Rejuvenation of aging skin fibroblast via restoration of disrupted proteostasis by Nelumbo Nucifera Germ Extract

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Abstract

Background: Aging is a complex multifunctional biological process that results in time-dependent attenuation of biological function and simultaneously induces the loss of various cellular functions. However, the sequence of aging-related changes and how they affect each other remain unclear. Mitochondria are important organelles for maintaining cellular homeostasis, and mitochondrial function is decreased over time due to their own production of abnormal proteins, which are generated by the loss of cellular proteostasis. Thus, many studies to determine how mitochondria can be reactivated during the aging process to suppress aging are still being conducted.

Methods: We determined mitochondrial function, RNA and protein expression level, AGEs level and collagen expression level by using three aging types of NB1RGB cells. We performed transmission electron microscopy to identify organelle structure such as autolysosomes and lipofuscin-like particles.

Results: This study reports that disruption of proteostasis attenuated mitochondrial function upon proliferative and replicative senescence before the induction of DNA damage signaling, and the clearance of abnormal substances via autophagy restored mitochondrial function in aging skin fibroblasts. We screened 75 plant extracts that restored mitochondrial function in aging skin fibroblast and discovered that Nelumbo Nucifera Germ Extract reduced the activity of senescence-associated β-galactosidase, a marker of aging, and activated mitochondria via induction of autophagy, which degraded lipofuscin aggregates and AGEs. Moreover, the treatment of aging skin fibroblast with Nelumbo Nucifera Germ Extract stimulated collagen production and contractile ability in three-dimensional cell culture.

Conclusion: Nelumbo Nucifera Germ Extract was found to rejuvenate aging skin fibroblasts and is thus a promising new antiaging material.

Keywords: Mitochondria; Aging; Autophagy; Nelumbo Nucifera Germ Extract.

Introduction.

We have been seeking for ways to keep young since the ancient times. In particular, maintaining the beauty of the skin is a symbol of youth. One of the characteristics of skin aging is the formation of wrinkles. Wrinkles are caused by a disruption in the collagen structure in the dermis, and the one factor is a decrease the fibroblasts function such as collagen production due to the aging.

Aging is a complex multifunctional biological process that results in time-dependent attenuation of biological function and simultaneously induces the loss of various cellular functions [1]. Aging is an important factor in the onset and exacerbation of various diseases; however, though the molecular mechanisms of its progression have been elucidated, a comprehensive understanding has yet to be reported [1,2].

Cellular senescence refers to the irreversible loss of cell division capacity due to DNA damage or telomere deletion caused by repeated cell division and several stressors [3]. Aging normal organs contain some senescent cells, and the removal of these senescent cells was found to restore organ function and extend life span in aged mice, indicating that cellular senescence induces whole-tissue functional decline [4,5]. Thus, suppressing the progression of cellular aging prevents aging-dependent organ dysfunction. Cells lose their original functions when their homeostasis is disrupted due to repeated proliferation and several stressors, leading to various time-dependent effects, such as the production of aging-dependent advanced glycation end products (AGEs), protein aggregation, epigenetic changes, the loss of organelle function, the induction of DNA damage signals, and cell cycle arrest [1,3,6]. Finally, senescent cells stop proliferating and produce senescence-associated secretory phenotype (SASP)-related factors, which impair the function of surrounding cells [3]. However, the sequence of aging-related changes and how they affect each other remain unclear.

Mitochondria play a crucial role in the aging process [7]. Furthermore, mitochondria are one of the most important organelles for maintaining cellular homeostasis, which is involved in ATP production and several metabolic pathways, and mitochondrial function is disrupted over time [8]. Several reports have indicated that damaged mitochondria exhibit decreased function due to their own production of reactive oxygen species (ROS) and abnormal proteins, which are generated by the loss of cellular proteostasis, accelerating the cellular aging process [7,9,10]. While reactivation of mitochondrial function can inhibit the aging process, there is also the concern that excessive mitochondrial activation might exacerbate aging by increasing ROS production. Thus, many studies to determine at what time point in the cellular aging process mitochondrial function declines and how mitochondria can be reactivated during the aging process to suppress aging are still being conducted.

In this study, we screened 75 plant extracts that restored mitochondrial function in aging fibroblasts and discovered that Nelumbo Nucifera Germ Extract reduced the activity of senescence-associated β-galactosidase (SA-β-gal), a marker of aging, and activated mitochondria via induction of autophagy, which degraded lipofuscin aggregates and AGEs, which increased the aging process. Thus, we analyzed the detailed mechanism of the anti-aging effect of Nelumbo Nucifera Germ Extract, focusing on the mechanism of time-dependent functional change of mitochondria due to aging.

Materials and Methods.

Plant extract

Freeze-dried Nelumbo Nucifera Germ Extract prepared by Maruzen Pharmaceuticals was stored in the dark at -30°C until use. Nelumbo Nucifera Germ Extract was dissolved in dimethyl sulfoxide (DMSO) and diluted with medium to appropriate concentrations, and the final volume was adjusted to 0.1% DMSO.

Cell lines and generation of stable cell lines

NB1RGB (Riken BRC) cells were maintained in MEMα supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. All cell cultures were passaged (1:4 to 3) and maintained at 37°C with 5% CO_2. We defined three types of cell line according to the number of days in culture: NB1RGB: young cells: from 8 to 20 days, middle passage: from 30 to 40 days, and aging cells: from 60 to 70 days.

Real-time quantitative PCR

Real-time quantitative PCR (qRT–PCR) was performed as previously described [11]. Total RNA was normalized in each reaction using *β-actin* cDNA as an internal standard.

Detection of SA-β-gal activity

Cellular SA-β-gal activity was determined using a senescence detection kit (Abcam, Cambridge, England) or 96-well cellular senescence assay (Cell Biolabs, San Diego, CA, USA) according to the manufacturer's protocols. All images were obtained under a microscope (Olympus, Tokyo, Japan) and processed using Adobe Photoshop software. Fluorescence was measured on a fluorescence microplate reader (Tecan, Tokyo, Japan) with 360 nm/485 nm filter pairs.

