Semiquantitative analysis of protein expression in heated rat lens using shotgun proteomics

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Abstract. Previous studies have reported that a strong correlation between the estimated cumulative thermal exposure in the crystalline lens and the incidence of nuclear cataracts; however, the precise relationship between temperature and cataracts remains to be fully elucidated. In the present study, the shotgun liquid chromatography/mass spectroscopy-based global proteomic approach was applied to investigate cataract-inducing factors in lens cultured at normal (35.0˚C) and slightly warmer (37.5˚C) conditions. In the rat lens, 190 proteins (total) were identified. Of these, 48 proteins (25.3%) were found in lenses cultured at both 35.0˚C and 37.5˚C. Moreover, 85 proteins (44.7%) were unique to lenses cultured at 35.0˚C, while 57 proteins (30.0%) were unique to lenses cultured at 37.5˚C. Protein expression changes in rat lenses cultured at 37.5˚C were examined using a label-free semiquantitative approach that uses spectral counting and Gene Ontology analysis. Filensin and vimentin protein expression, key factors in maintaining lens structure, were decreased. These findings may serve as a valuable indicator for elucidating the relationship between temperature and the onset of nuclear cataracts.

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Abbreviations: GO, Gene Ontology; LC-MS/MS, liquid chromatography with tandem mass spectrometry; NSAF, normalized spectral abundance factor; NSI, nanoelectrospray ionization; Rsc, log_2-transformed ratio of protein abundances

Key words: lens, temperature, shotgun proteomics, cataract, protein

Introduction

Nuclear cataracts usually develop slowly over time and represent the most common form of age-related cataracts globally (1,2). They are considered the primary form of senile cataracts, leading to visual disturbances characterized by increasing forward light scattering, higher-order aberrations, and backward light scattering, even in the early stages (3). Heister (4) reported cataracts arising in glassblowers due to infrared light in 1739. In addition, residents of regions with warmer climates tend to experience presbyopia at an earlier age (5).

A number of studies have investigated the relationship between environmental temperature and the development of nuclear cataracts, suggesting that prolonged exposure to hot environments may increase the risk of developing this condition (5-7). Moreover, as environmental temperature increases, UV exposure generally increases, in turn damaging eye tissue and increasing the risk of nuclear cataracts (8). Furthermore, exposure to hot environments exposes ocular tissues to thermal stress, which can lead to oxidative stress. Epidemiological studies have shown a markedly higher prevalence of grade 1 or higher nuclear cataracts, as per the World Health Organization cataract grading system, in tropical and subtropical regions compared with temperate and subarctic areas, irrespective of ethnicity (7,9). The relationship between environmental temperature and the development of nuclear cataracts is therefore complex, involving a myriad of factors.

In our previous study, using a comprehensive computational approach, it was observed that the temperature of the eye lens may rise to 37.5˚C or beyond with an increase in environmental temperatures. By setting the lens temperature threshold at 37.5˚C, we found a positive correlation between cumulative heat exposure, defined as the cumulative temperature difference exceeding 37˚C, and the incidence of nuclear cataracts over a decade. Additionally, there was a connection between the temporal pattern of mean lens temperature increase and the incidence of nuclear cataracts (10). However, the precise relationship between temperature and the development of nuclear cataracts remains to be elucidated.

The present study applied a proteomics approach to investigate the relationship between temperature and nuclear cataracts in the present study. Shotgun liquid chromatography/mass spectrometry-based global proteomic analysis has emerged as a valuable technique for various applications (11-13). Previously, we applied this technique to assess alterations in protein expression within the cornea and lens of streptozotocin-induced diabetic rats. The findings revealed that a reduction in superoxide dismutase levels contributes to the progression of diabetic cataracts. Additionally, upregulation of lumican was observed, which resulted in delayed corneal wound healing in corneas affected by diabetic keratopathy (14,15). This shotgun proteomic approach was applied to investigate the cataract-inducing factors in lens incubated at physiologically normal and warmer ocular temperatures in the present study.

Materials and methods

Materials. Urea was obtained from Cytiva. Thiourea and Triton X-100 were procured from Nacalai Tesque, Inc. All other reagents and solvents used were of analytical or HPLC grade.

