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

Namba, Takushi [1, 2*] Machihara, Kayo[1] Iwahashi, Hiroyasu [3]

[1] Research and Education Faculty, Multidisciplinary Science Cluster, Interdisciplinary Science Unit, Kochi University, Kochi, Japan.
[2] Department of Marine Resource Science, Faculty of Agriculture and Marine Science, Kochi University, Kochi, Japan.
[3] Research Center, Maruzen Pharmaceuticals Co., Ltd., Fukuyama City, Hiroshima, Japan.

Introduction:

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). 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 (2, 3). Thus, many studies to determine how mitochondria can be reactivated during the aging process to suppress aging are still being conducted .

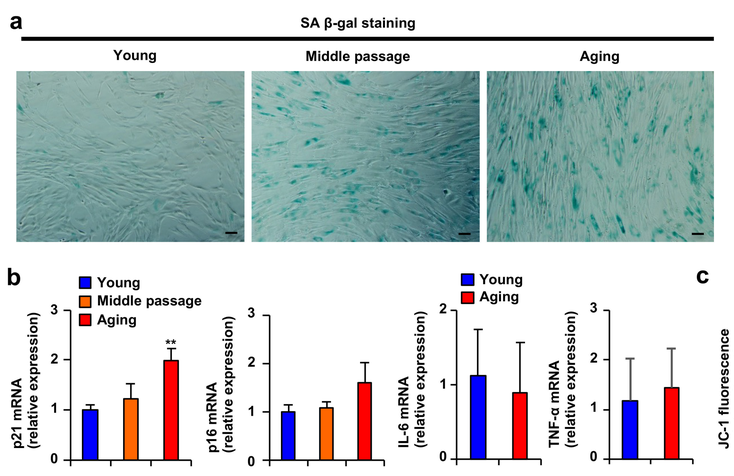

Figure 1. Replicative aging induced mitochondrial dysfunction.
(a) The indicated cells at several passages were stained as SA-β-gal-positive cells (scale bar, 300 μm). (b) Total RNA was extracted from NB1RGB cells at several passages and subjected to real-time quantitative PCR. Data are presented as 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; $^{*}P < 0.05$, $^{**}P < 0.01$.

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. 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. 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. Therefore, we hypothesized that restoring mitochondrial function would prevent cellular aging, because mitochondrial dysfunction occurs early in the aging process.

Materials & Methods:

Plant extract

Nelumbo Nucifera Germ Extract

Detection of SA-β-gal activity
Cellular SA-β-gal activity was determined using a senescence detection kit (Abcam, Cambridge, England).

Collagen assay
Cellular collagen levels were measured using a collagen quantitation kit (Cosmo Bio, Tokyo, Japan).

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.

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.

Results & Discussion:

Nelumbo Nucifera Germ Extract reactivated mitochondrial function in aging fibroblasts.