JC-1 staining

Mitochondrial membrane potential was assessed using JC-1 (Dojindo, Tokyo, Japan) staining according to the manufacturer's protocols. Fluorescence was measured on a fluorescence microplate reader (Tecan, Tokyo, Japan) with 535 nm/590 nm and 485 nm/535 nm filter pairs. The results are shown as the ratio of fluorescence measured at 535 nm/590 nm to that measured at 485 nm/535 nm (aggregate fluorescence to monomer fluorescence).

Cell viability assay

Cell viability was determined using the 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Cells were administered the indicated treatments and incubated with MTT solution (1 mg/ml for 2 h. Isopropanol and HCl were then added to final concentrations of 50% and 20 mM, respectively, and the absorbance at 570 nm was measured using a spectrophotometer.

Cell fluorescence staining

Cells were cultured on glass-bottom culture dishes with or without MitoTracker for 30 min and fixed in 4% formaldehyde. All images were obtained under a microscope (KEYENCE, Osaka, Japan) and processed using Adobe Photoshop software.

ATP assay

Intracellular ATP levels were measured using the CellTiter-Glo 2.0 assay kit (Promega, Madison, WI, USA) following the manufacturers' instructions.

Collagen assay

Cellular collagen levels were measured using a collagen quantitation kit (Cosmo Bio, Tokyo, Japan) following the manufacturers' instructions. Fluorescence was measured on a fluorescence microplate reader (Tecan, Tokyo, Japan) with a 360 nm/485 nm filter pair.

TEM

Cultured cells were fixed with 1% glutaraldehyde in PBS at pH 7.3 for 1 h at 4°C and then postfixed with 1% osmium tetroxide in 0.1 M phosphate buffer at pH 7.3 for 1 h at 4°C, followed by dehydration in a graded series of ethanol. Following dehydration, they were embedded in Epon 812 (TAAB Laboratories Equipment, Berkshire, England) and then observed under a JEM-1400 Plus electron microscope (JEOL, Japan).

siRNA-mediated gene targeting

NB1RGB cells were transfected with siRNA specific for siATG7 (the siRNA SMARTpool for human ATG7 from Dharmacon, Lafayette, CO, USA) and controls (Santa Cruz, Santa Cruz, CA, USA) using Lipofectamine RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions.

Immunoblotting analysis

Immunoblotting experiments were conducted as previously described [11]. The antibodies used for immunoblotting were specific for the following proteins: LC3b, ATG7, Beclin 1, p-S/T and BcL-2 (Cell Signaling); and β-actin (Sigma). The antibodies were diluted 1:1000, except for anti-β-actin (1:10000). Secondary antibodies were purchased from Promega (anti-rabbit and anti-mouse at 1:5000).

AGEs detection by ELISA

AGEs levels were measured using an OxiSelect AGE Competitive ELISA kit (Cell Biolabs, USA) following the manufacturer’s instructions. The concentrations and enzyme activities were standardized to the total protein levels.

Immunoprecipitation

Immunoprecipitation assays were conducted as previously described [12]. The primary antibody was covalently immobilized onto protein A/G PLUS-agarose, followed by immunoprecipitation using a crosslink immunoprecipitation kit (Pierce/Thermo Fisher

Scientific, Hampton, NH, USA) according to the manufacturer's protocol. Immunoprecipitated products were incubated with LDS sample buffer containing 50 mM DTT at 95°C for 10 min.

3-Dimensional collagen gel culture

Cells were suspended in type I atelocollagen gel medium (KOKEN, Tokyo, Japan), which was immediately warmed to 37°C to form a gel. After 24 h of incubation, the gels were detached from the culture plate, overlaid with a double volume of DMEM and incubated. Each gel area (pix) was measured using ImageJ (NIH, USA). Atelocollagen gels were digested by 0.2% collagenase and 0.1% trypsin treatment, and then the cells were counted.

Histological and immunohistochemical analyses

Atelocollagen gels were fixed in 4% buffered paraformaldehyde, embedded in O.C.T. compound, and cryosectioned. For histological examination [hematoxylin and eosin (H&E) staining], the sections were stained first with Mayer's hematoxylin and then with a 1% eosin alcohol solution. The sections were blocked with 5% goat serum for 15 minutes and incubated for 12 h with anti-collagen I primary antibody (Novotec, JZ Reuver, Netherlands) or biotinylated-hyaluronan-binding protein (HRBP) (Hokudo Co., Ltd., Hokkaido, Japan) in the presence of 5% bovine serum albumin and then incubated for 1 h with FITC-conjugated rabbit IgG (Molecular Probes, Hampton, NH, USA) or FITC-conjugated avidin (Vector Laboratories, Burlingame, CA, USA). Nuclei were stained with DAPI (Roche, Indianapolis, IN, USA). The samples were inspected with the aid of a microscope (Keyence, Osaka, Japan).

Statistical analyses

Differences in mean values were evaluated using two-way ANOVA, followed by Tukey's test, with $P < 0.05$ used to indicate statistical significance.

Results.

Mitochondrial dysfunction occurs before the induction of DNA damage signaling in proliferative and replicative senescence.

NB1RGB cells, which are normal human dermal fibroblasts, at different stages of proliferative and replicative aging showed several aging-related markers, such as SA-β-gal and genetic markers (DNA damage signal: p21 and p16, SASP factors: IL-6 and TNF-α), as shown in Figure 1a and b. Previously, p21 expression and SA-β-gal activity were reported to be increased in early senescence, and p16 and SASP factor gene expression was found to be induced in late senescence [13]. As shown in Figure 1a and b, aging fibroblasts exhibited SA-β-gal activity and increased p21 mRNA levels, but p16, IL-6 and TNF-α mRNA expression was no different than that in young fibroblasts. Mitochondrial transmembrane potential ($\Delta\Psi m$) reflects mitochondrial function. We analyzed the effect of these NB1RGB cells on $\Delta\Psi m$ using the fluorescent probe JC-1. Red fluorescence shows JC-1 aggregates that appear in the mitochondria after potential-dependent aggregation. Green fluorescence shows JC-1 monomers that appear in the cytosol after mitochondrial membrane depolarization.