Animals. Wistar rats (male; age, 6 weeks; weight, 214±3 g; n=3) were supplied by Sankyo Labo Service Corporation, Inc. All experiments were conducted in accordance with the regulations approved by the Ethics Committee of Kindai University Faculty of Pharmacy (approval no. KAPS-2021-004, April 1, 2021). The rats were kept in a room at 25˚C and 55±10% humidity with a 12-h light/dark cycle (3 rats per cage) with unlimited access to food and water. The present study adhered to the ARRIVE guidelines (16) and the Guiding Principles sanctioned by The Japanese Pharmacological Society (17). Euthanasia was performed by administering pentobarbital (200 mg/kg, i.p.) in line with the AVMA 2020 guidelines (18).

Ex-vivo culture of rat lenses. All experiments and animal handling were conducted in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research (19). Male Wistar rats purchased at 6 weeks of age were housed for 1 week, and those aged 7 weeks were used for the experiments (weight, 231±7 g; n=3). The three rats were housed in groups in suspended wire-bottomed cages with unlimited access to food and water, maintained under a 12-h light/dark cycle (temperature, 25˚C; humidity, 55±10%). Euthanasia was performed by administering pentobarbital (200 mg/kg, i.p.) in line with the AVMA 2020 guidelines (18). Lenses were then excised from the rats and were cultured as previously described (20), with some modifications. In brief, the six lenses extracted from the three rats were divided into two groups (3 lenses/group) and cultured in serum-free M199 medium (Thermo Fisher Scientific, Inc.), supplemented with 0.1% (w/v) bovine serum albumin, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml fungizone (Thermo Fisher Scientific, Inc.) at either 35.0˚C or 37.5˚C in a humidified atmosphere of 5% CO_2 for 48 h.

Tryptic digestion of proteins extracted from rat lenses. In the present study, three lenses were combined into a single sample and were homogenized. The proteins were extracted using RIPA Lysis Buffer (Santa Cruz Biotechnology, Inc.) and the supernatant was collected following centrifugation at 13,000 x g at 4˚C for 10 min. Protein concentrations were measured using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Inc.). Gel-free trypsin digestion was performed following a previously established protocol (21). In brief, 10 μg of protein extract from each sample was reduced at 37˚C for 30 min using tris (2-carboxyethyl) phosphine (20 mM) in ammonium bicarbonate buffer (50 mM) and dithiothreitol (45 mM). The proteins were then alkylated with iodoacetamide (100 mM) in ammonium bicarbonate buffer (50 mM) at 37˚C for 30 min. After alkylation, the samples were digested at 37˚C for 24 h with MS-grade trypsin gold (Promega Corporation) at a trypsin/protein ratio of 1:100 (w:w). Finally, the digested peptides were purified using PepClean C-18 Spin Columns (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions.

Liquid chromatography with tandem mass spectrometry (LC-MS/MS) identification of proteins. The semiquantitative analysis of protein expression by using LC-MS/MS analysis were performed following method of Kawamura *et al* (22). Peptide samples (2 μg) were injected using a peptide L-trap column (Chemicals Evaluation and Research Institute) with an HTC PAL autosampler (CTC Analytics AG). The peptides were then separated on a Paradigm MS4 system (AMR Inc.) fitted with a reverse-phase C18 column (Sunniest, 3 μm diameter gel particles, 120 Å pore size, 0.23x150 mm; Chromanik Technologies Inc.). The mobile phase comprised 0.1% formic acid in water (solution A) and acetonitrile (solution B), with a gradient increasing from 5-40% solution B over 120 min at a constant flow rate of 1 μl/min. The gradient-eluted peptides were directed into the mass spectrometer via a nanoelectrospray ionization (NSI) interface, with the separation column outlet directly connected to the NSI needle. Peptide analysis was performed using an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Inc.) without sheath or auxiliary gas. The mass spectrometry scan sequence involved a full-scan MS for precursor ions over a 300-2,000 m/z range at a resolution of 60,000, followed by MS/MS in normal/centroid mode. Positive ion mass spectra were acquired in a data-dependent manner, with MS/MS fragmentation of the most intense peaks in each MS scan using Collision-Induced Dissociation, and a dynamic exclusion window of 30 sec. All MS/MS data were analysed against the SwissProt Rattus database selected from SwissProt_2022_01 (https://www.uniprot.org/uniprotkb?query=*&facets=reviewed%3Atrue), which contains 566,996 peptides and 22,502 proteins using Mascot version 2.4.01 (Matrix Science). The search parameters included trypsin digestion with up to two missed cleavages, a mass tolerance of ±30 ppm, an MS/MS tolerance of ±0.8 Da, cysteine carbamidomethylation as a fixed modification and methionine oxidation as a variable modification.

