, Hijiya and Miyake (13) created  $\alpha$ -glucosyl Hsd (G-Hsd; Fig. 1C); the water solubility of G-Hsd was about 10,000 times higher than that of Hsd. High water solubility is a good advantage for creation of oral drugs and/or healthy food product. Thus, for the *in vivo* experiment in this paper, we used orally administered G-Hsd to assess the anti-cataract activity of Hsd.

Lens epithelial cells form a monolayer at the anterior surface, and the fiber cells are differentiated from the epithelial cells at the equatorial surface, are elongated, and compose the bulk of the lens. During their differentiation to become mature fiber cells, all cytoplasmic organelles, such as nucleus and mitochondria, are degraded (14). For these reasons, ROS are easier to generate in lens epithelial cells; the generated ROS are diffused within the fiber cells, inducing protein aggregation and causing cataract. Therefore, investigate the molecular mechanisms using lens epithelial cell lines is a useful tool for finding an anti-cataract drug; however, there are no reports about the effect of Hsd on the lens epithelial cells. In the current study, we investigated the anti-cataract activity of G-Hsd oral consumption using both *in vivo* and *in vitro* experiments.

## Materials and methods

**Materials.** G-Hsd (including >80%  $\alpha$ -glucosyl Hsd) was provided by Hayashibara Co. Sprague-Dawley (SD) rats were obtained from Japan SLC Inc., and balanced chow for rats (CE-2) was obtained from Clea Japan Inc. Isoflurane, sodium selenite, GSH, AsA, and metaphosphoric acid were purchased from Wako Pure Chemical industries, Ltd. Dithionitrobenzene (DTNB), trichloroacetic acid Penicillin-Streptomycin antibiotics mixture, and Annexin V-FITC apoptosis detection kit were purchased from Nakalai Tesque Inc. CAT assay kit was obtained from Cayman Chemical Inc. SOD assay kit-WST was purchased from Dojindo molecular Technologies, Inc. GlutaMAX and Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (DMEM/F-12) were obtained from Gibco; Thermo Fisher Scientific Inc.

**Animals.** SD rats had unlimited access to balanced chow CE-2 and drinking water and were housed in a temperature-controlled ( $23^{\circ}\text{C}\pm 5^{\circ}\text{C}$ ) environment with a 12-h regular light/dark cycle. Rats were sacrificed with isoflurane (5% inhalation). The Keio University Animal Research Committee approved all animal procedures performed in this study [12048-(4)]. All animals in this work were treated according to the National Institutes of Health guide for the care and use of laboratory animals.

**Selenite-induced cataract and G-Hsd treatment.** Rats were randomized into 4 groups (Table I). Group 1: PBS treatment group (control group: G1). Group 2: G-Hsd treatment group (G2). Group 3: Sodium selenite treatment group (G3). Group 4: Sodium selenite and G-Hsd treatment group (G4).

Rats in each group received 0.2 ml phosphate-buffered saline (PBS: 130 mM NaCl, 3 mM KCl, 10 mM  $\text{Na}_2\text{HPO}_4$ , 2 mM  $\text{KH}_2\text{PO}_4$ ; pH 7.4) or an equal volume of 200 mg/kg G-Hsd dissolved in PBS via a feeding tube 4 h before the sodium selenite injection, then once a day for two days (total of

3 days). G-Hsd (>80%) was administered in the rats in groups G2 and G4. After G-Hsd oral administration, G-Hsd is hydrolysed by  $\alpha$ -glucosidase in the intestine from G-Hsd to Hsd. In the blood, all Hsd-related compounds are Hsd or its aglycone, Hsd Sodium selenite in a dose of 20  $\mu\text{mol/kg}$  body weight was administered to the rats in groups G3 and G4, while rats in groups G1 and G2 received PBS as control. After euthanasia on day 6 (19-days old), enucleated eyes were analyzed for GSH and AsA levels and lens CAT activities.

**Cataract classification.** Rats eyes were photographed on day 6 and the opacity area was measured using ImageJ software. Cataract classification was performed as previously described (15).

**Measurement of GSH, AsA and catalase activities in the lens.** Levels of lens GSH and AsA were determined according to a previously described method (16,17). For the measurement of lens GSH, lenses were homogenized in 0.1 M sodium phosphate buffer (pH 8.0) and centrifuged. The supernatant fraction was deproteinized with trichloroacetic acid and centrifuged. The supernatant sample was mixed with DTNB and incubated at room temperature in dark. Absorbance at 412 nm was measured using infinite M200 microplate reader after 30 min of incubation. (Tecan Ltd.).

For AsA measurement, lenses were homogenized in 0.1 M phosphate buffered saline (PBS: pH 7.4) and mixed with metaphosphoric acid to deproteinize. After centrifugation, the supernatant sample was titrated with DCPIP. Absorbance at 540 nm was measured in a microplate reader infinite M1000 (Tecan Ltd.).

CAT activity was measured using the catalase assay kit (Cayman Chemical) following the manufacturer's protocol. Briefly, the lenses were homogenized in ice-cold 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and were centrifuged. The supernatant was mixed with  $\text{H}_2\text{O}_2$ , potassium hydroxide, and the catalase purpald. After 10-min incubation at room temperature, absorbance at 540 nm was measured using the infinite M1000 (Tecan Ltd.). The standard curve of catalase was determined using a preparation with formaldehyde.

**Measurement of plasma SOD and CAT activities.** Under 5% isoflurane inhalation, blood samples were immediately collected from the vena cava. Plasma samples and erythrocytes were separated by centrifugation of the whole blood with heparin. For SOD measurement, erythrocyte fractions were re-suspended in Millipore purification water to cause hemolysis and then an ethanol/chloroform mixture was added. After shaking, samples were centrifuged and the water/ethanol fraction was collected to measure the erythrocyte SOD activity. The SOD activity of erythrocytes and plasma were measured using the SOD assay kit-WST (Dojindo) with commercial methods. CAT activity in the plasma was measured following the manufacturer's protocol. Briefly, the plasma was mixed with  $\text{H}_2\text{O}_2$ , potassium hydroxide, and the catalase purpald. After 10-minute incubation at room temperature, absorbance at 540 nm was measured using the infinite M1000. The standard curve of catalase was determined using a preparation with formaldehyde.

