Hesperetin treatment attenuates glycation of lens proteins and advanced-glycation end products generation

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Abstract. Advanced glycation end products (AGEs) in lens proteins increase with aging, thus inducing cataracts and/or presbyopia. Hesperetin (Hst), which is an abundant plant flavanone largely derived from citrus species, and its derivatives attenuate cataracts and presbyopia *in vivo* and *in vitro*; however, no reports have described its effects on AGE formation in lens proteins. The present study demonstrated that AGEs in lens proteins increase with age in mice. Additionally, it showed that Hst can prevent AGEs and N(ε)-carboxymethyl-lysine generation and modification of lens proteins using *in vitro* in human lens epithelial cell lines and *ex vivo* in mouse lens organ cultures. Furthermore, treatment with Hst prevented lens hardening and decreased chaperone activity in lens proteins. These results suggested that Hst and its derivatives are good candidates for the prevention of presbyopia and cataracts.

Introduction

Cataracts, which comprise opacification of the lenses, are the leading cause of visual impairment worldwide. Due to the global extended life expectancy and increased aging population, the burden and impact of age-related cataracts are expected to become more significant. The main causes of cataracts are aging, ultraviolet radiation, inflammation, diabetes, smoking, and application of steroid drugs, which result in the generation of oxidative stress in the lenses (1-3). As there is no protein turnover in the majority of lens tissues, post-translational modifications occur in a number of lens proteins, such as deamidation (4-6), racemization (7), truncation (8), oxidation (5,6), and glycation (6). The lens has a high concentration of protein, which helps it maintain a high refractive index. The main and longest-living proteins in the human body are crystallins (9). Among them, α-crystallin is associated with chaperone activity that suppresses the aggregation of denatured proteins. However, the three-dimensional structure of α-crystallin changes with post-translational modifications, which may cause reduced chaperone activity and loss of lens transparency.

Advanced glycation end products (AGEs) in lens proteins increase with aging through the formation of carbonyl compounds attributable to reactions with decreasing sugars. AGEs in the lens are initiated by ascorbate, methylglyoxal (MGO), and glucose. Ascorbate undergoes oxidation to produce major glycation agents, such as erythrulose and 3-deoxythreosone. Furthermore, it has been reported that oxidative ascorbic acid increases because glycation agents also increase with aging (10). Although a number of proteins in the lens change to AGEs, there are no methodologies that explain all AGEs. Therefore, most studies have used N(ε)-carboxymethyl-lysine (CML) as an AGE indicator. The present study used the CML level as an indicator of AGEs.

Presbyopia is directly correlated with protein-linking AGE levels because of increasing lens stiffness (11). Furthermore, presbyopia is the earliest observable symptom of age-related nuclear cataracts (12). Recently, dysfunctional lens syndrome, which comprises natural lens changes, has become more commonly observed (13). Therefore, preventing AGE accumulation is one of the best strategies for preventing cataracts and presbyopia.

Hesperetin (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 a bioflavonoid because of its various biological activities, including anti-inflammatory, anti-oxidative, anti-diabetic,

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Abbreviations: AGE, advanced glycation end product; ALDH, aldehyde dehydrogenase; CML, N(ε)-carboxymethyl-lysine; ERT, erythrulose; Hst, hesperetin; MGO, methylglyoxal; TRPV, transient receptor potential vanilloid

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and anti-hypertensive activities (14,15). The authors previously reported that Hst and its derivates could prevent cataracts and presbyopia in mice and rats (16-20). Hst can prevent AGE formation *in vivo* and *in vitro* (21,22) and it can prevent the onset of cataracts and presbyopia, thus preventing AGE formation. To elucidate whether Hst could inhibit AGE formation in lens proteins, the present study used *in vitro* human lens epithelial cell lines and *ex vivo* mouse lens organ cultures.

Materials and methods

Materials. Experimental C57Black/6JJ (C57BL/6) mice were purchased from Japan SLC Inc. (Shizuoka, Japan) and fed standard rodent chow (cat. no. CE-2; Clea Japan Inc.). The AGE antibody was obtained from Trans Genic Inc. (cat. no. KAL-KH001-01). The CML antibody was obtained from Abcam (cat. no. ab27684). The α-crystallin and β-actin antibodies were obtained from Santa Cruz Biotechnology, Inc. (cat. nos. sc-28306 and sc-47778). Aldehyde dehydrogenase (ALDH) was purchased from MilliporeSigma. Paraformaldehyde was obtained from Nacalai Tesque, Inc.