Semiquantitative analysis of identified proteins. The data were presented as the cumulative values from three measurements and the fold change in expression was determined as the $\log_2$-transformed ratio of protein abundance (Rsc) and

assessed through spectral counting (23). The value of Rsc was determined utilizing the subsequent equation:

$$\mathrm{Rsc} = \log_2 \frac{n_s + f}{n_n + f} + \log_2 \frac{t_n + n_n + f}{t_s + n_s - f}$$

Where, n_n and n_s represent spectral counts for proteins in rat lenses cultured at 35.0˚C and 37.5˚C, respectively. The t_n and t_s denote the total numbers of spectra for all proteins in the respective samples (35.0˚C and 37.5˚C), respectively. The f is a correction factor set to 1.25.

For comparative analysis, relative protein abundance was computed by the normalized spectral abundance factor (NSAF) (24). The value of NSAF was determined using the subsequent equation:

$$\mathrm{NSAF} = \frac{\mathrm{SpC}_n / \mathrm{L}_n}{\mathrm{SUM}(\mathrm{SpC}_n / \mathrm{L}_n)}$$

Where, SpCn refers to the spectral count of proteins in rat lenses incubated at 35.0˚C and 37.5˚C, while Ln denotes the length of these proteins in the lenses at the same temperatures. Proteins with differential expression were identified when the ratio Rsc was >1 or <-1, indicating fold changes >2 or <0.5, respectively. Table SI shows the raw data of semiquantitative analysis in the present study.

Bioinformatics. The present study explored the role of proteins that exhibit notable changes in expression in nuclear cataracts. Their sequences were annotated using Gene Ontology (GO) terms related to molecular function, cellular component and biological process, as well as the Kyoto Encyclopedia of Genes and Genomes (KEGG) signalling pathways, using the Database for Annotation, Visualization, and Integrated Discovery (https://david.ncifcrf.gov/tools.jsp) (25-27). The P-values from the GO analysis were also calculated using this database tool.

Results

Changes in protein expression in cultured rat lenses. A total of 133 and 105 proteins were detected in the rat lenses cultured at 35.0˚C and 37.5˚C, respectively. Of these, 190 proteins were identified, including 48 (25.3%) present in both lenses cultured at 35.0˚C and 37.5˚C, 85 (44.7%) unique to those cultured at 35.0˚C, and 57 (30.0%) unique to those cultured at 37.5˚C (Fig. 1A). The proteins expressed in rat lenses were next assessed using a label-free semi-quantitative approach based on spectral counting. Fig. 1B shows the Rsc values for proteins identified in lenses cultured at 35.0˚C and 37.5˚C. A positive Rsc value indicates increased expression in lenses cultured at 37.5˚C, while a negative Rsc value signifies reduced expression. Additionally, the NSAF value was determined for each identified protein at both temperatures. Proteins with Rsc values >1 or <-1 were considered as candidates for differential regulation depending on the culture temperature. The levels of housekeeping proteins, such as α-crystallin A chain, remained unchanged across different culture temperatures (Fig. 1B).

GO analysis was conducted on the candidate proteins regulated in rat lenses cultured at 37.5˚C. This involved searching for GO terms associated with molecular function (Table I), cellular component (Table II) and biological process (Table III), and

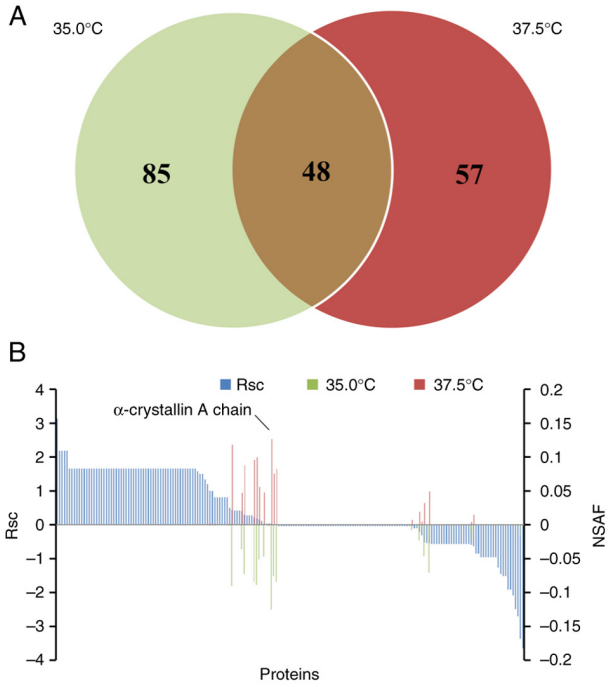

Figure 1. Identification and semiquantitative analysis of proteins with differential expression in rat lenses cultured at 35.0˚C and 37.5˚C. (A) Venn diagram showing the proteins identified in rat lenses at both 35.0˚C and 37.5˚C. (B) Semiquantitative comparison of proteins that are differentially expressed in rat lenses cultured at 37.5˚C. To evaluate protein expression levels in lenses cultured at 35.0˚C and 37.5˚C, Rsc and NSAF values were calculated for the identified proteins. Rsc, $\log_2$-transformed ratio of protein abundances; NSAF, normalized spectral abundance factor.