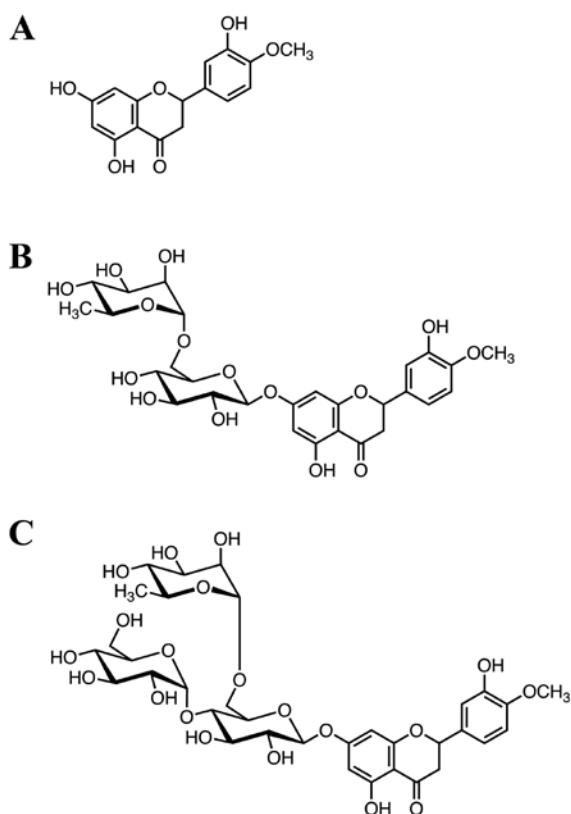


Figure 1. Structural formulas of hesperetin, hesperidin, and  $\alpha$ -glucosyl hesperidin. Structural formulas of (A) hesperetin, (B) hesperidin and (C)  $\alpha$ -glucosyl hesperidin.

**Cell culture.** Induced human lens epithelial cells (ihLECs) were established from human lens epithelial cells transfected with SV40 large T antigen, using the immortalized cell preparation method developed by Yamamoto *et al* (18). ihLECs were grown in DMEM/F-12 with 10% fetal bovine serum (FBS; Biosera), 4 mM glutamine (GlutaMAX; Gibco), and penicillin-streptomycin antibiotic mixture (100 U/ml and 100 mg/ml, respectively), under standard culture conditions of 5% CO<sub>2</sub> at 37°C. Cells were plated in a DMEM/F-12 medium 24 h before the experiment. Four hours before the sodium selenite or PBS treatment (10  $\mu$ M), Hst dissolved in DMSO or vehicle was added to the ihLECs at the concentrations of 50 or 100 mM, respectively. Twenty-four hours after the Hst treatment, cells were harvested and used for the following experiments.

**Cell viability and cytotoxicity assay.** Cell viability and cytotoxicity was measured by water-soluble tetrazolium (WST) dye. Briefly, cells were cultured in a 96-well plate treated with Hst and/or sodium selenite, and 24 h after Hst or PBS treatment, Cell Count Reagent SF (Nacalai Tesque) was added to each well and cells were incubated in normal culture conditions for 1 h. After incubation, absorbances at 450/490 nm were measured using the microplate reader, Infinite 200 Pro.

**Annexin V-FITC apoptosis detection.** Apoptotic cells were also analyzed utilizing an Annexin V-FITC apoptosis detection kit (Nacalai Tesque). Briefly, cells incubated with Hst and/or sodium selenite were harvested, washed with ice-cold

Table I. Experimental groups.

Group	Challenge	Test compound	Administration route
1	PBS	Vehicle	P.O
2	PBS	G-Hsd	P.O
3	Sodium selenite	Vehicle	P.O
4	Sodium selenite	G-Hsd	P.O

SD rats (age, 13 days) were divided into four groups randomly. PBS was injected in Groups 1 and 2, and sodium selenite was injected in Groups 3 and 4 on day 0. On days 0 (4 h after PBS or Se injection), 1 and 2, G-Hsd or vehicle was injected in each group subcutaneously (n=8 per each group). Se, sodium selenite; G-Hsd,  $\alpha$ -glucosyl hesperidin; P.O, per os.

PBS, and stained with Annexin V-FITC and PI according to the manufacturer's instructions. The resulting fluorescence was detected by BD FACS LSR II (BD Biosciences).

**Cell cycle parameter analysis.** Cell cycles were analyzed using propidium iodide (PI). Briefly, the cells treated with Hst and/or sodium selenite were harvested using rubber-police man and fixed using pre-cold 70% (v/v) ethanol at -20°C. After an overnight incubation, cells were centrifuged and treated with PBS containing 10  $\mu$ g/ml RNAase A (Nacalai Tesque). Subsequently, 100  $\mu$ g/ml PI were added and the cell cycle parameter was measured using FACS Caliber (BD Bioscience).

**DNA fragmentation assay.** DNA fragmentation assay was performed using DNA electrophoresis. Genomic DNA was prepared for electrophoresis on 1% (w/v) agarose gel and was visualized by staining with ethidium bromide after the electrophoresis.

**Statistical analysis.** All data are reported as the mean  $\pm$  standard error. Statistical analysis was performed using one-way analysis of variance (ANOVA) with a post-hoc Tukey's multiple comparison test with SPSS software, version 24 (IBM corporation). P-values less than 0.05 indicated statistical significance.