Animals. Nine-week-old of twelve male mice (average weigh was 23 g), 10-week-old of eight male mice (average weigh was 23 g), 25-week-old of eight male mice (average weigh was 28 g), and 75-week-old of six male mice (average weigh was 32 g) were used in this study. Animals were housed in a temperature-controlled environment with a 12/12-h regular light/dark cycle. The mice were sacrificed using 5% inhalational isoflurane for >3 min. The lenses were removed after confirming that the animals had stopped breathing and the heart had ceased to beat. All animal handling procedures were performed in accordance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research and the National Institutes of Health guidelines for the care and use of laboratory animals (arvo.org/About/policies/statement-for-the-use-of-animals-in-ophthalmic-and-vision-research/). The Keio University Animal Research Committee approved all animal experiments performed during this study [cat. no. 11014-(8)]. All animal experiments performed during this study was completed by October 2022.

High glucose stimulation to induce AGEs. Immortalized human lens epithelial cells (ihLECs) were established using transfection with SV40 large T antigen from cataract patient (23). The ihLECs were cultured under standard conditions in Dulbecco's modified Eagle medium/nutrient mixture F-12 (Nacalai Tesque, Inc.) containing glutamine (4 mM), penicillin-streptomycin antibiotic mixture (100 U/ml and 100 mg/ml, respectively) and 10% fetal bovine serum (Biosera) under the 5% CO_2 culture condition. The mice lenses were isolated after sacrifice and cultured in fresh equilibrated medium 199 with Earle's salts (Thermo Fisher Scientific, Inc.) with amphotericin B (2.5 mg/ml; FUJIFILM Wako Pure Chemical Corporation) and the penicillin-streptomycin antibiotic mixture containing 10% fetal bovine serum. To stimulate AGE formation, ihLECs or lens organs were treated with 31 mM glucose, 500 μM MGO, or 500 μM erythrose (ERT) for 2 days.

Immunoblot analysis. Lenses or cells were homogenized in ice-cold radioimmunoprecipitation buffer inhibitor cocktail for general use (Nacalai Tesque, Inc.). Protein concentrations were measured using Bradford assay dye. Denatured 10 μg protein samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Then, membranes were incubated with 5% skim milk solution (Morinaga co., Tokyo) for 1 h at room temperature, followed by incubated with the primary antibody. The primary antibodies in this study were anti-AGE (1:1,000), anti-CML (1:10,000) and anti-β-actin (1:1,000). At 1 h after incubation with the primary antibody at room temperature, the membranes were washed with Tween20 (0.1%) containing phosphate-buffered saline (T-PBS) more than three times. The corresponding secondary antibodies, either horseradish peroxidase-conjugated anti-mouse antibodies (cat. no. NXA931-1ML; 1:3,000; Cytiva) or horseradish peroxidase-conjugated anti-rabbit antibodies (cat. no. 7074; 1:3,000; Cell Signaling Technology, Inc.), were added and incubated at room temperature for 1 h. After washing the membranes, the protein signals were visualized using an enhanced chemiluminescence detection system (Cytiva). After exposure, NIH ImageJ 1.44 was used for gray analysis (National Institutes of Health).

Measurement of lens elasticity. Lens elasticity was measured using SoftMeasure HG1003-SL (Horiuchi Electronics Co., Ltd.) as previously described (19,20). Briefly, the lens was mounted soon after euthanasia and then a load was applied to the top of the lens to measure the force and indentation displacement. The mean strain (%) under 0.05 N of force was assessed using Young's modulus.

Immunohistochemistry. To perform immunohistochemistry, proteins in cultured ihLECs were fixed in 4% paraformaldehyde for 5 min at room temperature, followed by incubation with 0.2% Triton X-100 for 5 min to permeabilize the cell membranes at room temperature. The cells were incubated in a 3% bovine serum albumin/3% normal goat serum blocking solution for 5 min at room temperature. After blocking, the cells were first incubated with an anti-AGE antibody (1:100) or anti-CML antibody (1:1,000). After 1 h of incubation at room temperature, the cells were washed with phosphate-buffered saline and incubated with either anti-mouse or anti-rabbit Alexa Fluor 488 secondary antibody (cat. nos. A28175 and A11008, respectively; Thermo Fisher Scientific, Inc.) with 0.125 mg/ml 4',6'-diamidino-2-phenylindole (DAPI) for fluorescent labeling of the nuclei for 1 h. Organ culturing was performed by fixing the lenses in 0.75% paraformaldehyde and then they were prepared for sectioning using established protocols (24,25). The 12-μm-thick sections were obtained using a freezing microtome (cat. no. CM1950; Leica Microsystems GmbH), transferred onto microscope slides, incubated in blocking solution for 1 h, and incubated further in α-AGE antibody (1:100) or α-CML antibody (1:100) in blocking solution for 12 h at 4˚C. The slides were washed and incubated with either anti-mouse or anti-rabbit Alexa Fluor 488 secondary antibodies with DAPI and wheat germ agglutinin. Confocal images were obtained using a laser scanning microscope (FV-3000; Olympus Corporation).