KEGG pathways (Table IV), with resultant detected counts of 10, 17, 19 and 2, respectively. Among these, the most abundant terms in each category were 'protein binding,' 'cytoplasm,' 'intermediate filament organization,' and 'estrogen signaling pathway,' respectively. In addition, a cutoff value for spectral counting was set to 1, to exclude proteins with low expression levels, and listed proteins with expression changes following culture at 37.5˚C showed Rsc >1 or <-1 in the label-free semi-quantitative method based on spectral counting (Table V). A total of 22 proteins showed Rsc >1 or <-1 and, at 37.5˚C, the expression levels of 11 proteins was upregulated, while that of the other 11 was downregulated. The present study focused on the downregulated proteins at 37.5˚C since they were more likely to be affected than the overexpressed ones. In conjunction with GO analysis results, the factor associated with both 'protein binding' and 'intermediate filament organization' in the cytoplasm was filensin and vimentin. No factors related to 'estrogen signaling pathway' were identified.

Discussion

We previously reported that computer simulation analyses using a supercomputer and a biothermal transport equation have shown a strong relationship between the cumulative thermal dose estimation in the crystalline lens and the prevalence of nuclear cataracts (10). However, the precise relationship between temperature and nuclear cataracts remains unclear. The present study investigated the cataract-inducing factors associated with physiologically normal and warmer ocular

Table I. GO analysis of identified proteins in molecular function category.

Molecular function category	Relative abundance (%)	P-value
Protein binding	23.0	0.001
ATP binding	18.9	0.006
Structural constituent of cytoskeleton	8.11	0.065×10^{-5}
Ubiquitin protein ligase binding	8.11	0.010
GTPase activity	6.76	0.037
Structural constituent of epidermis	5.41	0.022×10^{-2}
Structural molecule activity	5.41	0.020
Double-stranded RNA binding	4.05	0.043
NAD binding	4.05	0.029
Keratin filament binding	2.70	0.024

GO, Gene Ontology; GTPase, guanosine triphosphate; NAD, nicotinamide adenine dinucleotide.

Table II. GO analysis of identified proteins in cellular component category.

Cellular component category	Relative abundance (%)	P-value
Cytoplasm	60.8	0.013×10^{-6}
Cytosol	40.5	0.032×10^{-3}
Synapse	12.2	0.008
Intermediate filament	10.8	0.077×10^{-7}
Keratin filament	9.46	0.012×10^{-4}
Axon	9.46	0.010
Cytoskeleton	8.11	0.015
Cell periphery	6.76	0.055×10^{-2}
Cornified envelope	6.76	0.042×10^{-3}
Cell body	5.41	0.012
Cell projection	5.41	0.027
Lamellipodium	5.41	0.031
Chloride channel complex	4.05	0.014
Filopodium	4.05	0.034
Intermediate filament cytoskeleton	4.05	0.019
Phagocytic vesicle	4.05	0.036
Mitotic spindle microtubule	2.70	0.050

GO, Gene Ontology.

temperatures using a shotgun proteomic approach and found a decrease in filensin and vimentin and an increase in chloride channel protein 2 under the latter temperature (37.5˚C).

Temperature is crucial in the formation of nuclear cataracts. In this regard, we previously reported that simulated lens temperatures ranged from 35.0-37.5˚C, which corresponds to ambient eye temperatures of 19-35˚C in a typical setting Based on these results, the experimental temperatures used in the present study were 35.0˚C and 37.5˚C, mirroring lens temperatures *in vivo*.