Aging and middle-passage fibroblasts exhibited decreased $\Delta\Psi m$, as shown by analysis with a microplate reader (Figure 1c). These results suggested that mitochondrial dysfunction and increased SA-β-gal activity were induced earlier than the upregulation of p21 mRNA expression and that aging fibroblasts were in the early phase of cellular senescence.

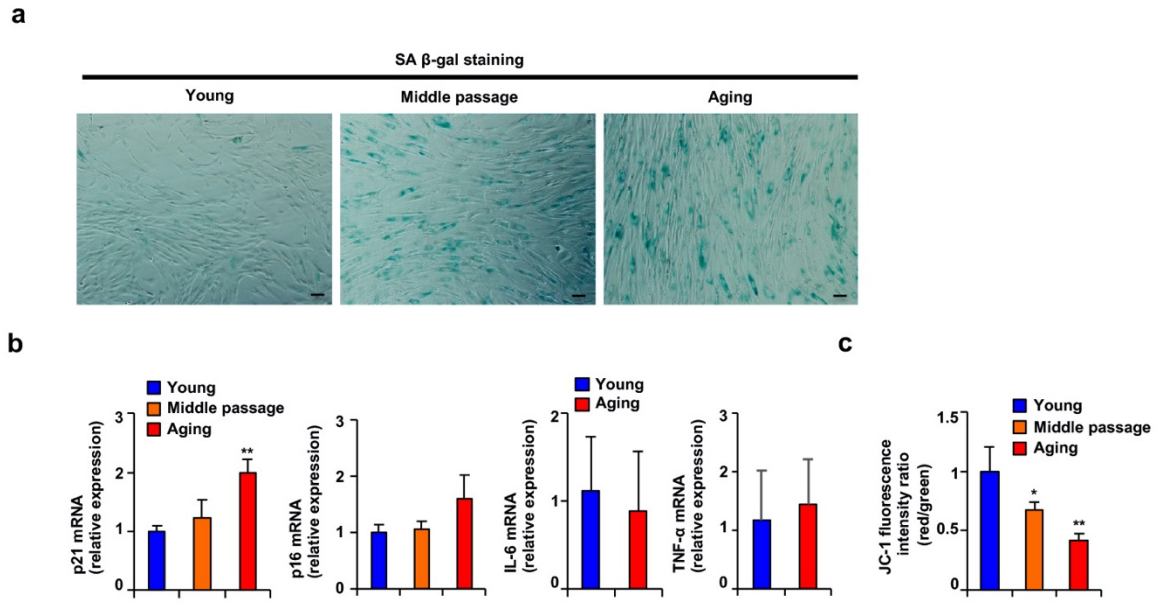

Figure 1. Replicative aging induced mitochondrial dysfunction.

(a) The indicated cells at several passages were stained as SA-β-gal-positive cells (scale bar, 300 μm). (b) Total RNA was extracted from NB1RGB cells at several passages and subjected to real-time quantitative PCR. Data are presented as the mean ± standard deviation (SD) (three different data sets). (c) The cells were incubated with JC-1 for 20 min and assessed with a microplate reader; $\Delta\Psi m$ was determined by the ratio between the red fluorescence intensity, indicating activated mitochondria, and the green fluorescence intensity, indicating deactivated mitochondria. The data are presented as the mean ± SD of three simultaneously performed experiments (b, c). Each *P value* was calculated using two-way ANOVA; $*P < 0.05$, $**P < 0.01$.

Nelumbo Nucifera Germ Extract reactivated mitochondrial function in aging fibroblasts.

Several reports have suggested that mitochondrial dysfunction stimulates cellular senescence [7,8]. Our results indicated that mitochondrial dysfunction occurred earlier than the induction of DNA damage signaling and SASP-related gene expression in the proliferative and replicative senescence steps, suggesting that reactivation of mitochondrial function suppressed the progression of cellular senescence in the early stage. We then screened 75 plant extracts to identify those that increase the $\Delta\Psi m$. As shown in Figure 2a, among these, Nelumbo Nucifera Germ Extract (#51) increased the $\Delta\Psi m$ the most in aging fibroblasts, and this increase was dose-dependent without the induction of cell death (Figure 2b, c). Extract from *Nelumbo nucifera* germ has several bioactive effects, such as its effects against cancer and obesity; however, the effect of extract from *Nelumbo nucifera* germ on mitochondrial function has not been elucidated [14]. Previously, it was reported that cycles of fusion and