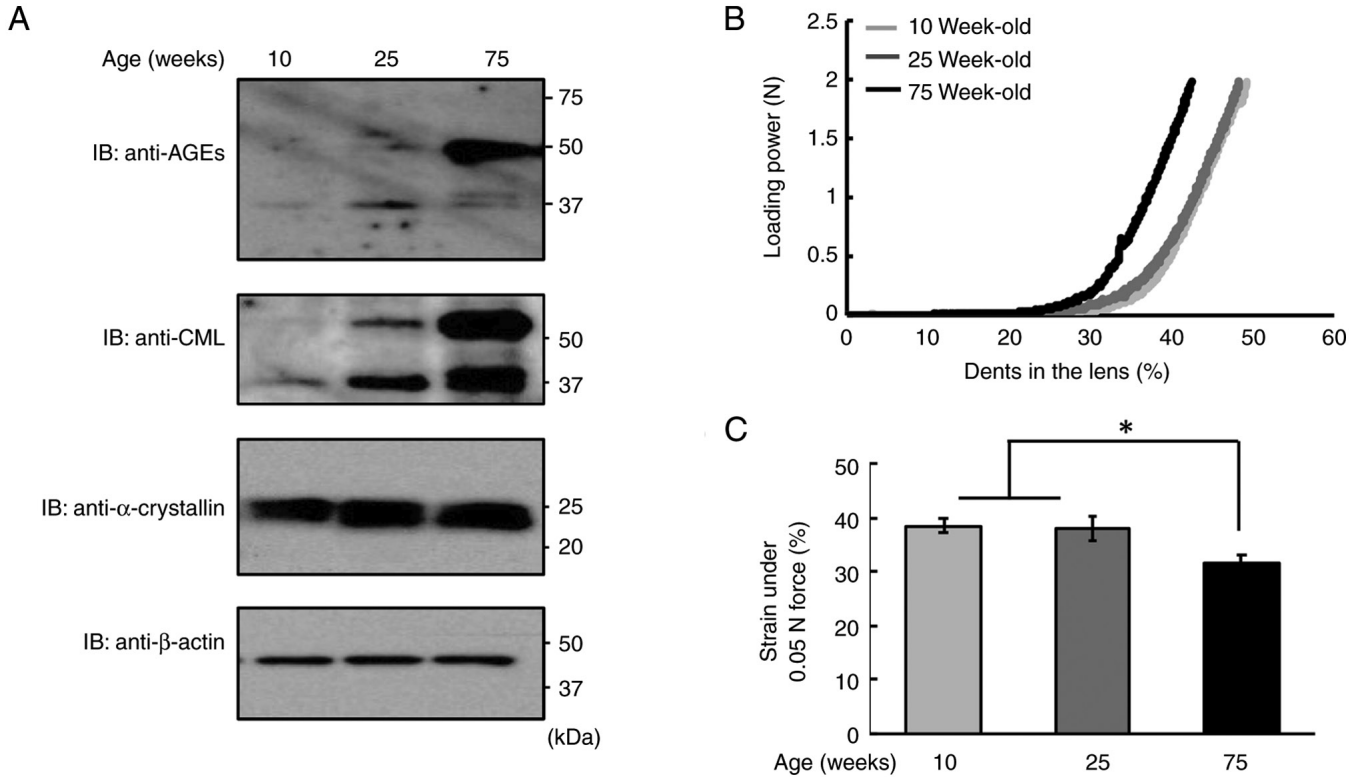

Figure 1. AGE modifications of lens proteins and lens elasticity changes with aging. (A) AGE and CML modifications of lens proteins of 10, 25, and 75-week-old mice were detected using an immunoblot analysis. (B) Lens elasticity was measured using SoftMeasure (Horiuchi Electronics Co., Ltd.) and (C) indentations of the lens were assessed under 0.05 N of force. The experiments used three to five independent samples per group. Data are presented as the mean ± standard error of the mean. *P<0.05 vs. the control group (n=6-8 mice/group). AGE, advanced glycation end product; CML, N(ε)-carboxymethyl-lysine.

Chaperone activity measurement. Chaperone activity was measured as previously reported, with minor modifications (26). Briefly, ALDH was aggregated by heating (42˚C) with 100 mM 1,10-phenanthroline (FUJIFILM Wako Pure Chemical Corporation) in 50 mM sodium phosphate buffer containing 100 mM NaCl (pH 7.0). Water soluble proteins were added 10 min before heat stimulation. The extent of aggregation was estimated by monitoring light scattering at 360 nm using a microplate reader (Infinite M200; Tecan Group, Ltd.). The rate of protein aggregation was expressed as $^{\Delta}$A360/60 min.