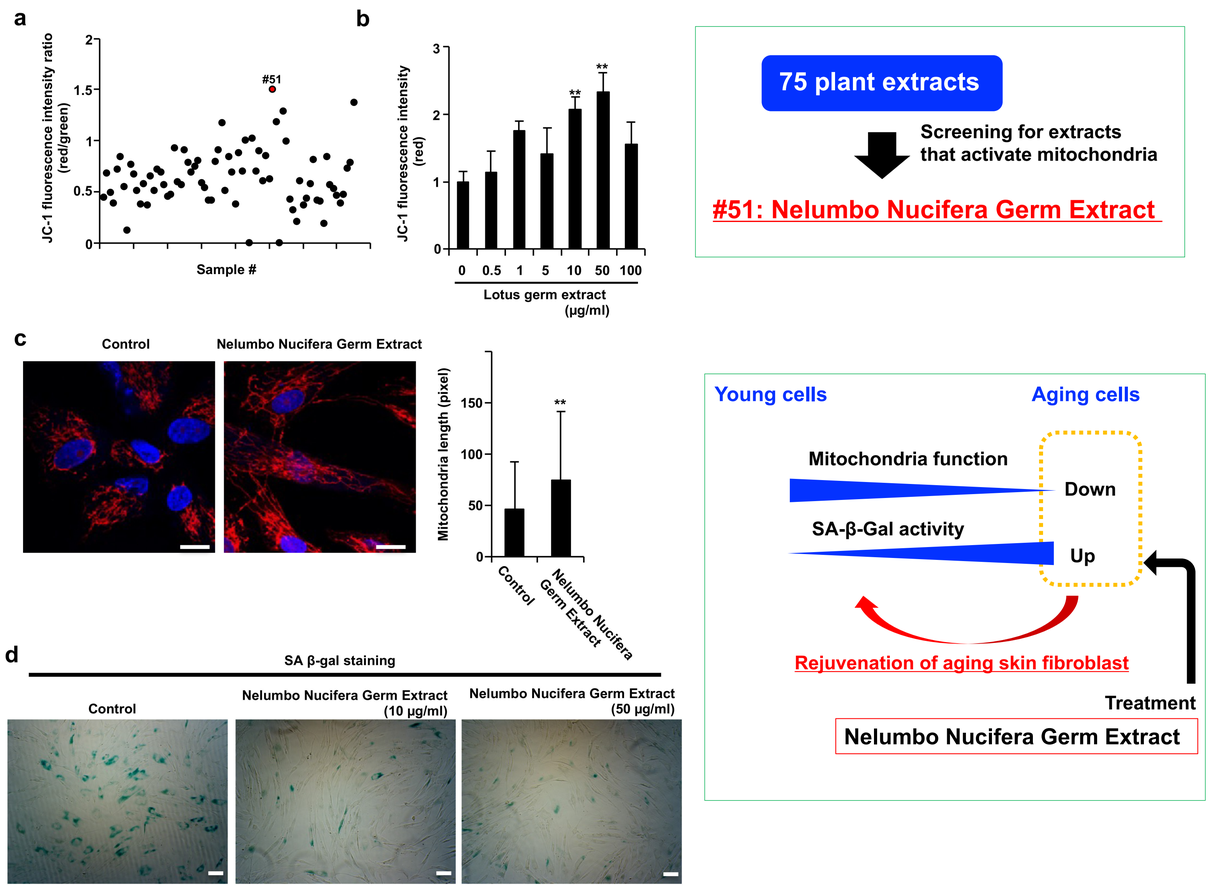

Figure 2. Nelumbo Nucifera Germ Extract restored mitochondrial function and suppressed the aging phenotype.
(a) Plant extract screening was performed in duplicate, and relative JC-1 activity was calculated (b) Aging NB1RGB cells were treated with Nelumbo Nucifera Germ Extract at the indicated concentration for 24 h. Relative JC-1 activity was calculated. (c) 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). (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. Data are presented as the mean ± SD of three simultaneously performed experiments. Each *P value* was calculated using two-way ANOVA; $^{**}P < 0.01$.

Acknowledgements:

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Results & Discussion:

Nelumbo Nucifera Germ Extract induces autophagy and degrades AGEs.