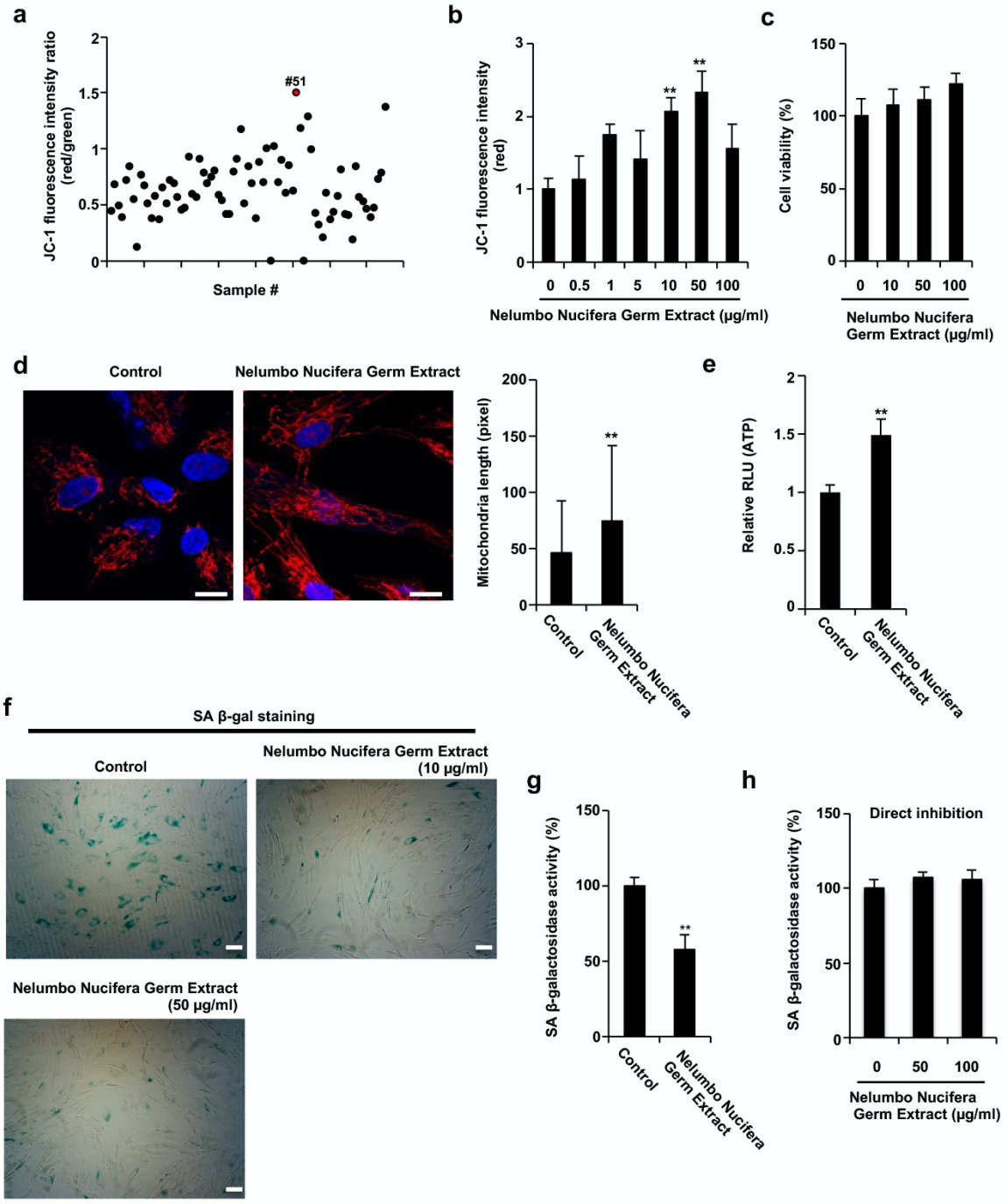

Figure 2. Nelumbo Nucifera Germ Extract restored mitochondrial function and suppressed the aging phenotype.

(a) Plant extract screening was performed in duplicate, and relative JC-1 activity was calculated using the procedure described in Figure 1c. (b, c) Aging NB1RGB cells were treated with Nelumbo Nucifera Germ Extract at the indicated concentration for 24 h. (b) JC-1 activity was determined by the red fluorescence intensity, indicating activated mitochondria. (c) Cell viability was determined using an MTT assay. (d) Aging NB1RGB cells were treated with or without 50 μg/ml Nelumbo Nucifera Germ Extract for 2 days, followed by treatment with MitoTracker Orange and fluorescence microscopy. Red represents mitochondrial MitoTracker staining, and blue represents nuclear DAPI staining (scale bar, 20 μm). The

length of the mitochondria within the cells was determined, and the data are presented as the mean ± SD (n = 30) (right panel). (e) Aging NB1RGB cells were treated with or without 50 μg/mL Nelumbo Nucifera Germ Extract for 24 h. (e) ATP levels were determined using a CellTiter-Glo assay. (f) Aging NB1RGB cells were treated with the indicated concentration of Nelumbo Nucifera Germ Extract for 3 days and stained to detect SA-β-gal-positive cells. (g) Aging NB1RGB cells were treated with 50 μg/ml of Nelumbo Nucifera Germ Extract for 3 days, and SA-β-gal activity was measured. (h) Nelumbo Nucifera Germ Extract at the indicated concentrations was added to cell lysates of aging NB1RGB cells, and SA-β-gal activity was measured. Data are presented as the mean ± SD of three simultaneously performed experiments. Each *P value* was calculated using two-way ANOVA; *$P < 0.05$, **$P < 0.01$.

fission modify the morphology of the mitochondrial compartment. Fragmented, short mitochondria have low respiratory activity than fused, long mitochondria [15]. As shown in Figure 2d, we performed imaging analyses using MitoTracker to visualize the mitochondrial morphology, which indicated many short mitochondrial forms in the aging cells (Control); however, treatment with Nelumbo Nucifera Germ Extract increased the abundance of the long form. ATP production is related to mitochondrial function; a luciferase assay revealed that treatment with Nelumbo Nucifera Germ Extract significantly increased ATP (Figures 2e).

Next, we examined whether Nelumbo Nucifera Germ Extract affects SA-β-gal activity in aging fibroblasts. As shown in Figure 2f, the number of SA-β-gal-positive cells was decreased by treatment with Nelumbo Nucifera Germ Extract in a dose-dependent manner. There are two possible explanations: Nelumbo Nucifera Germ Extract inhibits SA-β-gal activity directly or indirectly. As shown Figure 2g and h, aging cells were treated with Nelumbo Nucifera Germ Extract for 72 h, after which the cells were lysed, and SA-β-gal activity was measured. The results showed that Nelumbo Nucifera Germ Extract inhibited SA-β-gal activity; however, aging cells were lysed, and when SA-β-gal activity was measured upon the addition of Nelumbo Nucifera Germ Extract, it did not inhibit the activity, suggesting that the Nelumbo Nucifera Germ Extract suppresses the aging phenotype by altering cellular functions.

Nelumbo Nucifera Germ Extract induces autophagy and degrades aging-dependent increased lipofuscin-like particles.

To further understand the effect of Nelumbo Nucifera Germ Extract on cellular functions, we performed transmission electron microscopy (TEM) to identify any change in the cellular or organelle structure. As shown in Figure 3a, subsequent TEM indicated that Nelumbo Nucifera Germ Extract treatment induced the formation of autolysosome-like structures in aging fibroblasts, and aging fibroblasts exhibited increased lipofuscin-like particles in the cytosolic region compared to the abundance of the particles in young fibroblasts (Figure 3 a-c).