Statistical analysis. All data are reported as the mean ± standard error of the mean. The statistical analysis of data was performed using a one-way analysis of variance with Tukey's multiple comparison test using SPSS software (version 24; IBM Corp.). P<0.05 was considered to indicate a statistically significant difference.

Results

AGEs and lens elasticity with aging. The present study tested whether AGE modifications and lens elasticity increased with age in 10, 25, and 75-week-old mice. AGEs in mouse lenses were measured using an immunoblot analysis, and lens elasticity was evaluated using SoftMeasure (Horiuchi Electronics Co., Ltd.). Using immunohistochemistry, AGE-modified proteins were detected in the lenses of 75-week-old mice, but not in those of 10-week-old and 25-week mice. Similar to AGE-modified proteins, CML-modified proteins were detected in the lenses of 75-week-old mice, but not in those of 10 and 25-week-old mice. The protein levels of α-crystallin or β-actin were not significantly different in the lenses of 10, 25 and 75-week-old mice (Fig. 1A). Subsequently, it was elucidated that AGE formation could affect lens elasticity and the lens stiffness was measured immediately following sacrifice. The lenses of 75-week-old mice were harder than those of 10 and 25-week-old mice. There was no difference in the lens stiffness of 10 and 25-week-old mice (Fig. 1B). Lens elasticity was assessed under 0.05 N of force and was significantly increased in 75-week-old mice (Fig. 1C). These results suggested that generating AGEs formation and changes in the elasticity of lens with were observed in the mouse model.

Hst treatment and AGE formation in vitro. The present study investigated whether Hst treatment affected AGE formation in lens epithelial cell lines using ihLECs to assess AGE formation. The structural formula of Hst is shown in Fig. 2A. CML, which is the major AGE compounds that bind to proteins in ihLECs, was detected. High-glucose medium with MGO and ERT can stimulate the binding of AGEs and/or CML with proteins (10). AGE formation in proteins was significantly increased in ihLECs treated with MGO and ERT in high-glucose medium, and Hst treatment stopped AGE and CML formation in a dose-dependent manner (Fig. 2B). Immunohistochemistry staining with AGEs and CML also revealed that high-glucose stimulation increased AGE and CML signals in ihLECs and that these were cancelled by Hst treatment (Fig. 3A and B).