## Results

**Effect of G-Hsd on selenite-induced cataract formation in rats.** First, we assessed the effect of G-Hsd on selenite-induced cataract formation in rats. Thirteen-day-old SD rats were randomly divided into two groups and injected with either PBS (control group) or sodium selenite (cataract group). Each group was further divided into two subgroups and received either PBS or G-Hsd (200 mg/kg body weight) once a day for three days. In the control group, there were no rats that had cataract regardless of the G-Hsd treatment (Fig. 2A and B). Among the selenite treatment group, rats that were given PBS developed mature or premature nuclear cataracts; the cataract was classified as grade 6 in 44%, grade 5 in 50%, and grade 4

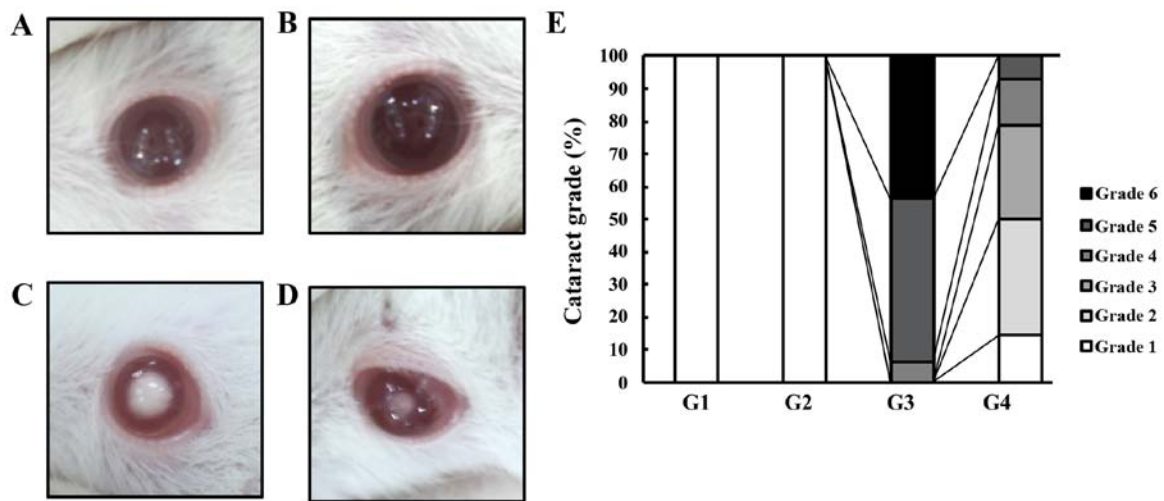


Figure 2. Images of lenses from selenite-induced cataract and cataract grade classification. Lenses from (A) PBS treatment group (G1), (B) G-Hsd treatment group (G2), (C) sodium selenite treatment group (G3) and (D) sodium selenite and G-Hsd treatment group (G4). (E) Cataract grade was classified in each group of rats (n=6 in groups G1 and G2, and n=8 in groups G3 and G4). Hst, hesperetin; Hsd, hesperidin; G-Hsd,  $\alpha$ -glucosyl hesperidin.

in 6% of the rats. Fig. 2C presents a grade 6 mature cataract in the selenite cataract group (G3). In contrast, rats treated with G-Hsd had delayed cataract development (cataract grade 3 in this group: Fig. 2D). The rats treated with selenite and G-Hsd did not have central opacity. Their cataract grades were as follows: Grade 5 in 7%, grade 4 in 14%, grade 3 in 29%, grade 2 in 36%, and grade 1 in 14% of the rats (Fig. 2E). These results indicated that G-Hsd had an anti-cataract effect in the selenite-induced cataract experimental model.

*G-Hsd ameliorates the reduction of GSH, AsA, and CAT activity induced by sodium selenite.* Thereafter, we measured the lens antioxidant compound levels (GSH and AsA) because ROS is thought to be a major cause of selenite-induced cataract. There were no changes in the lens GSH concentrations between the control group and the G-Hsd treated group (G1 vs. G2). The GSH concentrations in the rat lenses treated with sodium selenite were significantly decreased (G3); however, this reduction was reversed in the sodium selenite with G-Hsd treated group (G4) (Fig. 3A). Similarly, the lens AsA concentrations were not changed with or without G-Hsd treatment. These concentrations were significantly reduced in the rat lenses treated with sodium selenite. The G-Hsd treatment ameliorated the Se-induced AsA reduction (Fig. 3B). These results indicated that G-Hsd consumption inhibits the decrease in GSH and AsA concentrations in selenite-induced cataract. We also measured the CAT activity in the lenses of rats administered G-Hsd and/or sodium selenite. In the control groups (G1 and G2), CAT activities were not changed regardless of the G-Hsd treatment. In the groups of sodium selenite treatment, the CAT activity was significantly decreased compared with that in the controls; however, it was reversed with the G-Hsd co-treatment (Fig. 3C). These results suggested that G-Hsd consumption could prevent the onset of cataract due to maintaining a reduced state in the lens.

*G-Hsd treatment reverses the reduction of antioxidant enzyme activities in blood.* We measured the total-SOD activity (Cu/Mn SOD) in erythrocytes and plasma using the SOD assay kit-WST

(Dojindo). SOD activity in the erythrocytes was not changed in the control and G-Hsd treatment groups rats; however, it was significantly decreased in the rats treated with sodium selenite, but this reduction could be reversed by the co-treatment with G-Hsd and sodium selenite (Fig. 4A). Similarly, the SOD activity in the plasma was not different between the control group rats (G1) and G-Hsd treatment group rats (G2), and it was reduced in the sodium selenite group rats (G3); however, the SOD activity was recovered by the G-Hsd treatment in the sodium selenite cataract rats (G4) (Fig. 4B). Thereafter, we measured the plasma CAT activity in each group. CAT activity in plasma was significantly decreased in the rats treated with sodium selenite (G3); however, this phenotype was canceled with the G-Hsd oral consumption (Fig. 4C). These results suggested that G-Hsd administration could reverse the reduction of SOD and CAT activities in the blood.