Autophagy is an important cellular response that maintains proteostasis by the bulk degradation of abnormal proteins and organelles that have lost their function. Previously, autophagic dysfunction was reported to stimulate cellular aging, and the induction of

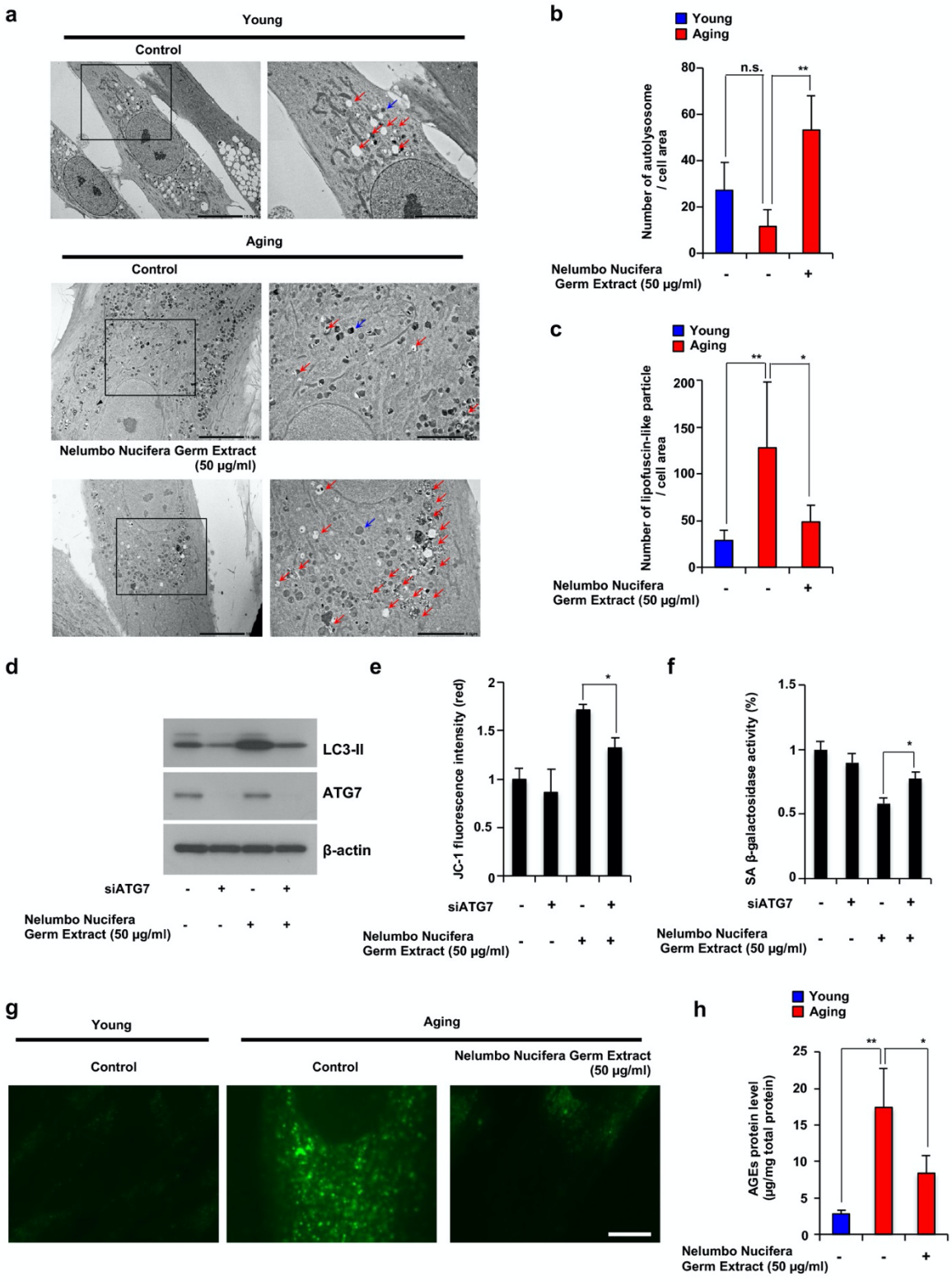

Figure 3. Nelumbo Nucifera Germ Extract induced autophagy and decreased aging-related accumulation of lipofuscin-like particles and AGEs.

(a) Young and aging NB1RGB cells with and without Nelumbo Nucifera Germ Extract (50 μg/ml) treatment were subjected to transmission electron microscopy. The scale bar in the

left panel represents 10 μm. Higher magnification images are shown in the right panel, with the scale bar representing 5 μm. Blue arrows indicate aged-related increased lipofuscin-like particles. Red arrows indicate autolysosomes. The numbers of lipofuscin-like particles (b) and autolysosomes (c) in each cell area were determined. Data are presented as the mean ± SD ($n = 5$). (d, e) Aging NB1RGB cells were transfected with siControl or siATG7 for 24 h, followed by treatment with or without 50 μg/ml Nelumbo Nucifera Germ Extract for 24 h. Cells were subjected to immunoblotting using the indicated antibodies (d). JC-1 activity was determined based on fluorescence intensity (e). (f) Aging NB1RGB cells were transfected with siControl or siATG7 for 24 h, followed by treatment with or without 50 μg/ml Nelumbo Nucifera Germ Extract for 3 days, and SA-β-gal activity was measured. (g, h) Young and aging NB1RGB cells were treated with or without 50 μg/ml Nelumbo Nucifera Germ Extract for 3 days and subjected to fluorescence microscopy analysis to detect autofluorescence due to lipofuscin-like particles (g) or AGEs levels by ELISA (h). Data are presented as the mean ± SD of three simultaneously performed experiments (e, f, h). *P value* was calculated using two-way ANOVA; n.s.: not significant, $*P < 0.05$, $**P < 0.01$.

autophagy suppresses senescence phenotypes such as the reactivation of mitochondrial function [16]. We further examined whether Nelumbo Nucifera Germ Extract treatment affects autophagic induction and whether induced autophagy is related to the reactivation of mitochondria and suppression of the aging phenotype. Aging fibroblasts were treated with or without siRNA for ATG7 and/or Nelumbo Nucifera Germ Extract, and levels of the autophagy marker LC3-II were monitored. As shown in Figure 3d, treatment with Nelumbo Nucifera Germ Extract increased LC3-II protein expression, and ATG7 knockdown by siRNA silencing suppressed Nelumbo Nucifera Germ Extract-induced LC3-II protein expression. ATG7 knockdown significantly suppressed Nelumbo Nucifera Germ Extract-induced upregulation of $\Delta\Psi m$ and SA-β-gal in aging fibroblasts (Figure 3e and f). These results indicate that Nelumbo Nucifera Germ Extract-induced autophagy plays an important role in the reactivation of mitochondria.