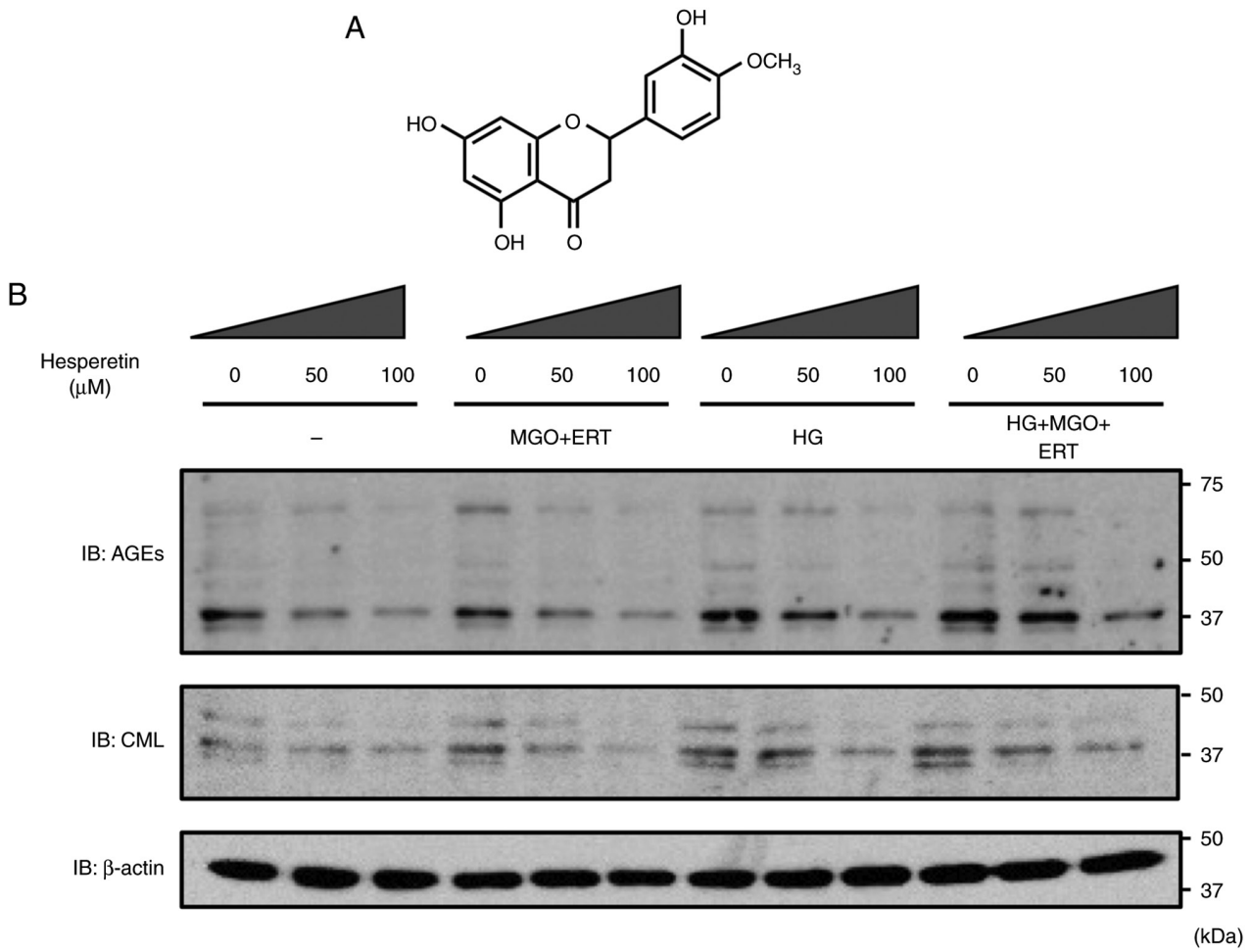

Figure 2. Effect of Hst treatment on AGE modifications of lens epithelial cell lines. (A) The structural formula of Hst. (B) Immunoblot analysis of AGE and CML modifications of lens proteins stimulated with high-glucose medium, MGO and CML administration with Hst. Hst treatment prevented AGE and CML modifications of lens proteins in a dose-dependent manner. The experiments used three independent samples per group. Hst, hesperetin; AGE, advanced glycation end product; CML, N(ε)-carboxymethyl-lysine; MGO, methylglyoxal; HG, High glucose stimulation (31 mM glucose).

These results suggested that Hst could prevent AGE and CML formation in the lens.

Hst affects lens chaperone activity and elasticity. To investigate whether Hst treatment could ameliorate lens sclerosis, which causes presbyopia, lens elasticity was measured using SoftMeasure (Horiuchi Electronics Co., Ltd.), and Young's modulus was calculated (Fig. 4A). Organ culturing of the lenses was performed with high-glucose medium and/or Hst. Prior to glucose stimulation, lenses were incubated at 42˚C for 6 h to promote AGE formation. There were no significant differences in the lenses incubated under the control glucose condition, regardless of Hst treatment. The elasticity of the lenses incubated with high-glucose medium at 42˚C was significantly decreased compared to those incubated under the control glucose condition; however, Hst treatment markedly inhibited the decrease in elasticity (Fig. 4B).

Lens proteins are associated with chaperone activity that prevents protein aggregation and cataracts. Following high-glucose stimulation, the lens chaperone activity was measured by evaluating the light scattering with ALDH at 360 nm (Fig. 4C). An increase in light scattering indicated ALDH aggregation and inhibition of protein aggregation, depending on the chaperone activity. Light scattering with ALDH was increased without lens proteins, and it was suppressed by the addition of water-soluble lens proteins at 37˚ under the control condition, normal glucose culture condition, and high-glucose culture condition with Hst; however, light scattering in the lens was not changed under the high-glucose culture condition without Hst. Data are shown as the relative $^{\Delta}$A360/180 min values of light scattering with ALDH without lens proteins (defined as 100%; Fig. 4D). Lens proteins subjected to high-glucose stimulation completely lacked the inhibition of light scattering; however, Hst treatment of lenses subjected to high-glucose stimulation protected against increased light scattering. These data suggested that Hst treatment prevents protein aggregation by maintaining the chaperone activity and preventing lens hardening and presbyopia.