*Hst cancels the cell death induced by sodium selenite.* From the above data, it is suggested that the anti-cataract activity of G-Hsd could be induced by the inhibition of lens epithelial cell damage. Therefore, we tested whether Hst, since cells do not have  $\alpha$ -glucosidase and Hst is an active ingredient of G-Hsd, could prevent the cell death from sodium selenite using the ihLEC line. First, we checked the cell toxicity of sodium selenite in ihLECs using WST-1 dye assay. The cell proliferation was significantly decreased in the cells that were exposed to 10  $\mu$ M of sodium selenite, which was used for further current study (Fig. 5A). We also tested the cell toxicity of Hst in ihLECs ranging from 25 to 400 mM (Fig. 5B). Thereafter, we tested the cell proliferation ability for ihLEC co-treatment with Hst and sodium selenite. After 24 h Hst treatment, cells were collected and measured for cell toxicity. The cell proliferation ability was significantly decreased in the ihLECs treated with sodium selenite, but it could be reversed in the cells treated with Hst (Fig. 5C). These results suggested that Hst could ameliorate cell death of ihLECs induced by sodium selenite in a dose-dependent manner. From these data, we have decided to use 10  $\mu$ M sodium selenite and 50 or 100 mM Hst for further studies.

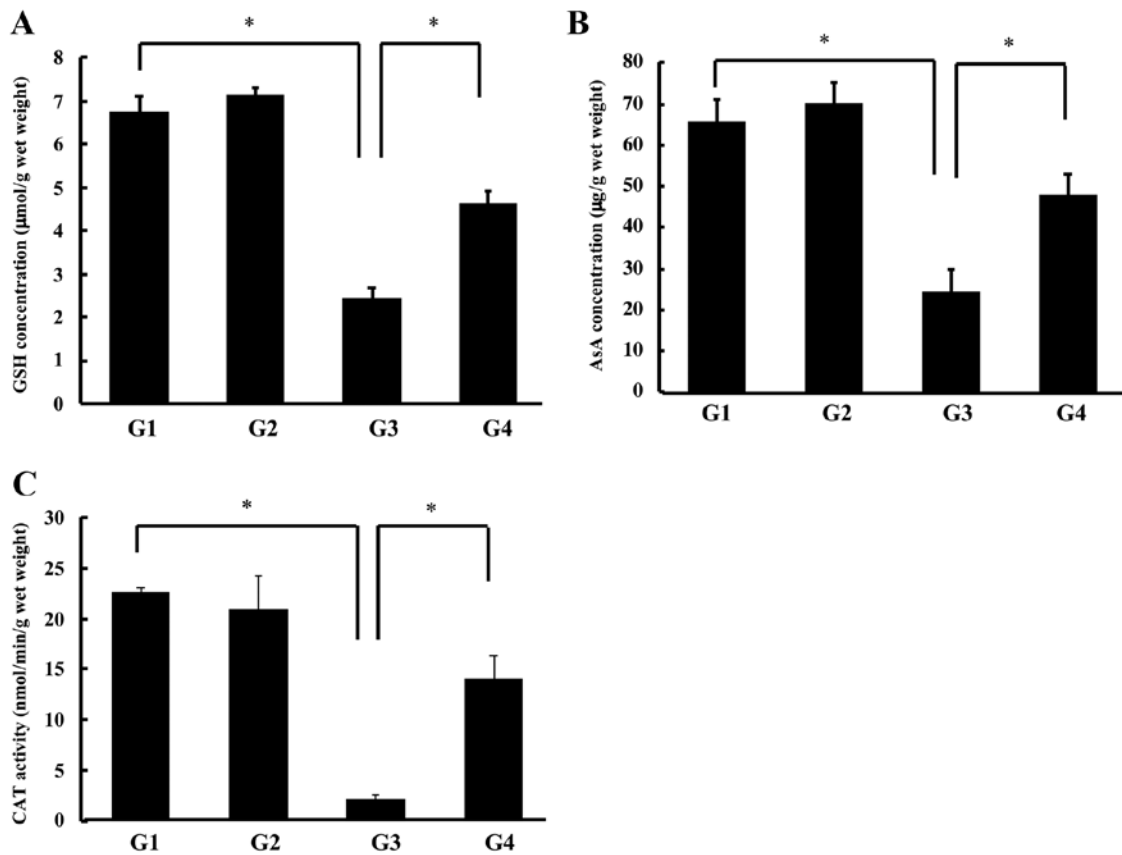


Figure 3. Effect on selenite-induced cataract lenses with oral administration of G-Hsd. (A) GSH levels in lenses of selenite-induced cataracts with or without G-Hsd treatment. (B) AsA levels in lenses of selenite-induced cataracts with or without G-Hsd treatment. (C) CAT activities in the lenses of selenite-induced cataracts with or without G-Hsd treatment. All results are presented as the mean  $\pm$  SEM (n=6 in groups G1 and G2, and n=8 in groups G3 and G4). \* $P < 0.05$ , as indicated. GSH, reduced glutathione; AsA, ascorbic acid; CAT, catalase; G-Hsd,  $\alpha$ -glucosyl hesperidin.