Although the quantitative values obtained *via* spectral counting may not be accurate, they reflect expression discrepancies and have been used in previous studies investigating novel diagnostic biomarkers and biological mechanisms (12,14,15,28-31). Thus, the present proteome analysis was semi-quantitative and, for quantitative analysis, more detailed investigations such as western blotting are essential. However, the present study aimed to elucidate how temperature changes, as determined by computer simulation analyses using a supercomputer and a biothermal transport equation, are related to cataract formation by conducting a comprehensive proteome analysis. Therefore, this method was chosen for its ability to provide insights into the connection between temperature changes and cataract development. First, the changes in proteins expression from rat lenses cultured at 35.0˚C and 37.5˚C were identified and their functions scrutinized through analysis against four GO terms. GO analysis revealed that the most prevalent factors identified in the

Table III. GO analysis of identified proteins in biological process category.

Biological process category	Relative abundance (%)	P-value
Intermediate filament organization	14.9	$0.031x10^{-12}$
Process positive regulation of gene expression	9.46	0.028
Cell division	8.11	0.002
Epithelial cell differentiation	6.76	$0.089x10^{-2}$
Keratinization	6.76	$0.015x10^{-2}$
Axonogenesis	5.41	0.009
Cell cycle	5.41	0.023
Mitotic cell cycle	5.41	0.014
Neuron projection development	5.41	0.033
Regulation of protein localization	5.41	0.008
Collagen fibril organization	4.05	0.019
Excitatory postsynaptic potential	4.05	0.047
Glucose metabolic process	4.05	0.037
Intermediate filament-based process	4.05	$0.049x10^{-2}$
Protein deubiquitination	4.05	0.039
Response to electrical stimulus	4.05	0.022
Sensory perception of pain	4.05	0.038
Lens fiber cell development	2.70	0.033
Regulation of protein complex stability	2.70	0.048

GO, Gene Ontology.

Table IV. Kyoto Encyclopedia of Genes and Genomes pathway analysis of identified proteins.

Pathway category	Relative abundance (%)	P-values
Estrogen signaling pathway	8.11	$0.048x10^{-2}$
Staphylococcus aureus infection	6.76	0.001

cellular component, biological Process and molecular function categories were 'cytoplasm,' 'intermediate filament organization' and 'protein binding' respectively. In addition, the effect on the expression system is generally more pronounced when a protein is underexpressed than when it is overexpressed. Taken together, the present study identified factors that satisfied two criteria: A significant number of proteins expressed in response to temperature changes and reduced protein expression levels to half or less at 37.5˚C. As a result, the factors associated with filensin and vimentin were identified in the 'cytoplasm' category and were related to both 'intermediate filament organization' and 'protein binding.' Therefore, variations in the expression of filensin and vimentin were the focus of the present study.

Decreased filensin expression was observed at 37.5˚C. Filensin is an important protein for maintaining water balance in the lens and its decrease affects lens function. Moreover, filensin interacts with aquaporin 0 and is involved in water transport and retention (32). Reduced filensin expression may weaken this interaction, resulting in reduced water transport and lens regulation. Thus, reduced expression of filensin and its weakened interaction with aquaporin 0 under temperature conditions >35˚C may disrupt the water balance within the lens and cause over-entry of water. This may be the key mechanism associated with lens dysfunction and pathology. On the other hand, it is known that phakinin often works in concert with filensin (33). Therefore, the changes in expression of phakinin protein was also measured. The Rsc value of phakinin was -0.85 and phakinin also decreased at 37.5˚C.

To prevent the excessive entry of water into the lens, the expression of voltage-dependent chloride channel protein 2 may be increased. Voltage-dependent chloride channel protein 2 plays an important role in regulating intracellular and extracellular salt concentration, as well as intracellular water content (34). Therefore, its increased expression is expected to regulate the amount and balance of water in lens cells and prevent excessive water entry. Through this mechanism, water balance within the lens may be maintained and damage to the lens and disease progression may be inhibited. In the present study, voltage-dependent chloride channel protein 2 expression was enhanced; therefore, decreased filensin expression may be related to water transport abnormalities, potentially compensated for by upregulating the expression of voltage-dependent chloride channel protein 2, which is responsible for expelling excess water from the lenses.

In addition, the expression of vimentin in 37.5˚C-incubated lenses was lower than that in those incubated at 35.0˚C. Vimentin is an intracellular, intermediate-diameter filament

Table V. Differentially expressed proteins in the rat lenses incubated with 35.0˚C and 37.5˚C.