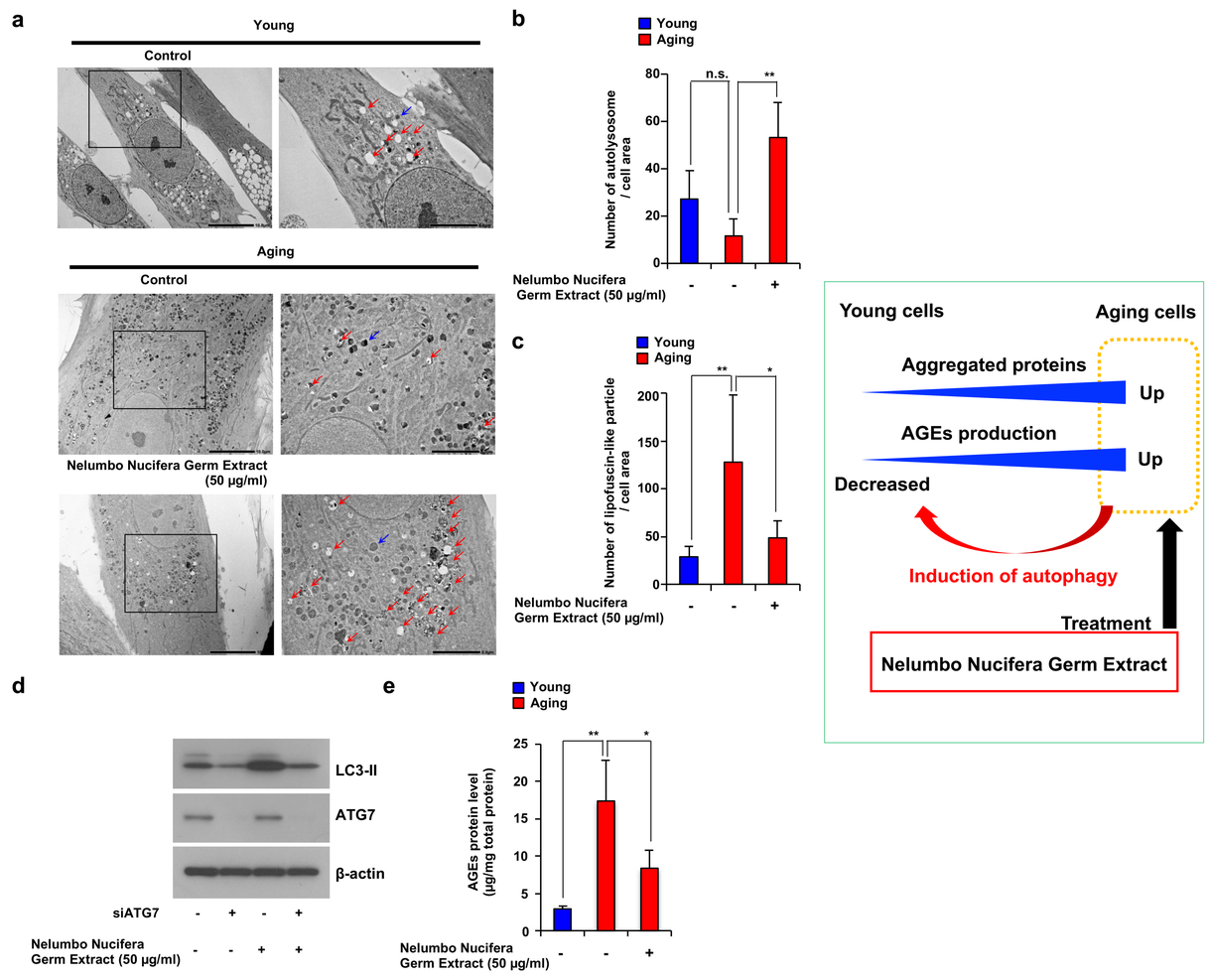


Figure 3. Nelumbo Nucifera Germ Extract induced autophagy and decreased aging-related accumulation of lipofuscin-like particles and AGEs.
(a) Young and aging NB1RGB cells with and without Nelumbo Nucifera Germ Extract (50 μg/ml) treatment were subjected to transmission electron microscopy. The scale 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). (e) Young and aging NB1RGB cells were treated with or without 50 μg/ml Nelumbo Nucifera Germ Extract for 3 days and subjected to AGEs levels by ELISA. Data are presented as the mean ± SD of three simultaneously performed experiments. *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.