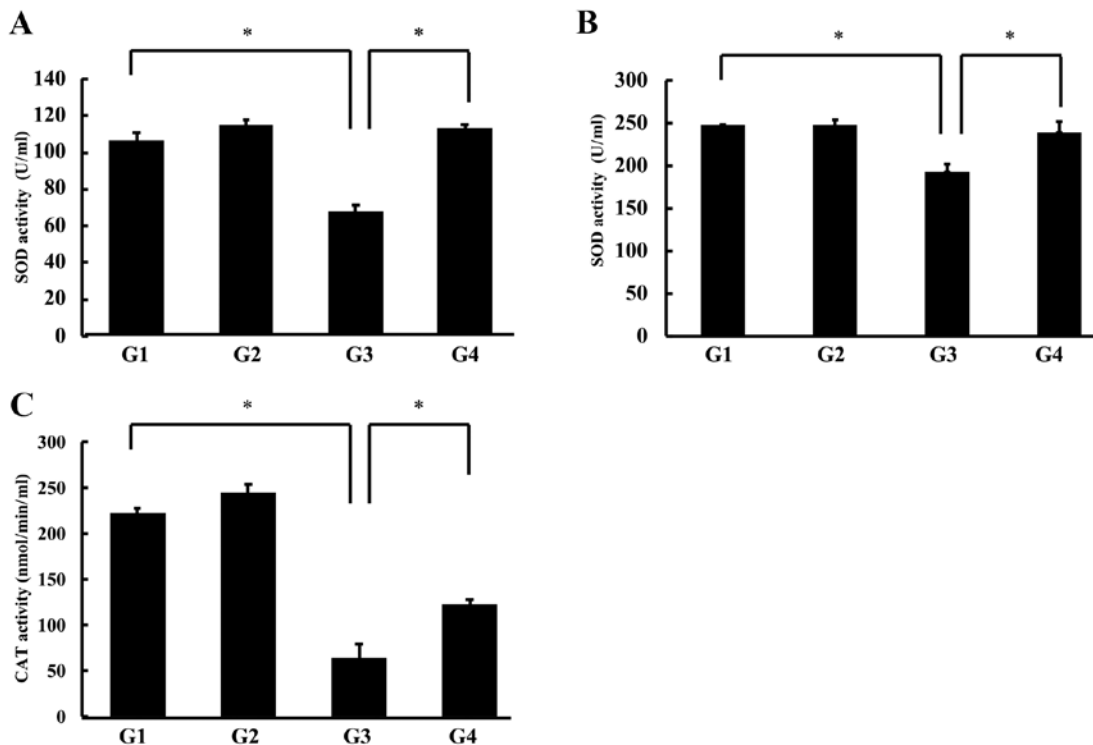


Figure 4. Effect on selenite-induced cataract rats of oral administration of G-Hsd. SOD activities in (A) erythrocytes and (B) blood plasma of rats administered with sodium selenite and/or G-Hsd. (C) CAT activities in blood plasma of rats treated with sodium selenite and/or G-Hsd. All results are presented as the mean  $\pm$  SEM (n=6 in groups G1 and G2, and n=8 in groups G3 and G4). \* $P < 0.05$ , as indicated. SOD, superoxide dismutase; G-Hsd,  $\alpha$ -glucosyl hesperidin; CAT, catalase.

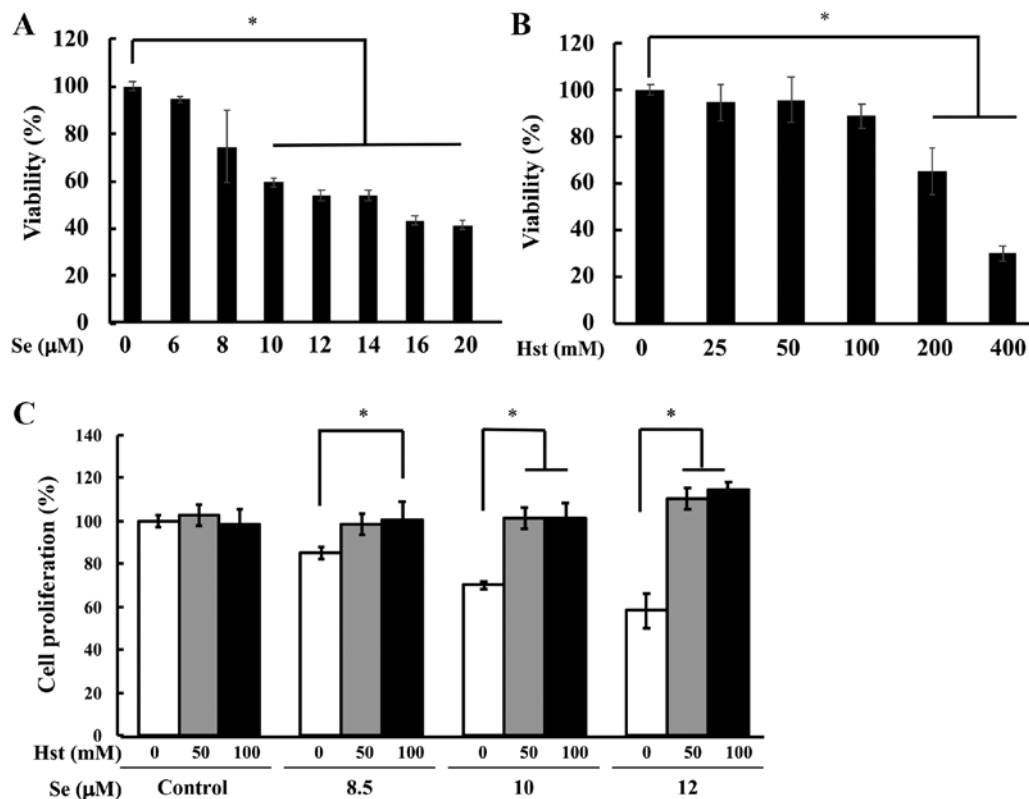


Figure 5. Cell toxicity of sodium selenite and Hst for lens epithelial cells. Cell toxicity of (A) sodium selenite and (B) Hst for ihLEC using WST-1 dye assay. (C) Effect of ihLEC cell proliferation ability following administration of sodium selenite and Hst. All results are presented as the mean  $\pm$  SEM (n=10 in groups G1 and G2, and n=12 in groups G3 and G4). \*P<0.05, as indicated. Hst, hesperetin; ihLECs, induced human lens epithelial cells; Se, sodium selenite.