Several reports indicated that lipofuscin is a fluorescent complex mixture composed of highly oxidized cross-linked protein residues, lipids, and sugars, and increased in senescent cells, and age-related lipofuscin aggregates included AGEs, which have also autofluorescence [17]. As shown in Figure 3a and c, Nelumbo Nucifera Germ Extract decreased age-related lipofuscin-like particles incorporated into autolysosomes. Thus, we confirmed whether autofluorescence and AGEs protein levels would be increased in aging fibroblasts and decreased by Nelumbo Nucifera Germ Extract treatment. As shown in Figure 3g and h, autofluorescence and AGEs protein levels were increased in aging fibroblasts compared to young fibroblasts, and treatment with Nelumbo Nucifera Germ Extract decreased the increase in aging fibroblasts. Therefore, Nelumbo Nucifera Germ Extract treatment could restore age-dependent disrupted protein homeostasis in aging cells.

Nelumbo Nucifera Germ Extract activates the DAPK1-Beclin1 signaling pathway.

Beclin1 plays a central role during the autophagy process [18]. Under normal conditions, Bcl-2 binds Beclin1 and inhibits autophagosome formation; however, the phosphorylation of Beclin1 releases it from Bcl-2, and Beclin1 can then carry out its function in autophagy [19]. Therefore, we tested whether Nelumbo Nucifera Germ Extract affects the binding of Beclin1 and Bcl-2 and the phosphorylation of Beclin1. Immunoprecipitation showed that Beclin1 did

not bind Bcl-2, and Beclin1 was phosphorylated under Nelumbo Nucifera Germ Extract treatment (Figure 4a). We aimed to identify other factors involved in Beclin1 phosphorylation. Death-associated protein kinase 1 (DAPK1) expression was induced by Nelumbo Nucifera Germ Extract, but DAPK2 expression was not (Figure 4b). DAPK1 promotes noncanonical autophagy via the phosphorylation of Beclin1 [20], and we determined whether the induction of DAPK1 expression is involved in Nelumbo Nucifera Germ Extract-induced autophagy. Treatment with a DAPK1 inhibitor (TC-DAPK6) suppressed Nelumbo Nucifera Germ Extract-induced upregulation of LC3-II expression and disintegration of the Beclin1 and Bcl-2 complex in aging fibroblasts (Figure 4c and d). Altogether, the data shown in Figure 4 indicate that Nelumbo Nucifera Germ Extract induces autophagy via the DAPK1-Beclin1 pathway, a noncanonical autophagy pathway.

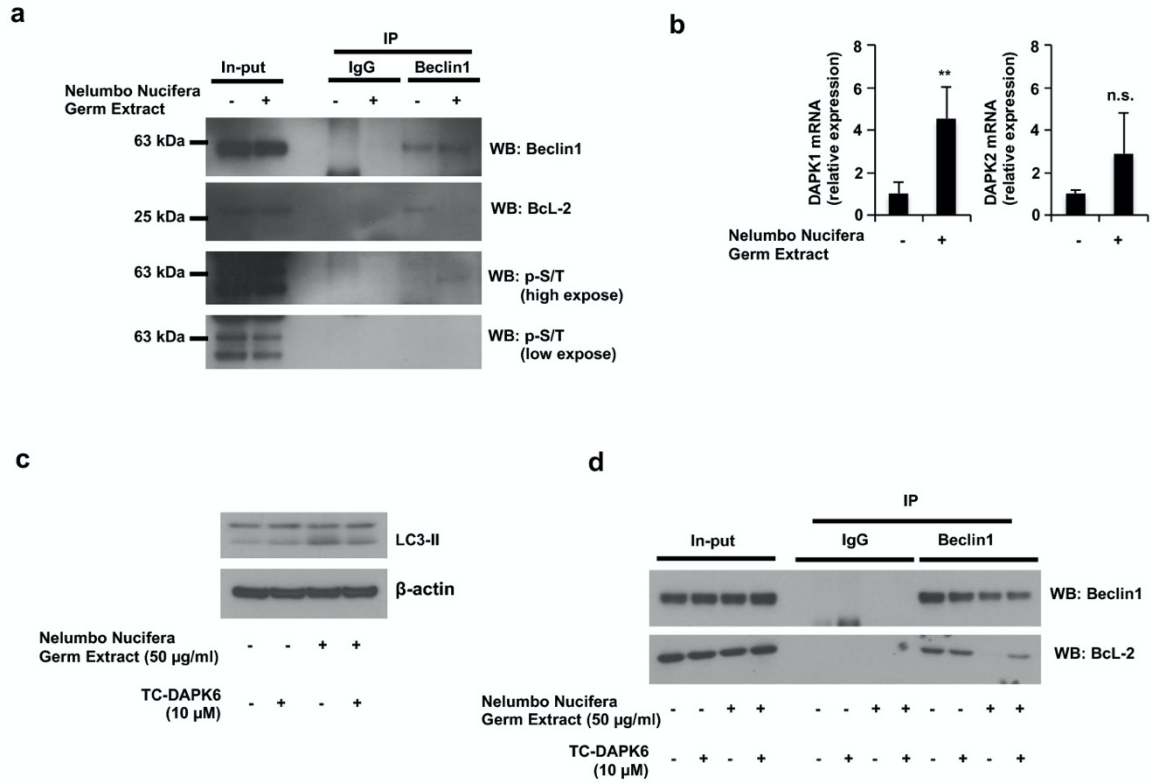

Figure 4. Nelumbo Nucifera Germ Extract activated the JNK and DAPK1-Beclin1 pathways.