Hst treatment reduces AGE formation ex vivo. The present study investigated whether lens hardening and decreased chaperone activity occurred based on AGE formation and examined the results of immunohistochemical staining with AGE-binding protein or CML-binding protein (Fig. 5). To stimulate AGE formation, lenses were incubated at 42˚C for

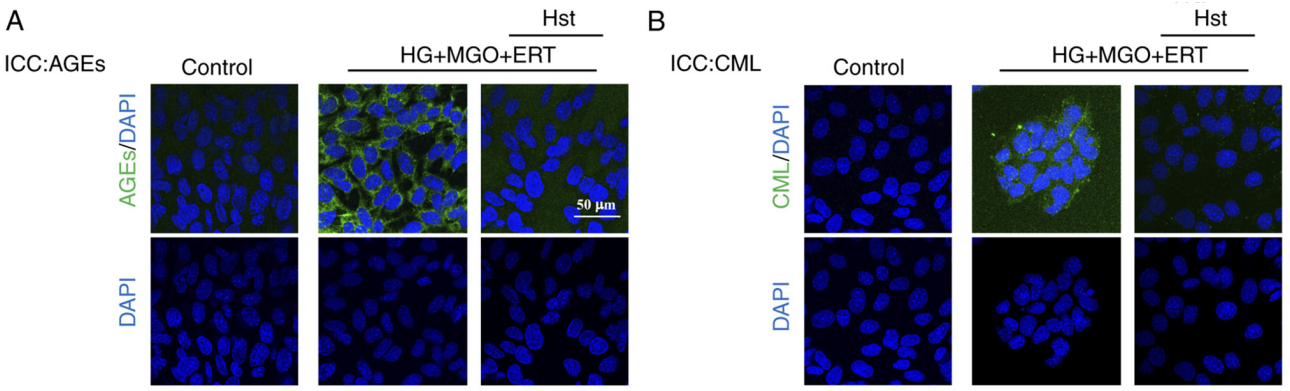

Figure 3. Effect of Hst treatment on AGE and CML formations in lens epithelial cell lines. The immortalized human lens epithelial cells were incubated using high-glucose medium with or without Hst for 5 days. After incubation, lens proteins were fixed with 4% paraformaldehyde and immunostained with (A) AGE antibody and (B) CML antibody. The nuclei are labeled with DAPI (blue). Hst, hesperetin; AGE, advanced glycation end product; CML, N(ε)-carboxymethyl-lysine.