*Hst cancels the cell death induced by sodium selenite.* To identify the effect of Hst on cell death in ihLECs, cells were treated with Annexin V and PI to measure the percentage of apoptotic cells using flow cytometry. The cells treated with PBS or Hst alone did not show apoptosis (Fig. 6A and B), and the number of apoptotic cells increased with exposure to sodium selenite (Fig. 6C); however, these were rescued by co-treatment with sodium selenite and Hst (Fig. 6D). The percentages of apoptotic cells are listed in Table II. Thereafter, to clarify the role of sodium selenite in ihLEC cell death, we measured the percentage of cells in the sub-G1 phase treated with sodium selenite and/or Hst using PI staining. This was found to be significantly increased after the sodium selenite treatment without Hst; however, Hst treatment inhibited the sub-G1 population in a dose-dependent manner (Fig. 6E). Next, we performed a genomic DNA fragmentation assay, which identified DNA fragmentation in the sodium selenite treatment cells, but it was weakened with Hst treatment in a dose-dependent manner (Fig. 6F). These results suggested that sodium selenite could induce cell death in lens epithelial cells, but Hst treatment could neutralize this action.

## Discussion

The aim of the current study was to evaluate the preventive effect of G-Hsd on cataract formation on selenite-induced cataract *in vivo* and *in vitro*, which is a classical and widely accepted method because of its effectiveness, the

reproducibility of cataract formation, and its similarity to human senile cataract characteristics (8,9). Using this animal model, we previously reported that Hst could prevent or delay the onset of cataract by a subcutaneous injection. To the best of our knowledge, this study was the first report that orally administered G-Hsd could prevent the cataract formation. We detected  $1.33 \pm 0.23$  nmol/g Hst in the eye after 1,000 mg/kg G-Hsd for 5 days administration orally. Hst and Hsd were reported to modulate expressions of apoptosis regulatory proteins such as Bax, Bcl-XL, and cleaved caspase-3 (19,20). These data suggested that orally administered G-Hsd had direct effect for anti-cataract to modulate apoptosis regulatory proteins expression in lens and indirect effect for anti-cataract to maintain the body redox state in blood. It has been reported that Hsd could permeate across the blood-brain barrier (BBB) (21,22). We hypothesised that Hst and/or Hsd could reach the lens through the vitreous body via the blood-retinal-barrier (BRB) or aqueous humor via the blood-aqueous-barrier (BAB) because the characteristics of BRB and BHB are almost the same as those of the BBB. Further studies are needed to investigate the route of the Hst from the blood to the lens.

It is vital to study the long-term safety of pharmacological therapies for cataract patients because drug therapeutics for these patients should be applied for a long period of time to get the effect of delay or prevent the development of cataract effectively. Furthermore, it would provide significant health and economic benefits to identify and develop effective anti-cataract agents in the human diet that can be consumed



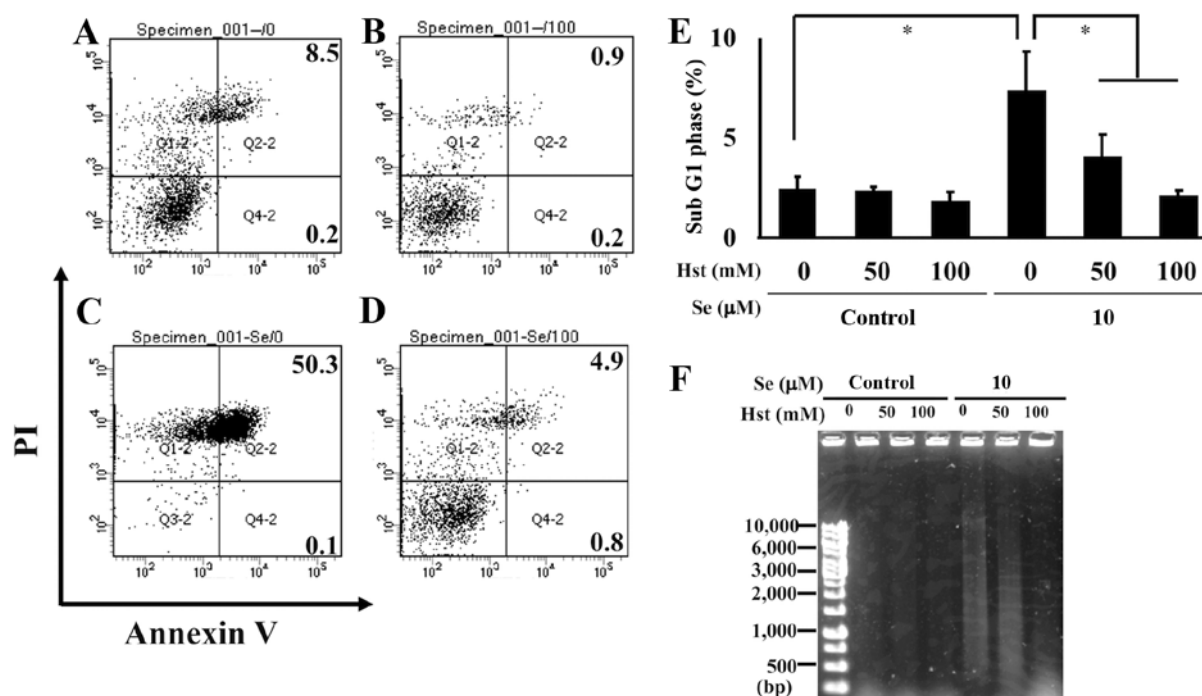


Figure 6. Apoptosis analysis for the lens epithelial cells. Annexin V-FITC analysis for apoptosis of ihLECs treated with (A) PBS, (B) Hst, (C) sodium selenite and (D) co-treatment of sodium selenite and Hst. (E) Percentage of sub-G1 population for ihLECs with sodium selenite and Hst co-treatment. (F) DNA ladder formation in ihLECs treated with sodium selenite and/or Hst. All results are presented as the mean  $\pm$  SEM (n=3). \*P<0.05, as indicated. PI, propidium iodide; Hst, hesperetin; ihLECs, induced human lens epithelial cells; Se, sodium selenite.

Table II. Percentage of apoptotic cells of induced human lens epithelial cells.