(a) Aging NB1RGB cells were treated with 50 μg/ml Nelumbo Nucifera Germ Extract for 24 h, and proteins were crosslinked with DSP prior to protein extraction. A coimmunoprecipitation assay was performed with cell lysates using the indicated antibodies, followed by western blotting. (b) Aging NB1RGB cells were treated with 50 μg/ml Nelumbo Nucifera Germ Extract for 24 h and subjected to real-time quantitative PCR. (c, d) Aging NB1RGB cells were treated with or without 10 μM TC-DAPK6 and/or 50 μg/ml Nelumbo Nucifera Germ Extract for 24 h and subjected to immunoblotting using the indicated antibodies (c), coimmunoprecipitation assay (d). Data are presented as the mean ± SD of three simultaneously performed experiments. Each *P value* was calculated using two-way ANOVA; n.s.: not significant, $*P < 0.05$, $**P < 0.01$.

Nelumbo Nucifera Germ Extract rejuvenated aging fibroblasts.

We showed that Nelumbo Nucifera Germ Extract restored several age-dependent changes in aging fibroblasts to more closely resemble younger fibroblasts. Thus, we hypothesized that Nelumbo Nucifera Germ Extract can rejuvenate the functions of aging fibroblasts. First, we focused on collagen production. As shown in Figure 5a, the production of total collagen protein was significantly increased by Nelumbo Nucifera Germ Extract. Next, we performed a 3-dimensional culture assay using collagen gel to evaluate the functions of aging fibroblasts (proliferation, reorganization and the contraction of extracellular matrix) in an environment that more closely simulated *in vivo* conditions. As shown in Figures 5b-e, young fibroblasts contracted collagen gel and proliferated in 3-dimensional culture, but aging fibroblasts showed decreased contraction activity and proliferation, and the cell number in 3-dimensional culture was lower for aging fibroblasts than for younger fibroblasts. Nelumbo Nucifera Germ Extract treatment promoted these activities in aging fibroblasts (Figures 5b-e). An immunofluorescence staining assay revealed that collagen and hyaluronan, a factor in the extracellular matrix, were increased in 3-dimensional culture upon treatment with Nelumbo Nucifera Germ Extract (Figure 5f), indicating that Nelumbo Nucifera Germ Extract restored extracellular matrix-remodeling activity in aging fibroblasts. Altogether, the data shown in Figure 5 indicate that Nelumbo Nucifera Germ Extract treatment rejuvenated aging fibroblasts by restoring the various phenotypes of aging via activation of DAPK1-Beclin1 signaling.

Discussion.

Here, we have identified Nelumbo Nucifera Germ Extract as a new anti-aging material. We discovered that mitochondrial dysfunction occurred earlier than the induction of DNA damage signaling, and reactivation of mitochondrial function rejuvenated aging fibroblasts, which improved collagen-production capacity and contractile ability, which were reduced over time via degradation of lipofuscin-like aggregation bodies, including AGEs. Through a pharmacological approach, we identified the DAPK1-Beclin1 pathway as an important autophagy induction signaling pathway in aging skin fibroblast, and the induction of autophagy plays a crucial role in the antiaging effects of Nelumbo Nucifera Germ Extract.

Numerous studies have suggested that mitochondrial dysfunction stimulates cellular aging, suggesting the involvement of mtDNA mutations, disruption of proteostasis and reduced mitochondrial biogenesis as possible mechanisms, but contradictory reports on the related mechanisms are available, and the issue remains unresolved [7,21]. In this study, we focused on the disruption of proteostasis in aging fibroblasts by electron microscopy analysis, and ELISA revealed that lipofuscin-like aggregates and AGEs were increased in fibroblasts in the early stage of aging. Li et al. reported that proteostatic stress induces mitochondrial dysfunction and cellular aging, leading to the direct accumulation of abnormal proteins in mitochondria [22]. The accumulation of lipofuscin in the cytosol also indicates increased unfolded protein levels via disruption of the proteostatic system, and lipofuscin is a strong target for autophagy; thus, attacking lipofuscin is ineffectual for lysosomal enzymes, and the number of defective mitochondria increases because the degradation of damaged mitochondria via autophagy has been impeded [23]. We showed that Nelumbo Nucifera