ID	Accession number	Description	Number of amino acids	Spectral counting		
				37.5˚C	35.0˚C	Fold change, Rsc
K1C27_RAT	Q6IFW8	Keratin, type I cytoskeletal 27	449	5	0	3.134039
LPAR3_RAT	Q8K5E0	Lysophosphatidic acid receptor 3	354	2	0	2.188507
PER1_RAT	Q8CHI5	Period circadian protein homolog 1	1,293	2	0	2.188507
VPS4A_RAT	Q793F9	Vacuolar protein sorting-associated protein 4A	437	2	0	2.188507
PLCD4_RAT	Q62711	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase delta-4	772	2	0	2.188507
K1C14_RAT	Q6IFV1	Keratin, type I cytoskeletal 14	485	6	3	1.582128
CLCN2_RAT	P35525	Chloride channel protein 2	907	4	2	1.502479
K2C1B_RAT	Q6IG01	Keratin, type II cytoskeletal 1b	519	4	2	1.502479
ACNT2_RAT	Q5FVR5	Acyl-coenzyme A amino acid N-acyltransferase 2	418	2	1	1.340108
UCHL1_RAT	Q00981	Ubiquitin carboxyl-terminal hydrolase isozyme L1	223	3	2	1.196919
K2C1_RAT	Q6IMF3	Keratin, type II cytoskeletal 1	625	15	13	1.003428
K1C17_RAT	Q6IFU8	Keratin, type I cytoskeletal 17	433	0	4	-1.263412
BFSP1_RAT	Q02435	Filensin (Fragment)	617	3	19	-1.449344
K2C5_RAT	Q6P6Q2	Keratin, type II cytoskeletal 5	576	2	15	-1.517975
TBB3_RAT	Q4QRB4	Tubulin β-3 chain	450	0	5	-1.515353
K1C15_RAT	Q6IFV3	Keratin, type I cytoskeletal 15	447	0	7	-1.916696
K2C7_RAT	Q6IG12	Keratin, type II cytoskeletal 7	457	0	7	-1.916696
TBB5_RAT	P69897	Tubulin β-5 chain	444	0	8	-2.082158
VIME_RAT	P31000	Vimentin	466	0	11	-2.488624
ACTC_RAT	P68035	Actin, α cardiac muscle 1	377	0	13	-2.707610
FABP5_RAT	P55053	Fatty acid-binding protein 5	135	1	39	-3.367463
ACTG_RAT	P63259	Actin, cytoplasmic 2	375	0	26	-3.648159

Rsc, $\log_2$-transformed ratio of protein abundances.

that plays an important role in maintaining cell morphology and motility. In particular, it plays an important role in intraocular tissues such as lens fibroblasts and dendritic cells (35,36). It is possible that decreased vimentin expression results in alterations in the intracellular skeleton within the lens. This may alter lens cell morphology and function and increase the risk of developing nuclear cataracts (35,36). Thus, reduced vimentin expression is associated with the development of nuclear cataracts and this mechanism may be primarily due to changes in the intracellular skeleton within the lens.

It is important to investigate whether overexpressed proteins and other decreased proteins at 37.5˚C are related to lens dysfunction. Further studies are required to investigate the relationship between the onset of nuclear cataracts and changes in vimentin and filensin levels. Additionally, the present proteome analysis is semi-quantitative and more detailed investigations such as western blotting are essential in a further study. Therefore, the authors are planning to determine the localization and expression of vimentin and filensin at 37.5˚C using western blotting and immunostaining.

In conclusion, shotgun proteomic analysis revealed that increased ocular temperatures decrease the expression of filensin and vimentin in rat lenses. The present study may serve as a valuable indicator for elucidating the relationship between temperature and onset of nuclear cataracts. Nonetheless, additional research, such as performing western blotting and immunostaining, is necessary to elucidate the molecular mechanisms that underpin the relationships among these factors.

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Availability of data and materials

The proteomics data generated in the present study may be found in the jPOSTrepo under accession number PXD056067

or at the following URL: https://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PXD056067. The other data generated in the present study may be requested from the corresponding author.

Author's contributions

The experiments were conducted by HO, SM, and NN. Data analysis and interpretation were performed by HO, SM, TY, YM, YN and NY. TY, YM, YN and NY further investigated the experimental methodologies. AT, HS, and NN made substantial contributions to the conceptualization and design of the present study. AT, HS, and NN devised the experimental framework and provided the final approval for the publication of the manuscript. NN, HO and TY confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were conducted in accordance with the regulations approved by the Ethics Committee of the Kindai University Faculty of Pharmacy (approval no. KAPS-2021-004; April 1, 2021). The present study adhered to the ARRIVE guidelines and the Guiding Principles endorsed by The Japanese Pharmacological Society. Animal sacrifice was performed following the AVMA 2020 guidelines.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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