Group	Apoptotic cells
1	8.03 $\pm$ 1.33
2	3.00 $\pm$ 2.48
3	49.03 $\pm$ 1.46
4	7.17 $\pm$ 2.46

Percentage of apoptotic cells of each group. The percentage was calculated from flow cytometry with Annexin V-FITC/propidium iodide staining. Results are presented as the mean  $\pm$  SD (n=4 per group).

daily. Several researchers have used this model for finding new compounds with anti-cataract effect, such as garlic and curcumin extracts (23,24). From our laboratory, we previously reported that daily coffee consumption could prevent the onset of selenite-induced cataract formation (15,17).

Hst has several general health benefits, such as anti-inflammatory, anti-hypertensive, and improvement of very low-density lipoprotein (VLDL) metabolic abnormality (10-12). Many health supplements containing G-Hsd and its related compounds are sold in Japan under the system of foods with functional claims. These supplements are claimed as functional substances because of their role in maintaining blood triglyceride levels, blood pressure, and peripheral blood flow. In the USA and other countries, some supplements

contain Hsd for its anti-inflammatory and anti-hypersusceptibility properties. However, currently, no health supplement claims to have an anti-cataract effect. Further studies are needed to understand the molecular mechanisms underlying the anti-cataract activity of G-Hsd.

Beside the lens GSH and AsA levels, we measured the SOD and CAT activities in plasma to indicate the redox state of the body in this study. It is difficult to measure the lens SOD activity because lens contains high concentrations of GSH and AsA, which are high antioxidant activity, and these compounds interfere with this assay system. In the current study, we were unable to detect plasma GSH levels using DTNB and AsA level using DCPIP because plasma GSH and AsA levels were below the detection limit. However, GSH and AsA in lens are came from blood through the vitreous body via the BRB and aqueous humor via BAB. So, it is important to evaluate plasma GSH and AsA levels to prevent cataractogenesis. Further studies are needed to detector or HPLC system with greater sensitivity to measure the GSH and AsA levels by G-Hsd consumption.

Selenium is an essential micronutrient involved in several important intracellular process, and low doses of selenite treatment are inversely correlated with the risk of cancer that acts as a pro-oxidant to induce tumor cell apoptosis (25,26). For some cancer cells, sodium selenite acts an antioxidant that protect cells against oxidative damage (27,28), however, it is also known to increase intracellular ROS levels. The environment of the lens epithelial cells is known to be in high redox state to prevent oxidative damage. For lens cell, sodium selenite may act as pro-oxidant and induced cell death. Further studies are needed to understand the function of sodium selenite in the lens.

In this current study, we measured and percentage of sub G1 phase population and Annexin V/PI double staining using flow cytometer. These methods are widely used for detection of apoptotic cells. The flow cytometric analysis and DNA ladder formation analysis also indicated that sodium selenite exposure induced apoptosis in ihLEC cells, and the apoptosis cells of ihLEC was significantly decreased by Hst treatment. It has been reported that apoptosis in lens epithelial cells occurs at an early stage and accelerates during the formation of selenite-induced cataract (29). Thus, to prevent the apoptosis in lens epithelial cells is one of the best ways to delay the onset of cataract. In the current study, we have found that Hst treatment could inhibit cell death in lens epithelial cells induced by sodium selenite.

In this study, we used the selenite-induced cataract model that is widely used for screening assay for anti-cataract agents. We have clearly shown that G-Hsd oral consumption can delay the onset of selenite- induced cataract *in vivo*, due to reversal of the selenite-induced cell death. After human clinical trial for humans, G-Hsd will be the first supplements that claimed for anti-cataract supplement in the near future.

### Acknowledgements

Not applicable.

### Funding

This work was supported by grants from the Keio Gijuku Fukuzawa Memorial Fund for the Advancement of Education and Research, and from the Japan Health Foundation.

### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Authors' contributions

YN and HT defined the research theme. YN, NM, SE, MFT and HT designed the methods. YN, MA, SI, NN and NY performed the laboratory experiments. YN, NN, MFT and HT analyzed and interpreted the data. YN was major contributor in the writing of the manuscript. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

All animal experiments were approved by the Keio University Animal Research Committee [approval no. 12048-(4)].

### Patient consent for publication

Not applicable.

### Competing interests

NM and SE are employees of Hayashibara Co., Ltd. (Okayama, Japan), and Hayashibara Co., Ltd. provided the alpha-glucosyl hesperidin (G-Hsd) for these experiments.