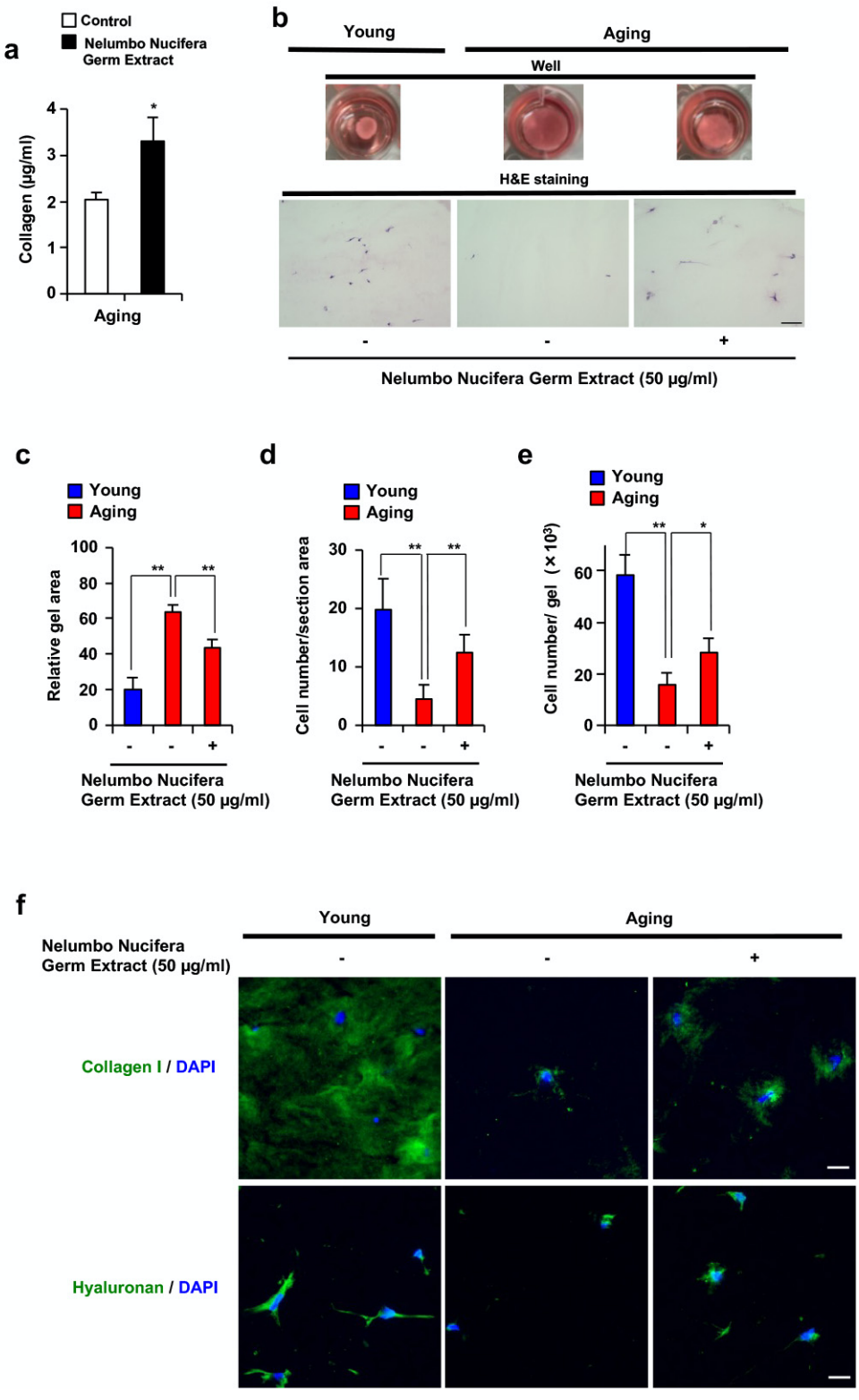

Figure 5. Nelumbo Nucifera Germ Extract stimulated collagen production and proliferative ability in aging cells in 3-dimensional gel culture.

(a) Aging NB1RGB cells were treated with 50 μg/ml Nelumbo Nucifera Germ Extract for 72 h and subjected to measurement of collagen content. (b-f) Aging NB1RGB cells were

treated with or without 50 μg/ml Nelumbo Nucifera Germ Extract for 72 h. Equal numbers of these cells and young NB1RGB cells were cultured in atelocollagen gel and treated with or with 50 μg/ml Nelumbo Nucifera Germ Extract for 10 days. (b) Representative image showing collagen gel contraction (upper panel). H&E staining of 3-dimensional atelocollagen gel sections. Scar bar: 100 μm (lower panel). (c) Gel surface area. (d) The cell density in a 3-dimensional gel was calculated by using H&E staining. (e) The cell number in the 3-dimensional gel was counted based on degradation of the atelocollagen gel. (f) Extracellular collagen and hyaluronan were measured by immunofluorescence staining. Scar bar: 20 μm. Data are presented as the mean ± SD of three simultaneously performed experiments. Each *P value* was calculated using two-way ANOVA; n.s.: not significant, **P* < 0.05, ***P* < 0.01.

Germ Extract degraded accumulated lipofuscin and AGEs, which inhibit mitochondrial function in aging fibroblasts. Together, the findings show that Nelumbo Nucifera Germ Extract restored aging-dependent mitochondrial dysfunction by restoring proteostasis via the induction of extensive autophagy.

Autophagy is an important biological system that maintains proteostasis by degrading abnormal proteins and dysfunctional organelles [18]. Autophagic disability is involved in stimulation of the aging process, and autophagy is known to decrease with increasing age [24]. We observed the downregulation of DAPK1 expression and a reduction in autophagy, which might relate to a decreased capacity to degrade abnormal substances in aging fibroblasts. DAPK1 induces a type of noncanonical autophagy, which is induced by the upregulation of DAPK1 expression induced via viral infection [20], and DAPK1 directly phosphorylates Beclin1, thereby releasing it from Bcl-2 [19]. Thus, Nelumbo Nucifera Germ Extract-induced DAPK1 expression is the main signal that activates autophagy in aging fibroblasts.

Many studies to determine the pharmacological effects of extract from *Nelumbo nucifera* germ have been conducted, and its anti-obesity, anticancer and anti-inflammatory effects have been identified [14]. Extract from *Nelumbo nucifera* germ has therapeutic effects on Alzheimer's disease, an aging-related disease, but its effects on time-dependent organismal and cellular aging have not been clarified.

Overall, these findings establish the rejuvenating effect of Nelumbo Nucifera Germ Extract on aging fibroblasts via the induction of autophagy to clear abnormal proteins and agglutinates. Moreover, DAPK1-Beclin1 signaling-induced autophagy might be a specific target for the antiaging effect of Nelumbo Nucifera Germ Extract in aging fibroblasts.

Conclusion.

In this study, we found that Nelumbo Nucifera Germ Extract has anti-aging effects by activating autophagy, promoting collagen and hyaluronic acid production from aging skin fibroblasts, and activating mitochondria, indicating the possibility of inhibiting the reduction of dermal functions due to aging. These results suggest that Nelumbo Nucifera Germ Extract may have anti-aging effects to prevent or improve wrinkles and dryness associated with aging.

Acknowledgments.

TEM analysis was performed by the Science Research Center of Kochi University. We thank the members of the Namba laboratory for helpful advice and suggestions during the project. This study was partially supported by Grants-in-Aid from the JSPS (19K07045), Takeda science foundation, Uehara memorial foundation, Nakashima foundation and Nakatomi foundation.

Conflict of Interest Statement.

None.

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