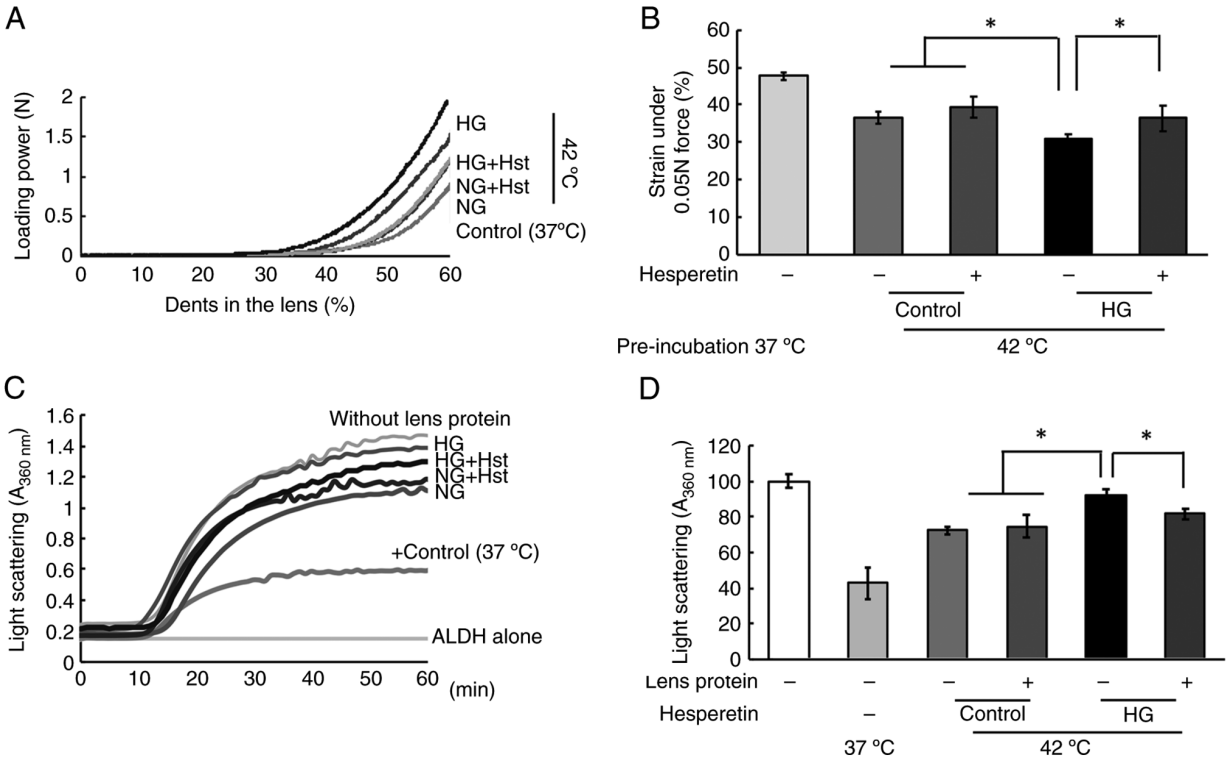

Figure 4. Lens elasticity and lens chaperone activity *ex vivo*. Lenses were incubated in M199 medium for up to 2 days. Lens elasticity and chaperone activity in the lens were measured. (A) Lenses incubated with high-glucose solution with or without Hst were mounted, and measurements of the pressure on and indentations of the lenses were performed. (B) Strain when lenses were pushed with 0.05 N of force is shown (bar graph). (C) The chaperone activity of water-soluble lens fractions was measured by light scattering with ALDH at 360 nm following the administration of high-glucose solution and/or Hst. Increased light scatting at 360 nm indicates ALDH aggregation, and inhibition of light scattering is dependent on chaperone activity. (D) Relative chaperone activity of lens proteins. The relative chaperone activity of cultured lens proteins with Hst was calculated using light scattering with ALDH for 60 min after the addition of 1,10-phenanthroline. The change in light scattering with ALDH without water-soluble lens proteins was defined as 100%. Bars represent the mean value. Error bars represent the standard error in (B) and (D). *P<0.05). Hst, hesperetin; NG, normal glucose stimulation (5.5 mM glucose); HG, high glucose stimulation (31 mM glucose); ALDH, aldehyde dehydrogenase.

6 h before glucose, MGO and ERT stimulation, with or without 100 μM Hst. As a result, AGE-binding and CML-binding proteins were not detected in the lenses incubated under the normal glucose condition either with or without Hst. AGE-binding and CML-binding proteins were found in the lenses stimulated under the high-glucose condition; however, these signals were diminished in the lenses treated with Hst (Fig. 5A and B). These results suggested that Hst treatment could attenuate lens sclerosis by preventing chaperone activity and AGE formation.

Discussion

Presbyopia is an age-related physiological reduction of accommodation that leads to unsatisfactory clarity of near vision (27,28). This condition usually begins after ~45 years. In

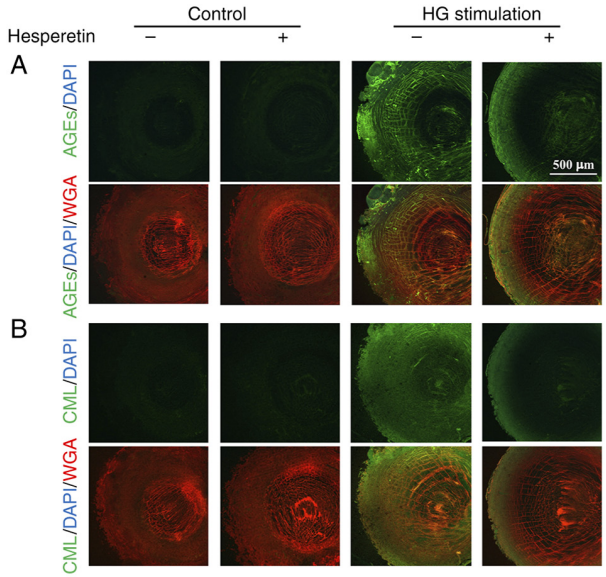

Figure 5. Effect of Hst treatment on AGE and CML formations in organ-cultured lenses with or without high-glucose stimulation. At two days following stimulation, lenses were sectioned and stained using AGE or CML antibody. (A) The effect of AGE formation on organ-cultured cells. (B) Effect of CML formation on organ-cultured cells. AGE and CML immunolabeling are presented in green. Membranes and nuclei are labeled with wheat germ agglutinin (WGA; red) and 4',6'-diamidino-2-phenylindole (DAPI; blue), respectively. Hst, hesperetin; AGE, advanced glycation end product; CML, N(ε)-carboxymethyl-lysine; HG, high glucose stimulation (31 mM glucose).