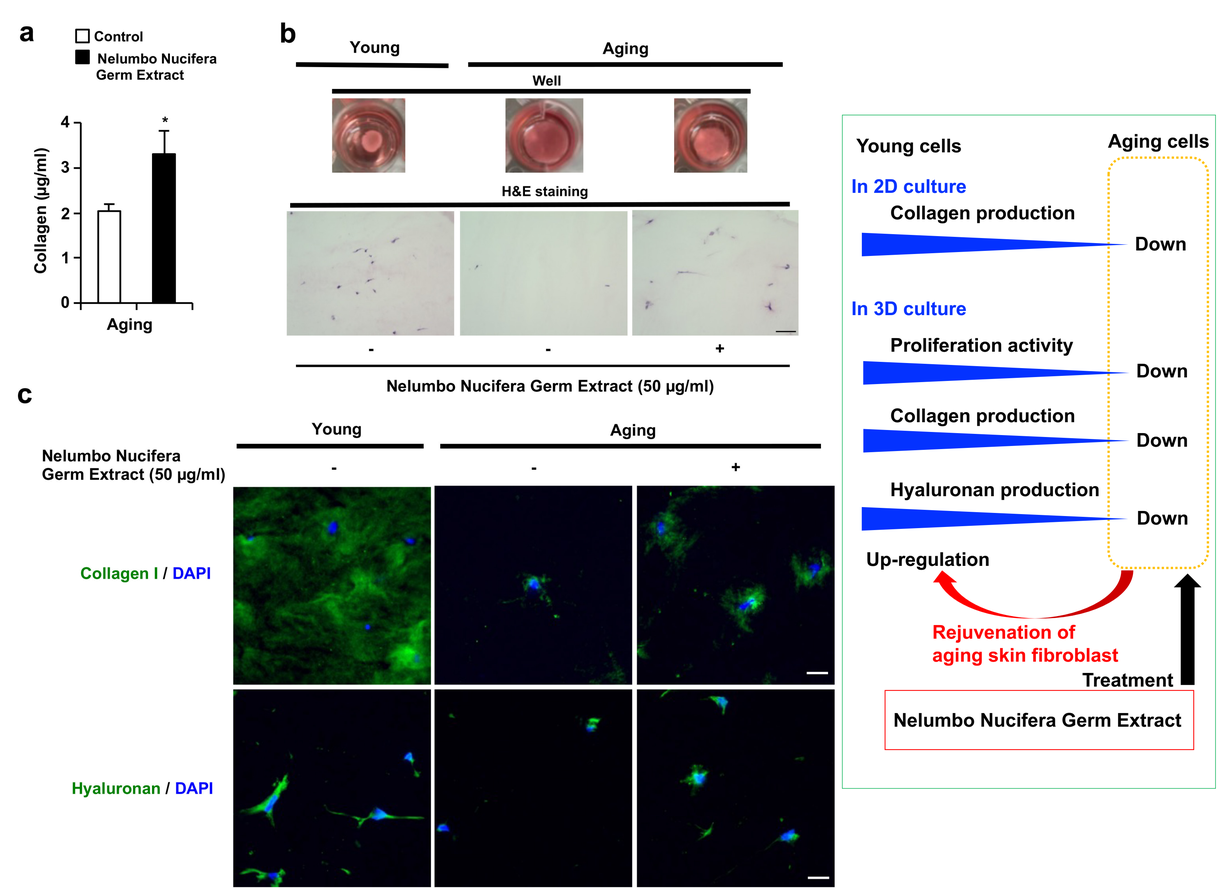
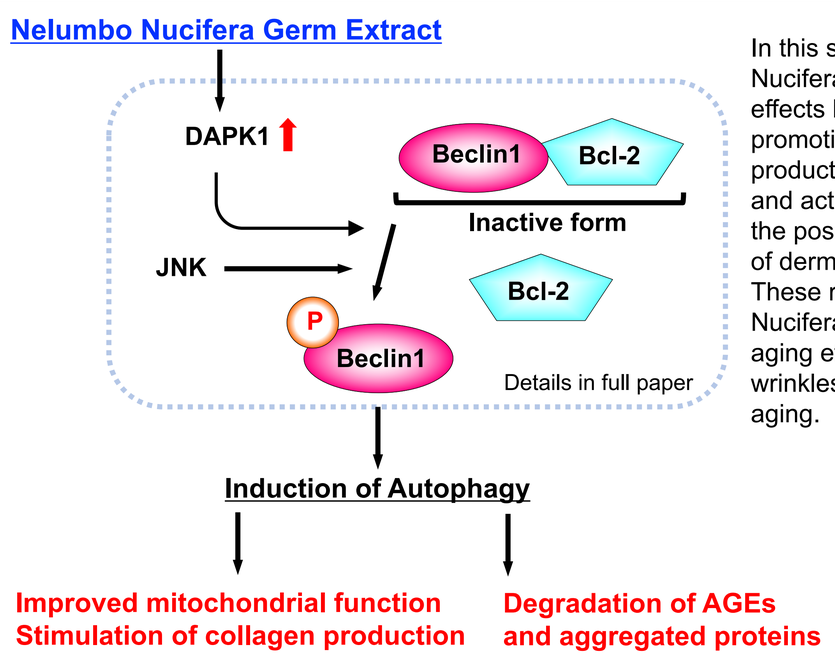


Figure 4. Nelumbo Nucifera Germ Extract stimulated collagen production and proliferative ability in aging cells in 3-dimensional gel culture.
(a) Aging NB1RGB cells were treated with 50 μg/ml Nelumbo Nucifera Germ Extract for 72 h and subjected to measurement of collagen content. (b) Aging NB1RGB cells 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 without 50 μg/ml Nelumbo Nucifera Germ Extract for 10 days. Representative image showing collagen gel contraction (upper panel). H&E staining of 3-dimensional atelocollagen gel sections. Scar bar: 100 μm (lower panel). (c) 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; $^{*}P < 0.05$

Conclusions:



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.

References:

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