### References

- Hegde KR and Varma SD: Protective effect of ascorbate against oxidative stress in the mouse lens. *Biochim Biophys Acta* 1670: 12-18, 2004.
- Zigman S, Reddan J, Schultz JB and McDaniel T: Structural and functional changes in catalase induced by near-UV radiation. *Photochem Photobiol* 63: 818-824, 1996.
- Fan X, Liu X, Hao S, Wang B, Robinson ML and Monnier VM: The LEGSKO mouse: A mouse model of age-related nuclear cataract based on genetic suppression of lens glutathione synthesis. *PLoS One* 7: e50832, 2012.
- Grey AC, Demarais NJ, West BJ and Donaldson PJ: A quantitative map of glutathione in the aging human lens. *Int J Mass Spectrom* 437: 58-68, 2019.
- Katta AV, Katkam RV and Geetha H: Lipid peroxidation and the total antioxidant status in the pathogenesis of age related and diabetic cataracts: A study on the lens and blood. *J Clin Diagn Res* 7: 978-981, 2013.
- Kim JH, Baik HW, Yoon YS, Joung HJ, Park JS, Park SJ, Jang EJ, Park SW, Kim SJ, Kim MJ, *et al*: Measurement of antioxidant capacity using the biological antioxidant potential test and its role as a predictive marker of metabolic syndrome. *Korean J Intern Med* 29: 31-39, 2014.
- Fridovich I: Superoxide radical and superoxide dismutases. *Annu Rev Biochem* 64: 97-112, 1995.
- Nakazawa Y, Oka M, Bando M and Takehana M: Hesperetin prevents selenite-induced cataract in rats. *Mol Vis* 21: 804-810, 2015.
- Nakazawa Y, Oka M, Tamura H and Takehana M: Effect of hesperetin on chaperone activity in selenite-induced cataract. *Open Med (Wars)* 11: 183-189, 2016.
- Miwa Y, Mitsuzumi H, Sunayama T, Yamada M, Okada K, Kubota M, Chaen H, Mishima Y and Kibata M: Glucosyl hesperidin lowers serum triglyceride level in hypertriglyceridemic subjects through the improvement of very low-density lipoprotein metabolic abnormality. *J Nutr Sci Vitaminol (Tokyo)* 51: 460-470, 2005.
- Akiyama S, Katsumata S, Suzuki K, Nakaya Y, Ishimi Y and Uehara M: Hypoglycemic and hypolipidemic effects of hesperidin and cyclodextrin-clathrated hesperetin in Goto-Kakizaki rats with type 2 diabetes. *Biosci Biotechnol Biochem* 73: 2779-2782, 2009.
- Alu'datt MH, Rababah T, Alhamad MN, Al-Mahasneh MA, Ereifej K, Al-Karaki G, Al-Duais M, Andrade JE, Tranchant CC, Kubow S and Ghazlan KA: Profiles of free and bound phenolics extracted from Citrus fruits and their roles in biological systems: Content, and antioxidant, anti-diabetic and anti-hypertensive properties. *Food Funct* 8: 3187-3197, 2017.
- Hijiya H and Miyake T:  $\alpha$ -glycosyl hesperidin, and its preparation and uses. *Eur Patent no. EP0402049A2*. Filed June 31, 1990; issued July 12, 1995.
- Cvekl A and Ashery-Padan R: The cellular and molecular mechanisms of vertebrate lens development. *Development* 141: 4432-4447, 2014.
- Ishimori N, Oguchi J, Nakazawa Y, Kobata K, Funakoshi-Tago M and Tamura H: Roasting enhances the anti-cataract effect of coffee beans: Ameliorating selenite-induced cataracts in rats. *Curr Eye Res* 42: 864-870, 2017.
- Nakazawa Y, Pauze M, Fukuyama K, Nagai N, Funakoshi-Tago M, Sugai T and Tamura H: Effect of hesperetin derivatives on the development of selenite-induced cataracts in rats. *Mol Med Rep* 18: 1043-1050, 2018.
- Nakazawa Y, Ishimori N, Oguchi J, Nagai N, Kimura M, Funakoshi-Tago M and Tamura H: Coffee brew intake can prevent the reduction of lens glutathione and ascorbic acid levels in HFD-fed animals. *Exp Ther Med* 17: 1420-1425, 2019.
- Yamamoto N, Kato Y, Sato A, Hiramatsu N, Yamashita H, Ohkuma M, Miyachi E, Horiguchi M, Hirano K and Kojima H: Establishment of a new immortalized human corneal epithelial cell line (iHCE-NY1) for use in evaluating eye irritancy by in vitro test methods. *In Vitro Cell Dev Biol Anim* 52: 742-748, 2016.
- Samie A, Sedaghat R, Baluchnejadmojarad T and Roghani M: Hesperetin, a citrus flavonoid, attenuates testicular damage in diabetic rats via inhibition of oxidative stress, inflammation, and apoptosis. *Life Sci* 210: 132-139, 2018.
- Hanchang W, Khamchan A, Wongmanee N and Seedadee C: Hesperidin ameliorates pancreatic  $\beta$ -cell dysfunction and apoptosis in streptozotocin-induced diabetic rat model. *Life Sci* 235: 116858, 2019.



21. Youdim KA, Dobbie MS, Kuhnle G, Proteggente AR, Abbott NJ and Rice-Evans C: Interaction between flavonoids and the blood-brain barrier: In vitro studies. *J Neurochem* 85: 180-192, 2003.
22. Takumi H, Mukai R, Ishiduka S, Kometani T and Terao J: Tissue distribution of hesperetin in rats after a dietary intake. *Biosci Biotechnol Biochem* 75: 1608-1610, 2011.
23. Javadzadeh A, Ghorbanihaghjo A, Arami S, Rashtchizadeh N, Mesgari M, Rafeey M and Omid Y: Prevention of selenite-induced cataractogenesis in Wistar albino rats by aqueous extract of garlic. *J Ocul Pharmacol Ther* 25: 395-400, 2009.
24. Cao J, Wang T and Wang M: Investigation of the anti-cataractogenic mechanisms of curcumin through in vivo and in vitro studies. *BMC Ophthalmol* 18: 48, 2018.
25. Schrauzer GN: Selenium and selenium-antagonistic elements in nutritional cancer prevention. *Crit Rev Biotechnol* 29: 10-17, 2009.
26. Terry PD, Qin B, Camacho F, Moorman PG, Alberg AJ, Barnholtz-Sloan JS, Bondy M, Cote ML, Funkhouser E, Guertin KA, *et al*: Supplemental selenium may decrease ovarian cancer risk in African-American Women. *J Nutr* 147: 621-627, 2017.
27. Woth G, Nagy B, Mérei Á, Ernyey B, Vincze R, Kaurics Z, Lantos J, Bogár L and Mühl D: The effect of Na-selenite treatment on the oxidative stress-antioxidants balance of multiple organ failure. *J Crit Care* 29: e7-e11, 2014.
28. Içe F, Gök G and Pandir D: Acute effects of lipopolysaccharide (LPS) in kidney of rats and preventive role of vitamin E and sodium selenite. *Hum Exp Toxicol* 38: 547-560, 2019.
29. Tamada Y, Fukiage C, Nakamura Y, Azuma M, Kim YH and Shearer TR: Evidence for apoptosis in the selenite rat model of cataract. *Biochem Biophys Res Commun* 275: 300-306, 2000.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.