2030, the number of individuals with presbyopia is expected to increase to 2.1 billion globally (29). There are two age-related biomechanical changes that have been hypothesized as causes of presbyopia: aging of the ciliary muscle, which exerts the force required to change the shape of the crystalline lens, and stiffening of the lens itself, which increases with age (30,31). Ostrin and Glasser (32) reported that muscle function is normal beyond the onset of presbyopia in the eyes of primates and humans. Furthermore, lens sclerosis is a major inducer of presbyopia, thus demonstrating that the replacement of a presbyopic lens with a soft polymer restores the accommodative ability (33). The present study revealed that AGE formation affects the lens stiffness *ex vivo*. The cause of presbyopia is lens hardening by several sources such as decreasing levels of α-crystallin water solubility and chaperone activity and increasing lens disulfides such as oxidative glutathione (34). The accumulation of denatured proteins and disulfides makes the lens cloudy, which is termed a cataract. It has also been reported that dysfunctional lens syndrome includes three stages: Stage 1, presbyopia or cataracts (age 42-50 years); stage 2, accommodation loss (from ≥50 years); and stage 3, lens opacity, poor vision quality, and full cataracts (from ≥65 years) (13). Therefore, maintaining the accommodation ability of the lens and preventing lens sclerosis are the best ways to improve the quality of vision in elderly individuals and prevent cataracts.

The pharmacological treatment of presbyopia has been studied recently. In November 2021, the United States Food and Drug Administration approved the use of 1.25% pilocarpine hydrochloride ophthalmic solution as the first eye drop treatment for presbyopia (35). This eye drop effectively increases the depth of focus and causes the pupils of the eye to constrict, thus producing a small pupil and creating a pinhole effect for ~6 h. However, retinal detachments and vitreofoveal traction were reported following pilocarpine eye drop treatment (36-38). Other candidate eye drops for presbyopia comprise the choline ester of lipoic acid (EV-06), which can replenish glutathione and decrease disulfide linkages in lens proteins of aged lenses (39). The study did not report any serious adverse effect from use of EV-06, drug development had been abandoned following phase 2b clinical trial in which the drug did not achieve a statistically significant dose response. In addition, mercaptoethylguanidine could increase lens stiffness by preventing AGE formation in lens proteins and increasing lens glutathione levels (40). It is suggested that oxidative stress and AGE formation with age may promote lens hardening and cause presbyopia (11). The authors previously reported that Hst and its derivatives have antioxidant and anti-cataract effects (18) and in the present study, it was found that Hst treatment could prevent AGE generation and AGE formation in the lens protein and lens hardening caused by presbyopia.

The lens is a large avascular tissue that has evolved an internal microcirculation system that compensates for the absence of a blood supply by generating circulating fluxes of ions and water that deliver nutrients and remove metabolic waste. This microcirculation system is generated by a circulating flux of sodium, which drives the flow of isotonic fluid that enters the lens at both the anterior and posterior poles via an extracellular pathway. Sodium and water cross the membranes of deeper fiber cells before returning to the surface via an intracellular pathway mediated by gap junction channels that direct sodium and water to the lens equator, which is regulated by a dual feedback system that utilizes transient receptor potential vanilloid (TRPV)1 and TRPV4 (41). Moreover, TRPV1 and/or TRPV4 in peripheral fiber cells of the lens alter their membrane trafficking by accommodating changes, meaning that TRPV channels located in the cytoplasm comprise a protein pool that meets physiological needs and accommodates changes. The authors previously reported that treatment with oral a-glucosyl hesperidin, which is Hst derivatives with a water solubility ~10,000 times higher than that of Hst, altered TRPV4 localization in the peripheral fibre cells of the lens to control water microcirculation (20). Additionally, oral treatment ameliorated lens fluid influx and osmotic imbalance in the lenses of individuals with diabetes and cataracts (42). These results suggest that TRPV channels could contribute to lens sclerosis and might be a target for presbyopia treatment.

The limitation to this study was *in vitro* and *ex vivo* experiments. In this study, we experienced Het treated culture cells *in vitro* study and mouse lens organ culture *ex vivo* study. *In vivo* experiments such as the suppression of AGEs formation by experimental animals fed Het or Hst derivatives will be needed.

Hst treatment attenuates lens hardening *ex vivo* and prevents AGE generation *in vitro*. The results of the present study revealed that Hst treatment may prevent lens sclerosis and cataract formation attributable to water influx, TRPV channel distribution and AGE generation. Further research is needed to evaluate the onset of presbyopia attributable to oxidative stress, AGE generation and TRPV channel localization.

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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' contribution

YN, HT, and MFT defined the study themes. YN, NN, MFT and HT designed the study. YD and YN performed laboratory experiments. YN, NM, SE, NN, NY and HT analyzed and interpreted the data. YD, YN and HT confirm the authenticity of all the raw data. YN was a major contributor in writing 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. 11014-(8)].

Patient consent for publication

Not applicable.

Competing interests

NM and SE are employees of Hayashibara Co., Ltd. (Okayama, Japan). The funders had no role in the design of the study; collection, analysis, or interpretation of data; writing of the manuscript; or decision to publish the results. The other authors also declare that they have no competing interests.

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